EJN fens EUROPEAN JOURNAL OF NEUROSCIENCE

European Journal of Neuroscience, Vol. 33, pp. 1751–1760, 2011 doi:10.1111/j.1460-9568.2011.07648.x

SYNAPTIC MECHANISMS

The endocannabinoid 2-arachidonoylglycerol inhibits long-term potentiation of glutamatergic synapses onto ventral tegmental area dopamine neurons in mice

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Keywords: 2-AG, glutamate, LTP, midbrain, plasticity

Abstract

Drugs of abuse cause changes in the mesocorticolimbic dopamine (DA) system, such as a long-term potentiation (LTP)-like phenomenon at glutamatergic synapses onto ventral tegmental area (VTA) DA neurons. Abolishing this LTP interferes with drug-seeking behavior. Endocannabinoids (ECs) can be released by DA neurons in response to repetitive activation, which can inhibit glutamate release. Therefore, we hypothesized that ECs may act as negative regulators of LTP. Here we tested the induction of LTP in DA neurons of the VTA in mice expressing enhanced green fluorescent protein under the control of the tyrosine hydroxylase promoter. Immunohistochemistry showed colocalization of CB1 receptors with vesicular glutamate transporter (VGLUT)1 in terminals near DA neuron dendrites, with less extensive colocalization with VGLUT2. In addition, a CB1 receptor agonist, as well as trains of stimulation leading to EC production, decreased glutamate release onto DA neurons. We found that blocking CB1 receptors or synthesis of the EC 2-arachidonoylglycerol (2-AG) was without effect on basal excitatory postsynaptic potential amplitude; however, it facilitated the induction of LTP. As previously reported, antagonizing γ-aminobutyric acid $(GABA)_A$ transmission also facilitated LTP induction. Combining $GABA_A$ and CB1 receptor antagonists did not lead to larger LTP. LTP induced in the presence of CB1 receptor blockade was prevented by an *N*-methyl-D-aspartate receptor antagonist. Our observations argue in favor of the hypothesis that 2-AG acts as a negative regulator of LTP in the VTA. Understanding the factors that regulate long-term synaptic plasticity in this circuit is critical to aid our comprehension of drug addiction in humans.

Introduction

Addiction is thought to be due, in part, to long-lasting changes in the mesocorticolimbic dopamine (DA) system. Behavioral sensitization, a long-lasting phenomenon thought to underlie the craving typical to addiction in humans (Berridge & Robinson, 1998), seems dependent on *N*-methyl-D-aspartate (NMDA) subtype glutamate receptor activation in the ventral tegmental area (VTA; Kalivas & Alesdatter, 1993), as does conditioned place preference for cocaine and morphine (Harris & Aston-Jones, 2003; Harris *et al.*, 2004). A single cocaine injection *in vivo* causes an increase in the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/NMDA ratio of glutamatergic synaptic currents, which is an indicator of long-term potentiation (LTP) *in vivo*, in the VTA (Ungless *et al.*, 2001). It was subsequently found that amphetamine, morphine, nicotine and ethanol all cause similar changes in the AMPA/NMDA ratio in VTA DA neurons (Saal *et al.*, 2003). Therefore, it seems that an induction of LTP at glutamatergic synapses onto DA neurons is a common denominator in the action of all major classes of drugs of abuse.

Chen *et al.* (2008) showed that this LTP occurs when cocaine or natural rewards are self-administered. LTP at excitatory synapses in the VTA may thus underlie natural forms of motivational learning. Self-administration of cocaine caused an LTP that was significantly more persistent than that induced by natural rewards. This suggests that drugs of abuse hijack a natural learning mechanism in the VTA (Chen *et al.*, 2008). Mice in which this LTP was abolished through selective inactivation of NMDA receptors in DA neurons exhibited a normal initial sensitization to cocaine, but showed significantly less drug-induced locomotor activation 21 days after cocaine withdrawal, indicating an aberrant delayed sensitization. These mice also displayed a lack of conditioned place preference (Zweifel *et al.*, 2008; but see Engblom *et al.*, 2008). Finally, reinstatement of cocaine seeking is abolished in similar knockout (KO) mice (Engblom *et al.*, 2008).

Because of the implication of LTP at glutamatergic synapses onto VTA DA neurons in the long-term actions of drugs of abuse, it seems essential to further understand the mechanisms that regulate the induction of LTP at these synapses. However, it has so far been difficult to reliably induce LTP in DA neurons *in vitro*. A robust, albeit

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Received 15 July 2010, revised 17 January 2011, accepted 7 February 2011

small, LTP was reported to be induced by a spike time-pairing protocol (Liu *et al.*, 2005; Luu & Malenka, 2008). This LTP could be induced when γ-aminobutyric acid (GABA) transmission was blocked. These observations suggest that the induction of LTP may be gated by various neurotransmitter systems.

Endocannabinoids (ECs), acting in a retrograde fashion, can modulate neurotransmission and synaptic plasticity by a presynaptic mechanism through activation of CB1 receptors (for review, see Chevaleyre *et al.*, 2006). ECs have been shown to inhibit glutamatergic transmission onto VTA DA neurons (Melis *et al.*, 2004a,b). DGL-alpha, the biosynthetic enzyme of the EC 2-arachidonoylglycerol (2-AG), is expressed in VTA DA neurons and colocalizes with CB1 receptors on both symmetric and asymmetric terminals in this region (Matyas *et al.*, 2008). Here we tested the hypothesis that ECs negatively regulate LTP induction in the VTA.

Materials and methods

Animals

All procedures were approved by the animal ethics committee (CDEA) of the Université de Montréal. Transgenic mice (P13–P24) of the TH-eGFP/21-31 line expressing the enhanced green fluorescent protein (eGFP) gene under the control of the tyrosine hydroxylase (TH) promoter (Sawamoto *et al.*, 2001) were used in all electrophysiological experiments to facilitate identification of VTA DA neurons. Although electrophysiological criteria are often used to identify presumed DA neurons in brain slice experiments, evidence has been presented that this may sometimes be unreliable (Jones & Kauer, 1999; Margolis *et al.*, 2006; Zhang *et al.*, 2010), thus justifying the use of alternate approaches. In the present work, eGFP expression was used to initially select DA neurons. Considering the existence of a modest amount of ectopic eGFP expression in these mice (Jomphe *et al.*, 2005), post-recording immunohistochemistry was performed in most cases to confirm the presence of TH in the recorded neurons.

Drugs

The following drugs were used in electrophysiological experiments: SR95531 (10 and 2 μM); WIN55, 212-2 mesylate (WIN; 1 μM; both from Tocris Bioscience, Ellisville, MO, USA); AP-5 (20 and 25 μM); AM251 (500 nM and 4 μM; Sigma, St Louis, MO, USA), tetrahydrolipstatin (2 μM; intra-pipette; Calbiochem, San Diego, CA, USA) and 6-cyano-2,3-dihydroxy-7-nitro-quinoxaline acid (CNQX; 20 μM; RBI, Natick, MA, USA).

Slice preparation

Mice were anesthetized with isoflurane and killed by decapitation. The brain was removed and placed in an ice-cold oxygenated (95% O_2 and 5% CO_2) sucrose solution, which contained (in mM): sucrose, 250; KCl, 2; $MgCl_2$, 7; $CaCl_2$, 0.5; $NaHPO_4$, 1.2; glucose, 11; $NaHCO_3$, 26; pH 7.35. Horizontal slices (250 μm) were cut in cold sucrose solution using a Leica VT1000S vibrating microtome (Leica Microsystems, Wetzlar, Germany) and transferred to a slice-saver containing oxygenated artificial cerebrospinal fluid (ACSF) at room temperature (about 22–23°C). ACSF contained (in mM): NaCl, 126; KCl, 3.5; $MgCl_2$, 1.3; $CaCl_2$, 2; $NaHPO_4$, 1.2; glucose, 11; $NaHCO_3$, 26; pH 7.35; 305 mOsm. Slices were rested in ACSF for at least 1 h before transfer to the recording chamber, which was continuously perfused with oxygenated ACSF (1.5 mL/min) heated to 30 ± 1 °C, using a TC-324B in-line heating unit (Warner Instruments, Hamden, CT, USA).

Electrophysiology

The stimulation electrode (concentric bipolar Pt/Ir; FHC, Bodoin, ME, USA) was positioned in the rostral part of the VTA, and recordings were started only after an equilibration period of at least 20 min. Recording electrodes (3.5–7 MΩ) were pulled from borosilicate capillaries (WPI, FL, USA) using a p-97 Flaming Brown micropipette puller (Sutter Instrument, Novato, CA, USA). Electrodes were filled with an intrapipette solution containing (in mM): potassium methylsulfate, 145; NaCl, 10; EGTA, 0.1; MgATP, 2; GTP, Tris salt, 0.6; HEPES, 10; phosphocreatine, 10; pH 7.35; 300 mOsm. Biocytin (Sigma) was added to pipette solutions for post-recording identification of the neurons using immunohistochemistry.

Whole cell current- and voltage-clamp recordings were performed using a MultiClamp 700A amplifier, filtered at 2 kHz, digitized at 20 kHz and acquired using a Digidata 1440A digitizer, PCLAMP 9 and 10 acquisition software, and analysed with CLAMPEX software (Molecular Devices, Sunnyvale, CA, USA). Drugs were bath-applied and, in case of recordings with the CB1 agonist WIN and EC release experiments, 2 μM SR95531 was added to the ACSF solution.

In experiments evaluating the effect of drugs on basal synaptic transmission, afferents were stimulated at 0.1 Hz for 40 min, or 32 min in the case of tetrahydrolipstatin (THL). Excitatory postsynaptic potentials (EPSPs) were recorded from eGFP-expressing neurons that were current-clamped at −70 mV. Drugs were applied after a baseline period of 4 min, and the application was maintained throughout the remaining 36 min. In LTP experiments, drugs were bath-applied 4 min before the start of the recordings, with the exception of experiments performed with THL in the intra-pipette solution. A 10-min baseline period of 0.1 Hz extracellular stimulation and EPSP recording was obtained before LTP induction took place. LTP was induced using the spike time-dependent pairing protocol described in Liu *et al.* (2005). Briefly, an extracellular stimulation was followed 5 ms later by a 3-ms intracellular current injection of 1–2 nA to produce an action potential. This single pairing was performed five times per train at 10 Hz, for 20 trains, each separated by 5 s. Following LTP induction, a post-pairing period of 20 min identical to the baseline period was performed. For experiments evaluating EC release induced by pairing trains, 15 baseline EPSPs were evoked at 0.1 Hz, following which one or 20 pairing trains were applied. Five seconds after the trains, 10 more EPSPs were recorded at 0.1 Hz.

In experiments evaluating the pharmacological profile of EPSPs, 10 EPSPs at 0.1 Hz were obtained in the absence of pharmacological agents. CNQX (20 μM) was then allowed to wash in for 5 min before a further 10 EPSPs were recorded in continued presence of the drug. SR95531 (2 μM) was then added and allowed to wash in for 5 min. A final 10 EPSPs were obtained in the presence of both drugs.

The bridge balance (current-clamp) and series resistance (voltage-clamp) were monitored throughout all recordings, and cells displaying a change of more than 30% in these parameters were discarded.

Cell culture and transfections

HEK 293 cells, used to test the specificity of the CB1 antibody, were plated on collagen/poly-L-lysine pre-coated 1.5-cm round glass coverslips and incubated in 100 × 15-mm Petri dishes (two coverslips per dish) containing 2.5 mL Dulbecco's modified Eagle's medium (DMEM) enriched with 1% penicillin-streptomycin, 1% glutamine and 10% fetal bovine serum (Wisent, St-Jean-Baptiste, Qc, Canada). Upon reaching ± 80% confluence, coverslips were transferred to 12-well plates containing 1 mL/well of basic DMEM, and the cells were transiently transfected with a Flag-tagged CB1 plasmid (provided by

Dr K. Mackie, Indiana University) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Twenty-four–48 h later, cells were fixed for 20 min in 4% paraformaldehyde (PFA) before immunocytochemistry.

Immunohisto- and immunocytochemistry

Immunolabeling was performed to confirm the recorded neuron's dopaminergic phenotype. Slices were fixed for at least 24 h in 4% PFA and incubated overnight with a mouse monoclonal anti-TH antibody (1 : 5000; Sigma). Slices were then incubated for 2 h with both a fluorescent anti-mouse secondary antibody (Alexa 546, 1 : 250–1 : 500) to visualize the TH primary antibody and a streptavidin-conjugated Alexa 488 antibody (1 : 250) to visualize biocytin.

Tissue used for the immunostaining shown in Fig. 2 was obtained by transcardial perfusion with 4% PFA in phosphate-buffered saline (PBS) of deeply anesthetized mice (P16). The brains were immediately removed afterwards and kept in 4% PFA for 4 h, before being transferred to PBS with 0.01% NaN_3 and stored for 24 h at 4 °C. Sections (25 μm) were then prepared using a vibrating microtome.

For eGFP labeling, performed to show colocalization of eGFP and TH (Fig. 1), a rabbit anti-GFP primary antibody (1 : 5000; Clontech, Mountain View, CA, USA) was used and visualized with an anti-rabbit fluorescent secondary antibody (Alexa 488, 1 : 250). For the labeling of CB1, vesicular glutamate transporter (VGLUT)1 and VGLUT2, to show the presence of these receptors on glutamatergic afferents, a rabbit anti-CB1 primary antibody (Nyiri *et al.*, 2005) was used (1 : 500, provided by Dr K. Mackie, Indiana University) and visualized by an anti-rabbit fluorescent secondary antibody (Alexa 647, 1 : 250) and a guinea pig anti-VGLUT1 (1 : 5000) or a mouse anti-VGLUT2 primary antibody (1 : 1000; Millipore, Billerica, MA, USA), visualized by an anti-guinea pig (Alexa 555, 1 : 250) or a mouse fluorescent secondary antibody (Alexa 546, 1 : 250). For labeling of transfected HEK cells, a mouse anti-FLAG antibody, conjugated to FITC, was used to confirm transfection and to determine colocalization with CB1 immunoreactivity, as previously described. All fluorescent secondary antibodies were obtained from Molecular Probes, Eugene, OR, USA.

For capture of epifluorescence images (Figs 1 and Supporting Information S1), an inverted Nikon Eclipse TE-200 fluorescence microscope equipped with a Hamamatsu Orca-II digital-cooled CCD camera (Hamamatsu, Bridgewater, NJ, USA) and an Olympus workstation using the IMAGE-PRO Plus 6.2 software suite was used. Fluorescence excitation was controlled through the ScopePRO module of Image-Pro Plus and a DG4 xenon lamp (Sutter Instruments). Fluorescence was using collected using a Nikon 4 × (0.13 N.A.) or 40 × objective (N.A. 0.75) after passing through 510–550-nm (Alexa 488) or 575–645-nm (Alexa 546) band-pass emission filters.

For capture of confocal images (Fig. 2), a laser-scanning confocal microscope (Fluoview FV1000; Olympus) was used together with an Olympus 60 × oil-immersion objective (N.A. 1.42). The 488-nm wavelength of an argon laser was used for excitation of eGFP. Fluorescence was collected after passing through a 500–530-nm band-pass emission filter. The 543-nm wavelength of a helium–neon laser was used for excitation of the Alexa 546-coupled and Alexa 555-coupled secondary antibodies used to detect VGLUT2 and VGLUT1 receptor antibodies, respectively. Fluorescence was collected after passing through a 555–625-nm band-pass emission filter. The 633-nm wavelength of a helium–neon laser was used for excitation of the Alexa 647-coupled secondary antibodies used to detect the CB1 antibodies. Fluorescence was collected after passing through a 655–755-nm band-pass emission filter.

Colocalization analysis

Image stacks were obtained from the VTA of a total of 12 immunostained slices, six for VGLUT1 and six for VGLUT2. A threshold was applied and three images per stack were analysed for percentage of colocalization of VGLUT and CB1 signals, and one average percentage of signal overlap per stack was thus obtained. No analysis of the absolute number of VGLUT terminals containing CB1 receptor was performed. It should be noted that because the colocalization analysis performed takes into account the surface of two signals, a value of 10% colocalization does not mean that 10% of terminals contain CB1 signal, but rather that 10% of the surface occupied by VGLUT signal is occupied by CB1 signal.

Statistical analysis

The amplitude of recorded EPSPs was measured using CLAMPEX software (Molecular Devices). In experiments evaluating basal synaptic transmission, the 4-min baseline period was normalized to the average of the first 2 min of this period. In LTP recordings, the last 5 min of the baseline period was normalized to the first 2.5 min of this same 5-min period. The last 10 min of all recordings was compared with these normalized baseline periods. For experiments evaluating EC release in response to pairing trains, all 15 EPSPs of the baseline period were normalized to the average of the first seven EPSPs evoked during that baseline period. The last 10 of these baseline EPSPS were compared with the 10 EPSPs that followed the pairing trains. Two-tailed paired and unpaired *t*-tests and ANOVAs with Tukey's *post hoc* tests were performed, where appropriate, using GRAPHPAD PRISM software (Graphpad Software, La Jolla, CA, USA). Results were considered statistically significant when $P < 0.05$. Values are presented as average ± SEM. All averages are expressed as percentages of baseline values.

Results

Identification of VTA DA neurons

Patch-clamp recordings were obtained from VTA DA neurons in horizontal brain slices. Because current electrophysiological methods to identify DA neurons, such as the presence or absence of the rectifying current I_h, may be unreliable (Jones & Kauer, 1999; Margolis *et al.*, 2006; Zhang *et al.*, 2010), we used a line of mice expressing eGFP under the control of the TH promoter (Sawamoto *et al.*, 2001; Fig. 1A–C). Post-recording anti-TH immunohistochemistry was performed to confirm the expression of TH in most recorded neurons (Fig. 1D–F). Out of 194 recorded eGFP-positive neurons that were identified by the presence of biocytin, 87.1% were confirmed to be immunopositive for TH (not shown). Neurons that were conclusively confirmed not to express TH were excluded from final analyses. Thirty-two neurons were identified solely by eGFP expression prior to recording.

CB1 receptors are present on VGLUT1- and VGLUT2-positive axon terminals in the VTA

To validate that CB1 receptors are indeed present and functional on glutamatergic afferents to the VTA in our mouse model and

determine whether these are VGLUT1-expressing terminals, predicted to be of cortical origin, and/or VGLUT2-expressing terminals, predicted to be of sub-cortical origin (Geisler *et al.*, 2007), we performed CB1 immunohistochemistry and used the CB1 receptor agonist WIN (1 μM). Using double-labeling immunohistochemistry, we evaluated the distribution of CB1 receptors on glutamatergic axon terminals in close proximity to DA neurons. We used the endogenous eGFP signal to identify the dendrites and cell body of DA neurons (Fig. 2A and E). CB1 receptors were localized using an antibody that otherwise strongly and selectively recognized CB1 receptors, as confirmed by overexpression of the receptor in HEK cells (Supporting Information Fig. S1). This specificity test was performed because it has been reported that commercially available CB1 receptor antibodies are often unreliable (Grimsey *et al.*, 2008).

Abundant VGLUT1- and VGLUT2-positive terminals were detected (Fig. 2B and F). CB1-immunoreactivity was also detected in punctate structures close to VTA DA neuron dendrites (Fig. 2C and G), and colocalization between the CB1 signal and the VGLUT signals was found in a subset of axonal-like varicosities, suggesting that the CB1 receptor is indeed present on glutamatergic terminals close to DAergic cell bodies and dendrites in the TH-eGFP/21-31 mouse line (Fig. 2D and H). Colocalization analysis on confocal images taken from thin slices (25 μm) of the mouse VTA (P16) revealed that 7.25 ± 1.5% of VGLUT1 signals colocalized with CB1 staining in the VTA, and 11.15 ± 1.08% of total CB1 staining colocalizes with VGLUT1 staining (N = 23 stacks of three images each, six slices). In comparison, although quite abundant, only 0.15 ± 0.04% of VGLUT2 signal colocalized with CB1 staining and 0.85 ± 0.13% of total CB1 staining colocalized with VGLUT2 staining (N = 18 stacks of three images each, six slices). There was thus significantly more CB1 signal that colocalized with VGLUT1 compared with VGLUT2 (t_{39} = 4.172, P = 0.0002).

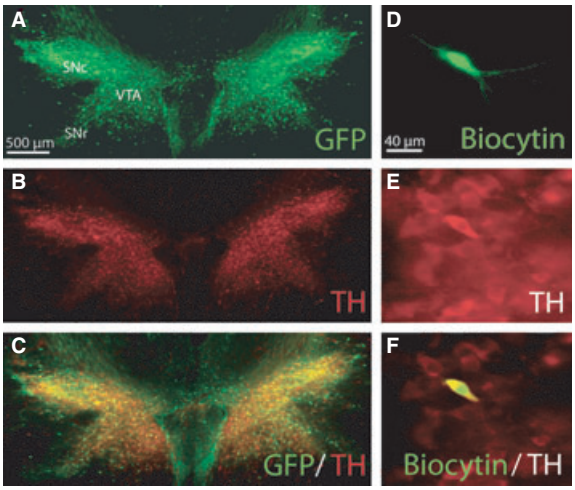

FIG. 1. Identification and localization of DA neurons in TH-eGFP/21-31 mice. (A) Enhanced green fluorescent protein (eGFP) amplified by anti-eGFP immunostaining identifying DA neurons of the ventral tegmental area (VTA) and substantia nigra comptacta (SNc) and reticulata (SNr) in a horizontal brain section prepared from a P15 mouse. (B) Anti-tyrosine hydroxylase (TH) immunostaining of the same brain section shown in (A). (C) Merged image showing the extensive colocalization of both signals. (D) A recorded neuron labeled for biocytin. (E) The same section, stained for TH. (F) The two images merged, confirming the expression of TH in the recorded neuron.

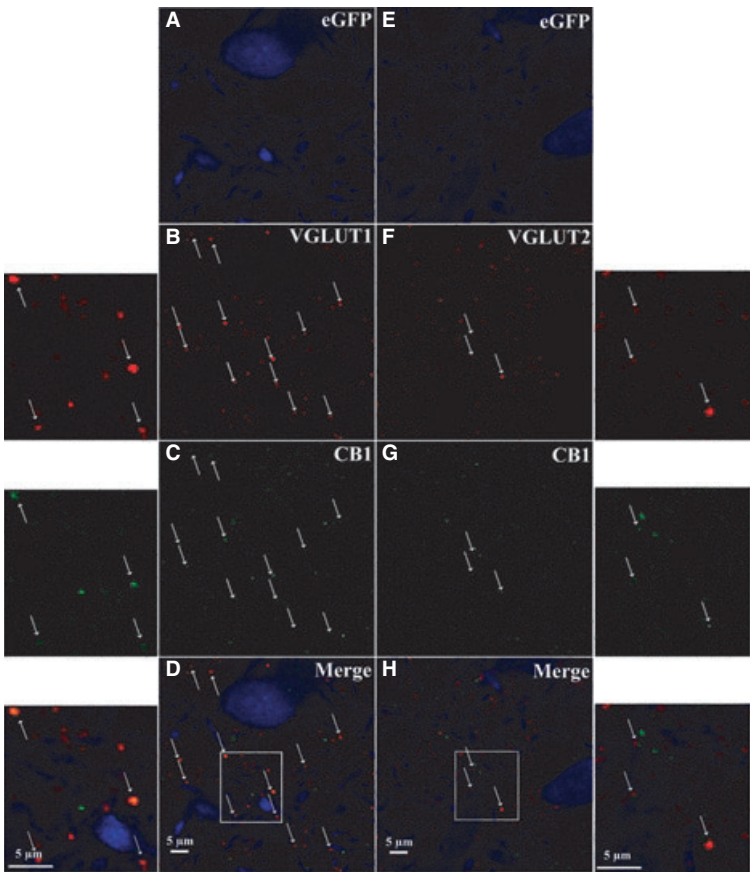

FIG. 2. Localization of CB1 receptors on vesicular glutamate transporter (VGLUT)1- and VGLUT2-positive axonal varicosities in the VTA. (A) Endogenous enhanced green fluorescent protein (eGFP) expression (shown in blue) in a section of the VTA in a horizontal midbrain slice. (B) VGLUT1 expression (red) in the same section. (C) CB1 receptor expression (green) in the same section. (D) The three images merged, showing colocalization of the CB1 receptor and VGLUT1-positive terminals in close proximity to DA neurons. (E–H) Same as (A–D), except that VGLUT2 labeling is shown in (F) and (H). The panels on the side show a higher magnification image of the signal identified by the white box in (D) and (H). White arrows indicate VGLUT1- or VGLUT2-positive terminals expressing CB1 receptors.

In support of these morphological data, we found that the CB1 receptor agonist WIN caused a robust decrease in evoked excitatory postsynaptic current (EPSC) amplitude (48.8 ± 14.9%, $N = 5$, $t_4 = 9.287$, $P = 0.0007$; Fig. 3A and B). This strongly suggests that CB1 receptors are indeed present on glutamatergic synapses onto DA neurons, the activation of which results in a decrease of glutamate release. Such receptors can also be activated by the release of ECs, as confirmed by our ability to induce depolarization-induced suppression of excitation, using a stimulation protocol that was previously used successfully in rat VTA DA neurons (Melis *et al.*, 2004a). This protocol consisted of a single train of 10 extracellular stimulations evoked at 5 Hz. We found that the amplitude of the first two EPSPs evoked after this stimulation protocol was significantly decreased compared with baseline (74.02 ± 3.7%, $N = 4$, $t_3 = 11.95$, $P = 0.0013$, not shown). This decrease was abolished by the CB1 receptor antagonist AM251 (4 μM; 106.2 ± 3.1%, $N = 4$, $t_3 = 1.199$, $P = 0.32$, not shown). Finally, we tested whether pairing trains such as those used in our LTP induction protocol also cause a similar decrease in glutamatergic transmission in DA neurons through release of ECs. Although a single pairing train did not produce a classical, short-lasting, decrease in the amplitude of the first two EPSPs following the train, this stimulation protocol induced a slowly-developing and significant overall decrease of EPSP amplitude, reaching 86.9 ± 4.6% of pre-train values ($N = 10$, $t_9 = 2.719$, $P = 0.024$; Fig. 3C). This decrease was abolished by the CB1 receptor antagonist AM251 (4 μM), with post-train values reaching 102.5 ± 3.3% of pre-train values ($N = 8$, $t_7 = 1.317$, $P = 0.23$; Fig. 3D). These experiments were performed in the presence of AP-5 (20 μM) to avoid NMDA receptor-dependent facilitation caused by the train. The average post-train amplitude was significantly decreased in the presence of AP-5 alone, but not in the presence of both AP-5 and AM251 ($t_{16} = 2.619$, $P = 0.019$; Fig. 3E). Similarly, when tested in the presence of AP-5, the 20 pairing trains used in our LTP induction protocol caused a small but statistically significant decrease in the amplitude of the first two EPSPs after the pairing trains, compared with the pre-train values ($N = 8$, $t_7 = 2.853$, $P = 0.025$; Fig. 3F).

As the decreases in EPSP amplitude presumably caused by EC release were rather small, we wondered whether the full magnitude of EC release was partly masked by non-NMDA-mediated facilitation of synaptic transmission. Similar experiments were therefore performed with unchanged extracellular stimulation parameters, but without postsynaptic action potentials. We found that after 20 trains of non-paired stimulation, the amplitude of the first two post-stimulation EPSPs significantly decreased to 69.79 ± 9.14% of pre-train values ($N = 10$, $t_9 = 3.228$, $P = 0.01$; Fig. 3G). This decrease was abolished when AM251 was present (104 ± 7.6%, $N = 8$, $t_7 = 0.409$, $P = 0.7$; Fig. 3H). The average amplitude of the first two EPSPS in the absence of AM251 was thus significantly smaller than the average in the presence of AM251 ($t_{16} = 2.795$, $P = 0.013$; Fig. 3I), showing that during stimulation trains, significant EC release and CB1 activation occurs.

Evidence for tonic inhibition of glutamatergic transmission onto DA neurons through $GABA_A$ but not CB1 receptors

We first evaluated the effect of $GABA_A$ and CB1 receptor antagonists, as well as the 2-AG synthesis inhibitor THL on basal synaptic transmission evoked at a rate of 0.1 Hz (Fig. 4A). The $GABA_A$ receptor antagonist SR95531 (10 μM) caused a gradually developing increase in evoked EPSP amplitude, reaching an average of 124 ± 8.4% of baseline values during the last 10 min of drug application ($N = 8$, $t_7 = 3.329$, $P = 0.013$). In comparison, in control experiments without drug application, no significant change in EPSP amplitude was detected (100.8 ± 13.4% of baseline value, $N = 5$, $t_4 = 0.04732$, $P = 0.97$). Similarly, the CB1 receptor antagonist AM251 (500 nM) caused no change in EPSP amplitude (101.4 ± 9.3%, $N = 6$, $t_5 = 0.478$, $P = 0.65$), nor did THL (2 μM) when present in the intrapipette solution (105 ± 10.2%, $N = 6$, $t_5 = 0.6081$, $P = 0.57$; Fig. 4A). These findings suggest that while $GABA_A$ receptor-mediated transmission tonically inhibits glutamatergic EPSPs under our conditions, CB1 receptor-mediated transmission does not.

The gradually developing increase in EPSP amplitude during application of SR95531 (Fig. 4B) may reflect a mechanism of synaptic plasticity. Alternately, it could simply suggest that under our recording conditions, a small overlapping GABA-mediated inhibitory postsynaptic potential (IPSP) was present and gradually blocked by SR95531. To test this possibility, we evoked EPSPs at −70 mV and applied CNQX (20 μM) to determine how much of the recorded postsynaptic response was AMPA receptor mediated. We found that after 5 min of CNQX, only 1.99 ± 2.9% of the initial EPSP amplitude remained. Subsequent application of SR95531 failed to further reduce that small remaining synaptic response (1.94 ± 1.98% of the initial EPSP amplitude; $N = 5$, $t_4 = 0.018$, $P = 0.99$; Fig. 4C). These findings show that the increase in EPSP amplitude observed after SR95531 was not due to the presence of overlapping IPSPs.

GABA and 2-AG negatively regulate the induction but not expression of NMDA-dependent LTP in the VTA

We next examined induction of LTP using a spike time-pairing protocol. As previously shown (Liu *et al.*, 2005), under drug-free conditions (ACSF), this LTP protocol was ineffective in inducing a change in EPSP amplitude (99.5 ± 6.4%, $N = 6$, $t_5 = 0.1057$, $P = 0.92$; Fig. 5A). In the presence of SR95531 (10 μM), significant LTP was induced (123.1 ± 8.9%, $N = 10$, $t_9 = 2.824$, $P = 0.02$; Fig. 5B). The effect of SR95531 alone did not interfere with the quantification of LTP because its effect had already reached a plateau by the pre-induction time period of LTP experiments, as revealed by an analysis of control experiments evaluating the effect of SR95531 on EPSP amplitude at periods that correspond to the analysed pre- and post-induction periods in the LTP group (Fig. 4B; $t_{14} = 0.966$, $P = 0.35$). This observation of LTP induced in the presence of $GABA_A$ blockade is compatible with previous findings obtained in rats and wild-type mice (Liu *et al.*, 2005; Luu & Malenka, 2008), and shows that similar LTP can be induced in eGFP-positive DA neurons of TH-eGFP mice.

Because DA neurons release ECs during the stimulation trains used to induce LTP, we reasoned that these ECs could partially counteract the induction of LTP. To test this hypothesis, we evaluated the induction of LTP in the presence of the CB1 receptor antagonist AM251. A significant LTP was induced under these conditions (115.7 ± 4.9%, $N = 11$, $t_{10} = 2.757$, $P = 0.02$ at 500 nM of AM251; 128.8 ± 11.2%, $N = 10$, $t_9 = 2.489$, $P = 0.034$ at 4 μM of AM251; Fig. 5C and D, respectively), compatible with the idea that activation of CB1 receptors occurs during stimulation trains, thus negatively influencing LTP induction or expression. We next evaluated whether ECs influence the expression rather than the induction of LTP. We bath-applied AM251 immediately after the LTP pairing trains had taken place. We found that no LTP occurred under these circumstances (97.69 ± 5.28%, $N = 6$, $t_5 = 0.0001$, $P = 0.99$; Fig. 5E), showing that ECs need to be blocked during and not after pairing stimuli. These findings show that ECs negatively regulate LTP induction and not LTP expression.

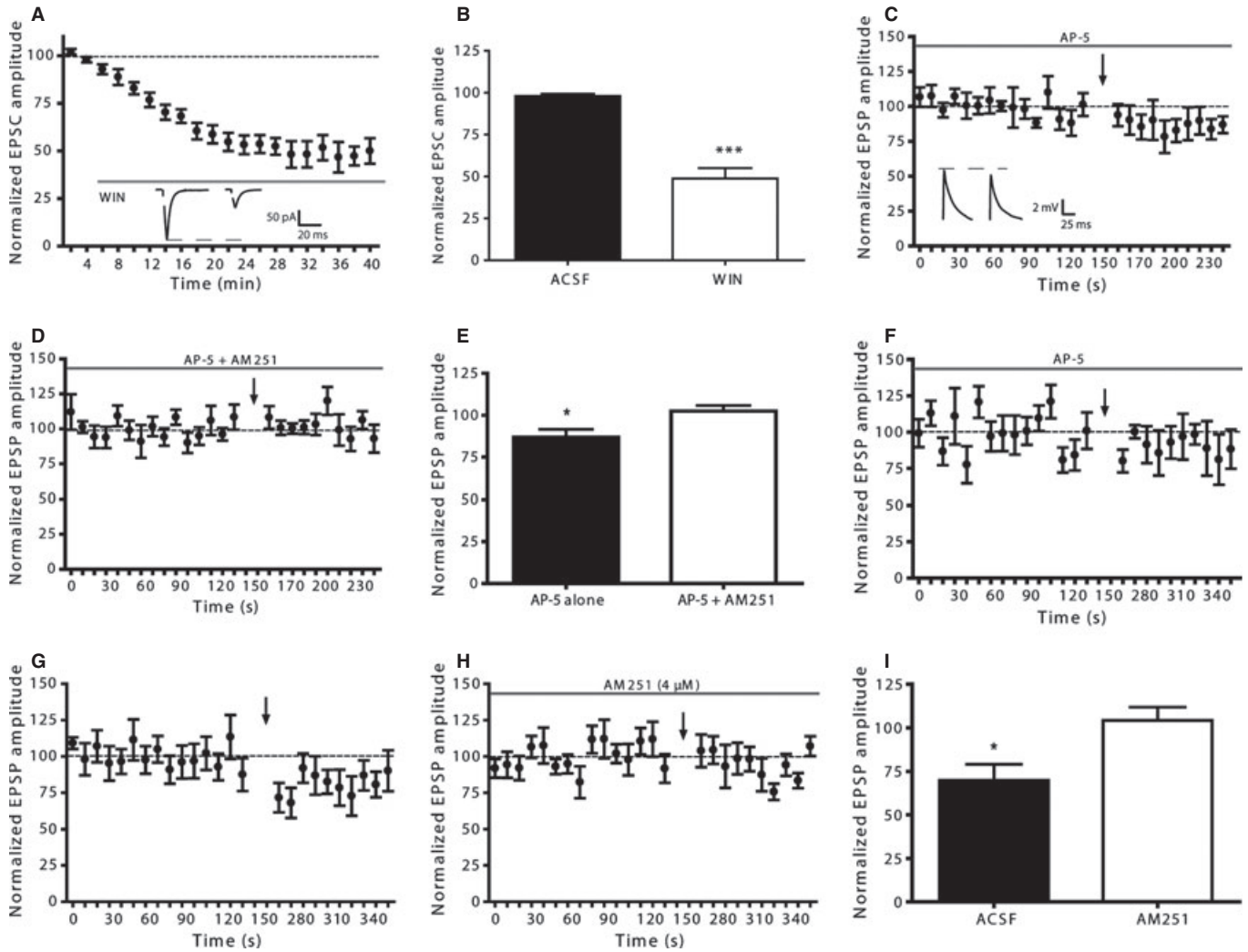

FIG. 3. CB1 receptors are functional and activated by a pairing train of pre- and postsynaptic stimulation. (A) Averaged time-course data of a voltage-clamp experiment measuring glutamate-mediated excitatory postsynaptic currents (EPSCs) in DA neurons. Application of the CB1 receptor agonist WIN55,212-2 mesylate (WIN; 1 μM) elicited a large decrease in EPSC amplitude. Insets represent averaged example traces before and after application of WIN. (B) Summary data of the experiments evaluating the effect of WIN on EPSC amplitude, presented in comparison to the period prior to drug application. (C) Averaged time-course data of the effects of a single pairing train used in LTP experiments on the amplitude of glutamatergic excitatory postsynaptic potentials (EPSPs) in the presence of AP-5 (20 μM). Arrows indicate the moment of application of the train. Insets represent averaged example traces before and after pairing stimulation. (D) Averaged time-course data of the effects of the single pairing train on the amplitude of glutamatergic EPSPs in the presence of AP-5 (20 μM) and AM251 (4 μM). (E) Summary data of the effects of the single pairing train on the amplitude of glutamatergic EPSPs. The average EPSP amplitude after the train without blockade of CB1 receptors differs significantly from the EPSP amplitude after the train when CB1 receptors are blocked with AM251 (4 μM). (F) Averaged time-course data of the effects of 20 pairing trains on the amplitude of glutamatergic EPSPs in the presence of AP-5 (20 μM). (G) Averaged time-course data of the effects of 20 trains without pairing with postsynaptic action potentials on the amplitude of glutamatergic EPSPs. (H) Averaged time-course data of the effects of 20 trains without pairing with postsynaptic action potentials on the amplitude of glutamatergic EPSPs in the presence of AM251 (4 μM). (I) Summary data of the effects of 20 trains without pairing with postsynaptic action potentials on the amplitude of glutamatergic EPSPs. The average EPSP amplitude of the first two EPSPs after the trains in the absence of CB1 blockade differs significantly from the EPSP amplitude of the first two EPSPs after the trains when CB1 receptors are blocked with AM251 (4 μM). Arrows indicate the moment of application of stimulation train(s). *$P < 0.05$; ***$P < 0.001$. ACSF, artificial cerebrospinal fluid.

In order to identify which EC was involved in the inhibition of plasticity at these synapses, we added the 2-AG synthesis inhibitor THL to the pipette solution (2 μM). In the presence of THL, LTP was induced (138.8 ± 14.8%, $N = 9$, $t_8 = 2.971$, $P = 0.018$; Fig. 5F). The magnitude of LTP induced in the presence of THL was not different than that induced in the presence of 500 nM or 4 μM of AM251 ($F_{2,27} = 1.203$, $P = 0.316$, ANOVA with Tukey's *post hoc* analysis). This observation suggests that 500 nM of AM251 is sufficient to block enough CB1 receptors to prevent the inhibitory effect of 2-AG on LTP induction. Also, this strongly suggests that 2-AG is likely to be the sole EC required for this CB1-mediated inhibition of LTP induction at glutamatergic synapses onto DA neurons.

To verify whether GABA and ECs affect LTP independently, we combined both SR95531 (10 μM) and AM251 (500 nM; Fig. 5G). In the presence of both antagonists, a robust LTP was induced (132 ± 8.2%, $N = 9$, $t_8 = 3.692$, $P = 0.004$), which, however, was not significantly different from that induced in the presence of either antagonist alone ($F_{2,29} = 2.437$, $P = 0.105$, ANOVA with Tukey's *post hoc* analysis).

Considering that NMDA receptors are necessary for the induction of behavioral sensitization to drugs of abuse and that an LTP-like

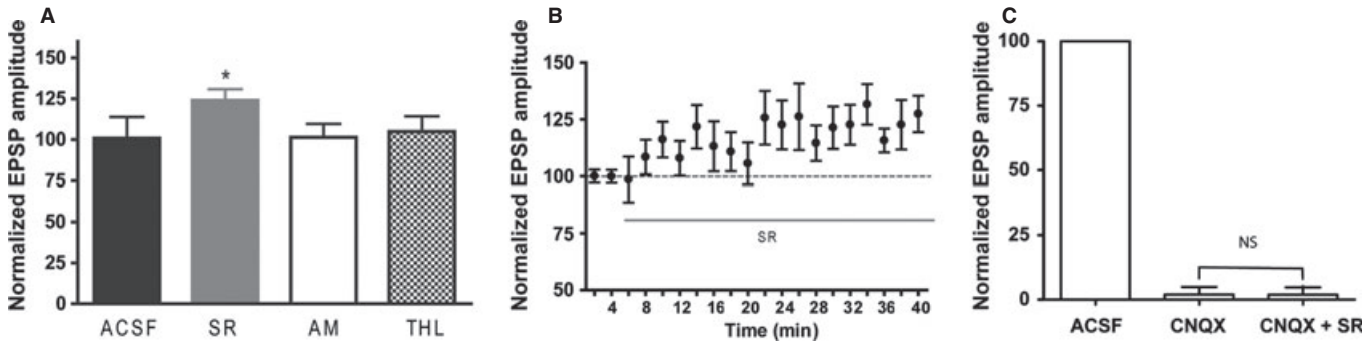

FIG. 4. $GABA_A$ receptor-mediated transmission, but not CB1 receptor-mediated transmission, tonically inhibits glutamatergic transmission onto VTA DA neurons. (A) Summary data representing average excitatory postsynaptic potential (EPSP) amplitude in response to bath application of the $GABA_A$ receptor antagonist SR95531 (SR; 10 μM), the CB1 receptor antagonist AM251 (AM; 500 nM) or the 2-AG synthesis inhibitor tetrahydrolipstatin (THL; 2 μM). While SR95531 caused an increase in EPSP amplitude, neither AM251 nor THL caused a significant change. The bars represent the relative change in EPSP amplitude normalized to the average of pre-application values. (B) Averaged time-course data of the effect of SR95531 on EPSP amplitude. (C) Averaged normalized EPSP amplitude after application of the AMPA receptor antagonist 6-cyano-2,3-dihydroxy-7-nitro-quinoxaline acid (CNQX; 20 μM) and after application of CNQX + SR95531 (2 μM). The EPSPs are thus largely AMPA receptor mediated, with no significant contamination by $GABA_A$-mediated responses. Error bars represent the SEM. NS, not significant, $^*P < 0.05$. ACSF, artificial cerebrospinal fluid.

phenomenon may be involved, we next evaluated whether LTP induced in VTA DA neurons by spike time-pairing in the presence of $GABA_A$ and CB1 receptor antagonists is also dependent on NMDA receptors. We found that no LTP could be induced in the presence of the NMDA receptor antagonist AP-5 (25 μM; 90.5 ± 5.4%, $N = 6$, $t_5 = 0.4517$, $P = 0.67$; Fig. 5H).

The summary data of all LTP experiments are shown in Fig. 5I.

Discussion

In the present work, we first showed that functional CB1 receptors are present on VGLUT1-positive terminals in close proximity of DA neuron dendrites, with less extensive colocalization found in VGLUT2-positive terminals. We found that in addition to $GABA_A$ receptors, EC CB1 receptors also negatively regulate LTP induction in the VTA through their activation by 2-AG. Moreover, the facilitation of LTP induced by antagonists of the two types of receptors was not additive, suggesting the implication of a final common pathway or the presence of a ceiling effect. Finally, ECs appear to act as negative regulators of LTP induction, rather than LTP expression.

Based on functional and structural data, glutamatergic fibers in the rat VTA have been previously suggested to possess CB1 receptors, activation of which can cause a decrease of EPSC amplitude (Melis *et al.*, 2004a,b; Matyas *et al.*, 2008). Here we confirmed these findings in our transgenic TH-eGFP mice. The region of origin of the stimulated glutamatergic afferents was not identified in the present study, but previous work has shown that they originate in large part from the prefrontal cortex and several subcortical regions (Geisler *et al.*, 2007). Our immunohistochemical localization of the CB1 receptor on VGLUT1 and VGLUT2-positive terminals in the VTA suggests that although CB1 receptors are present on glutamatergic afferents coming from both cortical and subcortical regions, they may be more abundant on VGLUT1-positive terminals, presumably coming from cortical regions.

The large population of VGLUT-negative terminals containing CB1 receptors that we observed can be presumed to be GABAergic, as a large proportion of intrinsic VTA neurons are GABAergic and this region also receives dense GABAergic projections. The presence of CB1 receptors on VTA axon terminals presumed to be GABAergic based on ultrastructural characteristics has been shown recently (Matyas *et al.*, 2008). The relative abundance of such CB1-expressing GABAergic terminals in the VTA may explain the previous observation that the summed effect of systemic and local administration of CB1 receptor agonists on VTA DA neurons is excitation (French *et al.*, 1997; Cheer *et al.*, 2000).

We showed that CB1 receptors on glutamatergic inputs to DA neurons are activated during stimulation and pairing trains used to induce LTP in VTA DA neurons. We detected an AM251-sensitive decrease in EPSP amplitude after both paired and unpaired trains of stimulation, suggesting that ECs are produced under conditions used to induce LTP. The specific signal leading to the activation of EC production was not identified in the present work, but calcium influx through dendritic voltage-dependent calcium channels or activation of metabotropic glutamate receptors (mGluRs) could be involved (Chevaleyre *et al.*, 2006).

Importantly, we found that blocking CB1 receptors allowed for induction of LTP, as does inhibiting the synthesis of 2-AG. Blocking CB1 receptors after the induction protocol had taken place was not sufficient to allow for LTP to occur. This suggests that the activation of CB1 receptors occurring during a pairing train interferes with induction, rather than expression, of LTP and that the EC released during the trains is 2-AG. Our finding of ECs acting as negative regulators of LTP induction in DA neurons is compatible with the finding that hippocampal LTP elicited by moderate stimulations or theta-burst stimulations is facilitated by CB1 receptor blockade (Slanina *et al.*, 2005; but see Carlson *et al.*, 2002), as well with the demonstration that 2-AG-dependent long-term depression of glutamatergic synapses can be induced in midbrain DA neurons during low-frequency stimulation combined with moderate postsynaptic depolarization (Haj-Dahmane & Shen, 2010).

Although previous work had shown that blocking $GABA_A$ receptors facilitates LTP induction in the rat VTA (Liu *et al.*, 2005) without inducing a change in basal synaptic transmission, in the present work we found that by itself, the $GABA_A$ receptor antagonist SR95531 caused a gradually developing increase in EPSP amplitude. The incongruity between our results and those of Liu *et al.* may perhaps be explained by the difference in species used (rats vs. mice) or in identification of DA neurons before recording. It is possible that by using I_h and firing rate parameters as criteria for DA neuron identification, these authors included a different sub-set of neurons with differential synaptic properties in comparison to DA neurons identified in the present work on the basis of TH promoter activity and

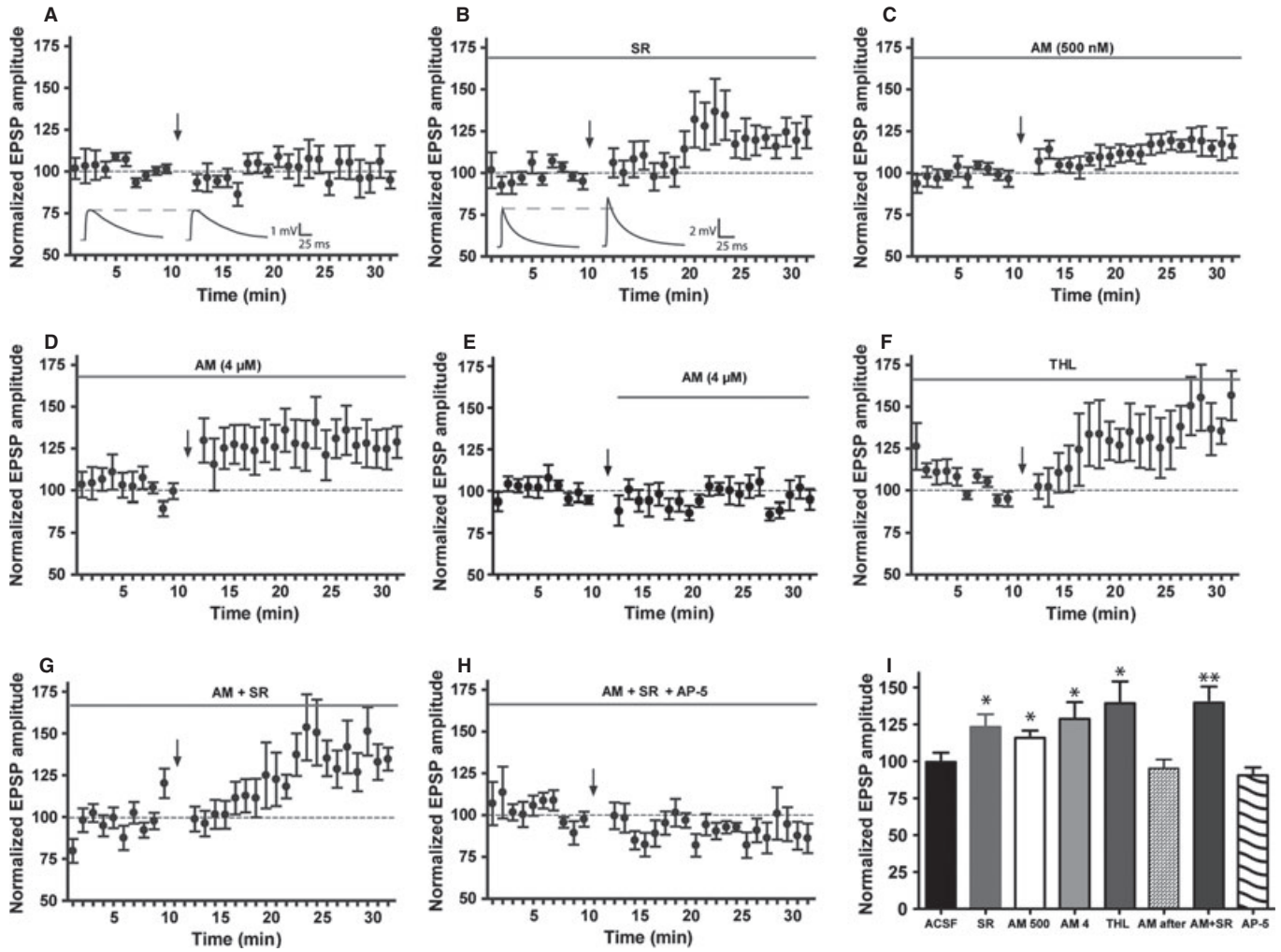

FIG. 5. $GABA_A$ receptor blockade, CB1 receptor blockade and inhibition of 2-AG synthesis allow for NMDA-dependent LTP induction. (A) Averaged time-course data illustrating excitatory postsynaptic potential (EPSP) amplitude prior to and after the spike time-dependent pairing stimulation (arrow). No significant LTP was induced in the presence of ACSF alone. Insets represent averaged example traces before and after pairing stimulation. (B) In the presence of SR95531 (SR; 10 μM), significant LTP was induced. Insets represent averaged example traces before and after pairing stimulation. (C and D) In the presence of AM251 (AM; 500 nM and 4 μM, respectively), significant LTP was induced. (E) Blocking CB1 receptors with AM251 (4 μM) after the LTP induction pairing trains had been delivered failed to allow for significant LTP to occur. (F) When tetrahydrolipstatin (THL; 2 μM) was added to the recording pipette solution, a significant LTP was induced. (G) In the presence of both SR95531 and AM251, significant LTP was induced, but its amplitude was not larger than when the individual antagonists were used alone. (H) The NMDA receptor antagonist AP-5 (25 μM) prevented the induction of LTP induced by spike time-pairing stimulation in the presence of both SR95531 and AM251. (I) Summary data illustrating normalized changes in EPSP amplitude relative to pre-pairing stimulation values. Arrows indicate the moment of LTP induction. Error bars represent the SEM. $*P < 0.05$; $**P < 0.01$.

post-recording TH immunohistochemistry. This latter approach is likely to have allowed us to sample a broader population of confirmed DA neurons. A recent extracellular recording study performed in the VTA reported that bath application of a $GABA_A$ antagonist increased the amplitude of evoked field potentials as well as EPSPs measured intracellularly, presumably by blocking simultaneously generated and overlapping IPSPs (Nugent *et al.*, 2008). We found that no significant overlapping IPSPs were present under our conditions. However, we cannot exclude a possible implication of $GABA_A$ receptor-mediated shunting or an increase in fiber excitability in mediating the increase in EPSP amplitude. Irrespective of the direct effect of $GABA_A$ receptor blockade, we confirmed here in TH/eGFP transgenic mice the previous conclusion that a significant LTP can indeed be induced in the VTA under such conditions (Liu *et al.*, 2005; Luu & Malenka, 2008).

Although both $GABA_A$ and CB1 receptor blockade facilitated the induction of LTP, we found that simultaneous blockade of both receptors did not lead to a statistically significant increase in the magnitude of LTP. One interpretation of this finding is that both of these manipulations facilitate LTP through a final common pathway. However, it is also possible that in VTA DA neurons, a ceiling effect prevents any further increase in the magnitude of LTP induced in this manner, thus preventing any additive effect of the two manipulations.

The NMDA receptor antagonist AP-5 prevented the induction of LTP when applied in the presence of both $GABA_A$ and CB1 receptor antagonists. This finding, compatible with recent reports (Liu *et al.*, 2005; Argilli *et al.*, 2008; Luu & Malenka, 2008; Mameli *et al.*, 2009), confirms that spike time-dependent LTP relies on activation of NMDA receptors. In this context, it is interesting to note that cell-specific KO of NMDA receptor NR1 subunits in mouse DA neurons prevents enhancement of glutamatergic transmission in the VTA by cocaine but fails to block the initial phase of locomotor sensitization to this drug (Engblom *et al.*, 2008; Zweifel *et al.*, 2008). However, other

adaptive changes to drugs of abuse such as reinstatement of drug seeking and the late phase of sensitization after prolonged drug withdrawal were abolished in these KO mice (Engblom *et al.*, 2008; Zweifel *et al.*, 2008).

The physiological impact of LTP of glutamatergic inputs to DA neurons on the activity of these neurons and on DA release is presently undetermined. Although facilitation of DA release could occur, it is important to consider that GABAergic transmission onto DA neurons can also undergo NMDA-dependent LTP through retrograde nitric oxide signaling (Nugent *et al.*, 2007). Such parallel potentiation of GABAergic synapses could act as a gain modulator in these circuits. Although the specific role and impacts of LTP at glutamatergic afferents onto DA neurons remains to be clarified, it is likely to be of great importance for long-term adaptations to drugs of abuse.

Additional work will also be required to explore the physiological significance of 2-AG as a negative regulator of LTP induction in the VTA. What signals lead to the production of 2-AG is presently unclear. We showed that under circumstances where AP-5 is blocking NMDA receptor signaling, pairing trains induce a small but significant AM251-sensitive decrease in glutamate release. However, in the absence of both AP-5 and postsynaptic action potentials, a more extensive AM251-sensitive decrease in EPSP amplitude was detected. One interpretation of these findings is that 2-AG production is partly, although not entirely, dependent on calcium influx through the NMDA receptor. An additional implication of mGluR receptors is possible. However, Luu & Malenka (2008) showed that a broad-spectrum mGluR antagonist fails to facilitate LTP induced in the presence of picrotoxin in VTA DA neurons. It is to be noted, nonetheless, that the ability of an mGluR antagonist to facilitate spike time-pairing-dependent LTP when applied alone has not been evaluated. It also remains to be determined whether other sources of calcium influx such as voltage-dependent calcium channels could participate.

Because blocking CB1 receptors or synthesis of 2-AG facilitated LTP induction, we can hypothesize that 2-AG acts through CB1 receptors to inhibit excitation of DA neurons and reduces DA release and behavioral responses to drugs of abuse. However, as mentioned previously, CB1 receptor activation enhances the firing of DA neurons, and previous work has shown that phasic DA release in response to drugs of abuse in the nucleus accumbens partly requires CB1 receptor activation (Cheer *et al.*, 2007). In addition, blockade or knockout of the CB1 receptor causes blunting of cocaine self-administration (Soria *et al.*, 2005). Such a paradox can only be resolved by considering the global effect of CB1 receptors on both GABAergic and glutamatergic terminals in the VTA, as well as the widespread distribution of CB1 receptors elsewhere in the brain. The implication of CB1 receptors in gating the induction of long-term depression of GABAergic inputs to DA neurons in response to repeated cocaine administration and activation of mGluRs by glutamate is likely to be a factor explaining the facilitatory role of ECs in addiction (Pan *et al.*, 2008a,b).

We showed in the present work that induction of LTP in the VTA can be facilitated when 2-AG action at CB1 receptors is blocked, through a pathway that at least partially overlaps with that induced by $GABA_A$ blockade. Considering recent work suggesting that other neuromodulators such as orexin-A can also regulate LTP in the VTA (Borgland *et al.*, 2006), it is possible that while LTP is difficult to induce under basal conditions in the VTA, a number of different signals exist that can gate its induction under appropriate physiological or pathological conditions. Because LTP at glutamatergic synapses onto VTA DA neurons represents a likely mechanism by which drugs of abuse cause long-lasting drug-related adaptive changes, further work will be critical to elucidate the conditions and mechanisms by which LTP can be induced in this structure.

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Validation of the CB1 antibody using transfection of a Flag-tagged CB1 receptor in HEK cells. (A) Phase contrast image of transfected HEK cells. Arrows indicate labeled cells. (B) Labeling of the same cells with an anti-Flag antibody (green) identifies cells expressing the CB1 receptor. (C) Labeling of the same cells with the CB1 antibody (red). (D) Merge showing perfect colocalization of both signals, confirming the specificity of the antibody. (E) Phase contrast image of untransfected cells. (F) Labeling of untransfected cells with an anti-Flag antibody. (G) Labeling of the same untransfected cells with the CB1 antibody. (H) Merge showing an absence of both the Flag and the CB1 signal in untransfected cells, showing that the antibodies did not recognize other epitopes.

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Acknowledgements

This work was supported by grants from the Canadian Institutes of Health Research to L.-E. Trudeau (MOP-49591) and to J.-C. Lacaille (MOP-10848), and from an infrastructure grant from the Fonds de la recherche en santé du Québec to the GRSNC. J.-C.L is the recipient of the Canada Research Chair in Cellular and Molecular Neurophysiology. The authors would like to thank Dr K. Kobayashi for the use of the TH-eGFP mice, Dr K. Mackie for graciously providing the CB1 antibody and plasmid, and Marie-Josée Bourque for her help in the immunohistochemical identification of dopamine neurons. The authors declare to have no conflicts of interest.

Abbreviations

2-AG, 2-arachidonoylglycerol; ACSF, artificial cerebrospinal fluid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CNQX, 6-cyano-2,3-dihydroxy-7-nitro-quinoxaline acid; DA, dopamine; DMEM, Dulbecco's modified Eagle's medium; EC, endocannabinoid; eGFP, enhanced green fluorescent protein; EPSC, excitatory postsynaptic current; EPSP, excitatory postsynaptic potential; GABA, γ-aminobutyric acid; IPSP, inhibitory postsynaptic potential; KO, knockout; LTP, long-term potentiation; mGluR, metabotropic glutamate receptor; NMDA, *N*-methyl-D-aspartate; PBS, phosphate-buffered saline; PFA, paraformaldehyde; TH, tyrosine hydroxylase; THL, tetrahydrolipstatin; VGLUT, vesicular glutamate transporter; VTA, ventral tegmental area; WIN, WIN55,212-2 mesylate.

References

Argilli, E., Sibley, D.R., Malenka, R.C., England, P.M. & Bonci, A. (2008) Mechanism and time course of cocaine-induced long-term potentiation in the ventral tegmental area. *J. Neurosci.*, **28**, 9092–9100.

Berridge, K.C. & Robinson, T.E. (1998) What is the role of dopamine in reward: hedonic impact, reward learning, or incentive salience? *Brain Res. Brain Res. Rev.*, **28**, 309–369.

Borgland, S.L., Taha, S.A., Sarti, F., Fields, H.L. & Bonci, A. (2006) Orexin A in the VTA is critical for the induction of synaptic plasticity and behavioral sensitization to cocaine. *Neuron*, **49**, 589–601.

Carlson, G., Wang, Y. & Alger, B.E. (2002) Endocannabinoids facilitate the induction of LTP in the hippocampus. *Nat. Neurosci.*, **5**, 723–724.

Cheer, J.F., Marsden, C.A., Kendall, D.A. & Mason, R. (2000) Lack of response suppression follows repeated ventral tegmental cannabinoid administration: an in vitro electrophysiological study. *Neuroscience*, **99**, 661–667.

Cheer, J.F., Wassum, K.M., Sombers, L.A., Heien, M.L., Ariansen, J.L., Aragona, B.J., Phillips, P.E. & Wightman, R.M. (2007) Phasic dopamine release evoked by abused substances requires cannabinoid receptor activation. *J. Neurosci.*, **27**, 791–795.

Chen, B.T., Bowers, M.S., Martin, M., Hopf, F.W., Guillory, A.M., Carelli, R.M., Chou, J.K. & Bonci, A. (2008) Cocaine but not natural reward self-administration nor passive cocaine infusion produces persistent LTP in the VTA. *Neuron*, **59**, 288–297.

Chevaleyre, V., Takahashi, K.A. & Castillo, P.E. (2006) Endocannabinoid-mediated synaptic plasticity in the CNS. *Annu. Rev. Neurosci.*, **29**, 37–76.

Engblom, D., Bilbao, A., Sanchis-Segura, C., Dahan, L., Perreau-Lenz, S., Balland, B., Parkitna, J.R., Lujan, R., Halbout, B., Mameli, M., Parlato, R., Sprengel, R., Luscher, C., Schutz, G. & Spanagel, R. (2008) Glutamate receptors on dopamine neurons control the persistence of cocaine seeking. *Neuron*, **59**, 497–508.

French, E.D., Dillon, K. & Wu, X. (1997) Cannabinoids excite dopamine neurons in the ventral tegmentum and substantia nigra. *Neuroreport*, **8**, 649–652.

Geisler, S., Derst, C., Veh, R.W. & Zahm, D.S. (2007) Glutamatergic afferents of the ventral tegmental area in the rat. *J. Neurosci.*, **27**, 5730–5743.

Grimsey, N.L., Goodfellow, C.E., Scotter, E.L., Dowie, M.J., Glass, M. & Graham, E.S. (2008) Specific detection of CB1 receptors: cannabinoid CB1 receptor antibodies are not all created equal!. *J Neurosci Methods*, **171**, 78–86.

Haj-Dahmane, S. & Shen, R.Y. (2010). Regulation of plasticity of glutamate synapses by endocannabinoids and the cyclic-AMP/protein kinase a pathway in midbrain dopamine neurons. *J. Physiol.*, **588**, 2589–2604.

Harris, G.C. & Aston-Jones, G. (2003) Critical role for ventral tegmental glutamate in preference for a cocaine-conditioned environment. *Neuropsychopharmacology*, **28**, 73–76.

Harris, G.C., Wimmer, M., Byrne, R. & Aston-Jones, G. (2004) Glutamate-associated plasticity in the ventral tegmental area is necessary for conditioning environmental stimuli with morphine. *Neuroscience*, **129**, 841–847.

Jomphe, C., Bourque, M.J., Fortin, G.D., St-Gelais, F., Okano, H., Kobayashi, K. & Trudeau, L.E. (2005) Use of TH-EGFP transgenic mice as a source of identified dopaminergic neurons for physiological studies in postnatal cell culture. *J. Neurosci. Methods*, **146**, 1–12.

Jones, S. & Kauer, J.A. (1999) Amphetamine depresses excitatory synaptic transmission via serotonin receptors in the ventral tegmental area. *J. Neurosci.*, **19**, 9780–9787.

Kalivas, P.W. & Alesdatter, J.E. (1993) Involvement of N-methyl-D-aspartate receptor stimulation in the ventral tegmental area and amygdala in behavioral sensitization to cocaine. *J. Pharmacol. Exp. Ther.*, **267**, 486–495.

Liu, Q.S., Pu, L. & Poo, M.M. (2005) Repeated cocaine exposure in vivo facilitates LTP induction in midbrain dopamine neurons. *Nature*, **437**, 1027–1031.

Luu, P. & Malenka, R.C. (2008) Spike timing-dependent long-term potentiation in ventral tegmental area dopamine cells requires PKC. *J. Neurophysiol.*, **100**, 533–538.

Mameli, M., Halbout, B., Creton, C., Engblom, D., Parkitna, J.R., Spanagel, R. & Luscher, C. (2009) Cocaine-evoked synaptic plasticity: persistence in the VTA triggers adaptations in the NAc. *Nat. Neurosci.*, **12**, 1036–1041.

Margolis, E.B., Lock, H., Hjelmstad, G.O. & Fields, H.L. (2006) The ventral tegmental area revisited: is there an electrophysiological marker for dopaminergic neurons? *J. Physiol.*, **577**, 907–924.

Matyas, F., Urban, G.M., Watanabe, M., Mackie, K., Zimmer, A., Freund, T.F. & Katona, I. (2008) Identification of the sites of 2-arachidonoylglycerol synthesis and action imply retrograde endocannabinoid signaling at both GABAergic and glutamatergic synapses in the ventral tegmental area. *Neuropharmacology*, **54**, 95–107.

Melis, M., Perra, S., Muntoni, A.L., Pillolla, G., Lutz, B., Marsicano, G., Di Marzo, V., Gessa, G.L. & Pistis, M. (2004a) Prefrontal cortex stimulation induces 2-arachidonoyl-glycerol-mediated suppression of excitation in dopamine neurons. *J. Neurosci.*, **24**, 10707–10715.

Melis, M., Pistis, M., Perra, S., Muntoni, A.L., Pillolla, G. & Gessa, G.L. (2004b) Endocannabinoids mediate presynaptic inhibition of glutamatergic transmission in rat ventral tegmental area dopamine neurons through activation of CB1 receptors. *J. Neurosci.*, **24**, 53–62.

Nugent, F.S., Penick, E.C. & Kauer, J.A. (2007) Opioids block long-term potentiation of inhibitory synapses. *Nature*, **446**, 1086–1090.

Nugent, F.S., Hwong, A.R., Udaka, Y. & Kauer, J.A. (2008) High-frequency afferent stimulation induces long-term potentiation of field potentials in the ventral tegmental area. *Neuropsychopharmacology*, **33**, 1704–1712.

Nyiri, G., Cserep, C., Szabadits, E., Mackie, K. & Freund, T.F. (2005) CB1 cannabinoid receptors are enriched in the perisynaptic annulus and on preterminal segments of hippocampal GABAergic axons. *Neuroscience*, **136**, 811–822.

Pan, B., Hillard, C.J. & Liu, Q.S. (2008a) D2 dopamine receptor activation facilitates endocannabinoid-mediated long-term synaptic depression of GABAergic synaptic transmission in midbrain dopamine neurons via cAMP-protein kinase A signaling. *J. Neurosci.*, **28**, 14018–14030.

Pan, B., Hillard, C.J. & Liu, Q.S. (2008b) Endocannabinoid signaling mediates cocaine-induced inhibitory synaptic plasticity in midbrain dopamine neurons. *J. Neurosci.*, **28**, 1385–1397.

Saal, D., Dong, Y., Bonci, A. & Malenka, R.C. (2003) Drugs of abuse and stress trigger a common synaptic adaptation in dopamine neurons. *Neuron*, **37**, 577–582.

Sawamoto, K., Nakao, N., Kobayashi, K., Matsushita, N., Takahashi, H., Kakishita, K., Yamamoto, A., Yoshizaki, T., Terashima, T., Murakami, F., Itakura, T. & Okano, H. (2001) Visualization, direct isolation, and transplantation of midbrain dopaminergic neurons. *Proc. Natl. Acad. Sci. U S A*, **98**, 6423–6428.

Slanina, K.A., Roberto, M. & Schweitzer, P. (2005) Endocannabinoids restrict hippocampal long-term potentiation via CBI. *Neuropharmacology*, **49**, 660–668.

Soria, G., Mendizabal, V., Tourino, C., Robledo, P., Ledent, C., Parmentier, M., Maldonado, R. & Valverde, O. (2005) Lack of CB1 cannabinoid receptor impairs cocaine self-administration. *Neuropsychopharmacology*, **30**, 1670–1680.

Ungless, M.A., Whistler, J.L., Malenka, R.C. & Bonci, A. (2001) Single cocaine exposure in vivo induces long-term potentiation in dopamine neurons. *Nature*, **411**, 583–587.

Zhang, T.A., Placzek, A.N. & Dani, J.A. (2010) In vitro identification and electrophysiological characterization of dopamine neurons in the ventral tegmental area. *Neuropharmacology*, **59**, 431–436.

Zweifel, L.S., Argilli, E., Bonci, A. & Palmiter, R.D. (2008) Role of NMDA receptors in dopamine neurons for plasticity and addictive behaviors. *Neuron*, **59**, 486–496.