Supplemental Figures:

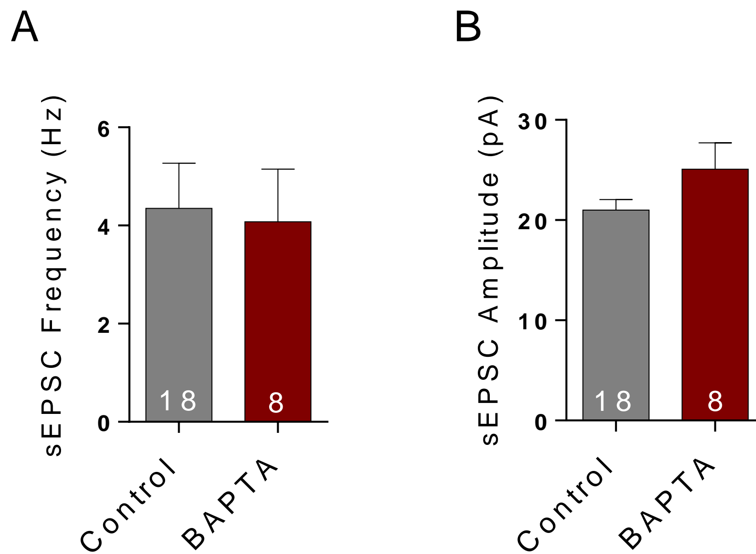

Figure S1. Effects of intracellular BAPTA on baseline glutamatergic transmission in the CeAL, related to Figure 3. Intracellular BAPTA does not affect CeAL spontaneous excitatory postsynaptic current (sEPSC) (A) frequency or (B) amplitude. (Frequency: control 4.35±0.9 Hz vs. BAPTA 4.1±1.0 Hz, $p>0.05$; Figure S1A); (Amplitude: control 21.0±1.0 pA vs. BAPTA 25.1±2.6 pA, $p>0.05$; Figure S1B). Statistical comparison performed using unpaired *t*-test.

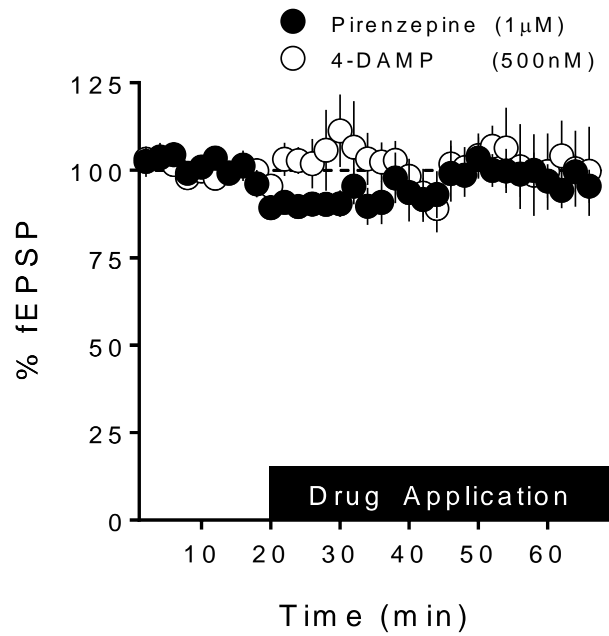

Figure S2. mAChR antagonists do not affect baseline glutamatergic transmission during prolonged drug exposure, related to Figure 4. Neither pirenzepine nor 4-DAMP affect fEPSPs in the CeAL.

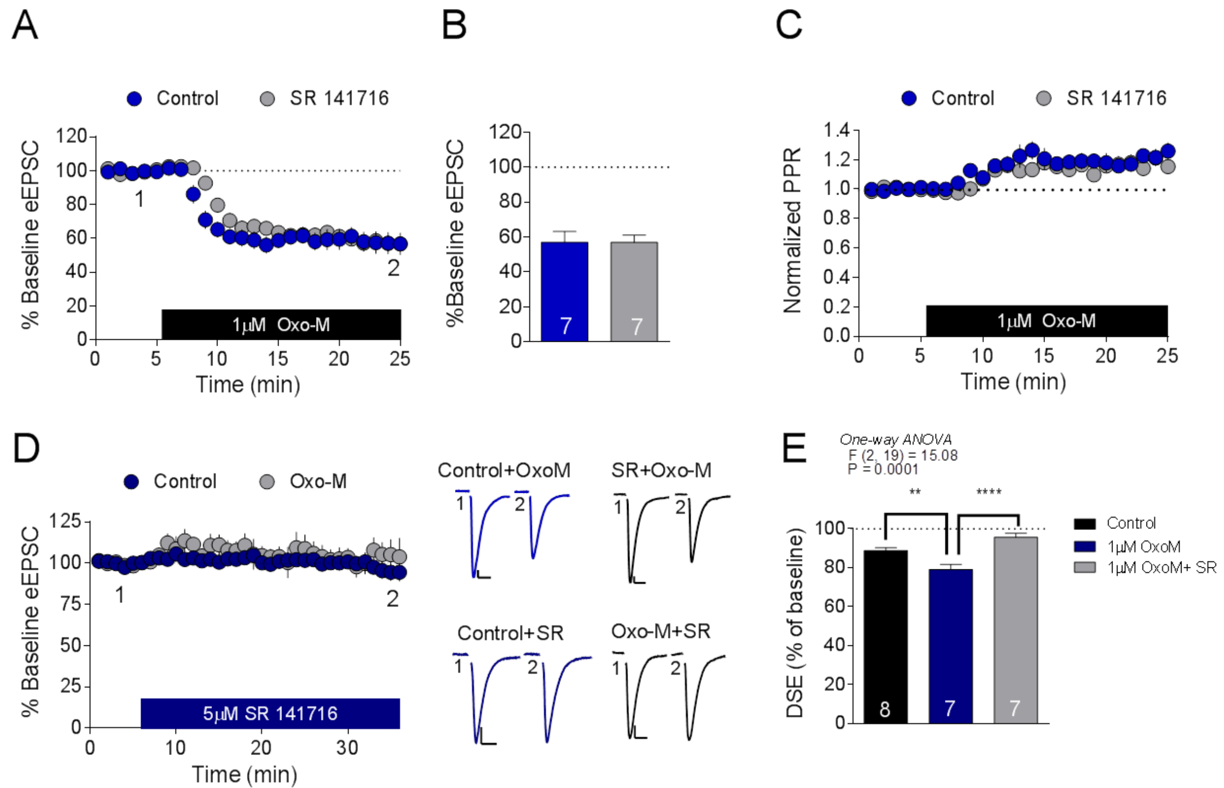

Figure S3. Oxo-M does not cause acute or tonic eCB release in dorsal striatum, related to Figure 7. (A-B) Acute application of Oxo-M causes synaptic depression in dorsal striatal neurons, which is not affected by pre-incubation with the CB_1 receptor antagonist, SR141716. (C) Oxo-M induced eEPSC depression is associated with an increase in PPR which is not affected by SR141716. (D) After continuous Oxo-M incubation, SR141716 does not induce synaptic potentiation. Top and bottom rows are representative current traces from experiments (A) and (D), respectively. (E) Oxo-M enhanced DSE magnitude in dorsal striatal neurons, which is blocked by the CB_1 antagonist, SR141716. **p<0.01, **** p<0.0001 by ANOVA followed by Sidak's multiple comparisons test.

Supplemental Experimental Procedures:

Animals

Anatomical experiments, with the exception of the double immunofluorescence labeling (ICR mice, Harlan Indianapolis, IN), were carried out in wild type and $CB_1^{-/-}$ C57BL/6 mice. All electrophysiology experiments were performed using male ICR mice (Harlan, Indianapolis, IN) 4-6 weeks old. Wild type and $CB_1^{-/-}$ mice, on the ICR background, were offsprings of wild type and homozygous $CB_1^{-/-}$ breeder (respectively), which were both previously obtained from a heterozygous breeding pair (kindly provided by Dr. C.J. Hillard, Medical College of Wisconsin, Milwaukee, WI). Mice were housed on a 12:12 light-dark cycle with food and water available *ad libitum*. All studies were approved by the Vanderbilt University Institutional Animal Care and Use Committee as well as the Committee of the Scientific Ethics of Animal Research (22.1/4027/0033/2009). Furthermore, all studies were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, as well as the institutional guidelines of ethical code, and the Hungarian Act of Animal Care and Experimentation (1998. XXVIII. Section 243/1998).

Drugs and Chemicals

SR141716 (Rimonabant) was a gift from the National Institute on Mental Health Drug Supply Program. JZL 184 was also a gift from the National Institute on Drug Abuse Drug Supply Program. Oxotremorine-M (Oxo-M) and 4-DAMP were purchased from Tocris Bioscience (Ellisville, MO) while Pirenzepine dihydrochloride and Atropine were obtained from Sigma-Aldrich (St. Louis, MO). PF-3845 was kindly provided by Dr. Douglas Johnson (Pfizer). Picrotoxin was purchased from Abcam Biochemicals (Cambridge, MA). All other drugs were

acquired from Cayman Chemicals (Ann Arbor, MI). Drugs sparingly soluble in aqueous solutions were first dissolved with DMSO and, thereafter, diluted in artificial cerebral spinal fluid (ACSF). For experiments utilizing lipophilic drugs, the ACSF was supplemented with 0.5g/L fatty acid-free bovine serum albumin (BSA; Sigma-Aldrich) to increase drug solubility and minimize nonspecific binding of lipophilic compounds. Equal amounts of DMSO and BSA were used in control solutions.

Brain Slice Preparation

Mice were anesthetized with isoflurane, then transcardially perfused with ice-cold high sucrose, low Na^+-containing ACSF and sacrificed by decapitation. Following decapitation, the brain was removed and a 3mm coronal block of the amygdala was cut using an ice-chilled, coronal brain matrix. Thereafter, hemisected coronal slices (200-300µm) were made using a Leica VT1000S vibratome (Leica Microsystems, Bannockburn, IL) in a 1-4°C oxygenated (95% v/v O_2, 5% v/v CO_2) high sucrose, low Na^+ - containing ACSF comprised of (in mM): 208 sucrose, 2.5 KCl, 1.6 NaH_2PO_4, 1 $CaCl_2{\cdot}2H_2O$, 4 $MgCl_2{\cdot}6H_2O$, 4 $MgSO_4{\cdot}7H_2O$, 26 $NaHCO_3$, 1 ascorbate, 3 Na-pyruvate, and 20 glucose. Once cut, slices were transferred to a 32°C oxygenated recovery buffer composed of (in mM): 100 sucrose, 60 NaCl, 2.5 KCl, 1.4 NaH_2PO_4, 1.1 $CaCl_2{\cdot}2H2O$, 3.2 $MgCl_2{\cdot}6H_2O$, 2 $MgSO_4{\cdot}7H2O$, 22 $NaHCO_3$, 1 ascorbate, 3 Na-pyruvate, and 20 glucose for 20 minutes followed by a minimum of 30 minutes in 24°C, oxygenated ACSF (in mM): 113 NaCl, 2.5 KCl, 1.2 $MgSO_4{\cdot}7H2O$, 1 NaH_2PO_4, 26 $NaHCO_3$, 1 ascorbate, and 3 Na-pyruvate, and 20 glucose. Thereafter, slices were placed in a submerged recording chamber where they were continuously perfused with oxygenated ACSF (30-32°C) at a flow rate of 2-3ml/min. For all electrophysiology experiments, other than those examining GABAergic currents, the ACSF was supplemented with the $GABA_A$ receptor antagonist, pircotoxin (25-

50μM), to isolate excitatory neurotransmission. To isolate eIPSCs, the ACSF was supplemented with AP-5 (50μM) and CNQX (20μM).

Field Potential Recordings

A bipolar stainless-steel stimulating electrode and a borosilicate glass recording electrode filled with ACSF were placed in the CeAL to elicit and record extracellular field responses (fEPSPs), respectively. fEPSPs were elicited at a rate of 0.05 Hz, with stimulation intensities ranging from ~100-200μA. Stable baseline fEPSPs were recorded for 20 min, followed by oxo-M (1μM) bath application for the indicated time period. For atropine + oxo-M (1μM) experiments, 1μM atropine was bath applied prior to (~30 min) and during baseline acquisition, as well as during 1μM oxo-M application. For all experiments, the N1 was monitored online and experiments that demonstrated a ≥ 20% change in N1 were discarded. Analyses measured the percent change of the N2, following drug application, relative to baseline.

Whole-Cell Voltage-Clamp Recordings

Whole-cell voltage-clamp recordings were performed on CeAL neurons easily identified visually by their medium-sized, spherical somata. Patch electrodes were pulled on a Flaming/Brown microelectrode puller (Sutter Instruments) and filled with solution containing (in mM): 120 K^+-gluconate, 4 NaCl, 10 HEPES, 20 KCl, 4 Mg-ATP, 0.3 Na-GTP, and 10 Na-phosphocreatine (pH 7.25-7.35, adjusted with KOH). For intracellular loading of the calcium chelator, BAPTA (Sigma-Aldrich), 20-40mM K^+-gluconate was replaced with 20-40mM BAPTA sodium salt. For all experiments, access resistance (Ra) was monitored online and cells that demonstrated a >20% change in Ra were excluded from analysis. Additionally, a time period

of ≥5 minutes, post break-in, was allowed for internal solution exchange and stabilization of membrane properties prior to initiation of experiments.

Monosynaptic evoked excitatory postsynaptic currents (eEPSCs) were elicited via constant-current stimulation of local glutamatergic fibers via an ACSF-filled glass electrode placed ~100µm from the cell soma. All recordings were carried out at a holding potential of -70mV. eEPSC amplitudes were typically adjusted to 200-1200pA, with stimulation intensities ranging from 10-70µA. For drug application studies, paired stimulations were elicited at a rate of 0.1Hz, with an interstimulus interval of 50ms, and six consecutive responses were averaged to generate one data point per minute. Following a five- six minute baseline, drugs of interest were bath applied. The magnitude of the drug effect was calculated as a percentage of averaged responses relative to baseline. Calculated paired-pulse ratios (PPRs) were defined as the ratio of the second eEPSC amplitude relative to that of the first.

Induction and Quantification of DSE

For DSE studies, responses were evoked with a single or paired stimulation delivered every 5 seconds to generate a 50 sec- and 100 sec- epoch prior to and following DSE induction, respectively. To induce DSE, a depolarizing pulse (-70 to 0mV) was applied to the postsynaptic neuron for 2-10 seconds as specified by the experimental conditions. The maximum DSE was classified as the first eEPSC following the depolarizing pulse. Within each DSE trial, eEPSC amplitudes were normalized to the averaged baseline response and data from two DSE trials, per cell, were averaged for analyses.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 6.01. Statistical significance between the means of two independent groups was assessed using two-tailed paired or unpaired *t*-test unless variance differed significantly (Bartlett's test for equal variances), in which case non-parametric Mann-Whitney (U) tests were used. Statistical comparisons between two or more groups were performed using one or two-way analysis of variance (ANOVA). F and P values for ANOVA are provided above individual figures. Post hoc analyses were conducted by Dunnett's or Sidak's test as indicated in the text. F-test for equality of variances between two independent groups was also used as indicated in the text. Cumulative probability plots were analyzed by Kolmogorov-Smirnov (KS) test. Statistical significance is indicated as follows: *$p<0.05$, ** $p<0.01$, ***$p<0.001$, ****$p<0.0001$. Averaged data are presented as means ± S.E.M.

Anatomical Experiments

For *in situ* hybridization, immunoperoxidase labeling, and electron microscopic analyses anesthetized male C57BL/6 mice (n=12) were perfused transcardially with 100 ml of 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (PB, pH: 7.4). After perfusion, the brain was removed from the skull, cut into blocks, and 50 µm thick coronal sections containing the amygdala were cut with a Leica Vibratome (Leica Microsystems, Weitzlar, Germany) and kept in washing buffer for further treatment. For confocal microscopy, male ICR mice (Harlan, Indianapolis, IN), 4 to 6 weeks old, were used. Mice were anesthetized using isoflurane and transcardially perfused with cold phosphate buffered solution (10ml) followed by ice-cold 20% formaldehyde. Brains were then removed, postfixed for 24 hours in the same fixative, and cryoprotected in 30% sucrose for 4 days. 40µm coronal sections of the amygdala were cut on a cryostat (Leica, CM 3050 S) and stored in an anti-freeze solution (0.1M phosphate buffer, ethylene glycol, and sucrose), at -20°C, before fluorescent immunohistochemical staining.

Non-radioactive free-floating *in situ* hybridization was carried out following the protocol as previously described in detail (Peterfi et al., 2012). We prepared antisense and sense riboprobes against a 1170 base pair (bp) long section (from position 1967 to 3136 in the open reading frame) of mouse DAGLα coding sequence using the following primers: forward, 5'-TCA TGG AGG GGC TCA ATA AG; reverse, 5'-CTA GCG TGC CGA GAT GAC CA (Katona et al., 2006). The CB_1 riboprobe was generated against a 738 bp long region of mouse CB_1 coding sequence (from position 548 to 1285 in the open reading frame; forward primer, 5'-CTA ATC AAA GAC TGA GGT TA; reverse primer, 5'- CAC AGA GCC TCG GCA GAC GT). Free-floating immunoperoxidase staining also followed the previously established protocol (Peterfi et al., 2012). The antibody (diluted 1:3000) against an internal segment of the DAGLα protein was described earlier (Katona et al., 2006), and its specificity was recently confirmed in DAGLα knockout mouse forebrain sections (Ludanyi et al., 2011). The antibody (diluted 1:200) against the C-terminus of CB_1 was previously described in Fukudome et al. (2004) and its specificity has been confirmed in many studies, including the present one. The DAGL-α, MAP2, and M_1 immunohistochemistry presented in Figure 2F-H and Figure 4E were generated using the rabbit anti-DAGLα polyclonal antibody (1:500), the mouse anti-MAP2 monoclonal antibody obtained from Millipore (1:2000), and the rabbit anti-M_1 receptor polyclonal antibody purchased from Alamone labs Ltd. (1:200). Thenceforth, brain sections were washed in 3 changes of tris-buffered saline (TBS), incubated in 10mM sodium citrate (pH=9; 80°C) for 30 minutes, followed by (3x10 min) TBS washes. Subsequently, slices were incubated in TBS+ (TBS supplemented with 4% horse serum and 0.2% Triton X-100) for 30 minutes and in primary antibody (room temperature) overnight. The next day, slices were washed in TBS+ (3x10 min) and incubated with Alexa Fluor 546 donkey anti-rabbit IgG (Life Technologies, 1:1000) and DyLight 488

donkey anti-mouse IgG (Jackson ImmunoResearch, 1:500) for 2.5 hours at room temperature. Stained slices were then washed using TBS (3x10 min), mounted unto slides with 0.15% gelatin solution, and imaged with a Zeiss LSM 710 confocal microscope. Images were analyzed with Zeiss LSM Image Browser software.

For electron microscopic analyses, after development of the immunostaining, the sections were treated with 0.5% OsO_4, dehydrated in an ascending series of ethanol and acetonitrile solutions, and finally embedded into Durcupan™ ACM Fluka (Sigma). During dehydration, sections were also treated with 1% uranyl acetate in 70% ethanol for 20 minutes. After overnight incubation in Durcupan, the sections were mounted onto glass slides and coverslips were sealed by polymerization of Durcupan at 56 ºC for 48 hours. From sections embedded in Durcupan, areas of interest from the CeAL were re-embedded and re-sectioned for electron microscopy. Sections were collected on Formvar-coated single-slot grids, stained with lead citrate, and examined with a Hitachi 7100 electron microscope (Hitachi High-Technologies Corporation, Tokyo, Japan).

Supplemental References:

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