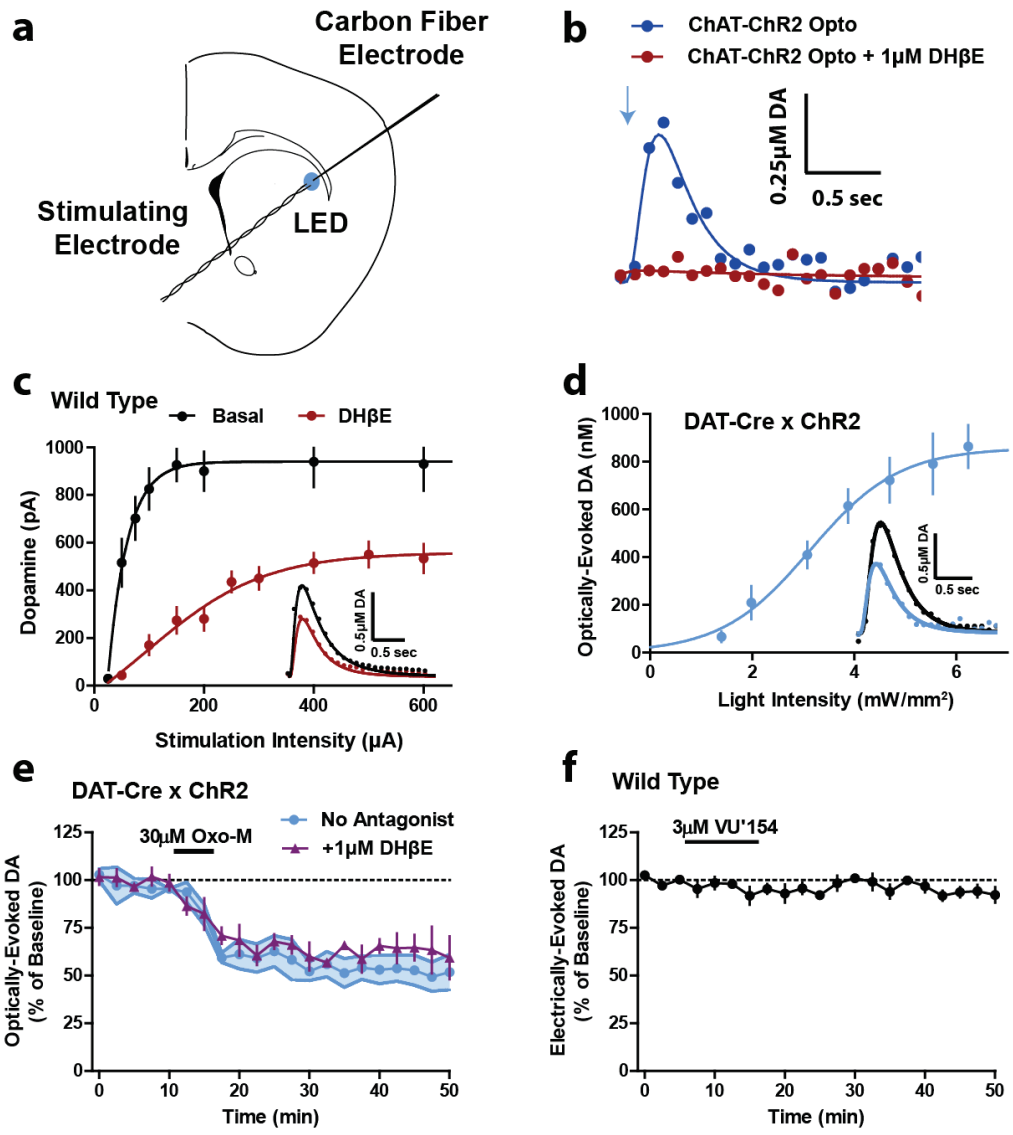

Figure S1, related to Figure 1. Isolation of nAChR-independent DA release using either electrical or optical stimulation. (**a**) Cartoon depicting the experimental set-up for fast scan cyclic voltammetry experiments. Carbon fiber electrodes were placed proximal to a stimulating electrode. In some experiments the area around the carbon fiber was periodically illuminated by a focused 470nm light source. (**b**) Pretreatment with 1 μM DHβE completely blocks optically-evoked DA release in slices from mice expressing ChR2 in ChAT-positive neurons. (**c**) Input / output curves displaying DA release observed with increasing intensity of electrical stimulation in the absence or presence of 1 μM DHβE (*n*=8-11). Experiments involving electrical stimulation were typically performed at 200-400 μA intensity so as to include both DHβE-dependent and DHβE-independent components. (**d**) Optically-evoked DA release from DAT-Cre x ChR2 mice was observed to be intensity-dependent (*n*=3) and displayed similar kinetics when compared to electrically-evoked DA release (inset: sample traces showing DA release evoked by 350 μA electrical stimulation, black; or 4.7 mW/mm^2 optical stimulation, blue). (**e**) Unlike DA release induced by electrical stimulation, pretreatment with 1 μM DHβE had no effect on Oxo-M mediated effects on optically-induced DA release (*n*=5). (**f**) Application of 3 μM VU'154 in the absence of agonist had little to no effect on DA release (*n*=5).

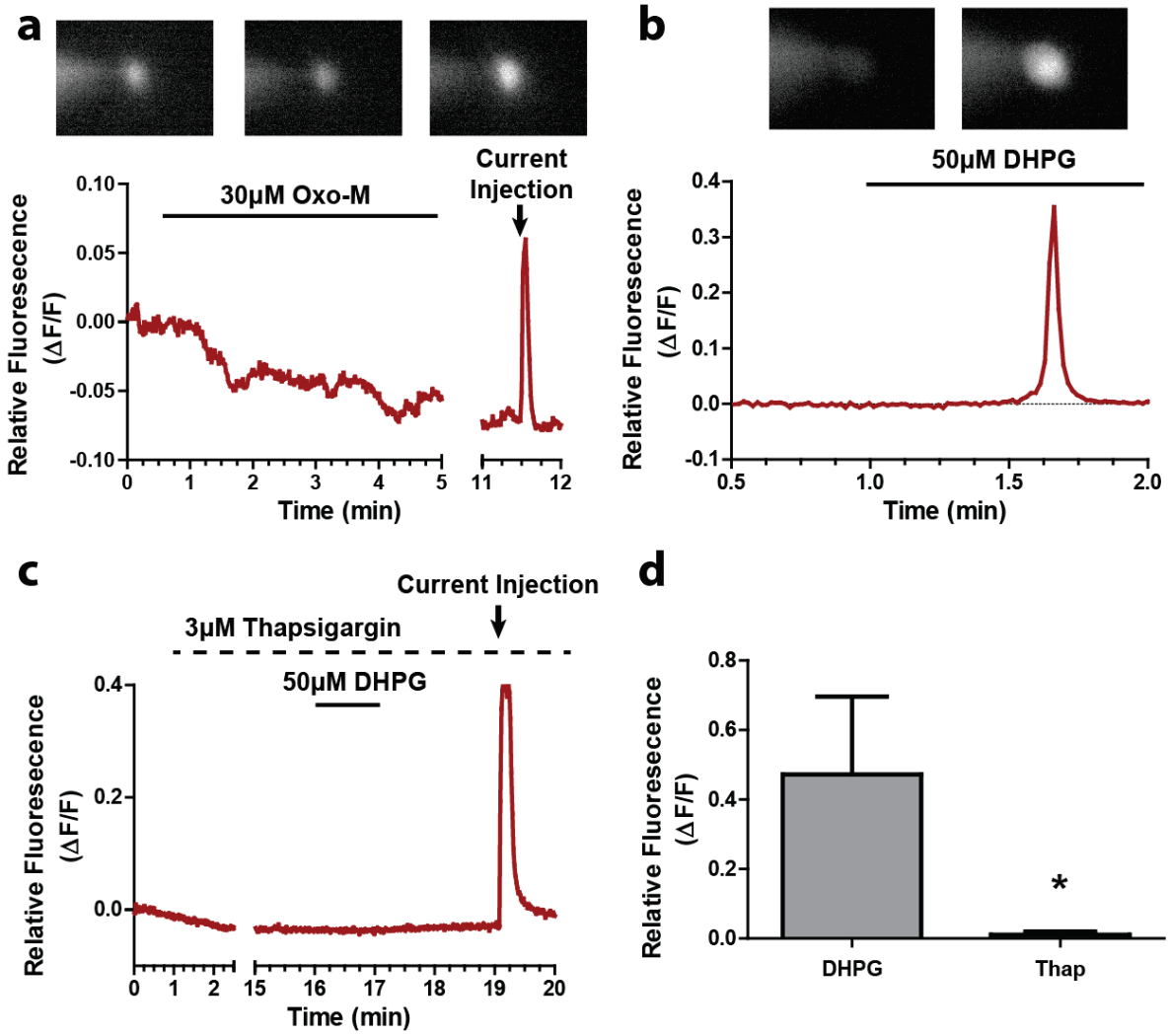

Figure S2, related to Figure 3. Activation of M_4 on direct pathway SPNs does not induce intracellular Ca^{2+} transients. M_1-KO mice that expressed the fluorescent reporter tdTomato in D_1-expressing neurons were used to identify direct pathway SPNs and to remove contributions of M_1 to Ca^{2+} signaling. SPNs were patched and loaded with the Ca^{2+} sensitive dye fluo-4. (**a**) Sample time-course and time-lapse images showing that application of the mAChR agonist Oxo-M did not induced any Ca^{2+} transients and reduced the basal fluorescence (*n*=5). Sample time-courses (**b,c**) and summarized data (**d**) demonstrating that application of the group I mGlu receptor agonist DHPG induced Ca^{2+} transients in the absence, but not the presence, of 3 μM thapsigargin which depletes intracellular Ca^{2+} stores (n=4, * Significant difference from DHPG, $p<0.05$; two-tailed Mann-Whitney test).

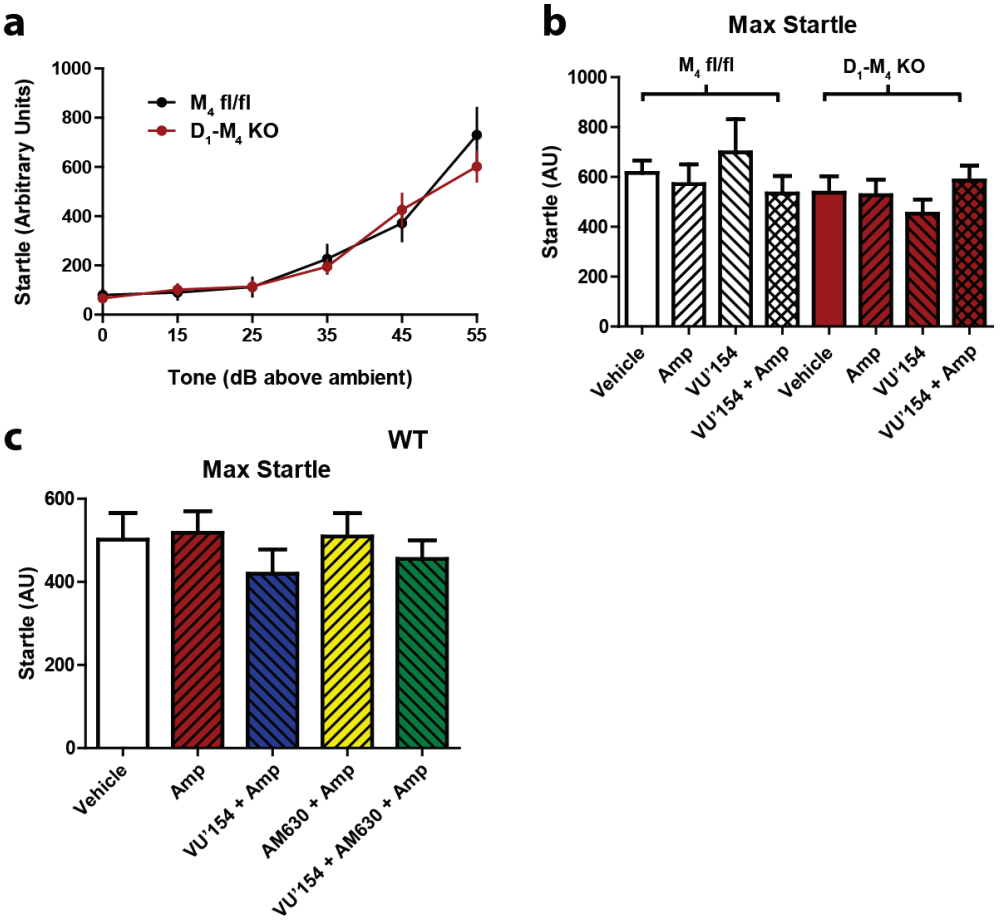

Figure S3, related to Figure 4. Neither deletion of M_4 from D_1 expressing neurons nor drug administration had an effect on the observed acoustic startle responses. (**a**) Startle response curves for D_1-M_4 KO mice and control littermates (M_4 fl/fl). For studying PPI, a 120 dB startle tone (55 dB above background noise) was used. (**b**) 4mg/kg amphetamine (Amp), and/or 10mg/kg VU0467154 (VU'154) had no effect on responses to 120 dB startle tones when compared to animals injected with vehicle in either M_4 fl/fl or D_1-M_4 KO mice (n=10-21). (**c**) 4mg/kg amphetamine (Amp), 10mg/kg VU0467154 (VU'154), and/or 10mg/kg AM630 had no effect on responses to 120 dB tones in WT mice when compared to vehicle (n=15-20).

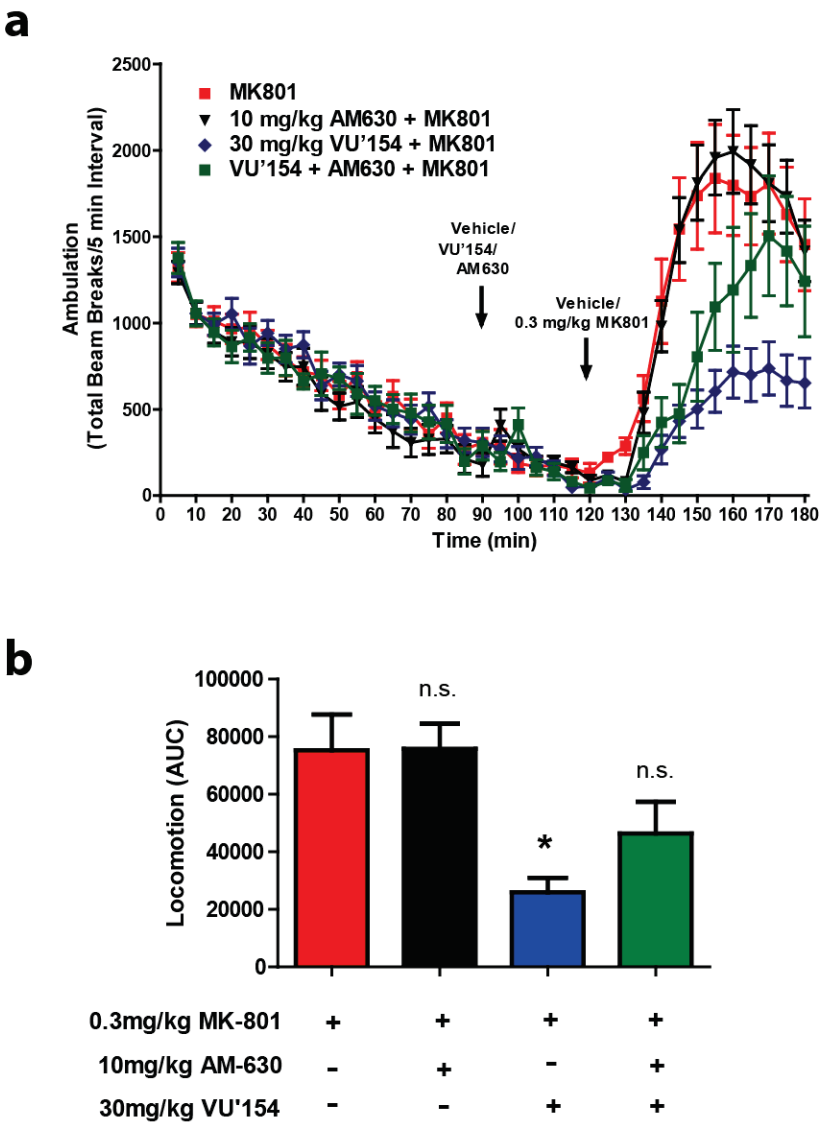

Figure S4, related to Figure 4. CB_2 antagonism significantly inhibits M_4 -mediated reversal of MK-801-induced hyperlocomotion. (**a**) Time-course of open field locomotor activity following various drug treatments. 0.3 mg/kg MK-801 robustly increased open field locomotor activity, an effect that was reduced when pre-treated with 10mg/kg VU0467154 (VU'154). Treatment with 10mg/kg AM630 alone did not alter MK-801-induced locomotion but significantly blunted the reversal observed with VU'154. (**b**) Averaged data depicting the total locomotion (area under the curve) observed with various treatments WT mice (*n*=9-18). * Significant difference from MK-801 alone group ($p<0.05$, one-way ANOVA with a *post hoc* Dunnett's test).

Supplemental Materials and Methods

Materials

Dihydro-beta-erythroidine hydrobromide (DHβE), kynurenic acid sodium salt, and AM630 were purchased from Tocris (Minneapolis, MN). Fluo-4 pentapotassium was purchased from Thermo Fisher Scientific. VU0467154 (VU'154) was synthesized as previously reported and has been demonstrated to be a positive allosteric modulator (PAM) at the murine M_4 receptor with low nanomolar potency (17nM), excellent subtype-selectivity (>30 μM potency at M_1, M_2, M_3, and M_5), and improved pharmacokinetic properties when compared to other M_4 PAMs (Bubser et al., 2014). DO34 was synthesized as previously reported (Ogasawara et al., 2016). All other compounds were purchased from Sigma-Aldrich (St. Louis, MO).

Experimental animals

All of the animals used in the present studies were group housed with food and water available *ad libitum*. Animals were kept under a 12h light/dark cycle with lights on from 6:00 AM to 6:00 PM and were tested during the light phase. All of the experimental procedures were approved by the Vanderbilt University Animal Care and Use Committee and followed the guidelines set forth by the *Guide for the Care and Use of Laboratory Animals*. All animals used were 8-15 weeks of age and maintained on a C57BL/6 background. For wild-type animals C57BL/6NTac wild-type mice were used (Taconic, Hudson, NY). The D_1-M_4 KO mice and control littermates (M_4 fl/fl) were maintained on a congenic C57BL/6NTac background (RRID: MGI:4442324; Jeon et al., 2010). For optical-stimulation of dopamine (DA) terminals slices were prepared from mice in which channelrhodopsin-2 (ChR2) was selectively expressed in DA transporter (DAT)-containing neurons by crossing $DAT^{IRES\mathit{cre}}$ mice (RRID: IMSR_JAX:006660) with Ai32 (RCL-ChR2(H134R)/EYFP) mice (RRID: IMSR_JAX012569). CB_2 receptor knock-out (KO) mice (RRID: IMSR_JAX:005786) and mice expressing ChR2/yellow fluorescent protein selectively in choline acetyltransferase (ChAT)-containing neurons (RRID: IMSR_JAX014545) were used and maintained on a C57BL/6NJ background. For the Ca^{2+} imaging studies M_1 KO mice (RRID: MGI:2655337) were crossed with mice that expressed the fluorescent reporter tdTomato in D_1-expressing neurons (RRID: IMSR_JAX:016204) and were used and maintained on a C57Bl/6NJ background.

Acute brain slice preparation

Acute coronal brain slices containing the striatum (300μm), were obtained via a variation of a reported brain slice methodology for adult mice (Foster et al., 2014). Mice were anesthetized by intraperitoneal injection of 0.2mL ketamine/xylazine (20mg/2mg per mL) and then transcardially perfused with ice-cold modified artificial cerebrospinal fluid (aCSF) [75mM sucrose, 87mM NaCl, 2.5mM KCl, 7mM $MgSO_4$, 0.5mM $CaCl_2$, 1.25mM NaH_2PO_4, 26mM $NaHCO_3$, 1mM kynurenic acid, and 25mM D-glucose, oxygenated with 95% O_2/5% CO_2]. Slices were initially recovered for 10-15 minutes at 32°C in protective artificial cerebrospinal fluid (aCSF) [92mM N-methyl-D-glucamine, 2.5mM KCl, 1.2mM NaH_2PO_4, 30mM $NaHCO_3$, 20mM HEPES, 25mM D-glucose, 5mM sodium ascorbate, 2mM thiourea, 3mM sodium pyruvate, 10mM $MgSO_4$, 0.5mM $CaCl_2$, pH 7.3, 305mOsM]. After the initial recovery the slices were transferred to aCSF containing 126mM NaCl, 2.5mM KCl, 2.45mM $CaCl_2$, 1.2mM $MgSO_4$, 1.25mM NaH_2PO_4, 26mM $NaHCO_3$, 0. 5mM sodium ascorbate, and 11mM D-glucose for at least 1 hour prior to recording. In the recording chamber slices were perfused with aCSF in the absence of ascorbate. All experiments on acute brain slices were performed at 32°C and drugs were bath-applied with a flow rate of 2mL/min.

Fast Scan Cyclic Voltammetry

Electrically-evoked DA overflow was monitored with carbon fiber electrodes with a 5 μm diameter as previously described (Schmitz et al., 2002). A triangular voltage wave (-400 to +1000 mV at 300V/sec) was applied to fresh cut carbon fiber electrodes every 100 msec. When monitoring electrically-evoked DA transients, stimulating electrodes

were placed 75 μm deep into the dorsolateral striatum and slices were electrically stimulated (30-600 μA) every 2.5 min via a bipolar stimulating electrode placed ~100 μm from the carbon fiber. For typical experiments the stimulation intensity used was 200-400 μA so as to induce both nAChR-dependent and –independent DA release (Figure S1c). When monitoring optically-evoked dopamine transients a 1msec 470nM light pulse was applied at different intensities (Figure S1d). The effects of Oxo-M on optically-induced dopamine release was monitored by alternating electrical and optical pulses every 75 seconds. Current was acquired using a Clampex9.2/Digidata1440A system (Molecular Devices; Sunnyvale, CA) with a low pass Bessell Filter at 10kHz and digitized at 100kHz. Background-subtracted cyclic voltammograms served both to calibrate the electrodes and to identify dopamine as the substance that was released following electrical stimulation. The best-fit simulation of electrically-evoked dopamine overflow was found by nonlinear regression. All time course data is presented as the mean ± SEM for individual time points. Acute inhibition was defined as the inhibition observed during the last time point that Oxo-M was still in the bath. Sustained inhibition was defined and the inhibition that was observed 15 minutes after Oxo-M had washed out. These data were plotted with box and whisker plots and analyzed using one-way analysis of variance (ANOVA) with a Dunnett's or Bonferroni post hoc test where appropriate. Statistical significance was determined as $p < 0.05$.

Single Cell Ca^{2+} Imaging

Fluorescence-based Ca^{2+} imaging in brain slices was performed as previously reported (Foster et al., 2014). Whole-cell recordings were made from visually identified D_1-expressing SPNs in acute brain slices from M_1 KO mice expressing the fluorescent reporter tdTomato. Cells were maintained in current clamp mode using an Axon Multiclamp 700B amplifier (Molecular devices). Patch pipettes were prepared from borosilicate glass (Word Precision Instruments) and had an electrode resistance of 3.5-6 MΩ when filled with intracellular solution containing the following (in mM): 120 $KMeSO_4$, 5 KCl, 10 HEPEs, 0.2 GTP-Na, 2 ATP-Mg, 5 phosphocreatine, and 0.1 Fluo-4 pentapotassium, pH 7.3, 290mOsM). After patching, cells were allowed to equilibrate with the intracellular solution for 15 minutes prior to imaging. Changes in fluorescence were monitored every 1 sec with exposures ranging from 1-3 msec using a Cool Snap HQ2 camera (Photometrics) Lambda 10-3 shutter and Lambda LS stand-alone xenon arc lamp and power supply (Sutter Instruements). Increases in Ca^{2+} mobilization were reported as changes in relative fluorescence divide by the baseline fluorescence (ΔF/F).

Prepulse Inhibition

The antipsychotic-like efficacy of M_4 activation on D_1-expressing neurons and role CB_2 receptors play in this phenomenon were assessed using the prepulse inhibition of the acoustic startle reflex assay (PPI). Studies were conducted using MedAssociates acoustic startle chambers (MedAssociates, St. Albans, VT). In M_4 fl/fl and D_1-M_4 KO mice, following a 30-minute pretreatment with vehicle or VU0467154 (10 mg/kg, 10% Tween 80 in sterile water, intraperitoneal (i.p.), 10 ml/kg), mice were subsequently injected with vehicle or amphetamine (4 mg/kg, subcutaneous (s.c.), saline, 10 ml/kg). For C57Bl/6 wild type mice, vehicle or AM630 (10 mg/kg, 10% Tween 80 in sterile water, i.p., 10 ml/kg) was administered followed 30 minutes later by vehicle or VU0467154 (10 mg/kg, 10% Tween 80 in sterile water, i.p., 10 ml/kg) and 30 minutes later by administration of vehicle or amphetamine (4 mg/kg, s.c., saline, 10 ml/kg). After an additional 25 minutes following amphetamine administration, the mice were then placed into individual startle chambers for the following testing paradigm. After a 5-minute acclimation period, the mice were presented with five presentations of a 120 dB startle stimulus alone, followed by nine rounds of psuedorandomized presentations of the following trials: no stimulus, startle pulse alone (120 dB, 40-msec broadband noise burst), highest prepulse noise alone (80 dB, 40-msec broadband noise burst), and three varying prepulses (70, 75, or 80 dB; 20 msec) followed by a startle pulse (120 dB, 40-msec broadband noise burst, 50 msec interstimulus interval). The intertrial interval varied pseudorandomly between 9 and 21 seconds. Background noise of 65 dB was presented continuously. Percent PPI was calculated as 100 x (mean acoustic startle reflex [ASR] - mean ASR in prepulse plus pulse trials) / mean ASR in startle pulse trials. %PPI is presented with 80dB prepulses, as this is the only condition at which amphetamine significantly disrupted PPI compared to vehicle. Data are expressed as mean ±

SEM and analyzed using one-way ANOVA with a Dunnett's post hoc test comparing all dosing groups to amphetamine-treated controls. Statistical significance was determined as $p < 0.05$.

MK-801 Hyperlocomotion

The effect of CB_2 antagonism on M_4-mediated reversal of MK-801-induced hyperlocomotion was assessed in 12 week old C57Bl/6 wild-type mice (Jackson Laboratories), using MedAssociates open field chambers (MedAssociates, St. Albans, VT). Mice were placed in the open arena and allowed to habituate for 90 min before being injected with either 10% Tween 80 in sterile water (vehicle), 10 mg/kg AM630, 30 mg/kg VU0467154, or a combination of 10 mg/kg AM630 and 30 mg/kg VU0467154 (i.p., 10 ml/kg). Following a 30 min pretreatment period, saline vehicle or 0.3 mg/kg MK-801 were administered (i.p., 10 ml/kg), and locomotor activity was recorded for an additional 60 min. Drug-induced changes in ambulation across time are expressed as distance traveled (cm per 5 min bins). Locomotion area under the curve data were calculated as the total distance traveled during the last 60 min of the experiment using Prism (Graphpad; San Diego, CA). Data are presented as means ± SEM and were analyzed by two-way or one-way ANOVA, respectively; post hoc comparisons were made by Dunnett's test with statistical significance determined as $p < 0.05$.

Data Analysis

ClampFit (Molecular Devices; Sunnyvale, CA), and Prism (Graphpad; San Diego, CA) were used for data analysis.

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