Development/Plasticity/Repair

Mediation of Amphetamine-Induced Long-Term Depression of Synaptic Transmission by CB_1 Cannabinoid Receptors in the Rat Amygdala

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The amygdala is thought to mediate memory consolidation of amphetamine-induced conditioned place preference, a behavioral paradigm that requires memory for an association between environmental cues and the affective state produced by the drug treatment. Here we show that amphetamine induces long-term synaptic depression (LTD) in the amygdala. Amphetamine LTD is not affected by dopamine, serotonin 1A, and norepinephrine α2 receptor antagonists but is blocked by the cannabinoid CB_1 receptor antagonist AM251. It is mimicked by the CB_1 agonist WIN55212-2 and facilitated and partially occluded by endocannabinoid uptake inhibitor AM404. Both amphetamine and WIN55212-2 LTDs are associated with an increase in the ratio of paired-pulse facilitation and a decrease in the frequency but not the amplitude of miniature EPSCs. They are also sensitive to block by P/Q type calcium channel blocker and occluded by each other, indicating that these two forms of synaptic plasticity share a common underlying mechanism. Loading postsynaptic neuron with calcium chelator blocked amphetamine LTD in some but not all neurons tested. However, in the presence of AM404, amphetamine LTD was present in all neurons recorded. These results suggest that amphetamine-induced endocannabinoid release depends on a rise in intracellular calcium and the incomplete block of LTD in some neurons may be attributable to the spillover of endocannabinoid from nearby cells. The finding that endocannabinoids underlie the synaptic actions of amphetamine may open a new avenue for the treatment of psychostimulants addiction.

Key words: **amphetamine; endocannabinoid; amygdala; LTD; synaptic plasticity; addiction**

Introduction

Repeated intermittent administration of psychostimulants results in a progressive enhancement of behavioral responses to these drugs, termed behavioral sensitization (Robinson and Berridge, 1993; Wolf, 1998). In humans, repeated treatments with amphetamine or cocaine have been shown to increase the vulnerability to self-administer drugs (Piazza et al., 1989). The correlation between behavioral sensitization produced by different drugs and their ability to reinstate drug-seeking behavior suggests that sensitization may model for the compulsive drug-seeking behavior in addiction (De Vries et al., 1998). Traditionally, dopaminergic pathway that projects from the ventral tegmental area to the nucleus accumbens is thought to be a critical site for the reinforcing effects of addictive drugs (Kalivas and Stewart, 1991; Wise, 1998). The amygdala receives substantial dopaminergic innervation from dopamine (DA)-containing perikarya within the ventral mesencephalon, via the mesoamygdaloid dopamine projection (Ungerstedt, 1971; Fallon et al., 1978). Because the induction of behavioral sensitization is thought to be attributable to DA release induced by psychostimulants, other DA projections may be involved in the sensitization process. Indeed, the mesoamygdaloid dopamine projection is sensitized after repeated exposure to amphetamine (Harmer and Phillips, 1999). Bilateral 6-hydroxydopamine lesion of dopaminergic terminals in the amygdala prevented the development of behavioral sensitization to amphetamine (Bjijou et al., 2002).

Amphetamine-induced conditioned place preference (CPP) is a behavioral paradigm that requires memory for an association between environmental cues and the affective state produced by the drug treatment (Bardo and Bevins, 2000). The amygdala is thought to mediate memory consolidation of amphetamine CPP because intra-amygdala infusion of the local anesthetic bupivacaine blocked amphetamine CPP (Hsu et al., 2002). Thus, some neurobiological effects produced by amphetamine involve the amygdala. Accumulating evidence indicates that glutamate receptors are important for the behavioral effects of amphetamine (Wolf, 1998). In the present study, we examine the effects of amphetamine on excitatory synaptic transmission in the amygdala. Our results show that amphetamine induces long-term depression (LTD) of synaptic responses, and, unexpectedly, this synaptic plasticity is not affected by the DA receptor antagonists but is mediated by endogenous cannabinoids.

Materials and Methods

Slice preparation. Male Sprague Dawley 4- to 5-week-old rats were decapitated, and their brains were rapidly removed and placed in cold oxygen-

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ated artificial CSF (ACSF) solution. Subsequently, the brain was hemisected and cut transversely posterior to the first branch and anterior to the last branch of the superior cerebral vein. The resulting section was glued to the chuck of a Vibroslice tissue slicer (Campden Instruments, Silbey, UK). Transverse slices of 450 μm thickness were cut, and the appropriate slices were placed in a beaker of oxygenated ACSF at room temperature for at least 1 hr before recording. ACSF solution had the following composition (in mM): 117 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 25 $NaHCO_3$, 1.2 NaH_2PO_4, and 11 glucose. The ACSF was bubbled continuously with 95%O_2–5%CO_2 and had the pH of 7.4.

Intracellular recording. A single slice was transferred to the recording chamber, in which it was held submerged between two nylon nets and maintained at 32 ± 1°C. The chamber consisted of a circular well of a low volume (1–2 ml) and was perfused constantly at a rate of 2–3 ml/min. EPSPs were recorded by electrical stimulation of the external capsule (EC), which contained fibers from the auditory cortex to the lateral amygdala, with a concentric bipolar stimulating electrode (SNE-100; David Kopf Instruments, Bern, Germany). Electrical stimuli (150 μsec in duration) were delivered at a frequency of 0.05 Hz. Intracellular recording microelectrodes were pulled from 1.0 mm microfiber capillary tubing on a Brown-Flaming electrode puller (Sutter Instruments, San Rafael, CA). The electrodes were filled with 3 M potassium acetate with resistance ranging from 70 to 130 MΩ. For chelating intracellular Ca^{2+}, the electrodes were filled with 50 mM BAPTA in addition to 3 M potassium acetate. When BAPTA-containing electrodes were used, loading of the cells with BAPTA was assayed by the blockade of Ca^{2+}-activated afterhyperpolarization (Huang et al., 1996). Bicuculline (1 μM) was present in the perfusion solution to eliminate the contamination of fast IPSP. Dopamine depletion experiments were performed according to the method reported previously (Spector et al., 1965; Jones and Kauer, 1999). Rats were given the tyrosine hydroxylase inhibitor α-methyl-*p*-tyrosine (300 mg/kg) intraperitoneally, and slice preparation was made 3 hr later.

Data were expressed as mean ± SE. The data were analyzed with ANOVA and Student's *t* test, and $p < 0.05$ was considered statistically significant. Drugs were applied directly to the ACSF using a continuous gravity-fed bath application, and the concentration of applied drug reached equilibrium within 2–3 min. Quinpirole, SCH23390, SKF81297, L-sulperide, and α-methyl-*p*-tyrosine were obtained from Sigma (Saint Louis, MO). AM251, AM404, bicuculline, CGP35348, and WIN55212-2 were obtained from Tocris Cookson (Bristol, UK).

Whole-cell patch-clamp recordings. Whole-cell patch-clamp recordings were made from the lateral nucleus of the amygdala (LA) thin slices (200 μm). Patch electrodes were pulled from thick-walled glass capillary (0.75 mm inner diameter, 1.5 mm outer diameter) to a tip resistance of 2–5 MΩ. The composition of the internal solution was as follows (in mM): 0.33 GTPtris, 125 K-gluconate, 5 KCl, 5 BAPTA, 0.5 $CaCl_2$, 5 MgATP, and 10 HEPES. The final pH of the internal solution was adjusted to 7.2 by adding 1 M KOH; the final osmolarity was adjusted to 280 mOsm by adding sucrose. Records were low-pass filtered at 2.5–20 kHz and digitized at 5–50 kHz. The signal is monitored and recorded with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Online analysis and control of experimental acquisition was accomplished via a Pentium 3-based personal computer clone and a Digidata 1320 computer interface.

Preparation of synaptosomes and glutamate release assay. Synaptosomes were prepared from the lateral and basolateral nucleus of the amygdala according to the method described previously (Wang et al., 2001). The final synaptosomal pellet was resuspended in incubation buffer consisting of 140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1 mM $MgCl_2 \cdot 6H_2O$, 1.2 mM Na_2HPO_4, 10 mM glucose, 10 mM HEPES, and 1 mg/ml BSA, pH 7.4, and protein concentration was determined using the Bradford assay. Synaptosomes were centrifuged in the final wash to obtain synaptosomal pellets with 0.3 mg of protein. Synaptosomal pellets were stored on ice and used within 2–3 hr.

Synaptosomal pellets were resuspended in incubation buffer in a stirred and thermostatted cuvette maintained at 37°C in a PerkinElmer Instruments (Norwalk, CT) LS-50B spectrofluorimeter. $NADP^+$ (1 mM), glutamate dehydrogenase (GDH) (50 U/ml), and $CaCl_2$ (1 mM) were added after 5 min. After an additional 10 min of incubation, 3 mM 4-aminopyridine (4-AP) was added to depolarize synaptosomes. Glutamate release was monitored by measuring the increase of fluorescence (excitation and emission wavelengths of 340 and 460 nm, respectively) as a result of NADPH being produced by the oxidative deamination of released glutamate by GDH. Data were accumulated at 2 sec intervals. A standard of exogenous glutamate (5 nmol) was added at the end of each experiment, and the fluorescence change produced by the standard addition was used to calculate the released glutamate as nanomoles of glutamate per milligrams of synaptosomal protein.

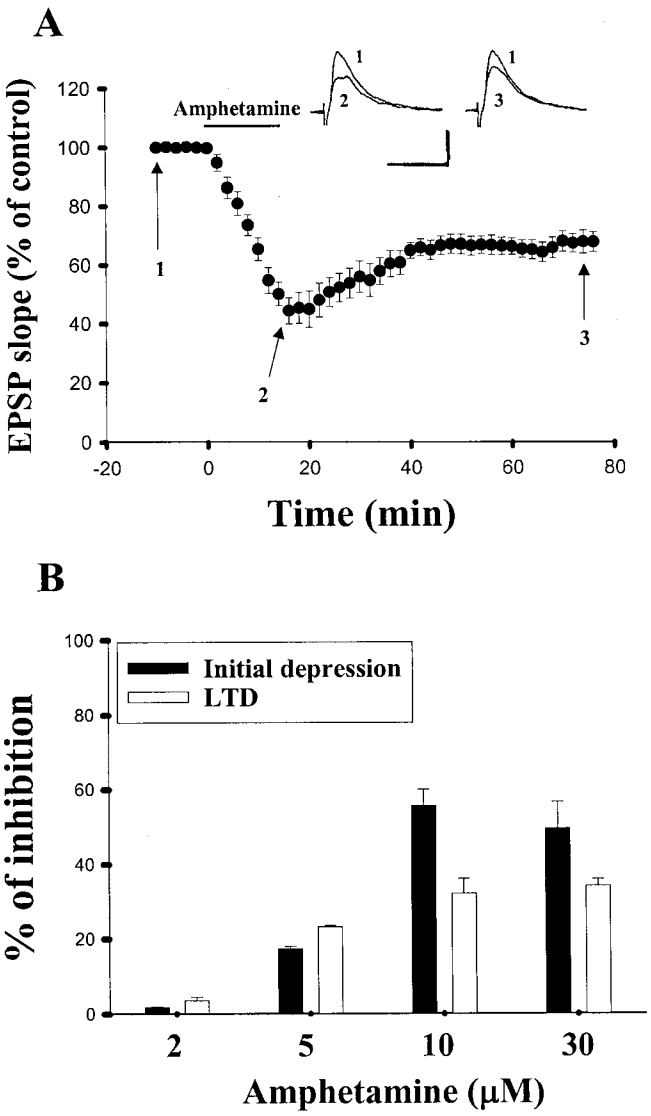

Figure 1. Concentration-dependent depression of EPSP by amphetamine. *A*, Application of amphetamine for 15 min resulted in an initial depression of EPSP, which was followed, after washout of the amphetamine, by LTD. Inset shows superimposed traces taken at the time points indicated. Calibration: 10 mV, 40 msec. *B*, Concentration-dependent effects of amphetamine on the initial depression and LTD.

Results

Amphetamine induces LTD in the LA neurons

EPSPs in the LA were elicited by stimulating the EC, which carries axons from the secondary auditory and perirhinal cortices to the amygdala. Superfusion of amphetamine for 15 min caused a rapid depression of EPSP. After washout of amphetamine, the EPSP was only partially recovered to a stable response that was depressed relative to the initial baseline value (Fig. 1*A*). This amphetamine-induced LTD lasted for at least 60 min, and we

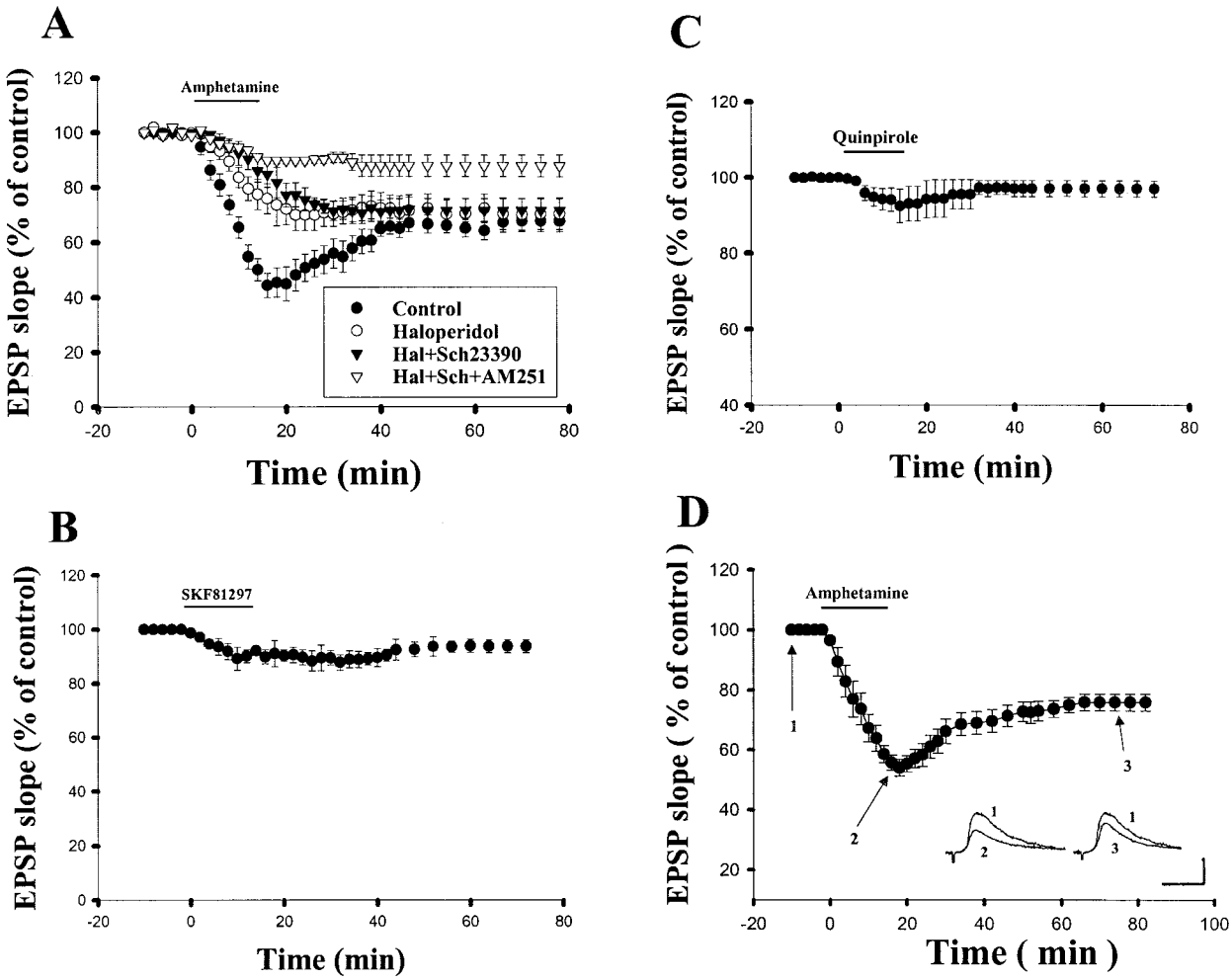

Figure 2. Effects of dopamine receptor antagonists on amphetamine-induced LTD. *A*, Time course data showing the effects of haloperidol (2 μM), haloperidol (Hal) plus SCH23390 (20 μM; Sch), and haloperidol plus SCH23390 plus AM251 (2 μM) on amphetamine-induced LTD. *B*, *C*, Effects of SKF82958 (50 μM; *B*) and quinpirole (20 μM; *C*) on the EPSP. *D*, Effect of amphetamine (10 μM) on the EPSP in animals pretreated with α-methyl-*p*-tyrosine. Inset shows superimposed traces taken at the time points indicated. Calibration: 10 mV, 40 msec.

measured the slope of EPSP at this time point for statistical comparison. The effect of amphetamine was concentration dependent ($F_{(3,21)} = 101.4$, $p < 0.001$ for acute depression and $F_{(3,21)} = 49.7$, $p < 0.001$ for LTD). Two, 5, 10, and 30 μM produced an initial depression measuring $-1.7 \pm 0.2\%$ ($n = 6$), $-17.3 \pm 0.6\%$ ($n = 6$), $-55.6 \pm 4.4\%$ ($n = 7$), and $-53.1 \pm 2.6\%$ ($n = 6$), respectively, and LTD measuring $-3.6 \pm 0.9\%$ ($n = 6$), $-23.8 \pm 0.5\%$ ($n = 6$), $-32.1 \pm 4.0\%$ ($n = 7$), and $-34.2 \pm 1.8\%$ ($n = 6$), respectively (Fig. 1*B*). Amphetamine (10 μM) LTD was not attributable to an alteration of resting membrane potential (RMP) or neuronal input resistance (IR) of the LA neurons (RMP and IR were -67.0 ± 1.6 mV and 45.8 ± 5.0 MΩ before and -66.0 ± 1.3 mV and 46.0 ± 6.0 MΩ 60 min after the washout of amphetamine; $n = 6$).

Block of amphetamine LTD by CB_1 cannabinoid receptor antagonist

Amphetamine is thought to exert its pharmacological actions by promoting nonvesicular release of DA, thereby increasing extracellular level of DA (Wise and Bozarth, 1987; Seiden et al., 1993; Sulzer et al., 1995). DA receptors are classified into two pharmacologically and biochemically distinct classes, the D_1-like (D_1 and D_5) and D_2-like (D_2, D_3, and D_4) receptors (Civelli et al., 1993). To characterize the receptors responsible for the actions of amphetamine, selective D_1 and D_2 receptor agonists and antagonists were used. Figure 2*A* shows that the D_2 receptor antagonist haloperidol (2 μM) attenuated amphetamine-induced acute depression without significantly affecting the magnitude of LTD. The acute depression and LTD in the presence of haloperidol were $-30.0 \pm 5.6\%$ ($n = 6$, $p < 0.01$ vs control) and $-29.6 \pm 5.8\%$ ($n = 6$, $p = 0.43$), respectively. Similar effect was observed with another D_2 receptor antagonist, L-sulperide. The acute depression and LTD in the presence of L-sulperide (3 μM) were -28.4 ± 6.1 and $-26.9 \pm 7.0\%$ ($n = 3$), respectively. A combination of both D_2 and D_1 (SCH23390, 20 μM) antagonists did not further influence the effect amphetamine (acute depression, $-29.2 \pm 3.9\%$, $n = 6$; LTD, $-28.3 \pm 4.3\%$, $n = 6$). These results suggest that the acute depression induced by amphetamine is only partly mediated by the DA receptors, and DA receptors are not involved in LTD induction. In agreement with this interpretation, both the D_1 agonist SKF82958 (30 μM; $-10.1 \pm 2.6\%$; $n = 6$) (Fig. 2*B*) and the D_2 agonist quinpirole (20 μM; approximately -10%; $n = 4$) (Fig. 2*C*) produced transient depression of EPSP.

If the effect of amphetamine was mediated by DA, then it would be attenuated when the DA level was reduced. We tested this possibility by intraperitoneal injection of rats with the tyrosine hydroxylase inhibitor α-methyl-*p*-tyrosine (300 mg/kg), and slice preparation was made 3 hr later. Figure 2*D* shows that

amphetamine-induced acute depression (−45.9 ± 2.8%; $n = 6$) and LTD (−24.0 ± 2.8%; $n = 6$) was not significantly altered in animals treated with α-methyl-*p*-tyrosine.

Amphetamine could induce LTD via its known effect on transporters for either norepinephrine (NE) or serotonin (5-HT). To elucidate the involvement of 5-HT_{1A} or NE α2 receptors, we examined the action of amphetamine in the presence of 5-HT_{1A} or NE α2 receptor antagonists. We found that the effect of amphetamine was not significantly inhibited by the 5-HT_{1A} receptor antagonist WAY100135 (5 μM), causing −29.4 ± 6.3% LTD in the presence of WAY100135 ($n = 3$, $p > 0.5$ vs control). Similarly, the NE α2 receptor antagonist yohimbine (1 μM) did not influence amphetamine LTD (−26.0 ± 5.2%; $n = 5$; $p > 0.2$).

In the amygdala, DA receptor activation increases the firing rate of local inhibitory interneurons (Ben-Ari and Kelly, 1976; Rosenkranz and Grace, 1999). Thus, the depressant effect could be indirect and attributable to DA receptor-mediated increase in extracellular GABA levels, resulting in activation of presynaptic $GABA_B$ receptors. To test whether such a mechanism accounted for the effect of amphetamine, we applied amphetamine in the presence of the $GABA_B$ receptor antagonist CGP35348 (500 μM). Amphetamine induced the same extent of LTD (−35.3 ± 4.6%, $n = 7$, $p > 0.5$ vs control) whether or not the antagonist was present, indicating that $GABA_B$ receptor was not involved.

Several studies have indicated that endogenous cannabinoids function as rapid, retrograde signaling molecules in the CNS (Kreitzer and Regehr, 2001b; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001; Carson et al., 2002). To test the involvement of cannabinoids in amphetamine-induced synaptic plasticity, we applied the CB_1 antagonist AM251 (2 μM) in the presence of D_1 and D_2 antagonists to determine whether it affected the actions of amphetamine. As illustrated in Figure 2*A*, both acute depression and LTD were significantly reduced by further addition of AM251 (acute depression, −12.1 ± 3.9%, $n = 6$; LTD, −12.0 ± 4.0%, $n = 6$). We therefore directly assessed the involvement CB_1 receptor in the action of amphetamine. AM251 by itself did not affect EPSP significantly (97.8 ± 3.5% of control, $n = 6$, $p > 0.1$). However, as illustrated in the Figure 3*A*, it blocked initial depression as well as LTD in a concentration-dependent manner ($F_{(2,14)} = 67.4$, $p < 0.001$ for acute depression and $F_{(2,14)} = 23.1$, $p < 0.001$ for LTD).

If an endogenous cannabinoid mediates the effect of amphetamine, then a CB_1 agonist should depress synaptic transmission by the same mechanism. Figure 3*B* shows that the synthetic CB_1 agonist WIN55212-2 induced an initial depression followed by LTD in a concentration-dependent manner. Preincubation of slices with AM251 blocked both initial depression and LTD. In the presence of AM251, the initial depression and LTD induced by 0.5 μM WIN55212-2 were only −7.9 ± 1.8% ($n = 6$) and −7.9 ± 2.0% ($n = 6$), respectively, which were significantly less than that without AM251 pretreatment (−29.9 ± 6.2 and −47.6 ± 6.1%, $n = 6$, $p < 0.005$ and $p < 0.0001$, respectively). To exclude the possibility that the observed decline of EPSP was related to the slow washout of WIN55212-2, experiments were performed in which AM251 was applied for 15 min at 30 min after the washout of WIN55212-2. In four neurons, post-WIN55212-2 application of AM251 did not reverse LTD (−40.4 ± 7.2%, $n = 4$, $p > 0.1$ vs without AM251 treatment), suggesting that LTD was not caused by the continued presence of WIN55212-2.

To elucidate possible presynaptic versus postsynaptic expression of amphetamine LTD, we tested the effects of amphetamine and WIN55212-2 on paired-pulse facilitation (PPF), which is commonly thought to be a presynaptic phenomenon (Zucker, 1989). A pair of synaptic responses was elicited with an interstimulus interval of 60 msec, and the ratios of PPF were compared among control, during amphetamine-induced acute depression, and 60 min after cessation of application in the same cell. In five experiments, control PPF was 1.08 ± 0.09, which was increased significantly to 1.51 ± 0.12 during acute depression and 1.31 ± 0.15 during LTD ($p < 0.05$). We repeated the experiments with WIN55212-2 (0.5 μM). PPF ratio was increased from 1.48 ± 0.05 in control to 1.93 ± 0.08 during acute depression and 1.88 ± 0.06 during WIN55212-2-induced LTD ($n = 4$; $p < 0.01$). To further characterize the site of action, we used whole-cell voltage clamp ($V_h = -70$ mV) to record miniature EPSCs (mEPSCs) in the presence of the Na^+ channel blocker tetrodotoxin (TTX) (1 μM) and the $GABA_A$ receptor antagonist bicuculline (1 μM). A decrease in the frequency of mEPSCs is interpreted to be a result of presynaptic action (e.g., a reduction in the probability of neuro-

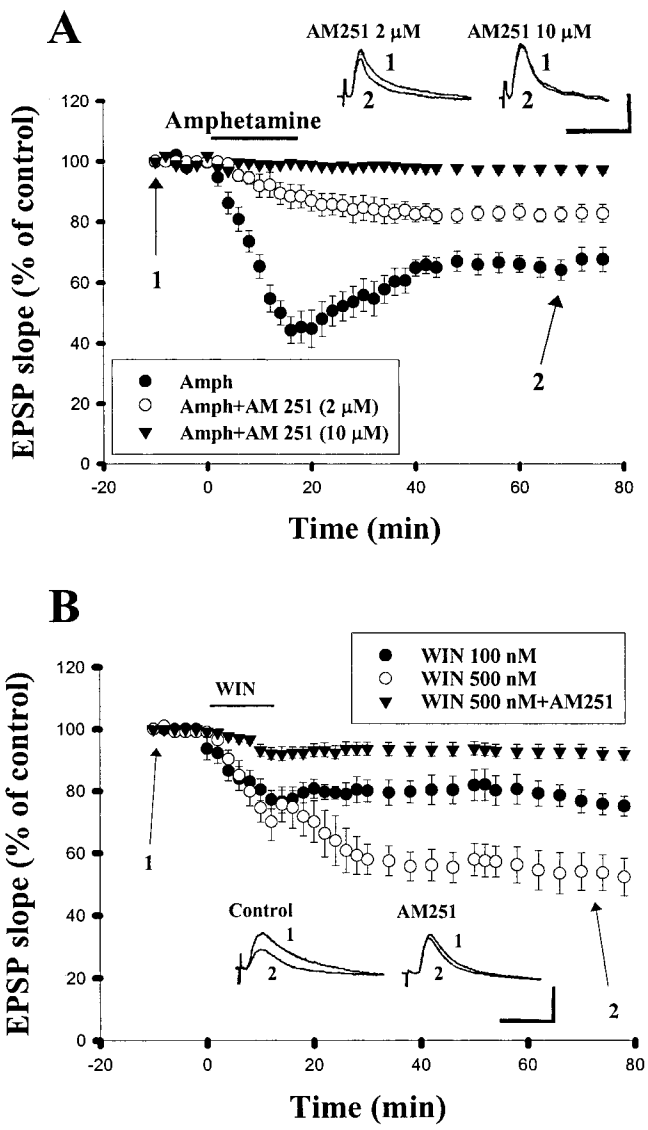

Figure 3. Mimicry and block of amphetamine LTD by respective CB_1 receptor agonist and antagonist. *A*, Time course data showing the concentration-dependent effect of AM251 on amphetamine (10 μM; Amph) LTD. *B*, Concentration-dependent depression of EPSP by WIN55212-2 (WIN) and its inhibition by AM251 (2 μM). Inset shows superimposed traces taken at the time points indicated. Calibration: 10 mV, 40 msec.

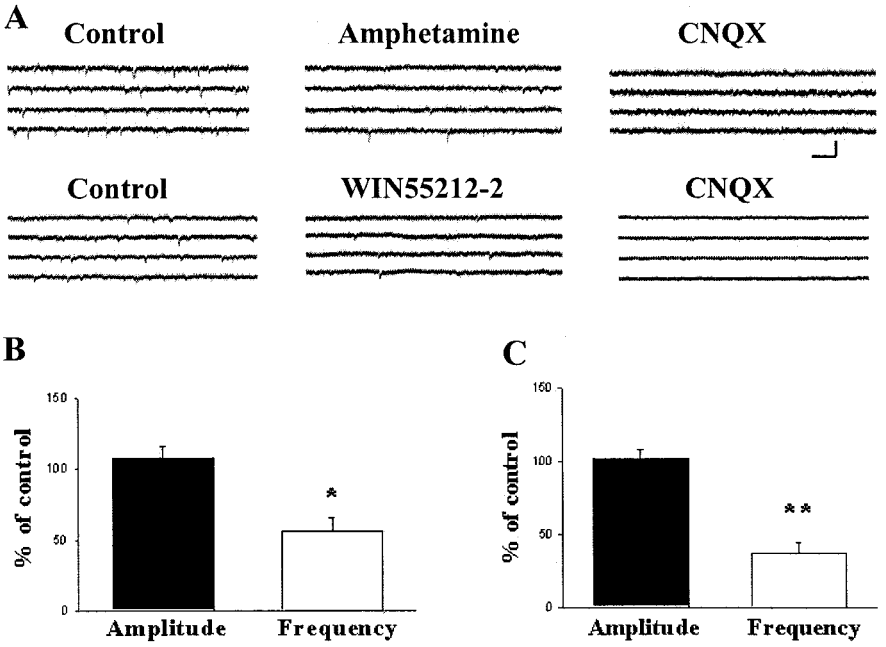

Figure 4. Amphetamine and WIN55212-2 LTDs are associated with a decrease in the frequency but not the amplitude of mEPSCs. *A*, Sample traces of mEPSCs recorded in the presence of TTX (1 μM) and bicuculline (1 μM). Traces were taken before the application of amphetamine or WIN55212-2 and 30 min after washout of the drugs. Amphetamine (10 μM) or WIN55212-2 (0.5 μM) was superfused for 15 min. At the end of experiments, CNQX (10 μM) was applied to confirm that mEPSCs were mediated by the non-NMDA receptors. Calibration: 20 pA, 200 msec. *B*, *C*, The frequency of mEPSCs after treatment with amphetamine or WIN55212-2 was significantly lower than that of control, whereas the amplitude of mEPSCs was unaffected. *$p < 0.02$ versus control; **$p < 0.01$ versus control.

transmitter release or in the number of quanta available for release), whereas a decrease in the amplitude normally indicates a reduction in the postsynaptic sensitivity to the transmitter. The result is summarized in Figure 4*A* in which amphetamine (10 μM) was applied for 15 min, and the frequency and amplitude of mEPSCs measured at 30 min after washout of amphetamine were 56 ± 10% ($n = 5$; $p < 0.02$) and 108 ± 8% ($n = 5$; $p > 0.1$) of control, respectively. In the same vein, WIN55212-2 (0.5 μM) reduced mEPSC frequency to 37 ± 7% ($n = 5$; $p < 0.01$) of control without affecting mEPSC amplitude (101 ± 6%; $n = 5$; $p > 0.1$) (Fig. 4*B*). The mEPSCs were mediated by non-NMDA glutamate receptors because they were abolished by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (10 μM). Thus, both amphetamine and WIN55212-2 LTDs were caused by a presynaptic mechanism.

Glutamate release from purified amygdala synaptosomes was monitored online continuously using an assay utilizing exogenous glutamate dehydrogenase and $NADP^+$ to couple the oxidative deamination of released glutamate to the generation of NADPH. Figure 5*A* shows that, under control conditions, 4-AP (3 mM) evoked glutamate release of 54.4 ± 4.0 nmol/mg per 4 min ($n = 10$). Preincubation of synaptosomes with WIN55212-2 (0.5 μM) significantly reduced the release of glutamate to 31.5 ± 4.1 nmol/mg per 4 min ($n = 5$, $p < 0.01$ vs control). On the other hand, preincubation of synaptosomes with amphetamine (10 μM) was without effect (46.5 ± 6.8 nmol/mg per 4 min; $n = 6$; $p = 0.30$) (Fig. 5*B*), suggesting that amphetamine did not directly act on synaptic terminals to reduce glutamate release.

Blocking uptake of endocannabinoids enhances and occludes the effect of amphetamine

Endocannabinoid diffusion is limited by high-affinity cellular reuptake (Beltramo et al., 1997; Piomelli et al., 1999). To further determine the involvement of endocannabinoids in the action of amphetamine, we blocked endocannabinoid transporter activity with the competitive inhibitor AM404 and examined its influence on the action of amphetamine. Figure 6*A* shows that application of AM404 (10 μM) gradually reduced the slope of EPSP. The EPSP slope was 79.5 ± 1.9% of control ($n = 4$) at 40 min after the application of drug. In addition, pretreatment of slices with AM251 blocked the effect of AM404 (97.3 ± 1.5% of control; $n = 4$; $p < 0.001$). We next tested whether amphetamine LTD would be influenced by a decrease in transporter activity. To perform these experiments, slices were incubated in 10 μM AM404 for at least 1 hr before being transferred to the recording chamber in which the same concentration of AM404 was maintained. As shown in Figure 6*B*, 2 μM amphetamine that normally was without effect (−3.0 ± 1.4%; $n = 6$) now induced LTD (−14.0 ± 1.8% measured at 60 min after the washout of amphetamine; $n = 6$; $p < 0.01$). However, under the same condition, high concentration of amphetamine (10 μM) produced a less degree of LTD (−15.6 ± 4.7%; $n = 11$) compared with that without AM404 treatment (−32.1 ± 4.0%; $n = 7$; $p < 0.05$) (Fig. 6*C*). These results suggest that endogenous substrate for the AM404-sensitive transporter is a cannabinoid that mimics and occludes the effect of amphetamine.

Amphetamine LTD and WIN55212-2 LTD are mutually occluded

We tested whether LTDs induced by amphetamine and WIN55212-2 shared the same mechanism by performing occlusion experiments. In the first set of experiments, LTD was induced to a near-maximal level by giving a concentration of 10 μM amphetamine. We then compared the magnitude of WIN55212-2 LTD under this condition with that of without amphetamine pretreatment. A summary of five experiments is shown in Figure 7*A* in which amphetamine reduced the slope of EPSP by 52.8 ± 5.7%. The stimulus intensity was increased to restore the EPSP slope to near-control level, and subsequent application of WIN55212-2 (0.5 μM) reduced EPSP only by 15.5 ± 5.7%, which was significantly less than when WIN55212-2 was applied alone ($p < 0.005$; unpaired t test). Thus, although WIN55212-2 was still able to elicit LTD, its magnitude was primarily attenuated in the presence of amphetamine.

To further address this question, we performed a reverse occlusion experiment, in which LTD was induced by WIN55212-2 before amphetamine was applied. As shown in Figure 7*B*, WIN55212-2 (0.5 μM) reduced the slope of EPSP by 37.6 ± 2.5%. Raising the stimulus intensity restored the EPSP slope to near pre-WIN55212-2 level. Under this condition, amphetamine (10 μM) elicited only an average of 12.6 ± 4.6% reduction, a magnitude significantly less than that without WIN55212-2 pretreatment ($n = 5$; $p < 0.01$; unpaired t test).

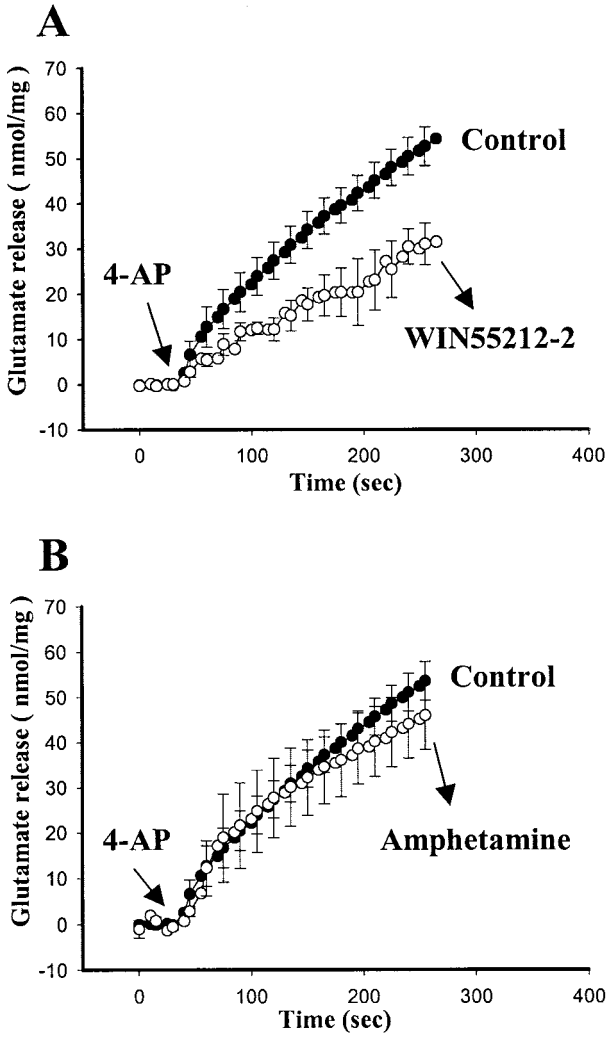

Figure 5. Inhibition of 4-AP-evoked glutamate release by WIN55212-2 but not by amphetamine. Rat synaptosomes (0.3 mg/ml) were preincubated for 10 min in the absence or presence of WIN55212-2 (0.5 μM; *A*) or amphetamine (10 μM; *B*), followed by the addition of a stimulator (4-AP, 3 mM). Glutamate release was assayed by online fluorimetry.

The role of postsynaptic Ca^{2+} in the action of amphetamine

As the trigger for cannabinoid synthesis is cytoplasmic Ca^{2+}, we determined whether a rise in postsynaptic Ca^{2+} is required for the action of amphetamine. Neurons were recorded with electrodes containing a fast Ca^{2+} chelator, BAPTA (50 mM). After impalement, the cells were allowed to stabilize for at least 30 min to let the cell fill with BAPTA, which was manifested by block of slow afterhyperpolarization (Huang et al., 1996). Baseline responses were then obtained for an additional 10 min before the application of amphetamine (10 μM). In 6 of 13 neurons, inclusion of BAPTA in the recording electrodes significantly attenuated amphetamine LTD ($-6.8 \pm 3.0\%$; $p < 0.001$) (Fig. 8*A*). In the remaining seven neurons, amphetamine evoked a normal LTD ($-36.9 \pm 6.6\%$; $p = 0.54$). These results suggest that amphetamine-induced release of endocannabinoid is not strictly dependent on the rise of intracellular Ca^{2+}. Alternatively, the failure of BAPTA to completely block the effect of amphetamine raised the possibility that cannabinoid that was released from nearby cells was responsible for the induction of LTD. If some "spillover" of cannabinoid onto the terminals of excitatory synapses occurred, then the effect should be enhanced after blockade

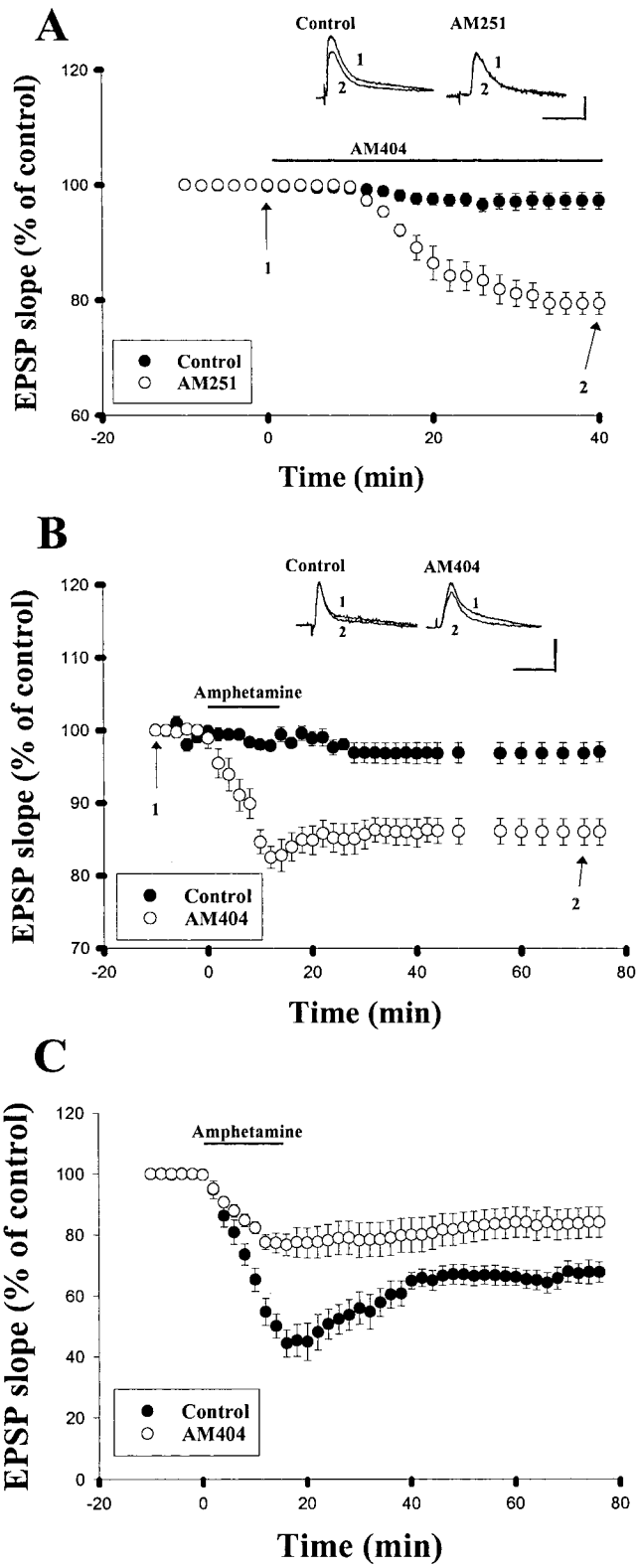

Figure 6. Enhancement and occlusion of amphetamine effect by blocking uptake of endogenous CB_1 ligands. *A*, Application of AM404 (10 μM) gradually reduced the slope of EPSP, and preincubation of slices in AM251 (2 μM) abolished the effect of AM404. *B*, Low concentration of amphetamine (2 μM), normally producing minimal effect on the EPSP, caused a significant LTD in AM404-treated slices. Slices were incubated in 10 μM AM404 for at least 1 hr before being transferred to the recording chamber in which the same concentration of AM404 was maintained. Inset shows superimposed traces taken at the time points indicated. Calibration: 10 mV, 40 msec. *C*, Under the same condition as in *B*, high concentration of amphetamine (10 μM) produced a less degree of LTD compared with that without AM404 treatment.

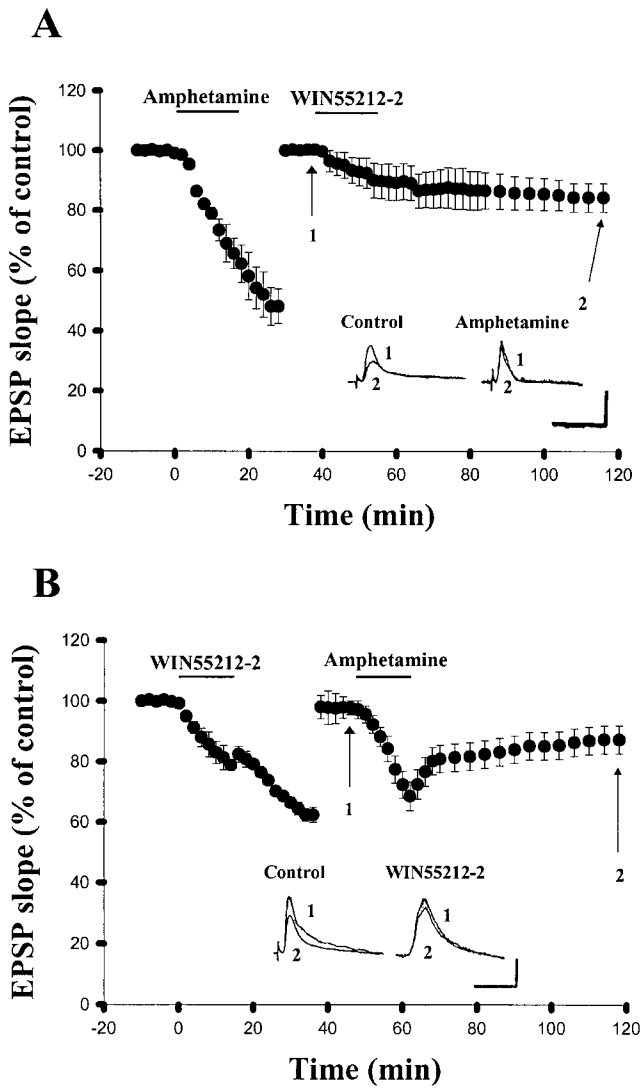

Figure 7. Amphetamine and WIN55212-2 LTDs are mutually occluded. *A*, Application of 10 μM amphetamine for 15 min induced LTD. Sixty minutes after washout of amphetamine, the stimulus intensity was increased to restore the EPSP slope to near-control level. Subsequent application of WIN55212-2 reduced EPSP slope by only 15.5 ± 5.7%, which was significantly less than when WIN55212-2 was applied alone. *B*, Reverse occlusion experiments in which LTD was induced by WIN55212-2 before amphetamine was applied. WIN55212-2 (0.5 μM) reduced the slope of EPSP by 37.6 ± 2.5%. Raising the stimulus intensity restored the EPSP slope to near-pre-WIN55212-2 level. Under this condition, amphetamine (10 μM) elicited only an average of 12.6 ± 4.6% reduction, a magnitude significantly less than that without WIN55212-2 pretreatment. Inset shows superimposed traces taken at the time points indicated. Calibration: 10 mV, 40 msec.

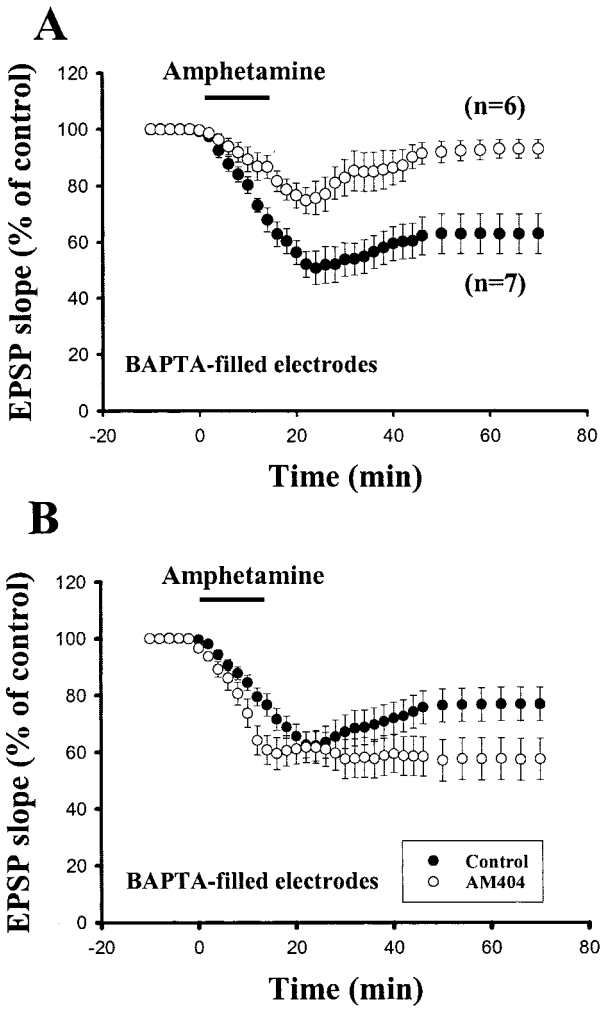

Figure 8. The role of postsynaptic Ca^{2+} in the action of amphetamine. Neurons were recorded with electrodes containing BAPTA (50 mM). After impalement, the cells were allowed to stabilize for at least 30 min to let the cell fill with BAPTA, which was manifested by block of slow afterhyperpolarization. *A*, In 6 of 13 neurons, inclusion of BAPTA in the recording electrodes significantly attenuated amphetamine LTD. In the remaining seven neurons, amphetamine evoked a normal LTD. *B*, Summary of the data obtained from neurons recorded with BAPTA-filled electrodes in the presence or absence of AM404. In the absence of AM404, the average of LTD was −23.0 ± 5.7% ($n = 13$), which is significantly different from that in the presence of AM404 (−44.1 ± 5.5%; $n = 6$; $p < 0.05$).

of cannabinoid uptake. Indeed, this was the case. Amphetamine induced LTD (−44.1 ± 5.5%) in the presence of AM404 (10 μM) in all six neurons recorded with BAPTA-containing electrodes. Figure 8 *B* summarized the data obtained from neurons recorded with BAPTA-filled electrodes in the presence or absence of AM404. In the absence of AM404, the average of LTD was −23.0 ± 5.7% ($n = 13$), which is significantly different from that in the presence of AM404 (−44.1 ± 5.5%; $n = 6$; $p < 0.5$).

Differential involvement of presynaptic voltage-dependent Ca^{2+} channels in endocannabinoid action

Neurotransmitter release is initiated by an elevation of intracellular Ca^{2+} triggered by depolarization of presynaptic nerve terminals and an influx of Ca^{2+} through voltage-dependent Ca^{2+} channels (VDCCs) (Llinas et al., 1981). Synaptic depression by endocannabinoids in the hippocampus and cerebellum is cause by inhibition of presynaptic VDCCs (Hoffman and Lupica, 2000; Kreitzer and Regehr, 2001a). We, therefore, asked whether amphetamine- and endocannabinoid-mediated inhibition showed any specificity for a particular presynaptic Ca^{2+} channel subtype. We found that the selective L-type Ca^{2+} channel blocker nimodipine (2 μM) had no significant effects on either normal synaptic transmission (97.0 ± 1.3% of control; $n = 10$) or amphetamine and WIN55212-2 LTDs. The magnitudes of amphetamine and WIN55212-2 LTDs in the presence of nimodipine were −26.6 ± 2.5% ($n = 6$) and −38.4 ± 6.0% ($n = 4$), respectively, which were not significantly different from that without nimodipine treatment.

Application of the N-type Ca^{2+} channel blocker ω-conotoxin-GVIA (ω-CTX-GVIA) (1 μM) for 15 min reduced EPSP slope by 52.8 ± 3.9%. Amphetamine (10 μM) was then

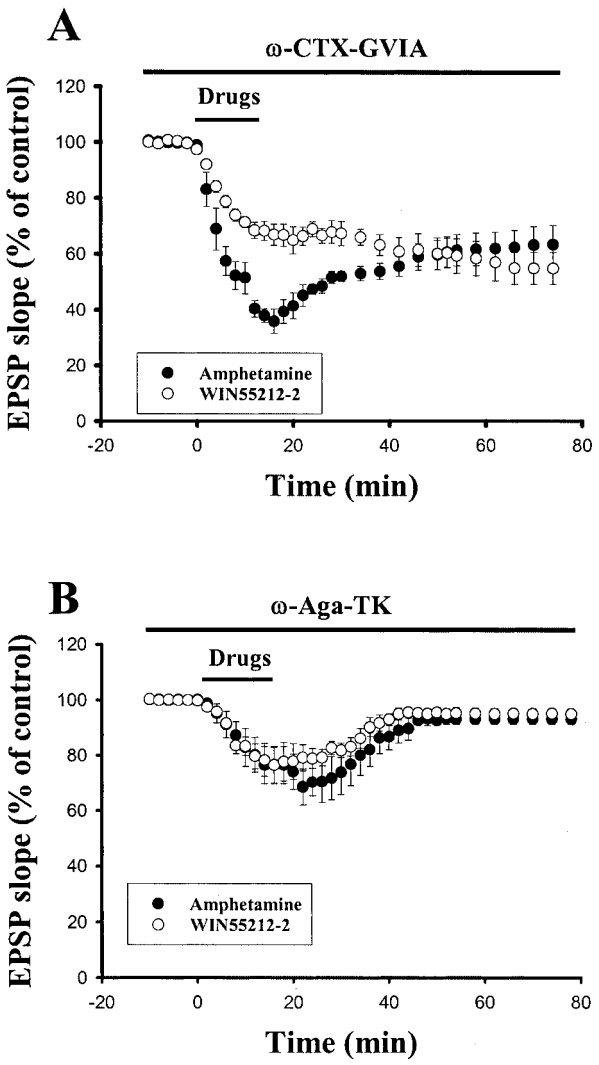

Figure 9. Pretreatment with ω-Aga-TK but not ω-CTX-GVIA blocks amphetamine and WIN55212-2 LTD. Time course of the effects of amphetamine (10 μM) and WIN55212-2 (0.5 μM) on the EPSP. ω-CTX-GVIA (1 μM; *A*) and ω-Aga-TK (200 nM; *B*) were present throughout the experiments. In the presence of ω-CTX-GVIA, amphetamine and WIN55212-2 still induced LTD. Conversely, in the presence of ω-Aga-TK, amphetamine and WIN55212-2 failed to elicit LTD.

given at 15 min after ω-CTX-GVIA perfusion, and both drugs were removed after 15 min application of amphetamine. Under this condition, amphetamine induced LTD of −52.7 ± 5.1% ($n = 5$), a magnitude greater than that without ω-CTX-GVIA treatment ($p < 0.01$). The larger LTD could be attributable to the slow washout of ω-CTX-GVIA; therefore, additional four experiments were performed in which ω-CTX-GVIA was present throughout the experiment. As depicted in Figure 9*A*, under such conditions, amphetamine induced an LTD of −36.5 ± 6.8%, which was not significantly different from that without ω-CTX-GVIA treatment ($p = 0.52$). Similar experiments were performed with WIN55212-2 (0.5 μM), and, under the same condition, WIN55212-2 induced an LTD of −45.3 ± 5.0% ($n = 5$), which was not significantly different from that without ω-CTX-GVIA treatment ($p > 0.05$).

We repeated the experiments using ω-agatoxin TK (ω-Aga-TK), a selective blocker for P/Q type Ca^{2+} channels. ω-Aga-TK (200 nM) alone reduced the EPSP slope by 58.0 ± 8.6%. Figure 9*B* shows that application of amphetamine (10 μM) in the presence of ω-Aga-TK failed to induce LTD (−4.9 ± 1.7%; $n = 6$). A similar result was seen with WIN55212-2. WIN55212-2 (0.5 μM) application in the presence of ω-Aga-TK induced LTD of −6.3 ± 1.9% ($n = 6$), which was significantly different from that without ω-Aga-TK treatment ($p < 0.001$) (Fig. 8*B*). Together, these results indicate that presynaptic P/Q type Ca^{2+} channels are primarily responsible for the action of amphetamine and cannabinoids.

Discussion

This work is the first demonstration of acute and long-term effects of amphetamine on the excitatory synaptic transmission in the amygdala. We show that application of amphetamine evokes acute depression followed by LTD at the EC–LA synapse, a crucial pathway for fear memory (Davis et al., 1994; LeDoux, 2000). One common feature shared by the psychostimulants is their interactions with DA-containing nerve terminals. Our results, however, unexpectedly reveal the mediation by endocannabinoid that may modulate the development of drug addiction as well as fear-related learning.

The effects of amphetamine are concentration dependent, with a maximal acute depression of ~60% and LTD of ~30%. We made some progress in understanding the receptor subtypes involved in the acute effect, but the complete picture remains to be worked out. Pharmacological analysis suggests that DA receptors are involved in the mediation of acute depression. The D_2 receptor antagonist haloperidol inhibited the action of amphetamine. A combination of D_2 and D_1 receptor antagonists failed to exert further inhibition. On the other hand, addition of CB_1 receptor antagonist in the presence of D_1 and D_2 antagonists nearly abolished acute depression. These results suggest that acute depression is mediated in large part by the DA and CB_1 receptors. Consistent with these results, both D_1 and D_2 receptor agonists caused only slight depression on the synaptic responses.

Amphetamine induces LTD in this synapse, and, surprisingly, DA receptors are not involved because these receptor antagonists did not affect LTD. This conclusion is supported by the observation that amphetamine LTD was not different in animals given dopamine depletor compared with littermate controls. In addition, 5-HT or NE apparently are not involved in amphetamine LTD because 5-HT_{1A} and NE α2 receptor antagonists were without effect. Amphetamine did release dopamine in this preparation because, as aforementioned, amphetamine evoked DA receptor-mediated acute depression. In addition, amphetamine-induced acute depression was nearly abolished by high concentration of AM251, suggesting the possibility that some portion of released endocannabinoid was linked to DA receptor activation. In this respect, using microdialysis measurement in dorsal striatum of freely moving rats, it has been shown that local administration of D_2 receptor agonist increased endogenous cannabinoid release (Giuffrida et al., 1999).

The intriguing finding of this study is that amphetamine LTD is mediated by cannabinoid CB_1 receptors. Amphetamine LTD was blocked in a concentration-dependent manner by the CB_1 receptor antagonist AM251 and was mimicked by the synthetic CB_1 agonist WIN55212-2. Both CB_1 and amphetamine LTDs were associated with an increase in PPF and a decrease in the frequency but not the amplitude of mEPSCs. Inhibition of endocannabinoid transporter activity facilitated low-concentration amphetamine-induced LTD but occluded higher-concentration amphetamine-induced LTD. Moreover, in synaptosome preparation, activation of CB_1 receptor by WIN55212-2 reduced glutamate release, whereas amphetamine was without effect, suggesting that amphetamine is not directly acting on the nerve

terminals. Finally, CB_1 and amphetamine LTDs were mutually occluded, and both were sensitive to block by P/Q type but not by N type calcium channel blockers. Recently, a role for endocannabinoid as a retrograde signal to induce LTD has been demonstrated in the striatum (Gerdeman et al., 2002).

In rat amygdala, there is a subpopulation of nonpyramidal neurons corresponding to large cholecystokinin-positive cells that has intense immunoreactivity for CB_1 receptor (Ong and Mackie, 1999; McDonald and Mascagni, 2002). Activation of CB_1 receptors in this area resulted in LTD of GABA-mediated IPSCs (Marsicano et al., 2002). Although pyramidal cells exhibited weak expression of CB_1 receptor mRNA and much less CB_1-immunoreactive boutons (McDonald and Mascagni, 2002), our results show that endocannabinoids released by amphetamine are capable of inducing LTD of excitatory synaptic transmission. In fact, dense staining of CB_1 receptor was observed not only in nonpyramidal neurons but also in putatively glutamatergic pyramidal neurons of the monkey amygdala (Ong and Mackie, 1999). Retrograde inhibition of excitatory synaptic transmission by endocannabinoids has also been demonstrated in cerebellar Purkinje cells (Kreitzer and Regehr, 2001a; Maejima et al., 2001).

The estimated caudate-putamen amphtamine levels were 2.5–5 μM after single doses (1 and 2.5 mg/kg) that caused hyperactivity and stereotypic behavior. Exposed to neurotoxic doses of amphetamine (four doses at 5 mg/kg), caudate-putamen amphetamine levels rose to 8 μM after the first dose and peaked at 15 μM after the third dose, with no additional increases after the fourth dose (Clausing et al., 1995). In other studies, amphetamine concentrations 1 hr after a single injection of 15 mg/kg were 86 and 110 μM for striatum and brainstem (Garattini et al., 1976) and a whole-brain concentration of 120 μM 15 min after injection (Lokiec et al., 1978). These are all in the range of concentrations necessary to elicit LTD in the present study. The finding that endocannabinoids underlie the synaptic actions of amphetamine may help explain some of the emotionally relevant behavioral effects of amphetamine and suggest a therapeutic target for the treatment of psychostimulants addiction. Especially, endocannabinoids play an important role in extinction of aversive memory in the amygdala (Marsicano et al., 2002).

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