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Metabotropic Glutamate Receptor 1 (mGluR1) Antagonism Impairs Cocaine-Induced Conditioned Place Preference via Inhibition of Protein Synthesis

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Antagonism of group I metabotropic glutamate receptors (mGluR1 and mGluR5) reduces behavioral effects of drugs of abuse, including cocaine. However, the underlying mechanisms remain poorly understood. Activation of mGluR5 increases protein synthesis at synapses. Although mGluR5-induced excessive protein synthesis has been implicated in the pathology of fragile X syndrome, it remains unknown whether group I mGluR-mediated protein synthesis is involved in any behavioral effects of drugs of abuse. We report that group I mGluR agonist DHPG induced more pronounced initial depression of inhibitory postsynaptic currents (IPSCs) followed by modest long-term depression (I-LTD) in dopamine neurons of rat ventral tegmental area (VTA) through the activation of mGluR1. The early component of DHPG-induced depression of IPSCs was mediated by the cannabinoid CB_1 receptors, while DHPG-induced I-LTD was dependent on protein synthesis. Western blotting analysis indicates that mGluR1 was coupled to extracellular signal-regulated kinase (ERK) and mammalian target of rapamycin (mTOR) signaling pathways to increase translation. We also show that cocaine conditioning activated translation machinery in the VTA via an mGluR1-dependent mechanism. Furthermore, intra-VTA microinjections of mGluR1 antagonist JNJ16259685 and protein synthesis inhibitor cycloheximide significantly attenuated or blocked the acquisition of cocaine-induced conditioned place preference (CPP) and activation of translation elongation factors. Taken together, these results suggest that mGluR1 antagonism inhibits *de novo* protein synthesis; this effect may block the formation of cocaine–cue associations and thus provide a mechanism for the reduction in CPP to cocaine.

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INTRODUCTION

Glutamatergic transmission plays a critical role in learning, memory, and drug addiction (Bird and Lawrence, 2009; Bliss and Collingridge, 1993; Kalivas, 2004). Glutamate signals through both ionotropic and metabotropic glutamate receptors (mGluRs) (Conn and Pin, 1997). Group I mGluRs (mGluR1 and mGluR5) are expressed in the mesolimbic dopamine system (Hubert *et al*, 2001; Shigemoto *et al*, 1992, 1993), a major component of the reward circuit of the brain (Leshner and Koob, 1999). Since the discovery that mGluR5 knockout mice do not self-administer cocaine and do not show hyperlocomotive responses to cocaine (Chiamulera *et al*, 2001), mGluR5 has received considerable attention as a therapeutic target for cocaine addiction (Herzig and Schmidt, 2004; Kenny *et al*, 2005; Kumaresan *et al*, 2009; Lee *et al*, 2005; McGeehan and Olive, 2003; Olive, 2009). However, fewer studies have investigated the role of mGluR1 in cocaine addiction. Selective mGluR1 antagonists reduce cocaine self-administration in monkeys (Achat-Mendes *et al*, 2012) and psychomotor sensitization in rodents (Dravolina *et al*, 2006; Xie *et al*, 2010). These studies suggest that mGluR1 is critically involved in behavioral effects of cocaine. However, the molecular and circuit mechanisms for group I mGluR antagonism-induced impairment of addictive behavior remain poorly understood.

Group I mGluRs are coupled to $G_{q/11}$, which activates the phospholipase C (PLC) pathway and induces Ca^{2+} release from internal stores (Conn and Pin, 1997). In addition, they are also coupled to extracellular signal-regulated kinase (ERK) and mammalian target of rapamycin (mTOR) signaling pathways (Gallagher *et al*, 2004; Grueter *et al*, 2006; Hou and Klann, 2004; Luscher and Huber, 2010), the activation of which leads to translation initiation and protein synthesis (Banko *et al*, 2005; Hoeffer and Klann, 2010; Huber *et al*, 2000; Ronesi *et al*, 2012). The activation of group I mGluRs, mGluR5 in particular, increases protein synthesis at synapses (Weiler and Greenough, 1993), and

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mGluR5-induced excessive protein synthesis has been implicated in the pathology of fragile X syndrome (Krueger and Bear, 2011). The formation and consolidation of memories require *de novo* protein synthesis (Nader *et al*, 2000a, b; Schafe *et al*, 2001). The development of drug-associated memories and addictive behavior also requires new protein synthesis (Kuo *et al*, 2007; Mierzejewski *et al*, 2006; Milekic *et al*, 2006). However, the upstream signaling mechanisms by which learning and drug exposure lead to protein synthesis remain poorly understood. One possibility is that learning and cue–drug pairing cause the release of glutamate, which activates group I mGluRs to increase protein synthesis. By coupling to ERK and mTOR signaling pathways (Gallagher *et al*, 2004; Grueter *et al*, 2006; Hou and Klann, 2004; Luscher and Huber, 2010), group I mGluRs are well positioned to initiate or increase protein synthesis. However, to our knowledge, no previous studies have demonstrated a role of group I mGluR-dependent protein synthesis in mediating behavioral effects of any drugs of abuse.

The ventral tegmental area (VTA) of the midbrain and its dopaminergic projection play a critical role in reward processing and addictive behavior (Kauer, 2004). Both mGluR1 and mGluR5 are expressed in midbrain dopamine neurons, although mGluR1 is expressed at much higher density (Hubert *et al*, 2001). We hypothesized that mGluR1-dependent protein synthesis in the VTA is required for cocaine-induced conditioned place preference (CPP). In the present study, we tested this hypothesis using a combination of electrophysiological, biochemical, and behavioral approaches. We report here that cocaine conditioning activated protein synthesis machinery in the VTA through mGluR1 activation, while intra-VTA microinjections of an mGluR1 antagonist or a protein synthesis inhibitor blocked the acquisition of cocaine CPP. These results provide evidence that mGluR1-dependent protein synthesis is critically involved in behavioral effects of cocaine.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Charles River, Wilmington, MA) were used for slice electrophysiology (P18–30 of age, 50–100 g), behavior (10–11 weeks old, 300–350 g) and western blotting (VTA slices, P18–30; *in vivo* VTA samples, 10–11 weeks old). Animal maintenance and use and all experimental procedures were approved by the Institution's Animal Care and Use Committees of the Medical College of Wisconsin, USA, and Shandong University, China.

Brain Slice Preparation

Midbrain slices (250 μm) from male Sprague Dawley rats (P18–30) were prepared as described in our previous studies (Pan *et al*, 2008a, 2011b; Zhong *et al*, 2012). Briefly, slices were cut in an ice-cold solution (4–6 °C) containing (in mM): 110 choline chloride, 2.5 KCl, 1.25 NaH_2PO_4, 0.5 $CaCl_2$, 7 $MgSO_4$, 26 $NaHCO_3$, 25 glucose, 11.6 sodium ascorbate, and 3.1 sodium pyruvate. After cutting, slices were immediately transferred and incubated in oxygenated (95% O_2/5% CO_2) artificial cerebrospinal fluid (ACSF) containing (in mM): 125 NaCl, 3 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 glucose and were allowed to recover at room temperature for $\geqslant$2.5 h.

Electrophysiology

Whole-cell recordings were made using patch clamp amplifiers (Multiclamp 700B) under infrared-differential interference contrast microscopy. Data acquisition and analysis were performed using DigiData 1440A digitizer and analysis software pClamp 10 (Molecular Devices, Sunnyvale, CA). Dopamine neurons were identified by the width of action potential in cell-attached configuration ($>$1.2 ms) (Chieng *et al*, 2011) and the presence of large I_h currents, rhythmic firing at low frequency and prominent afterhyperpolarization (Johnson and North, 1992; Jones and Kauer, 1999; Liu *et al*, 2005). Glass pipettes were filled with a solution containing (in mM): 100 K-gluconate, 50 KCl, 10 HEPES, 0.2 EGTA, 2 $MgCl_2$, 4 Mg-ATP, 0.3 Na_2GTP, and 10 Na_2-phosphocreatine at pH 7.2 (with KOH). Series resistance (15–25 MΩ) was monitored throughout the recordings and data were discarded if the resistance changed by $>$20%. All recordings were performed at 32 ± 1 °C by using an automatic temperature controller (Warner Instrument).

Western Blotting

In experiments shown in Figure 5, VTA slices were maintained in a static incubation chamber in oxygenated ACSF at 32 ± 1 °C. Slices were treated with vehicle or various drugs (see Results section), the VTA was dissected out and then homogenized in 0.2 ml lysis buffer (pH 7.6) containing 50 mM Tris-acetate, 50 mM NaF, 10 mM EDTA, 10 mM EGTA, 0.01% Triton-X, protease inhibitors (Research Product International, Mount Prospect, IL) and protein phosphatase inhibitors I and II (Sigma-Aldrich, St Louis, MO). In experiments shown in Figure 7 and Supplementary Figure S1, rats were anesthetized with isoflurane and rapidly decapitated ~1 h after the CPP tests. The brains were immediately removed and placed in oxygenated ACSF at 4 °C. Midbrain slices were prepared, and the VTA was dissected out and homogenized in lysis buffer. Western blotting was performed as we have described (Pan *et al*, 2011b). Blots were blocked in solution containing 5% (w/v) milk and 0.1% (v/v) Tween-20 in tris-buffered saline (TBS-T) for 2 h at room temperature, and incubated overnight at 4 °C with anti-phospho-ERK (1 : 1000), anti-ERK (1 : 1000), anti-phospho-mTOR (1 : 1000), anti-mTOR (1 : 1000), anti-phospho-p70S6K antibody (1 : 500), anti-p70S6K antibody (1 : 500, Abcam, Cambridge, MA), anti-phospho-S6 antibody (1 : 1000), anti-S6 antibody (1 : 1000), anti-phospho-eIF4E (1 : 500), anti-eIF4E (1 : 500), anti-eEF1A (1 : 10000, Millipore, Billerica, MA), or anti-GAPDH (1 : 1000) antibodies. The above antibodies were purchased from Cell Signaling (Danvers, MA) except stated otherwise. Blots were then washed three times with TBS-T and probed with horseradish peroxidase-conjugated secondary antibody (1 : 3000, Bio-Rad Laboratories, Hercules, CA) for 2 h at room temperature before being developed using ECL immunoblotting detection system (Thermo Scientific, Rockford, IL). The intensity of western blots

was quantified by densitometry using the ImageJ software (NIH, Bethesda, MD).

Animal Surgery, Intracranial Microinjections and CPP

Male Sprague-Dawley rats (300–350 g) were anesthetized with ketamine (90 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) and placed in a stereotaxic device (David Kopf Instruments, Tujunga, CA). Guide cannulae (26 gauge) (Plastics One, Roanoke, VA) were bilaterally implanted 2.8 mm above the VTA or substantia nigra (SN) using aseptic techniques. The stereotaxic coordinates for VTA were: anteroposterior, −5.6 mm; mediolateral, ±2.4 mm (10° angle); dorsoventral, −7.8 mm. The stereotaxic coordinates for the SN were: anteroposterior, −5.6 mm; mediolateral, ±2.4 mm (0° angle); dorsoventral, −7.8 mm (Paxinos and Watson, 1986). The cannulae were anchored to the skull with three stainless-steel screws and dental cement. After the surgery, rats received subcutaneous injections of analgesic (buprenorphine, 0.05 mg/kg) three times daily for 2 days. Rats were allowed to recover for about 1 week. CPP experiments were performed using three-chamber CPP apparatus (Med Associates, St Albans, Vermont) (Pan *et al*, 2011b). The apparatus has two conditioning chambers (28 cm × 21 cm × 21 cm) and a center chamber (12 cm × 21 cm × 21 cm). The left chamber has stainless-steel mesh floor with white walls, while the right chamber has stainless-steel grid floor with black walls. The three chambers were separated by manual guillotine doors. The CPP protocol consisted of the following sessions:

Pre-test (day 1): animals were allowed to explore both chambers for 20 min and time spent in each side was recorded. Rats showing unconditioned side preference (⩾180 s disparity) were excluded ($n=4$).

Conditioning (day 2–9): Rats received bilateral intra-VTA infusions of vehicle, JNJ16259685 or cycloheximide via the pre-implanted cannulae. Injector cannulae (33-gauge) were inserted into the guide cannulae. The intra-VTA infusions were made via C313C connectors to 2 μl-Hamilton microsyringes. Vehicle (1 μl per side), JNJ16259685 (0.1 ng, 1 μl per side) or cycloheximide (100 ng, 1 μl per side) was manually injected at a rate of 1 μl over 2 min, and the injectors were kept in place for an additional 2 min to ensure adequate drug diffusion from the injector tip. Thirty minutes after the microinjections, rats received cocaine or saline conditioning.

Cocaine conditioning. Rats received saline injection (0.9% NaCl, 1 ml/kg, i.p.) on days 2, 4, 6, and 8 and were immediately confined to one chamber for 20 min. On days 3, 5, 7, and 9, rats received cocaine injection (15 mg/kg, i.p.) and were immediately confined to the opposite chamber for 20 min.

Saline conditioning. Rats received daily saline injection and were immediately confined to one chamber for 20 min on days 2, 4, 6, and 8 and were confined to the opposite chamber for 20 min on days 3, 5, 7, and 9.

CPP test (day 10): all of the animals were allowed to explore freely for 20 min among the three chambers and time spent on each side is recorded.

In a subset of experiments in which we examined the target specificity of intra-VTA infusions (Figure 9), cocaine conditioning was performed, while the same amount of JNJ16259685 or cycloheximide as described above was microinjected into the SN bilaterally 30 min before each place conditioning, and CPP test was performed on day 10. In another subset of experiments in which we examined the environmental cues on the expression of p-ERK1/2 and p-mTOR levels (Supplementary Figure S1), the place conditioning was conducted without saline or cocaine injections.

Histological Verification of VTA Cannula Placements

Intra-VTA cannula placement was histologically verified by Cresyl Violet staining (Figure 8a) except the animals used in the experiment of western blotting (Figure 7), in which cannula placement was verified by visualizing VTA sections under dissecting microscope. After completion of the CPP experiments, animals were anesthetized with pentobarbital sodium (100 mg/kg, i.p.) and then perfused with phosphate-buffered saline following by 4% paraformaldehyde. The brains were cut into 40 μm sections, stained with Cresyl Violet and then examined with light microscopy. Based on the stereotaxic atlas of Paxinos and Watson (1986), 7 of 72 rats with misplaced cannulae were excluded from behavioral analysis.

Chemicals

Cocaine hydrochloride, CNQX-Na_2, and AP-5 were obtained from Sigma-Aldrich (St Louis, MO). Anisomycin, cycloheximide, JNJ16259685, U0126, U0124, MTEP, and rapamycin were obtained from Tocris Bioscience (Ellisville, MO).

Statistics

Data are presented as the mean ± SEM. The initial depression of evoked inhibitory postsynaptic currents (IPSCs) (%) was calculated as follows: 100 × (mean amplitude of IPSCs at last 5 min of drug application/mean amplitude of baseline IPSCs). Long-term depression (I-LTD) (%) was calculated as follows: 100 × (mean amplitude of IPSCs during the final 10 min of recording/mean amplitude of baseline IPSCs). The amplitude of IPSCs (evoked at 10–20 s intervals) was first averaged for every minute for each cell and then averaged from a group of cells that received the same treatment. The overall mean of the last 5 min of drug application (initial depression) or the final 10 min of recording (I-LTD) for each group was compared. The CPP score was calculated by the time spent in the cocaine-paired chamber minus the time spent in the saline-paired chamber. Data sets were compared with either Student's *t*-test (electrophysiology), one-way or two-way analyses of variance (ANOVA) followed by Tukey *post hoc* analysis (western blotting and CPP). For statistical analysis of locomotor activity, a mixed-design ANOVA, with the between-subjects factors of place conditioning (saline *vs* cocaine conditioning) and intra-VTA infusions (vehicle, JNJ16259685, or cycloheximide) and repeated measures on the conditioning days, was used. Results were considered to be significant at $p<0.05$.

RESULTS

The Early Component of DHPG-Induced Depression of IPSCs is CB_1 Receptor-Dependent

Whole-cell voltage-clamp recordings (holding potential −70 mV) were made from VTA dopamine neurons in midbrain slices prepared from 18- to 30-day-old rats. IPSCs were evoked by stimulating inhibitory synaptic afferents at 0.05–0.1 Hz. Glutamate receptor antagonists CNQX (20 μM) and AP-5 (50 μM) were present in the ACSF throughout the experiments to block excitatory synaptic transmission. Bath application of group I mGluR agonist DHPG (100 μM) for 10 min induced more pronounced initial depression of IPSCs (61.4 ± 7.9% of baseline, 5–10 min of DHPG application, $n = 7$, $p < 0.01$) followed by modest I-LTD (71.8 ± 6.1% of baseline, 40–50 min of the recording, $n = 7$, $p < 0.01$; Figure 1a). Group I mGluRs consist of mGluR1 and mGluR5, and both subtypes of receptors are expressed in the VTA (Hubert *et al*, 2001). Previous studies have shown that mGluR1-selective antagonists LY367385 (100 μM) and JNJ16259685 (100 nM) completed blocked DHPG-induced mGluR1 responses in brain slices (Mameli *et al*, 2007; Shin *et al*, 2009). LY367385 (100 μM) significantly attenuated the early component of DHPG-induced depression of IPSCs (84.3 ± 6.8% of baseline, $n = 7$, $p < 0.05$ *vs* control) and I-LTD (93.1 ± 5.6% of baseline, $n = 7$, $p < 0.05$ *vs* control; Figure 1b). Similarly, JNJ16259685 (100 nM) blocked DHPG-induced depression of IPSCs as well (early component: 85.4 ± 7.2% of baseline, $p < 0.05$ *vs* control; I-LTD: 93.6 ± 7.5% of baseline, $n = 6$, $p < 0.05$ *vs* control; Figure1c). In contrast, the mGluR5-selective antagonist MTEP (10 μM) did not significantly affect DHPG-induced depression of IPSCs (early component: 71.9 ± 6.3% of baseline, $p > 0.05$ *vs* control; I-LTD: 82.2 ± 6.1% of baseline, $n = 8$, $p > 0.05$ *vs* control; Figure1d). MTEP (1 μM) fully blocked DHPG-induced increase in neuronal excitability in amygdala slices (Li *et al*, 2011). The inability to block DHPG-induced I-LTD in the VTA by MTEP cannot be attributed to insufficient concentration. These results indicate that DHPG-induced depression of IPSCs in the VTA is mediated mainly by mGluR1 activation. Although both mGluR1 and mGluR5 are expressed in the VTA and SN, mGluR1 is expressed at much higher density than mGluR5 (Hubert *et al*, 2001), which may explain why DHPG-induced depression of IPSCs in the VTA is mediated mainly by mGluR1.

The activation of postsynaptic mGluRs induces release of endocannabinoids (eCBs), which activate presynaptic CB_1 receptors to produce retrograde synaptic depression (Gerdeman *et al*, 2002; Maejima *et al*, 2001; Robbe *et al*, 2002; Varma *et al*, 2001). We examined whether the CB_1 receptor antagonist AM251 affected DHPG-induced depression of IPSCs. To determine whether the depression of

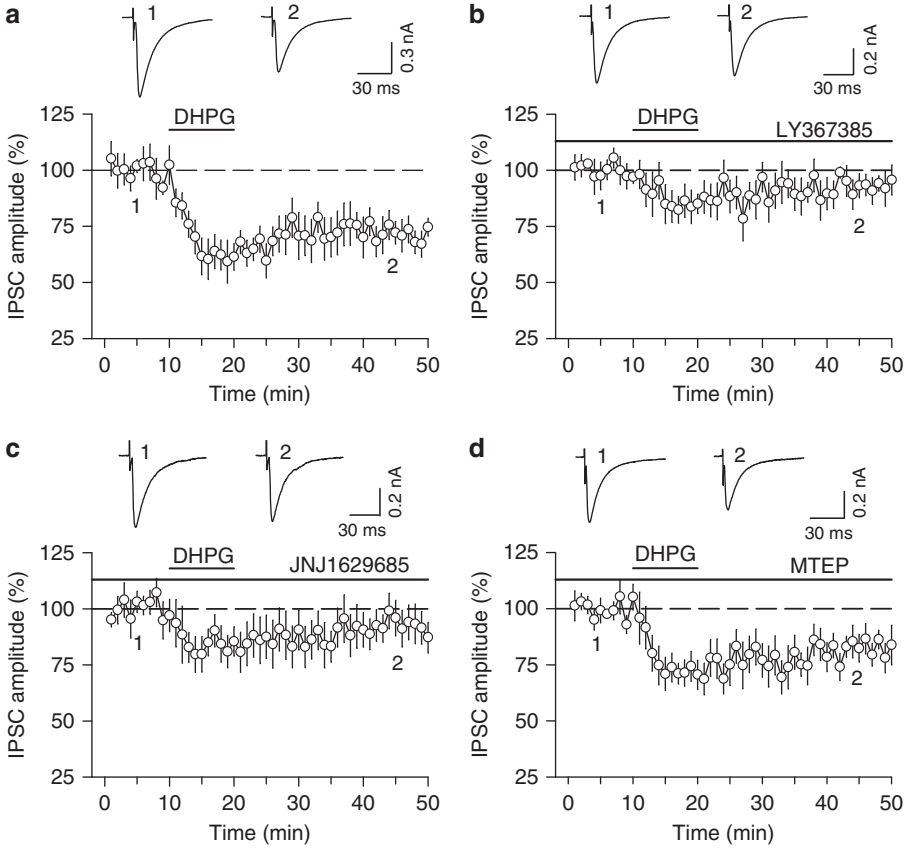

Figure 1 DHPG-induced depression of IPSCs in VTA dopamine neurons is mediated mainly by mGluR1. (a) Bath application of group I mGluR agonist DHPG (100 μM, 10 min) induced long-term depression (LTD) of evoked IPSCs ($n = 7$, $p < 0.01$ *vs* baseline). (b) The mGluR1-selective antagonist LY367385 (100 μM) blocked DHPG-induced depression of IPSCs ($n = 7$, $p < 0.05$ *vs* control). (c) Another mGluR1-selective antagonist, JNJ16259685 (100 nM) blocked DHPG-induced depression of IPSCs ($n = 6$, $p < 0.05$ *vs* control). (d) The mGluR5-selective antagonist MTEP (10 μM) did not affect DHPG-induced depression of IPSCs ($n = 8$, $p < 0.05$ *vs* control). Error bars indicate SEM.

IPSCs was presynaptic or postsynaptic, we measured the paired-pulse ratio (PPR) by evoking IPSCs with paired-pulse stimuli at an inter-pulse interval of 50 ms. In the continuous presence of AM251 (2 μM), the initial component of DHPG-induced depression of IPSCs was significantly attenuated (82.8 ± 6.4% of baseline, $n = 6$, $p < 0.05$ *vs* control; Figure 2a), while DHPG-induced I-LTD was not changed. There was a significant increase in the PPR during the initial depression of IPSCs (129.4 ± 8.7%, $n = 6$, $p < 0.05$), and the increase in PPR was blocked by AM251 (Figure 2b). In contrast, AM251 had no significant effect on DHPG-induced I-LTD (75.7 ± 7.4% of baseline, $n = 6$, $p > 0.05$ *vs* control; Figure 2b). Thus, the increase in the PPR during DHPG-induced initial depression is caused by CB_1 receptor activation. Taken together, these results suggest that the CB_1 receptor activation contributes to DHPG-induced initial depression of IPSCs, but not I-LTD.

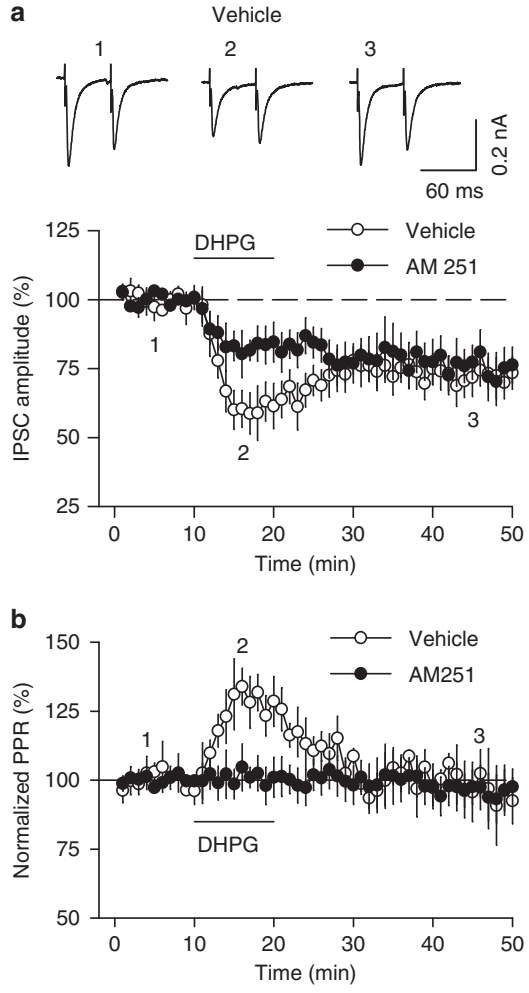

Figure 2 The early component of DHPG-induced depression of IPSCs is CB_1 receptor-dependent. (a) The CB_1 receptor antagonist AM251 (2 μM) attenuated the early component of DHPG-induced depression of IPSCs ($n = 6$, $p < 0.05$ *vs* control, $n = 6$) but did not affect DHPG I-LTD ($p > 0.05$). The amplitude of the first IPSCs was normalized. (b) DHPG increased the PPR during the early component of DHPG-induced depression of IPSCs ($p < 0.05$ *vs* control) but did not affect the PPR during I-LTD ($p > 0.05$). Error bars indicate SEM.

DHPG-Induced I-LTD is Dependent on Protein Synthesis

Group I mGluRs are known to be expressed on postsynaptic neurons (Hubert *et al*, 2001; Shigemoto *et al*, 1992, 1993). Group I mGluRs-mediated LTD, but not the initial depression of excitatory synaptic transmission, requires protein synthesis (Huber *et al*, 2000; Mameli *et al*, 2007; Mockett *et al*, 2011; Yin *et al*, 2006). However, it is unknown whether mGluR-mediated I-LTD also requires protein synthesis. We examined the effects of protein synthesis inhibitors on DHPG-induced I-LTD in the VTA. The protein synthesis inhibitor anisomycin (30 μM) or cycloheximide (80 μM) was included in the ACSF throughout the experiments while IPSCs were recorded. We found that either anisomycin (92.0 ± 7.1% of baseline, $n = 8$, $p < 0.05$ *vs* control) or cycloheximide (91.0 ± 6.2% of baseline, $n = 6$, $p < 0.05$ *vs* control) blocked the mGluR1-LTD but did not significantly affect the initial component of DHPG-induced depression of IPSCs (anisomycin: 68.1 ± 6.9% of baseline, $n = 8$, $p > 0.05$ *vs* control; cycloheximide: 66.8 ± 7.9% of baseline, $n = 6$, $p > 0.05$ *vs* control; Figure 3). These results indicate that mGluR1-mediated I-LTD in the VTA requires protein synthesis.

DHPG Activated ERK1/2 and mTOR Signaling Pathways in the VTA

Group I mGluRs are coupled to the mTOR and ERK signaling pathways (Banko *et al*, 2006; Gallagher *et al*, 2004; Mao *et al*, 2005), which are known to be key regulators of protein synthesis (Cammalleri *et al*, 2003; Huo *et al*, 2011; Kelleher *et al*, 2004). We examined the effects of inhibition of ERK1/2 and mTOR signaling pathways on DHPG-induced depression of IPSCs in the VTA. ERK1/2 is phosphorylated and activated by MAPK/ERK kinase (MEK) (Nakielny *et al*, 1992). The MEK inhibitor U0126 (20 μM)

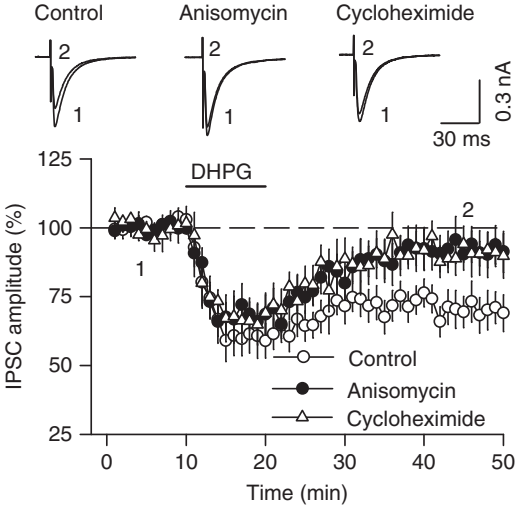

Figure 3 Protein synthesis is required for DHPG-induced I-LTD in the VTA. The protein synthesis inhibitor anisomycin (30 μM, $n = 8$, $p < 0.05$ *vs* control) or cycloheximide (80 μM, $n = 6$, $p < 0.05$ *vs* control) blocked DHPG-induced I-LTD but did not significantly affect the early component of DHPG-induced depression of IPSCs ($p < 0.05$ *vs* control). Error bars indicate SEM.

partially attenuated DHPG-induced I-LTD but the attenuation did not reach statistical significance (control: 72.4 ± 6.7% of baseline, $n = 6$; U0126: 86.1 ± 6.4% of baseline, $n = 8$, $p > 0.05$ *vs* control; Figure 4a). U0124 (20 μM), an inactive analog of U0126, did not affect DHPG-induced I-LTD (73.1 ± 7.5% of baseline, $n = 7$, $p > 0.05$ *vs* control; Figure 4a). The mTOR inhibitor rapamycin (100 nM) showed a trend toward attenuation of DHPG-induced I-LTD (81.3 ± 6.4% of baseline, $n = 7$, $p > 0.05$ *vs* control; Figure 4b). The combination of U0126 (20 μM) and rapamycin (100 nM) blocked DHPG-induced I-LTD (93.1 ± 6.0% of baseline, $n = 8$, $p < 0.05$ *vs* control) without significantly affecting the early component of DHPG-induced depression of IPSCs (71.1 ± 7.2% of baseline, $n = 8$, $p > 0.05$ *vs* control; Figure 4b). U0126, U0124, and rapamycin were present in the ACSF throughout the experiments. These results indicate that activation of both ERK1/2 and mTOR signaling is required for DHPG-induced I-LTD in VTA dopamine neurons.

To further investigate whether DHPG activates ERK1/2 in the VTA, we examined the effect of DHPG on the levels of phosphorylated ERK1/2 in isolated VTA slices, using phospho-specific antibodies recognizing the dually phosphorylated (Thr202/Tyr204) and active ERK (Dalby *et al*, 1998). VTA slices were incubated with DHPG (100 μM) for 10 min in the continuous presence of vehicle or mGluR1 antagonist JNJ16259685 (100 nM). Western blots of VTA homogenates were performed. Two-way ANOVA revealed significant effects of DHPG ($F_{(1,20)} = 16.06$, $p < 0.001$) and JNJ16259685 treatment ($F_{(1,20)} = 11.15$, $p < 0.01$) on p-ERK1/2 levels, as well as a significant interaction between DHPG and JNJ16259685 treatment ($F_{(1,20)} = 13.48$, $p < 0.01$). Tukey's *post hoc* analysis showed that DHPG significantly increased p-ERK1/2 levels ($p < 0.001$), which was blocked by JNJ16259685 ($p < 0.001$, $n = 6$ each group; Figure 5a). Similarly, DHPG ($F_{(1,24)} = 14.99$, $p < 0.001$) and JNJ16259685 treatment ($F_{(1,24)} = 12.14$, $p < 0.01$) had significant effects on p-mTOR levels, and there was a significant interaction between DHPG and JNJ16259685 treatment ($F_{(1,24)} = 4.39$, $p < 0.05$). Tukey's *post hoc* analysis showed that DHPG significantly increased p-mTOR levels ($p < 0.001$), and JNJ16259685 blocked DHPG-induced increase in p-mTOR ($p < 0.001$, $n = 7$ each group; Figure 5b). These results indicate that DHPG induced ERK1/2 and mTOR phosphorylation and activation in the VTA in an mGluR1-dependent manner.

Intra-VTA Infusions of JNJ16259685 and Cycloheximide Blocked Cocaine CPP

The above results suggest that mGluR1 activation with DHPG induced protein synthesis-dependent I-LTD in the VTA. To test whether this finding is relevant to behavioral effects of cocaine, we examined the effects of intra-VTA microinjections of selective mGluR1 antagonist JNJ16259685 or protein synthesis inhibitor cycloheximide on cocaine CPP. The timeline for baseline preference test (pre-test), cocaine or saline conditioning, intra-VTA microinjections and CPP test is described in Figure 6a. During pre-test, four rats showing unconditioned side preference (⩾180 s disparity) were excluded. The remaining rats did not exhibit significant difference in the time spent in each chamber ($p > 0.05$; Figure 6b). Then, cocaine or saline place conditioning was conducted once daily for 8 days (see Materials and methods). Vehicle (0.01% DMSO in saline, 1 μl per side), JNJ16259685 (0.1 ng, 1 μl per side), or cycloheximide (100 ng, 1 μl per side) was bilaterally microinjected into the VTA via the pre-implanted cannulae 30 min prior to each cocaine- or saline-pairing with a particular chamber. Twenty-four hours after the last pairing, CPP was tested without any drug or vehicle administration. Two-way ANOVA revealed that cocaine treatment ($F_{(1,45)} = 40.29$, $p < 0.001$) and intra-VTA microinjections ($F_{(2,45)} = 17.10$, $p < 0.001$) had significant main effects on CPP scores and there was a significant interaction between cocaine and intra-VTA infusions ($F_{(2,45)} = 7.06$, $p < 0.01$). Tukey's *post hoc* test showed that intra-VTA microinjections of JNJ 16259685 produced a significant decrease in the CPP score in cocaine-conditioned rats ($p < 0.001$) but did not affect the CPP score in

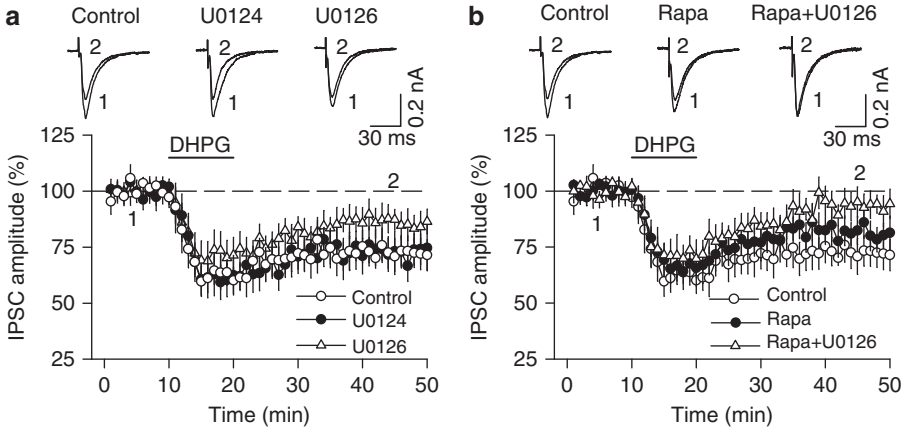

Figure 4 DHPG-induced I-LTD requires the activation of ERK1/2 and mTOR signaling pathways. (a) The MEK inhibitor U0126 (20 μM) exerted only a modest effect on DHPG-induced I-LTD that did not reach statistical significance ($n = 8$, $p > 0.05$ *vs* control). U0124 (20 μM), an inactive analog of U0126, had no effect on DHPG I-LTD ($n = 7$, $p > 0.05$ *vs* control). (b) The mTOR inhibitor rapamycin (Rapa, 100 nM) also had no significant effect on DHPG-induced I-LTD ($n = 7$, $p > 0.05$ *vs* control), whereas co-application of rapamycin (100 nM) and U0126 (20 μM) blocked DHPG-induced I-LTD ($n = 8$, $p < 0.05$ *vs* control). Error bars indicate SEM.

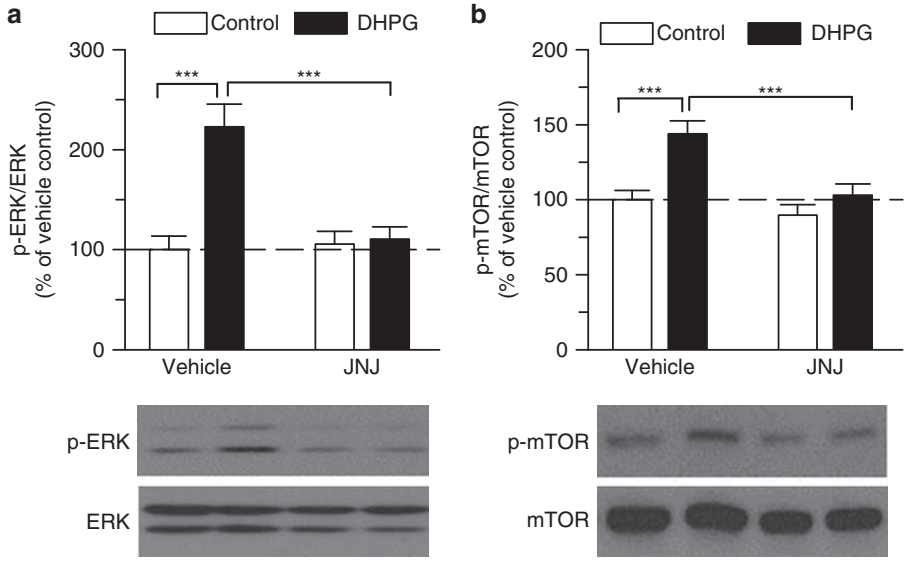

Figure 5 DHPG induced ERK and mTOR phosphorylation and activation in the VTA via mGluR1. (a) DHPG (100 μM, 10 min) increased p-ERK1/2 levels in VTA homogenates compared, this effect was blocked by the JNJ16259685 (JNJ, 100 nM, ***$p<0.001$, Tukey *post hoc* test). (b) DHPG also increased p-mTOR levels in VTA homogenates compared with control, this effect was blocked by JNJ16259685 (100 nM, ***$p<0.001$). Each group of data was obtained from 6 to 7 VTA slices prepared from 3 to 4 rats. Error bars indicate SEM.

saline-conditioned rats ($p>0.05$). In addition, intra-VTA microinjection of cycloheximide blocked the acquisition of CPP to cocaine ($p<0.001$) without affecting CPP score in saline-conditioned rats ($p>0.05$; Figure 6c). Thus, JNJ16259685 and cycloheximide attenuated CPP to cocaine without inducing CPP or conditioned place aversion in saline-conditioned rats.

During place conditioning, locomotor activities in the conditioning chambers were tracked by infrared photobeam breaks. We compared locomotor activities in saline- or cocaine-conditioned rats that received intra-VTA infusions of vehicle, JNJ16259685, and cycloheximide over the 4 days of cocaine conditioning or correspondent saline conditioning (i.e., day 3, 5, 7, 9). Locomotor activities were analyzed with a mixed ANOVA that included between-subjects factors of place conditioning and intra-VTA infusions and within-subject factor of conditioning days. Cocaine significantly increased locomotor activities over the 4 days of conditioning ($F_{(1,180)}=183.67$, $p<0.001$), and there was a significant interaction between cocaine and conditioning days ($F_{(3,180)}=6.06$, $p<0.001$). However, intra-VTA infusions of JNJ16259685 or cycloheximide did not significantly alter locomotor activities in saline- and cocaine-conditioned rats ($F_{(2,180)}=0.05$, $p>0.05$) and there was no significant interaction between place conditioning and intra-VTA infusions ($F_{(2,180)}=0.08$, $p>0.05$) (Figure 6d).

Cocaine Conditioning Activated Protein Synthesis Machinery in the VTA Through mGluR1

We determined whether the acquisition of CPP to cocaine was accompanied with mGluR1-dependent phosphorylation and activation of ERK1/2 and mTOR in the VTA. About 1 h after the CPP tests, some of saline- or cocaine-conditioned rats (shown in Figure 6) were euthanized and the VTA was dissected out bilaterally from midbrain slices. Western blotting was performed using antibodies against p-ERK1/2, ERK1/2, p-mTOR, and mTOR. Two-way ANOVA indicated that place conditioning and intra-VTA infusions had significant effects on p-ERK1/2 levels (place conditioning: $F_{(1,20)}=24.06$, $p<0.001$; intra-VTA infusion: $F_{(1,20)}=30.14$, $p<0.001$; place conditioning × intra-VTA infusion interaction: $F_{(1,20)}=38.34$, $p<0.001$) and p-mTOR levels (place conditioning: $F_{(1,20)}=8.66$, $p<0.01$; intra-VTA infusion: $F_{(1,20)}=9.95$, $p<0.01$; place conditioning × intra-VTA infusion interaction: $F_{(1,20)}=6.95$, $p<0.05$). Tukey's *post hoc* test showed that p-ERK1/2 and p-mTOR levels were significantly increased in cocaine-conditioned rats compared with those in saline-conditioned rats ($p<0.001$ for both p-ERK1/2 and p-mTOR), while intra-VTA infusions of JNJ16259685 significantly attenuated p-ERK1/2 and p-mTOR levels in cocaine-conditioned rats ($p<0.001$ for both p-ERK1/2 and p-mTOR; $n=6$ each group; Figure 7a and b). These results indicate that the acquisition of CPP to cocaine is accompanied by increases in ERK and mTOR phosphorylation and activation in the VTA, and mGluR1 activation contributes to cocaine-induced ERK and mTOR activation.

To test whether exposure to novel cues (the conditioning chamber) caused the increases in p-ERK1/2 and p-mTOR, we compared p-ERK1/2 and p-mTOR levels in the VTA between rats raised in home cages and rats that received place conditioning alone. The place conditioning was performed following the timeline of cocaine conditioning but the rats did not receive any saline or cocaine treatment. Place conditioning alone had no significant effect on p-ERK1/2 or p-mTOR levels in the VTA compared with control rats raised in home cages ($p>0.05$, $n=3$ rats each group; Supplementary Figure S1). These results suggest that place conditioning without cocaine injections does not affect p-ERK1/2 or p-mTOR levels in the VTA.

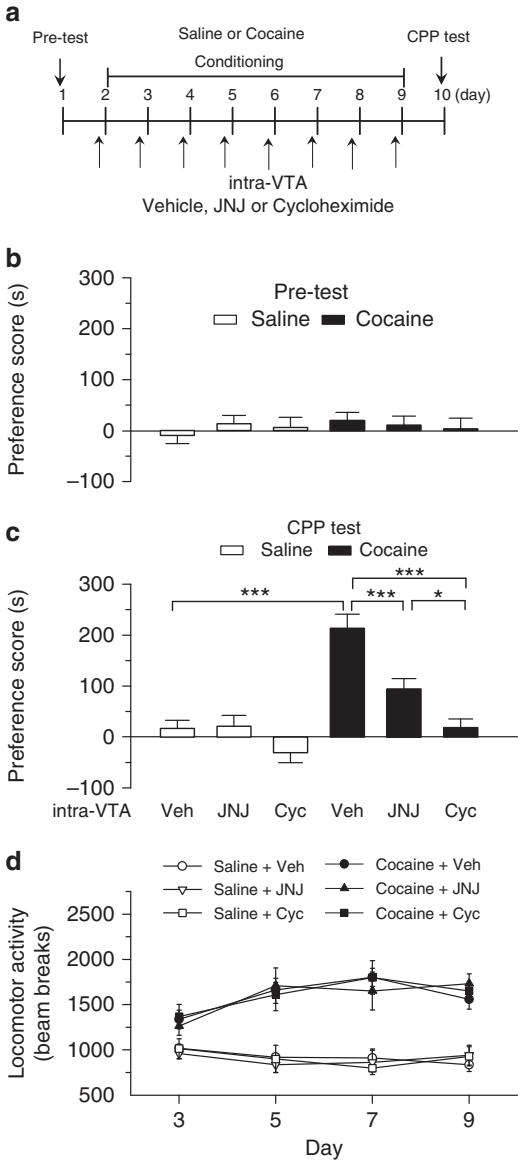

Figure 6 Intra-VTA infusions of the mGluR1 antagonist JNJ16259685 or protein synthesis inhibitor cycloheximide during the conditioning phase attenuated the acquisition of CPP to cocaine. (a) Timeline of drug treatment and behavioral paradigm. Groups of rats received saline and cocaine place conditioning once daily for 8 days. Vehicle (Veh), JNJ 16259685 (JNJ) or cycloheximide (Cyc) was bilaterally infused into the VTA 30 min prior to each saline- or cocaine-pairing. (b) Pre-test indicates that rats did not exhibit baseline bias in place preference in all groups ($n=7$–10, $p>0.05$). (c) Intra-VTA infusions of JNJ16259685 or cycloheximide significantly attenuated CPP in cocaine-conditioned rats but did not affect CPP scores in saline-conditioned rats ($n=7$–10, $^{*}p<0.05$, $^{***}p<0.001$, Tukey *post hoc* test). (d) Intra-VTA infusions of JNJ16259685 or cycloheximide did not significantly affect locomotor activity in cocaine- or saline-conditioned rats ($p>0.05$, $n=7$–10). Error bars indicate SEM.

Both ERK1/2 and mTOR control protein synthesis at the level of translation (Cammalleri *et al*, 2003; Huo *et al*, 2011; Kelleher *et al*, 2004). We examined whether cocaine-induced activation of ERK1/2 and mTOR signaling pathways in turn activated the protein translation machinery in the VTA. mTOR phosphorylates the 70-kDa ribosomal protein S6 kinase (p70S6K) at T389 site (Jefferies *et al*, 1997). To determine whether cocaine CPP affected phosphorylation of p70S6K in the VTA, we performed western blotting to detect phosphorylated p70S6K (p-p70S6K) at T389 site and total p70S6K in VTA homogenates from saline- and cocaine-conditioned rats shown in Figure 6. Two-way ANOVA indicated that place conditioning ($F_{(1,20)}=21.20$, $p<0.001$) and intra-VTA infusions ($F_{(1,20)}=40.38$, $p<0.001$) had significant effects on p-p70S6K levels and there was a significant interaction between place conditioning and intra-VTA infusions ($F_{(1,20)}=25.26$, $p<0.001$). Tukey's *post hoc* test showed that the levels of p-p70S6K at T389 site were significantly increased in cocaine-conditioned rats compared with those of saline-conditioned rats ($p<0.001$), while intra-VTA infusions of mGluR1 antagonist JNJ16259685 blocked the increase in p-p70S6K ($p<0.001$; Figure 7c).

The activated p70S6K phosphorylates its target substrate ribosomal protein S6 at Ser235/236. S6 phosphorylation initiates 5′ terminal oligopyrimidine (5′ TOP) mRNA translation (Proud, 2007). Two-way ANOVA indicated that place conditioning ($F_{(1,20)}=12.89$, $p<0.01$) and intra-VTA infusions ($F_{(1,20)}=4.69$, $p<0.05$) had significant effects on phosphorylated S6 (p-S6) levels and there was a significant interaction between place conditioning and intra-VTA infusions ($F_{(1,20)}=7.44$, $p<0.05$). Tukey's *post hoc* test showed that p-S6 was significantly increased in cocaine-conditioned rats ($p<0.001$), and this increase was also blocked by mGluR1 antagonist JNJ16259685 ($p<0.001$; Figure 7d).

ERK1/2 indirectly phosphorylates eukaryotic translation initiation factor 4E (eIF4E) by activating the MAPK-interacting kinase (Proud, 2007). Two-way ANOVA indicated that place conditioning ($F_{(1,20)}=22.85$, $p<0.001$) and intra-VTA infusions ($F_{(1,20)}=17.32$, $p<0.001$) had significant effects on phosphorylated eIF4E (p-eIF4E at S209) levels and there was a significant interaction between place conditioning and intra-VTA infusions ($F_{(1,20)}=14.14$, $p<0.001$). Tukey's *post hoc* test showed that cocaine conditioning increased p-eIF4E levels in the VTA ($p<0.001$), and this effect was attenuated by JNJ16259685 ($p<0.001$; Figure 7e). In addition, we found that place conditioning ($F_{(1,30)}=14.69$, $p<0.001$) and intra-VTA infusions ($F_{(2,30)}=94.27$, $p<0.001$) had significant effects on protein levels of eukaryotic elongation factor 1A (eEF1A), a 5′ TOP mRNA-encoded protein, and there was a significant interaction between place conditioning and intra-VTA infusions ($F_{(2,30)}=16.75$, $p<0.001$). Tukey's *post hoc* test showed that cocaine conditioning significantly increased protein levels of eEF1A ($p<0.001$), and this increase was blocked by intra-VTA infusions of JNJ16259685 ($p<0.001$) or protein synthesis inhibitor cycloheximide ($p<0.001$; Figure 7f). These results suggest that cocaine conditioning activates mGluR1, which is coupled to ERK1/2 and mTOR signaling pathways to stimulate *de novo* protein synthesis.

VTA cannula placement was histologically verified by Cresyl Violet staining except the animals used in the experiment of western blotting (Figure 7), in which cannula placement was verified by visualizing VTA slices under dissecting microscope. Figure 8 shows the representative coronal sections containing the VTA and the sites of the

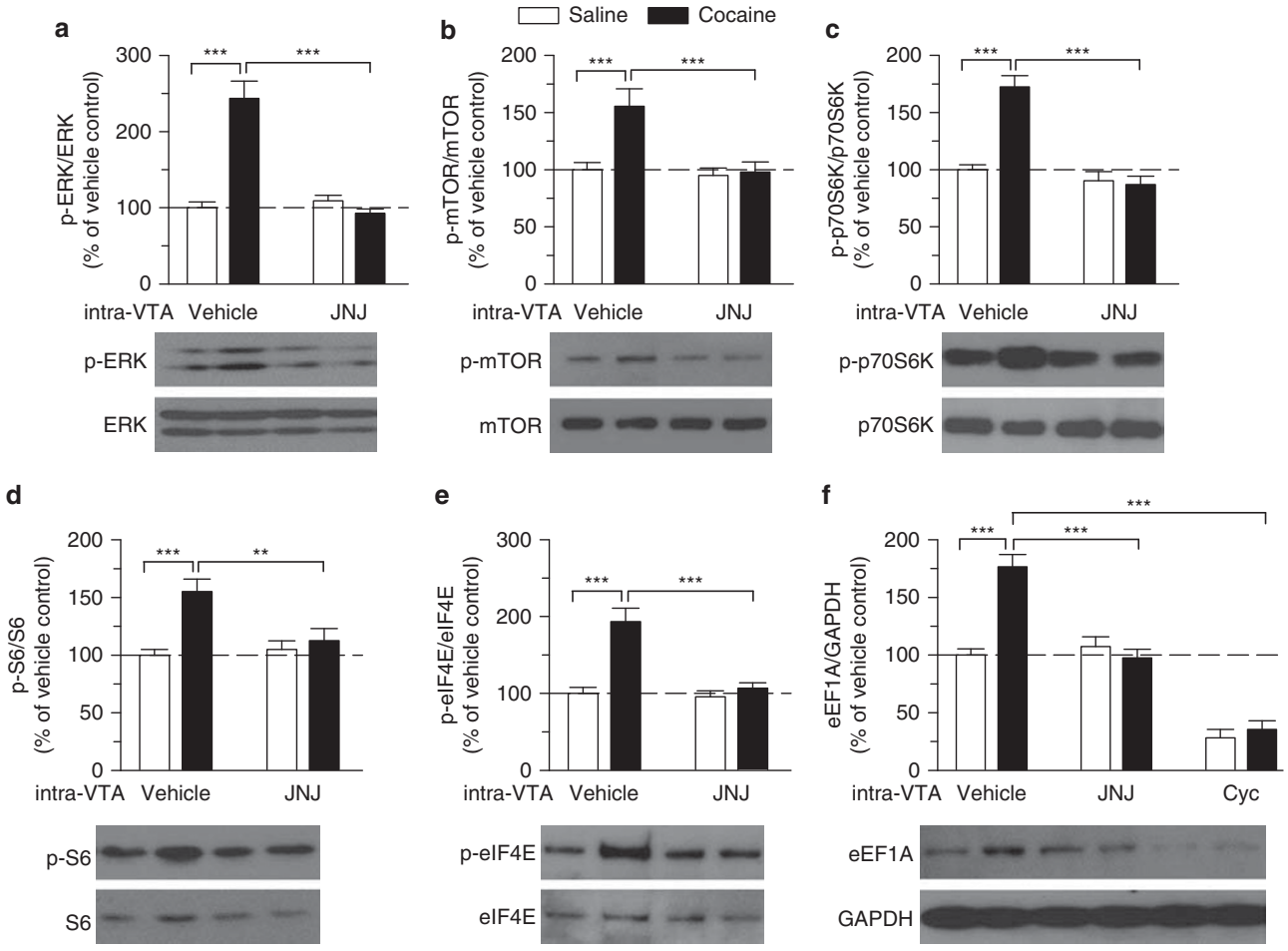

Figure 7 Cocaine CPP activates ERK and mTOR signaling pathways and increases the synthesis of the 5′TOP-encoded protein eEF1A through mGluR1 activation. (a, b) p-ERK1/2 (a) and mTOR levels (b) in the VTA were significantly increased in cocaine-conditioned rats compared with those in saline-conditioned rats, and the increase was blocked by intra-VTA infusions of JNJ16259685 (JNJ) (***$p<0.001$, Tukey *post hoc* test). (c, d, e) p-p70S6K (c), p-S6 (d), and p-eIF4E (e) levels in the VTA were significantly increased in cocaine-conditioned rats compared with those in saline-conditioned rats, and these increases were blocked by intra-VTA infusions of JNJ16259685 during the conditioning phase (**$p<0.01$, ***$p<0.001$). (f) eEF1A was significantly increased in cocaine-conditioned rats compared with that in saline-conditioned rats (***$p<0.001$), and this increase was blocked by intra-VTA infusions of JNJ16259685 or cycloheximide (Cyc) during the conditioning phase (***$p<0.001$). Western blotting was performed on VTA samples collected ~1 h after the CPP tests. $n=6$ rats each group. Error bars indicate SEM.

cannula tips for intra-VTA infusions from the experiments in Figures 6 and 7.

Intra-SN Infusions of JNJ16259685 and Cycloheximide Did not Affect Cocaine CPP

Intra-VTA infusions of JNJ16259685 or cycloheximide may affect the neighboring area (i.e., SN) due to drug diffusion. We examined whether intra-SN infusions of JNJ16259685 or cycloheximide affected cocaine CPP, the experiments were similar to those for intra-VTA infusions described above. Cocaine conditioning was performed as shown in Figure 9a. During pre-test, two rats showing unconditioned side preference ($\geq$180 s disparity) were excluded. The remaining rats did not exhibit significant difference in the time spent in each chamber ($p>0.05$; Figure 9b). JNJ16259685 (0.1 ng, 1 μl per side) or cycloheximide (100 ng, 1 μl per side) was bilaterally microinjected into the SN via the pre-implanted cannulae 30 min prior to each cocaine- or saline-pairing with a particular chamber. Twenty-four hours after the last pairing, CPP was tested without any drug or vehicle administration. One-way ANOVA showed that intra-SN infusions of JNJ16259685 and cycloheximide had no significant effects on the acquisition of cocaine CPP compared with intra-SN infusions of vehicle ($F_{(2,21)}=0.61$, $p>0.05$). The sites of the cannula tips for intra-SN infusions were shown in Figure 9c.

DISCUSSION

Both mGluR1 and mGluR5 antagonists reduce behavioral responses to drugs of abuse, including cocaine (Achat-Mendes *et al*, 2012; Bird and Lawrence, 2009; Chiamulera *et al*, 2001), but the underlying mechanisms remain largely unknown. We demonstrated here that cocaine conditioning activated protein synthesis machinery in the VTA through mGluR1 activation, while intra-VTA microinjections of an mGluR1 antagonist or a protein synthesis inhibitor blocked the acquisition of cocaine CPP. These results suggest that

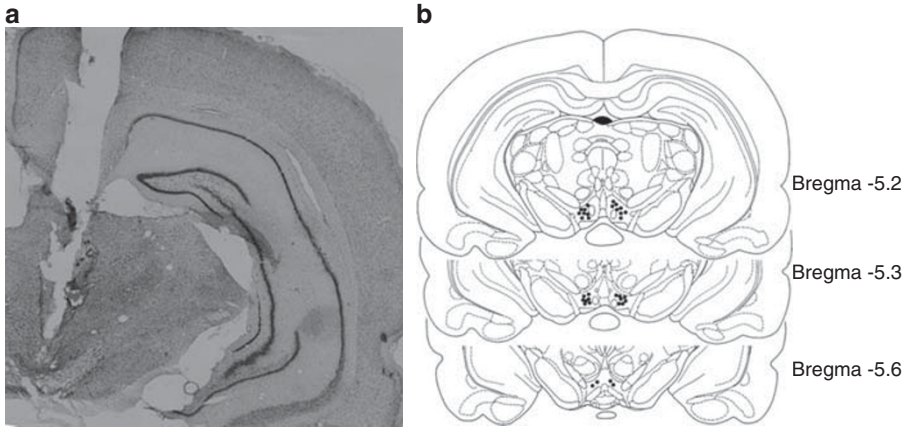

Figure 8 Histological verification of intra-VTA cannula placements. (a) Cresyl Violet-stained coronal section of typical bilateral intra-VTA cannula placements. (b) Representative sites of cannula tips in the VTA from data shown in Figures 6 and 7.

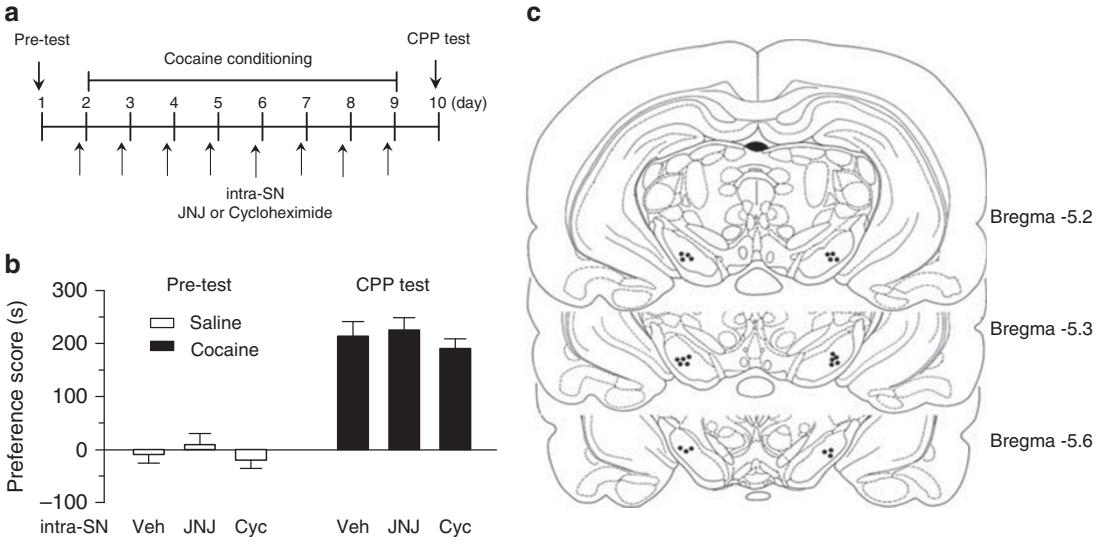

Figure 9 Intra-SN infusions of JNJ16259685 or cycloheximide did not affect cocaine CPP. (a) Timeline of drug treatment and behavioral paradigm. (b) Pre-test indicated that rats did not exhibit baseline, non-conditioning place preference ($n = 7–8$, $p > 0.05$). Intra-SN infusions of JNJ16259685 (JNJ) or cycloheximide (Cyc) did not affect CPP to cocaine ($n = 7–8$, $p > 0.05$). The group for intra-VTA infusions of vehicle (Veh) was obtained from Figure 6 and was re-plotted here for the purpose of comparison. Error bars indicate SEM. (c) Representative sites of cannula tips in the SN.

mGluR1-dependent protein synthesis in the VTA is required for acquisition of cocaine CPP. Since mGluR1 and mGluR5 belong to the same group I mGluR family and share same signal transduction mechanisms (Conn and Pin, 1997), it is likely that inhibition of *de novo* protein synthesis represents a common mechanism by which group I mGluR antagonists reduce addictive behavior.

Group I mGluR agonist DHPG induced protein synthesis-dependent LTD in the VTA

We showed that DHPG induced more pronounced initial depression of IPSCs followed by modest I-LTD in the VTA. The CB_1 receptor antagonist AM251 significantly attenuated the early component of DHPG-induced depression of IPSCs without affecting I-LTD, whereas protein synthesis inhibitors anisomycin and cycloheximide did not affect the early component of the depression of IPSCs but attenuated I-LTD. At excitatory synapses, protein synthesis inhibitors attenuated or blocked group I mGluR-mediated LTD without affecting the early component of DHPG-induced depression (Banko *et al*, 2005; Hoeffer and Klann, 2010; Huber *et al*, 2000; Mameli *et al*, 2007; Mockett *et al*, 2011; Ronesi *et al*, 2012; Yin *et al*, 2006). In contrast, CB_1 receptor antagonists blocked the early component of DHPG-induced depression, but not LTD (Bellone and Luscher, 2005; Grueter *et al*, 2008; Rouach and Nicoll, 2003). These results indicate that group I mGluR-LTD and I-LTD are protein synthesis-dependent, while DHPG-induced initial depression is mediated by the CB_1 receptor. However, it should be noted that some forms of DHPG-induced LTD was not dependent on protein synthesis (Grueter *et al*, 2007; Jung *et al*, 2012). Brain regions and methodological approaches (Sajikumar *et al*, 2005) might explain the difference in protein synthesis dependence.

We have shown that pairing cocaine application (3 μM) with repetitive stimulation at 10 Hz induced synaptic activation of D_2 dopamine receptor and group I mGluRs to induce I-LTD, which was also CB_1-dependent (Pan *et al*,

2008a, b). Besides synaptic activation of group I mGluRs, repetitive synaptic stimulation induced persistent increase in presynaptic Ca^{2+} level, which is required for CB_1-dependent I-LTD (Heifets *et al*, 2008; Pan *et al*, 2008b). The 10-Hz repetitive synaptic stimulation was not applied during DHPG application, which might explain why DHPG-induced activation of CB_1 receptors induced acute depression, but not I-LTD in the VTA. It is known that reduced $GABA_A$ receptor-mediated inhibition induces metaplasticity of the excitatory synapses and facilitates long-term potentiation and learning (Cui *et al*, 2008; Pan *et al*, 2011a). Dopamine neurons receive continuous synaptic bombardment *in vivo*; many synaptic connections are severed in the VTA slice preparation. We speculate that synaptic activation of group I mGluRs in the presence of cocaine causes I-LTD-like depression of inhibitory synaptic transmission *in vivo*, the resulting reduction of $GABA_A$ receptor-mediated inhibition may facilitate cue–cocaine associations and acquisition of CPP to cocaine.

ERK1/2 and mTOR signaling pathways play key roles in regulating protein synthesis (Costa-Mattioli *et al*, 2009) and have been implicated in group I mGluR-mediated LTD (Banko *et al*, 2005; Cammalleri *et al*, 2003; Hoeffer and Klann, 2010; Huber *et al*, 2000; Huo *et al*, 2011; Ronesi *et al*, 2012). We found that DHPG-induced I-LTD in the VTA was blocked by the combination of MEK inhibitor U0126 and mTOR inhibitor rapamycin, but not by either inhibitor alone. Western blotting showed that DHPG increased the p-ERK1/2 and p-mTOR levels in the VTA, and these increases were attenuated by mGluR1 antagonist JNJ16259685. These results indicate that mGluR1 activation in the VTA activates both ERK1/2 and mTOR signaling pathways to induce I-LTD. We suspect that the activation of either pathway is subthreshold for the induction I-LTD in the VTA, while activation of both ERK1/2 and mTOR signaling pathways reaches the threshold for LTD induction. Previous studies have shown that group I mGluR agonist DHPG induces LTD in the hippocampus, and this LTD is blocked by either MEK inhibitor U0126 (Gallagher *et al*, 2004) or mTOR inhibitor rapamycin (Hou and Klann, 2004), while mGluR-LTD in the VTA was blocked by rapamycin but was unaffected by U0126 (Mameli *et al*, 2007). Thus, ERK1/2 and mTOR signaling pathways are recruited differentially by group I mGluR activation, depending upon the synapses (excitatory *vs* inhibitory), brain regions or experimental conditions. It is thus likely that group I mGluR activation increases protein synthesis in the VTA through the activation of ERK1/2 and mTOR signaling pathways.

mGluR1-dependent activation of ERK1/2 and mTOR signaling pathways induced by cocaine conditioning

We have shown that cocaine conditioning increased p-ERK1/2 and p-mTOR levels in the VTA, and this increase was blocked by intra-VTA infusions of mGluR1 antagonist JNJ16259685. Thus, ERK1/2 and mTOR signaling pathways were not only activated by exogenous mGluR agonist DHPG, but also activated by cocaine conditioning. In contrast, repeated exposure to the conditioning chambers without cocaine injections did not activate ERK1/2 or mTOR, suggesting that environmental cues become effective in activating ERK1/2 or mTOR only after being paired with cocaine injections. How did cocaine conditioning lead to mGluR1 activation in the VTA? The environmental cues associated with cocaine injection during cocaine conditioning likely activate sensory inputs through glutamatergic excitatory afferents. Repetitive synaptic activity activates group I mGluRs in VTA dopamine neurons (Fiorillo and Williams, 1998). It is likely that cocaine conditioning activates excitatory afferents to induce the release of glutamate, which activates group I mGluRs (mainly mGluR1) on VTA dopamine neurons, and mGluR1 activation in turn leads to phosphorylation and activation of ERK1/2 and mTOR.

Systemic or local administration of ERK1/2 inhibitors blocked incubation of cocaine craving and CPP to cocaine (Girault *et al*, 2007; Lu *et al*, 2006; Miller and Marshall, 2005; Pan *et al*, 2011b). mTOR inhibitors blocked the expression of cocaine CPP and cue-induced reinstatement of cocaine seeking (Bailey *et al*, 2012; Wang *et al*, 2010). These studies suggest that ERK1/2 and mTOR signaling pathways are critically involved in cocaine- or cue-induced addictive behavior. However, the upstream activators and downstream effectors of ERK1/2 and mTOR in mediating addictive behavior remain poorly defined. Group 1 mGluRs activate translation regulatory factors via ERK1/2 and mTOR (Page *et al*, 2006). The translation regulatory factor p70S6K and its downstream target ribosomal protein S6 can be phosphorylated and activated by mTOR and ERK (Cammalleri *et al*, 2003; Jaworski *et al*, 2005; Lawrence *et al*, 2004; Page *et al*, 2006). We found that cocaine conditioning increased p-p70S6K, p-S6, and p-eIF4E in the VTA, and these increases were blocked by JNJ16259685. The activation of the translation regulatory factors is indicative of *de novo* protein synthesis. Thus, cocaine conditioning induces mGluR1 activation, which activates ERK1/2 and mTOR signaling pathways to increase translation and protein synthesis.

mGluR1-dependent protein synthesis is required for the acquisition of cocaine CPP

We found that intra-VTA infusions of mGluR1 antagonist JNJ16259685 or protein synthesis inhibitor cycloheximide significantly attenuated cocaine CPP, suggesting that mGluR1-dependent protein synthesis is required for the acquisition of cocaine CPP. mGluR5-dependent protein synthesis has been implicated in the pathology of fragile X syndrome (Krueger and Bear, 2011; Osterweil *et al*, 2010; Ronesi *et al*, 2012). Less is known whether mGluR1 activation is involved in protein synthesis and whether this process is required for the formation or drug-associated memories. Protein translation from mRNA can be divided into three critical steps: initiation, elongation, and termination (Taylor *et al*, 2006). During elongation, cognate aminoacyl-tRNA is delivered to the A site of the ribosome by eEF1A, which greatly speeds up the process. eEF1A is therefore required for translation elongation (Mateyak and Kinzy, 2010; Taylor *et al*, 2006). We found that cocaine conditioning increased the levels of eEF1A, and this increase was blocked by JNJ16259685 or cycloheximide. These results suggest that mGluR1 antagonism blocked cocaine CPP through inhibition of *de novo* protein synthesis. Previous studies have shown that systemic administration of mGluR5 antagonists reduces cocaine-induced locomotor

sensitization, CPP, and self-administration (Herzig and Schmidt, 2004; Kenny *et al*, 2005; Lee *et al*, 2005; McGeehan and Olive, 2003). Since mGluR1 and mGluR5 share common signal transduction mechanisms (Conn and Pin, 1997), we speculate that inhibition of protein synthesis may constitute a mechanism for mGluR5 antagonism-induced reduction of addictive behavior as well. mGluR1 and mGluR5 are often expressed in non-overlapping fashion in the central nervous system (Hubert *et al*, 2001; Shigemoto *et al*, 1992, 1993). mGluR5 is abundantly expressed in the nucleus accumbens (Shigemoto *et al*, 1993). Inhibition of protein synthesis in the nucleus accumbens and other parts of the reward circuitry may account for the effects of mGluR5 antagonists on addictive behavior (Chiamulera *et al*, 2001; Herzig and Schmidt, 2004; Kenny *et al*, 2005; Kumaresan *et al*, 2009; Lee *et al*, 2005; McGeehan and Olive, 2003; Olive, 2009).

Intra-VTA microinjections of JNJ16259685 and cycloheximide did not significantly alter cocaine-induced increase in locomotor activities. Although it is thought that a common brain circuitry underlies both drug reward and locomotor stimulation (Wise and Bozarth, 1987), locomotor sensitization and development of CPP are dissociable effects (Shimosato and Ohkuma, 2000; Veeneman *et al*, 2011). The attenuation of cocaine CPP by JNJ16259685 and cycloheximide could be explained by decreased rewarding effects of cocaine or decreased ability to learn the association between environmental cues and rewarding effects of cocaine. More recent studies indicate that group I mGluRs, mGluR5 in particular, are critically involved in learning and memory. Group I mGluR antagonists attenuated the acquisition of hippocampus-dependent contextual and reference memories (Gravius *et al*, 2006; Naie and Manahan-Vaughan, 2004). Long-term memory requires transcription and protein synthesis as protein synthesis inhibitors disrupt memory consolidation (Morris *et al*, 2006; Santini *et al*, 2004). The decreased associative learning might explain why JNJ16259685 and cycloheximide attenuated the acquisition of CPP to cocaine. A recent study has shown that non-pharmacological manipulation of cue–drug associated memories through extinction within the reconsolidation window can reduce drug craving and seeking in rats and humans (Xue *et al*, 2012). Both pharmacological and non-pharmacological intervention of drug–cue memories might provide new avenues for treating drug addiction.

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DISCLOSURE

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

All authors designed the experiments. FY, PZ, XL, and DS performed the experiments, collected and analyzed the data. FY, PZ, and QSL drafted the manuscript.

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