Localization and Mechanisms of Action of Cannabinoid Receptors at the Glutamatergic Synapses of the Mouse Nucleus Accumbens

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Despite the role of excitatory transmission to the nucleus accumbens (NAc) in the actions of most drugs of abuse, the presence and functions of cannabinoid receptors (CB1) on the glutamatergic cortical afferents to the NAc have never been explored. Here, immunohistochemistry has been used to show the localization of CB1 receptors on axonal terminals making contacts with the NAc GABAergic neurons. Electrophysiological techniques in the NAc slice preparation revealed that cannabimimetics [WIN 55,212,2 (WIN-2) and CP55940] strongly inhibit stimulus-evoked glutamate-mediated transmission. The inhibitory actions of WIN-2 were dose-dependent (EC_{50} of 293 ± 13 nM) and reversed by the selective CB1 antagonist SR 141716A. In agreement with a presynaptic localization of CB1 receptors, WIN-2 increased paired-pulse facilitation, decreased miniature EPSC (mEPSC) frequency, and had no effect on the mEPSCs amplitude. Perfusion with the adenylate cyclase activator forskolin enhanced glutamatergic transmission but did not alter presynaptic CB1 actions, suggesting that cannabinoids inhibit glutamate release independently from the cAMP–PKA cascade. CB1 did not reduce evoked transmitter release by inhibiting presynaptic voltage-dependent Ca^{2+} currents through N-, L-, or P/Q-type Ca^{2+} channels, because CB1 inhibition persisted in the presence of ω-Conotoxin-GVIA, nimodipine, or ω-Agatoxin-IVA. The K^+ channel blockers 4-aminopyridine (100 μM) and $BaCl_2$ (300 μM) each reduced by 40–50% the inhibitory actions of WIN-2, and their effects were additive. These data suggest that CB1 receptors are located on the cortical afferents to the nucleus and can reduce glutamate synaptic transmission within the NAc by modulating K^+ channels activity.

Key words: nucleus accumbens; cannabinoid; glutamate; CB1 receptors; presynaptic inhibition; K^+ channels; mice

Derivatives of *Cannabis sativa* (L.), such as marijuana and hashish, have been used for centuries for therapeutic and recreational purposes. The psychopharmacologically active component of *C. sativa*, (−)-*trans*-delta9-tetrahydrocannabinol, as well as cannabimimetics and endocannabinoids, mediate their actions in the CNS through specific interactions with a G_i/G_o-protein-coupled receptor [cannabinoid receptor (CB1)] (Mechoulam et al., 1996). The CB1 receptor is widely expressed in the brain and has been shown to inhibit adenylate cyclase (AC), activate mitogen-activated protein kinases, reduce Ca^{2+} currents, and modulate several K^+ conductances (Mackie and Hille, 1992; Mackie et al., 1993, 1995; Bouaboula et al., 1995; Twitchell et al., 1997; Schweitzer, 2000). Activation of CB1 receptors inhibits synaptic transmission in the hippocampus (Sullivan, 1999; Hoffman and Lupica, 2000), substantia nigra pars compacta (Chan and Yung, 1998), the cerebellum (Levenes et al., 1998), and the prefrontal cortex (Auclair et al., 2000).

The mesolimbic–mesocortical dopaminergic system and particularly the nucleus accumbens (NAc) are essential to the reinforcing properties of addictive drugs (Hyman, 1996; Koob, 1996). Drugs of abuse, such as psychostimulants, opiates, nicotine, alcohol, and cannabinoids, alter dopamine levels in the NAc (Kalivas and Duffy, 1990; Self and Nestler, 1995; Tanda et al., 1997; Pontieri et al., 1998). Because of intrinsic and network properties, the projection cells of the NAc, the GABAergic medium-spiny neurons, depend on glutamatergic excitatory afferents to generate action potentials (Pennartz et al., 1994). Glutamatergic transmission in the NAc participates in the effects of opiates and psychostimulants (Pulvirenti et al., 1989, 1991, 1992; Pap and Bradberry, 1995; Cornish and Kalivas, 2000) and is altered by chronic drug treatment (Nie et al., 1994; Pierce et al., 1996a,b; Manzoni et al., 1998). Although cannabis derivatives are the most common illicit drugs, little is known of their actions in the mesolimbic regions and the NAc. In particular, the potential effects of cannabinoids on the excitatory afferents to the NAc have never been explored.

The specific purpose of this study was to identify the localization and functions of CB1 receptors at the glutamatergic cortical–NAc synapses. Using immunohistochemical techniques, we identified CB1 receptors on afferents making synaptic-like contacts with GABAergic neurons (presumably medium spiny neurons) of the NAc. It was found that CB1 receptors inhibit glutamatergic excitatory synaptic transmission through the modulation of presynaptic K^+ conductances.

MATERIALS AND METHODS

Immunohistochemistry. After deep anesthesia with sodium pentobarbital (50 mg/kg), five C57BL/6 male mice were perfused through the ascending aorta with PBS, pH 7.4, followed by 300 ml of fixative composed of 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. The brain was then dissected and fixed by immersion in the fixative without glutaraldehyde for 12 hr at 4°C. It was then cut sagittally with a vibratome into 40- to 50-μm-thick sections. These were carefully rinsed in PBS and treated for single- or double-fluorescence immunostaining. Sections were incubated for 48 hr at 4°C with either one

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or two specific antibodies including (1) a rabbit polyclonal antibody against CB1 cannabinoid receptor (diluted 1:500; kindly provided by Dr. K. Mackie, Department of Anesthesiology, University of Washington, Seattle, WA), or (2) both the anti-CB1 and a mouse monoclonal antibody against GABA (diluted 1:1000; Chemicon, Temecula, CA). After careful rinsing in PBS, sections were incubated for 2 hr with an anti-rabbit IgG conjugated with Cy3 (diluted 1:1000; The Jackson Laboratory, Bar Harbor, ME), alone (single immunostaining), or in combination with an anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) (diluted 1:200; Sigma, St. Louis, MO). After rinsing, the sections were mounted in mowiol (Calbiochem, La Jolla, CA) and examined under a Bio-Rad (Hercules, CA) MCR 1024 confocal laser scanning microscope equipped with a krypton–argon mixed gas laser. Two laser lines emitting at 488 and 568 nm were used for exciting the FITC- and Cy3-conjugated secondary antibodies, respectively. The organization of immunostained (IS) structures was studied either 1/ on single confocal images 1- to 2-μm thick, or 2/ on reconstructed sections made by projecting z-series of 20–40 consecutive confocal images 1 μm apart, collected throughout the thickness of the vibratome section. The background noise of each confocal image was reduced by averaging five image inputs. Unaltered digitized images were transferred to a personal computer, and PowerPoint (Microsoft, Seattle, WA) was used to prepare and print final figures. The specificity of the antibodies has been assessed previously by the absorption test (see Tsou et al., 1998 for anti-CB1 and Szabat et al., 1992 for anti-GABA). Additional controls consisted of omitting the primary antibodies and applying the secondary antibodies alone.

Electrophysiology. Whole-cell patch-clamp and extracellular field recordings were made from medium spiny neurons in parasagittal slices of mouse NAc. This method has been described previously (Manzoni et al., 1998). In brief, mouse (male C57BL/6, 4–6 weeks) were anesthetized with fluorene and decapitated. The brain was sliced (300–400 μm) in the parasagittal plane using a vibratome and maintained in physiological saline at 4°C. Slices containing the NAc were stored at least 1 hr at room temperature before being placed in the recording chamber and superfused (2 ml/min) with artificial CSF (ACSF) that contained (in mM): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 18 $NaHCO_3$, 1.2 NaH_2PO_4, and 11 glucose, equilibrated with 95% O_2–5% CO_2. All experiments were done at room temperature. The superfusion medium contained picrotoxin (100 μM) to block $GABA_A$ receptors. All drugs were added at the final concentration to the superfusion medium.

For field potential recordings, the recording pipette was filled with a 3 M NaCl solution, and both the field EPSP (fEPSP) slope (calculated with a least square method) and fEPSP amplitude were measured.

For patch-clamp experiments, cells were visualized using an upright microscope with infrared illumination, and recordings were made with whole-cell electrodes containing the following (in mM): 128 Cs-gluconate, 20 NaCl, 1 $MgCl_2$, 1 EGTA, 0.3 $CaCl_2$, 2 Mg-ATP, 0.3 GTP, and 0.2 cAMP buffered with 10 HEPES, pH 7.3. Electrode resistance was 4 MΩ, acceptable access resistance was <15 MΩ, and the holding potential was −70 mV. An Axopatch-1D (Axon Instruments, Foster City, CA) was used to record the data, which were filtered at 1–2 kHz, digitized at 5 kHz on a DigiData 1200 interface (Axon Instruments), and collected on a personal computer using ACQUIS-1 software (Bio-Logic, St. Egreve, France). To evoke synaptic currents, stimuli (100–150 μsec duration) were delivered at 0.033 Hz through bipolar tungsten electrodes placed at the prefrontal cortex–accumbens border (Manzoni et al., 1997, 1998). Recordings were made in the rostromedial dorsal accumbens close to the anterior commissure. Evoked EPSC amplitudes were measured by averaging a 5 msec window around the peak and subtracting the average value obtained during a 10 msec window immediately before the stimulus. Two stimuli were applied at an interval of 50 msec, and the paired-pulse ratio (PPR) was calculated by dividing the amplitude of the EPSC evoked by the second stimulus by the amplitude of the first EPSC evoked by the first stimulus. A change in the paired-pulse ratio is thought to result from the alteration in transmitter release caused by a presynaptic mechanism (Manabe et al., 1993).

Miniature EPSCs (mEPSCs) were recorded in the presence of tetrodotoxin (TTX) (300 nM) using Axoscope 1.1.1. mEPSC amplitude and inter-interval time were measured using Axograph 3.6. For this analysis, a template of mEPSCs having the width and time course of a typical synaptic event [a double exponential: $f(t) = \exp(-t/\text{Rise}) - \exp(-t/\text{Decay})$, where rise is 0.5 msec, and decay is 3 msec] was slid along the data trace one point at a time. At each position, this template is optimally scaled and offset to fit the data, and a detection criterion is calculated. The detection criterion is the template scaling factor divided by the goodness-of-fit at each position. An event is detected when this criterion exceeds a threshold and reaches a sharp maximum. The limit of detection was 2 pA (Manzoni and Williams, 1999).

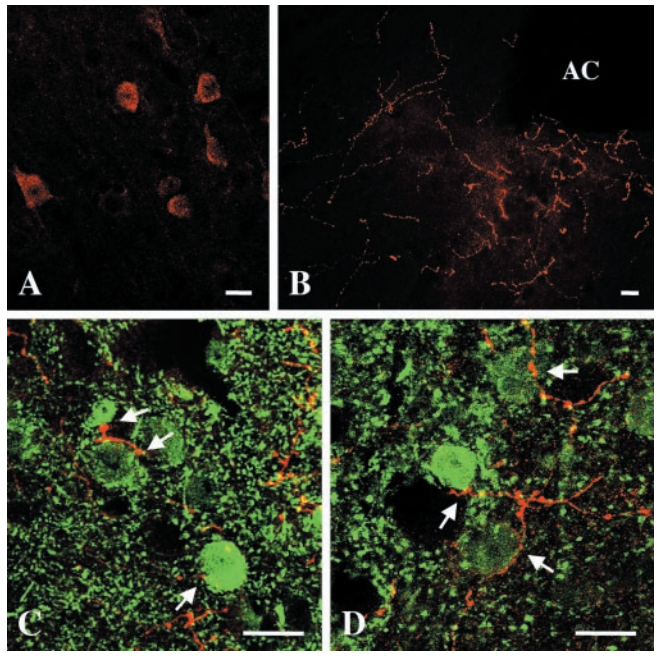

Figure 1. Localization of CB1 receptors in the prelimbic cortex and the nucleus accumbens. Confocal images of single- or double-immunostained sections. *A*, Single immunostaining for CB1 shows that intense labeling is associated with perikarya located in the prelimbic cortex. *B*, CB1 labeling was also associated with large varicose axonal fibers that extend throughout the core of the NAc surrounding the anterior commissure. *C*, *D*, Double immunostaining for both CB1 (*red*) and GABA (*green*) shows that, within the NAc, CB1-IS fibers form terminal (*arrows* in *C*) or en passant (*arrows* in *D*) synaptic-like contacts with GABA-IS perikarya. *AC*, Anterior commissure. Scale bars, 25 μm.

The fitting of concentration–response curves were calculated according to $y = \{y_{max} - y_{min}/1 + (x/EC_{50})n\} + y_{min}$ (where y_{max} is response in the absence of agonist, y_{min} is response remaining in the presence of maximal agonist concentration, x is concentration, EC_{50} is concentration of agonist producing 50% of the maximal response, and n is the slope) with Kaleidagraph software (Abelbeck Software, Readinig, PA). All values are given as mean ± SEM. Statistical analyses were done with the Mann–Whitney U test or the Kolmogorov–Smirnov tests using Statview (Abacus Concepts, Calabasas, CA), and $p < 0.05$ was taken as indicating statistical significance. The drugs used were as follows: WIN 55,212,2 (WIN-2), WIN 55,212,3, and CP 55940 were from Tocris Cookson (Bristol, UK); TTX, picrotoxin, $BaCl_2$, $CdCl_2$, forskolin, adenosine, 4-AP, and nimodipine were from Sigma (St. Quentin Fallavier, France); ω-Agatoxin-IVA and ω-Conotoxin GVIA were from Alomone Labs (Jerusalem, Israel); and SR 141716A was generously provided by Sanofi Recherche (Montpellier, France).

WIN-2, WIN 55,212,3, CP 55940, and SR 141716A were prepared as (10 mM) stock solutions in DMSO. Final concentrations were <0.1% DMSO.

RESULTS

Throughout the brain, the organization of the CB1-IS structures conformed to previous descriptions in the rat (Tsou et al., 1998). Dispersed CB1-IS perikarya were observed throughout the cortex, the hippocampus, and the olfactory bulb. More specifically, numerous CB1-IS perikarya were detected in the prelimbic cortex area (Fig. 1*A*), which knowingly massively projects to the NAc (Wright and Groenewegen, 1995). Dense plexuses of intensely labeled CB1-IS fibers were detected throughout the cortex, the hippocampus, the anterior olfactory nucleus, and the olfactory bulb. Although more dispersed than in these regions, a number of CB1-IS fibers were also detected throughout the striatum. Within

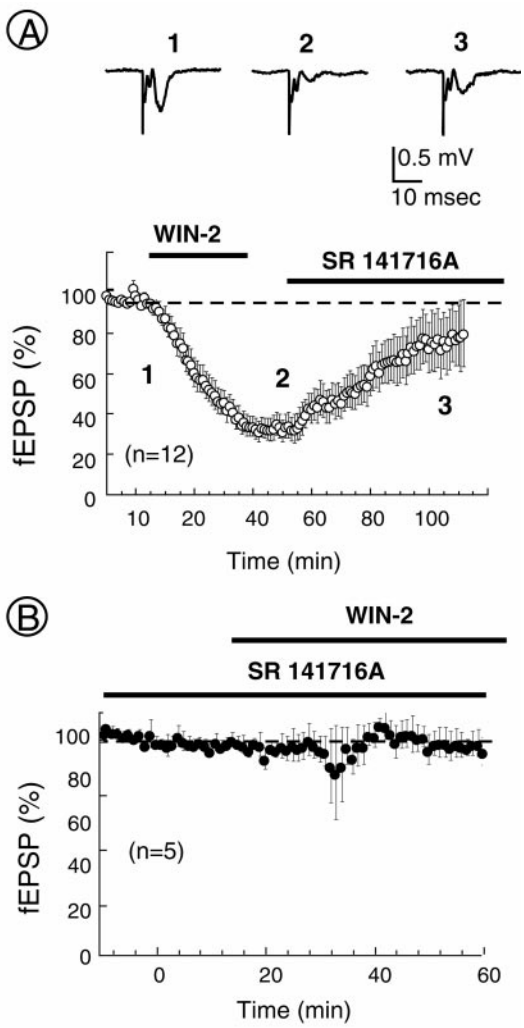

Figure 2. CB1 receptor-mediated inhibition of evoked excitatory synaptic transmission in mice nucleus accumbens. *A*, The cannabimimetic WIN-2 (10 μM) reduced the fEPSP on average to 34 ± 5% (n = 12) of its basal value. *Traces* represent averages of 10 consecutive EPSPs. The effects of WIN-2 were reversed by the selective CB1 antagonist SR 141716A (10 μM). *B*, Preincubation of the slices with 10 μM of the CB1 antagonist SR 141716A was without effect on basal synaptic transmission (data not shown) but completely prevented the WIN-2-induced inhibition (n = 5).

the NAc, these CB1-IS fibers were mostly localized to the core of the nucleus in which they appeared as large, poorly branched fibers exhibiting a large number of intensely immunostained varicosities (Fig. 1*B*). Double-immunostaining experiments indicated that, throughout the NAc, these CB1-IS fibers established frequent en passant or terminal synaptic-like contacts with GABA-IS perikarya or dendritic processes (Fig. 1*C*,*D*).

The present immunohistochemical identification of presynaptic CB1 receptors on axonal fibers in the NAc prompted us to explore the effects of cannabimimetics at the glutamatergic synapses between the prelimbic cortex and the NAc.

Extracellular field potential recordings were performed to measure the effects of CB1 agonists on synaptic responses evoked by stimulating prelimbic cortex fibers (Manzoni et al., 1997, 1998). It was found that fEPSPs, in the core of the NAc, were strongly inhibited by CB1 agonists. Bath-applied WIN-2 (10 μM) reduced the fEPSP to 35.3 ± 5.8% of its basal value (n = 12) (Fig. 2*A*). This depression was strongly reversed by the selective CB1 antagonist SR 141716A (10 μM) (Rinaldi-Carmona et al., 1994), suggesting the implication of cannabinoid receptors of the CB1 subtype (Fig. 2*A*). We also verified that the WIN-2-induced inhibition was totally prevented by preincubation with the CB1 antagonist SR 141716A (Fig. 2*B*).

To further confirm the implication of CB1 receptors, the following experiments were performed. First, we determined that the effects of WIN-2 were dose-dependent with an EC_{50} of 291 ± 13 nM (Fig. 3*A*). Second, because high doses (1–10 μM) of WIN-2 were routinely used to ensure fast and complete drug penetration into the slices, we studied the effects of WIN 55,212,3, the enantiomer of WIN-2, which lacks affinity for cannabinoid binding sites. Figure 3*B* shows that, contrary to the WIN-2-induced inhibition, the effects of WIN 55,212,3 were not blocked by a selective CB1 antagonist. Finally, the WIN-2 effect on fEPSP was not modified by preincubation with saturating doses of WIN 55,212,3 (Fig. 3*B*). These experiments confirmed the specificity of WIN-2 for CB1 receptors in the NAc slice preparation. Third, it was verified that the CB1 agonist CP 55940 (2 μM), which does not relate structurally to WIN-2, also reduced the fEPSP (Fig. 3*C*). Together, these data strongly suggest that the inhibitory effects of WIN-2 can be attributed to the activation of CB1 receptors.

Our immunohistochemical data demonstrated the association of CB1 immunostaining with axonal fibers innervating the NAc and naturally suggest a presynaptic site of action. In a first attempt to functionally assess the origin of the CB1-mediated depression, the variation of the PPR of excitatory transmission, a presynaptic phenomenon thought to reflect changes in transmitter release and sensitive to presynaptic manipulations, was measured. Bath application of 10 μM WIN-2 induced a depression of EPSCs recorded in the whole-cell patch-clamp configuration (Cs-gluconate-based intracellular medium, holding potential of −70 mV). The depression of evoked release was accompanied by an increase of the PPR. At the peak of the depression induced by WIN-2, the EPSC was reduced to 51 ± 11% of its control value, whereas the PPR was up to 169 ± 46% of its control value (n = 6). This finding suggested that CB1 receptors could decrease presynaptic neurotransmitter release.

To further characterize the site of action of CB1 receptors, mEPSCs were recorded. A decrease in the frequency of the mEPSCs is interpreted to be a result of a presynaptic action (e.g., a reduction in transmitter release), whereas a decrease in mEPSCs amplitude classically reveals a postsynaptic site of action (Katz, 1966). We first verified that a high concentration of the Ca^{2+} channel blocker cadmium (100 μM) did not affect mEPSCs frequency or amplitude recorded in the presence of TTX (Fig. 4*A*,*B*). This showed that, in the NAc, mEPSCs are totally independent of external Ca^{2+} entry. What are the effects of WIN-2 on the Ca^{2+}-independent mEPSCs? A typical experiment shows that, in the presence of TTX, the mEPSCs frequency was depressed by WIN-2, whereas the mEPSCs amplitude remained unaffected (Fig. 4*C*). Accordingly, the distribution of the mEPSCs amplitude was not modified by WIN-2 (Fig. 4*D*), whereas the time interval distribution was shifted to the right (Fig. 4*E*). These data suggest that CB1-mediated inhibition of action potential-independent synaptic transmission does not require interactions with presynaptic Ca^{2+} channels. In all cases, the experiments demonstrated that the CB1-mediated inhibition is mediated by presynaptic receptors.

What are the presynaptic targets of CB1 receptors? How do

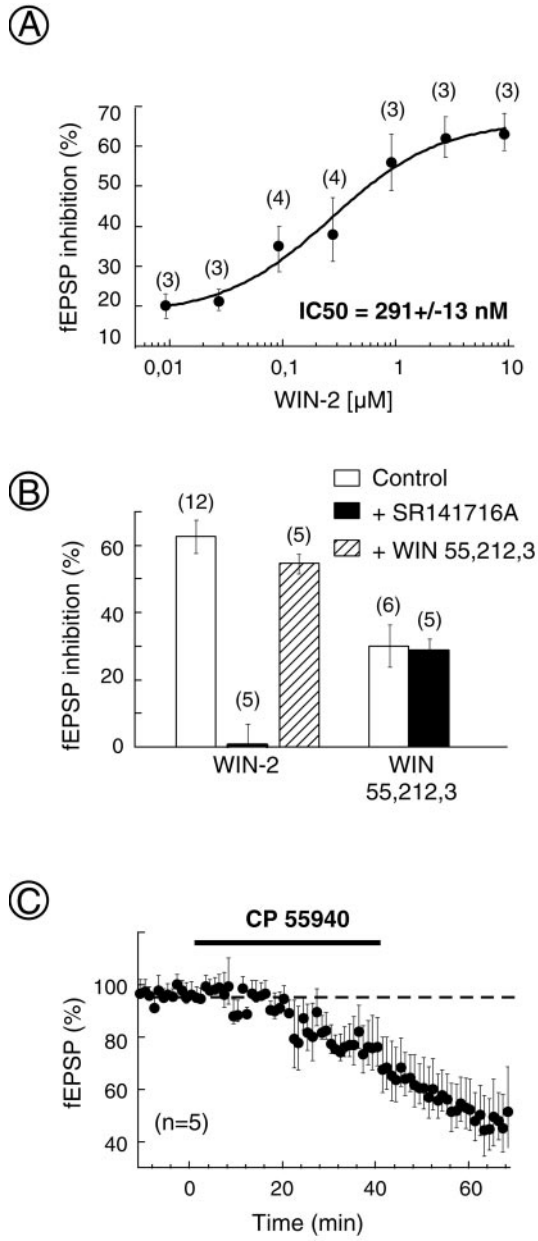

Figure 3. Pharmacological characterization of the CB1-mediated inhibition. *A*, Dose–response curve for the CB1 agonist WIN-2. The EC_{50} for WIN-2 was 291 ± 13 nM. Each *point* is expressed as the percentage of inhibition of basal evoked transmission, and the error bar represents the SEM. *B*, WIN 55,212,3 (10 μM), an enantiomer of WIN-2 inactive at CB1 binding sites, caused a small but significant fEPSP inhibition that, contrary to the WIN-2-induced inhibition, was not reversed by SR 141716A (10 μM; *black bar*). WIN-2-mediated fEPSP reduction was additive to the WIN 55,212,3 effect (*hatched bar*). These data are in agreement with the idea that WIN-2 at concentrations up to 10 μM is selective for CB1 receptors. *C*, The cannabinoid agonist CP 55940 (2 μM; $n = 5$), which is not structurally related to WIN-2, also inhibited fEPSP.

they inhibit synaptic transmission at the cortical afferents to the NAc? Theoretically, CB1 receptors could inhibit synaptic release of glutamate because of their negative coupling to AC types I, III, V, VI, and VIII (Rhee et al., 2000) and the resulting decrease in intracellular cAMP levels, either through the inhibition of presynaptic voltage-sensitive calcium channels (VSCCs), which would decrease intraterminal Ca^{2+} levels and reduce evoked synaptic transmission (Mackie and Hille, 1992; Chan and Yung, 1998; Sullivan, 1999; Hoffman and Lupica, 2000) or through the activation of potassium channels that would cause a strong hyperpolarization of the synaptic terminal ("synaptic shunt") (Deadwyler et al., 1995; Mackie et al., 1995; McAllister et al., 1999).

To determine whether the AC–cAMP pathway interacted with the CB1-induced synaptic depression, the effects of forskolin, a powerful activator of AC (Seamon and Daly, 1986), were examined. Bath application of forskolin (10 μM) reliably enhanced NAc fEPSPs of ~50% (Fig. 5*A*). When the forskolin effect had reached its plateau, WIN-2 was perfused and induced a depression identical to what was observed in control conditions (Fig. 5*B*). Thus, in the NAc, CB1 receptors inhibit glutamate synaptic release independently of cAMP levels.

The fact that CB1 activation could inhibit the Ca^{2+}-independent release of glutamate in the NAc (Fig. 4) did not exclude an additional interaction with the Ca^{2+} channels underlying evoked synaptic release. In fact, in the hippocampus, CB1 receptors have been shown to inhibit evoked glutamatergic and GABAergic synaptic transmission via an interaction with VSCCs (Chan and Yung, 1998; Sullivan, 1999; Hoffman and Lupica, 2000). It was therefore decided to alter presynaptic Ca^{2+} entry by blocking L-, N-, or P/Q-type Ca^{2+} channels with nimodipine, ω-Conotoxin-GVIA, or ω-Agatoxin-IVA, respectively. Figure 6*A* shows in a representative experiment that, similar to what we described previously in the rat NAc (Manzoni et al., 1997), ω-Conotoxin-GVIA-sensitive Ca^{2+} channels (presumably of the N-type) are responsible for most of the evoked transmission in mice NAc. All of the experiments performed with ω-Conotoxin-GVIA (1 μM, 15–20 min) gave similar results and are summarized Figure 6*B*. In the presence of ω-Conotoxin-GVIA (1 μM), WIN-2 depressed the fEPSP to a similar extent as in control conditions. Similarly, bath perfusion with 200 nM ω-Agatoxin-IVA or 1 μM nimodipine to selectively block the P/Q or L-type channels, respectively, reduced by 15–20% synaptic transmission but did not affect the inhibitory actions of WIN-2 (Fig. 6*C*). It was concluded that, in the NAc, the CB1-induced depression does not require the reduction of presynaptic Ca^{2+} entry through N-, P/Q-, or L-type channels.

Voltage-dependent and -independent K^+ channels are modulated by CB1 receptors (Deadwyler et al., 1995; Henry and Chavkin, 1995; Mackie et al., 1995; Garcia et al., 1998; McAllister et al., 1999). Therefore, the actions of K^+-channels blockers on the cannabimimetics-induced presynaptic inhibition were assessed. Bath application of 4-AP (100 μM), $BaCl_2$ (300 μM), or the combination of both caused a large enhancement of the duration and the size of the evoked fEPSP. When voltage-dependent K^+ conductances were blocked by 4-AP (100 μM), the WIN-2-induced inhibition was significantly reduced (Fig. 7*A*). Blockade of K^+ conductances with $BaCl_2$ (300 μM) also caused a clear reduction in the inhibitory actions of the cannabimimetic (Fig. 7*A*), consistent with the involvement of G-protein-gated inwardly rectifying K^+ channel-like conductances (Coetzee et al., 1999). When the K^+ channels blockers were applied together, the inhibitory actions of WIN-2 were completely prevented (Fig. 7*A*). Notably, in the same experiments, adenosine (200 μM) could still inhibit fEPSPs, suggesting that presynaptic inhibitory processes were still intact [43 ± 3 ($n = 3$) and 45 ± 6% ($n = 5$) inhibition of the fEPSP in control and 4-AP–$BaCl_2$, respectively] (Fig. 7*A*). Nonetheless, an important limitation in the interpretation of these experiments is that blockade of presynaptic K^+ channels augments presynaptic action potential duration and could cause the saturation of the Ca^{2+}-dependent release process (Colmers et al., 1988; Hoffman and

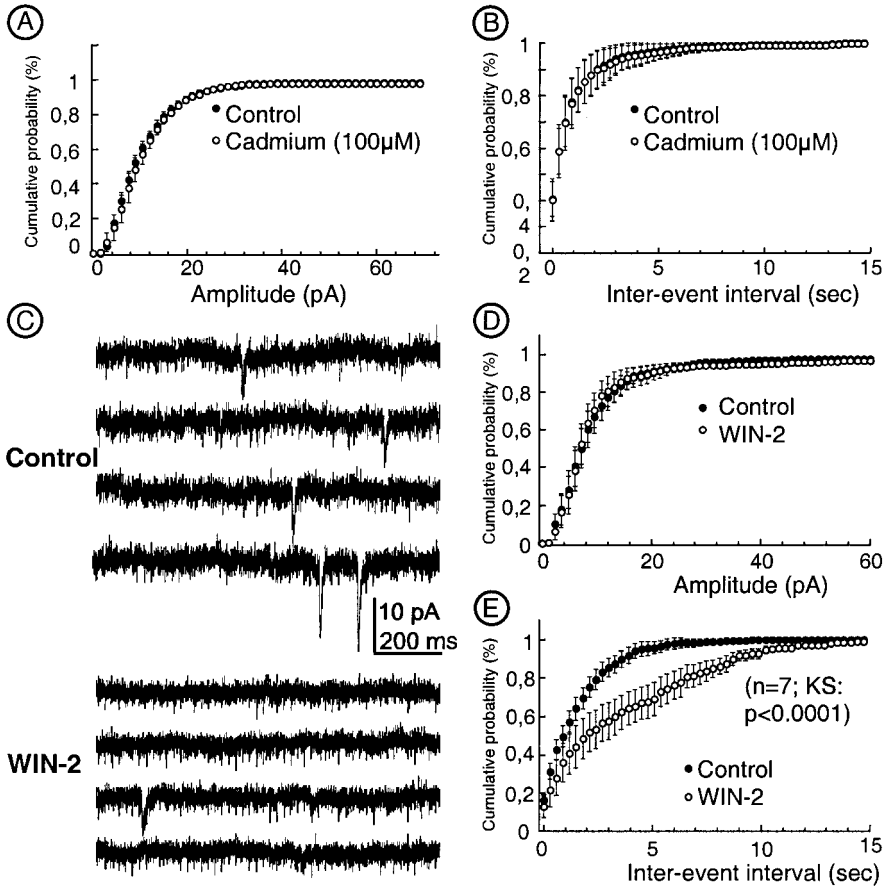

Figure 4. CB1-induced inhibition of action potential-independent glutamate release reveals a presynaptic site of action. *A*, *B*, Miniature EPSCs recorded in the presence of TTX are independent of Ca^{2+} channels activation. Bath application of cadmium chloride (100 μM) to block voltage-sensitive Ca^{2+} channels changed neither the amplitude distribution (*A*) nor the frequency distribution of mEPSCs (*B*). *C*, Representative consecutive 1 sec current sweeps from a cell (holding potential of −70 mV) in which mEPSCs were recorded in the absence or presence of 10 μM WIN-2. *D*, The distribution of mEPSC amplitude before and during the application of the CB1 agonist, in the seven cells recorded, was unchanged after 20 min bath perfusion of WIN-2. *E*, The distribution of the time intervals between successive mEPSCs in all of the neurons recorded (same as above) revealed that the mEPSCs frequency was reduced during WIN-2 application. For control conditions, a total of 1849 events were detected over a period of 9.14 ± 1.14 min (n = 7; range, 6–14 min). In the same neurons, a total of 1004 events were collected after 10–15 min WIN-2 perfusion over a period of 11.86 ± 2.20 min (range, 6–20 min).

Lupica, 2000). Thus, it is possible that the lack of effect of WIN-2 in the presence of K^+ channels blockers is attributable to indirect actions on some Ca^{2+}-dependent processes. To address this question, $[Ca^{2+}]_o$ was lowered (from 2.4 to 0.3–1.2 mM, with corresponding increases in Mg^{2+} concentrations to correct for the osmolarity change) to reduce the size of evoked EPSPs in the presence of 4-AP and $BaCl_2$. Lowering $[Ca^{2+}]_o$, although efficient at reducing fEPSPs evoked in the presence of 4-AP and $BaCl_2$, did not restore WIN-2-induced inhibition (Fig. 7*B*,*C*). We verified that, in low $[Ca^{2+}]_o$, the WIN-2 inhibition was identical to what was observed in standard extracellular medium (Fig. 7*C*). Together, these data demonstrate that presynaptic CB1 receptors modulate synaptic transmission through the modulation of K^+ conductances.

DISCUSSION

The results show that, in the mouse NAc, CB1 receptors are present on large fibers making synaptic-like contacts with GABA containing perikarya or processes. It was found that cannabimimetics, with a pharmacology consistent with the involvement of CB1 receptors, caused a profound inhibition of glutamatergic transmission at the synapses between the prelimbic cortex and the NAc. The electrophysiological analysis corroborated the anatomical data and pointed out a presynaptic site of action for CB1 receptors. It is also shown that the CB1 receptor-mediated presynaptic inhibition of glutamatergic transmission is independent of the cAMP–PKA cascade and of the inhibition of VSCCs. These data thereby are in striking contrast to those showing that the modulation of presynaptic VSCCs is responsible for the CB1 receptor-mediated inhibition of transmitter release in the hippocampus (Sullivan, 1999; Hoffman and Lupica, 2000) and substantia nigra pars compacta (Chan and Yung, 1998). Finally, we provide evidence for a role of voltage-sensitive and -insensitive K^+ channels in mediating CB1 presynaptic inhibition of glutamate release because low concentrations of the potassium channel blockers 4-AP and $BaCl_2$ completely prevented the CB1 effect.

The effects of CB1 receptors are presynaptic

CB1 receptors have been found in axons, cell bodies, and dendrites (Herkenham et al., 1991; Tsou et al., 1998) and could theoretically modulate synaptic transmission at presynaptic and postsynaptic sites via a variety of cellular effectors. Practically, when CB1-mediated inhibition of synaptic transmission was observed in the CNS, it always resulted from presynaptic actions (Levenes et al., 1998; Shen and Thayer, 1998; Sullivan, 1999; Auclair et al., 2000; Hoffman and Lupica, 2000). Before this study, no data were available on the presence of CB1 receptors at excitatory or inhibitory synapses of the NAc. We present compelling evidences for a presynaptic site of action of CB1 receptors at the excitatory synapses to the NAc medium spiny neurons. First, we identified immunostaining for CB1 receptors on axons making synaptic-like contacts to GABAergic neurons of the NAc (Fig. 1*C*,*D*). Intense CB1 immunostaining was located within the cytoplasm of a number of perikarya dispersed throughout the prelimbic cortex (Fig. 1*A*). Although suggestive of a cortical localization, our observation does not exclude other origins for the CB1 receptors containing neurons making synapses in the NAc (e.g., hippocampus, amygdala. . .). Second, electrophysiological analysis showed an increase in the paired-pulse ratio of evoked EPSCs and a decrease of the mEPSC frequency (but not their amplitude) during CB1 inhibition

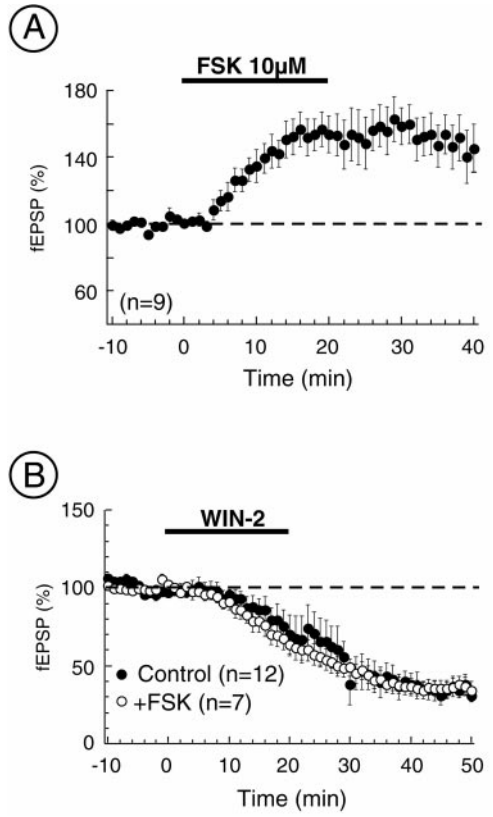

Figure 5. CB1-mediated inhibition is independent of cAMP levels. *A*, The adenylate cyclase activator forskolin (*FSK*; 10 μM, 20 min) caused a robust augmentation of the fEPSP in NAc slices ($n = 9$). *B*, The WIN-2-mediated fEPSP inhibition was not affected when cAMP levels were augmented by bath application of forskolin ($n = 7$). Forskolin was applied 20 min before and during the WIN-2 application (10 μM).

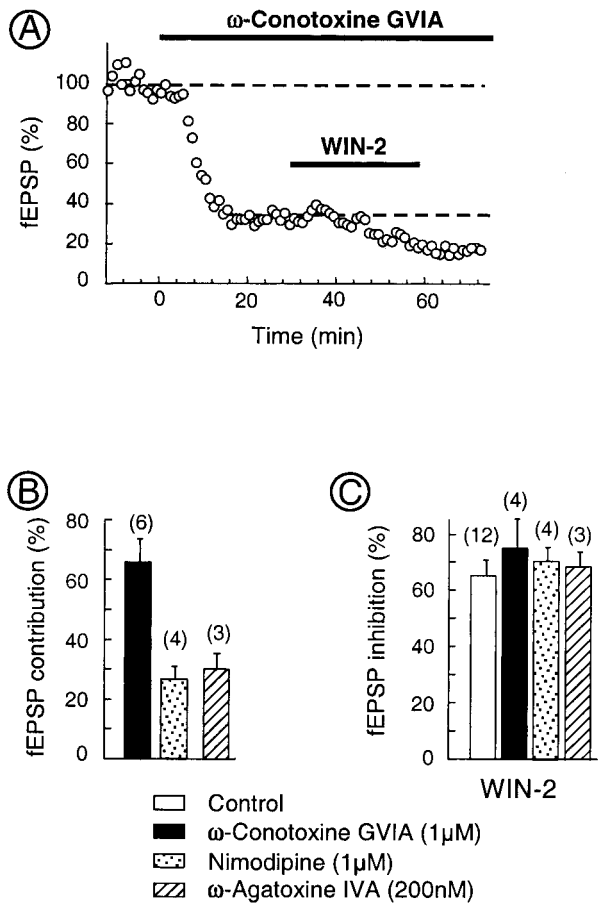

Figure 6. The WIN-2-induced inhibition does not require N-, L-, or P/Q-type Ca^{2+} channels modulation. *A*, Typical experiment in which the slice was perfused with 1 μM ω-Conotoxin-GVIA, which blocked ~60% of the fEPSP. It is clear that the fraction of synaptic transmission insensitive to ω-Conotoxin-GVIA was still sensitive to the CB1 agonist WIN-2 (10 μM). *B*, Summary of all of the experiments performed as above with specific blockers of N-type (ω-conotoxin-GVIA, 1 μM), L-type (nimodipine, 1 μM); and P/Q-type (ω-Agatoxin-IVA, 200 nN) voltage-sensitive Ca^{2+} channels. The histogram of the maximum inhibitions caused by perfusion of selective agents reveals how the different types of voltage-sensitive Ca^{2+} channels contribute to the evoked release of glutamate in the NAc. *C*, Histogram of the maximum 10 μM WIN-2-mediated inhibition in the presence of Ca^{2+} channel inhibitors. All experiments were performed as in *A*.

(Fig. 4). Thus, the simplest interpretation of our anatomical and electrophysiological data are that a presynaptic mechanism is responsible for the actions of the cannabinoid agonists at the excitatory synapses to the NAc.

As shown previously for $GABA_B$ and adenosine A1 receptors (Scanziani et al., 1992; Wu and Saggau, 1994; Dittman and Regehr, 1996), the CB1 receptor seems to activate two separate and inhibitory mechanisms in the terminal; one is likely to involve a direct action on the release machinery and is responsible for the reduction of action potential independent transmitter release, whereas the other one would involve one or more K^+ conductances and participates to the inhibition of evoked transmitter release (see below).

The CB1 effect is independent of the cAMP–PKA cascade

Like many other receptors coupled to pertussis toxin-sensitive G-protein, CB1 receptor actions include inhibition of adenylyl cyclase. Some of the effects of cannabinoid withdrawal depend on the activation of the cAMP–PKA cascade (Tzavara et al., 2000), and chronic treatment with CB1 agonists causes a superactivation of several adenylyl cyclases isozymes (Rhee et al., 2000), underlying the importance of the cAMP–PKA cascade in the long-term effects of cannabinoids. The acute inhibition of adenylyl cyclase by CB1 receptors can have several synaptic consequences, mainly through the modulation of presynaptic ion channels or the direct inhibition of transmitter release. In the guinea pig myenteric plexus, elevation of cAMP levels by the adenylyl cyclase activator forskolin significantly reduced the maximum inhibitory response to WIN-2 (Coutts and Pertwee, 1998). In agreement with the idea that presynaptic inhibition mediated through the adenylyl cyclase cascade are not universal at CB1 sensitive synapses, the CB1 inhibition was not occluded in forskolin-treated slices (Fig. 5*B*).

The CB1-mediated inhibition of synaptic transmission does not require voltage-sensitive Ca^{2+} channels

One of the most documented actions of CB1 receptors in neuronal cells is their inhibitory coupling with voltage-sensitive Ca^{2+} channels (Caulfield and Brown, 1992; Mackie and Hille, 1992; Mackie et al., 1993, 1995; Pan et al., 1996; Twitchell et al., 1997; Shen and Thayer, 1998). Not surprisingly, recordings from substantia nigra pars reticulata neurons showed that cannabinoids exert a presynaptic inhibition on GABAergic transmission via the CB1 inhibition of Cd^{2+}-sensitive presynaptic Ca^{2+} channels (Chan and Yung, 1998). More recently, it was shown that Cd^{2+}

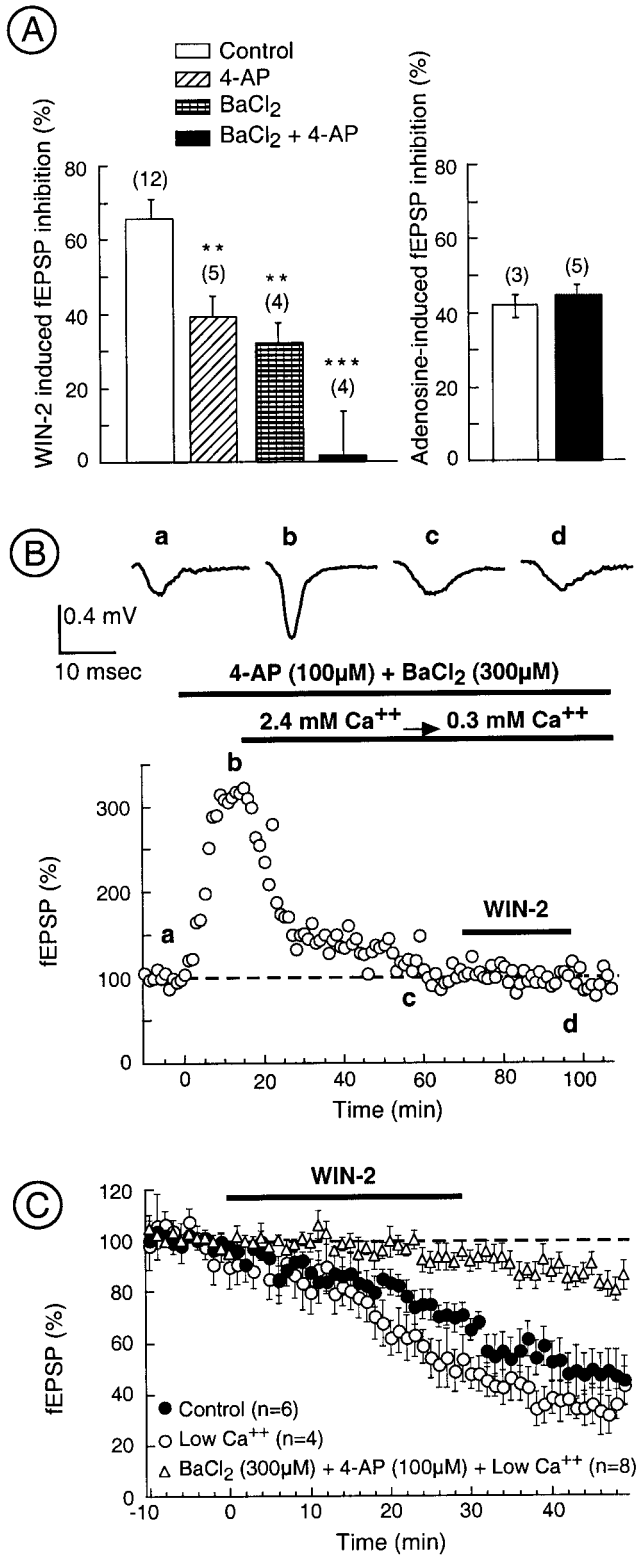

Figure 7. Effects of K^+ channels blockade on the CB1-induced inhibition of glutamatergic synaptic transmission in the NAc. *A*, In standard ACSF (2.4 mM CaCl2; *white bar*), 4-AP (100 μM; *hatched bar*), and $BaCl_2$ (300 μM; *dotted bar*) reduced the WIN-2-induced fEPSP inhibition. WIN-2 reduced fEPSP by 39.1 ± 5.5% ($n = 5$; $p = 0.015$) and 32.3 ± 9.4% ($n = 4$; $p = 0.029$) in the presence of 4-AP and $BaCl_2$, respectively, compared with 65.6 ± 5.4% ($n = 12$) in control. When added together, 4-AP and $BaCl_2$ (*black bar*) completely prevented the WIN-2 (10 μM) inhibition (1.7 ± 11%; $n = 4$; $p = 0.002$). In contrast, the adenosine (200 μM; *white bar*)-induced inhibition was not affected by pretreatment with 4-AP and $BaCl_2$ (*black bar*). *B*, Representative experiment in which the large enhancement of the fEPSP caused by the combination of 4-AP and $BaCl_2$ was reduced by lowering extracellular Ca^{2+} concentration from 2.4 to 0.3 mM. In this condition, 4-AP and $BaCl_2$ still prevented the 1 μM WIN-2-induced inhibition. *C*, Summary of all the experiments performed as above ($n = 8$). It is clear that 4-AP (100 μM) and $BaCl_2$ (300 μM) blocked the CB1-mediated inhibition in low external Ca^{2+}. Lowering the external Ca^{2+} affected neither the time course nor the amplitude of the WIN-2 (1 μM)-mediated inhibition of the fEPSP [compare the inhibition in control ACSF (*filled circles*) with the inhibition in low external Ca^{2+} (*open circles*)].

prevented cannabinoids actions on spontaneous GABA release (Hoffman and Lupica, 2000) and that CB1 receptors can inhibit evoked glutamate release via the modulation of N- and P/Q-type Ca^{2+} channels (Sullivan, 1999). In marked contrast, we found that CB1 receptors inhibit action potential-independent and evoked synaptic transmission independently from the modulation of presynaptic voltage-sensitive Ca^{2+} channels (Fig. 6).

A role for K^+ channels in mediating the CB1 induced inhibition of glutamate release

Modulation of voltage-dependent and -independent potassium conductances is another well described effect of CB1 receptors (Deadwyler et al., 1995; Henry and Chavkin, 1995; Mackie et al., 1995; Garcia et al., 1998; McAllister et al., 1999; Schweitzer, 2000). Moreover, recent reports have underlined the importance of K^+ conductances in mediating some opioid receptor actions on inhibitory (Vaughan et al., 1997) and excitatory synaptic transmission (Simmons and Chavkin, 1996; Manzoni and Williams, 1999). Surprisingly, it was found that blockade of presynaptic K^+ channels hampered the cannabinoid effects in standard conditions but also when $[Ca^{2+}]_o$ was lowered to reduce the size of evoked EPSPs and control for indirect actions of K^+ channel blockers on Ca^{2+}-dependent processes (Fig. 7). Similar experiments performed at the GABAergic synapses of the CA1 region of the hippocampus led to opposite results because lowering $[Ca^{2+}]_o$ restored the inhibitory properties of WIN-2 (Colmers et al., 1988; Hoffman and Lupica, 2000). It is likely that this apparent discrepancy is simply an example of the diversity of the cellular effectors of CB1 receptors at the synapses of the CNS.

Conclusions

How do cannabinoids activate mesolimbic dopamine neurons (Gessa et al., 1998) and increase dopamine levels in the NAc (Tanda et al., 1997; Szabo et al., 1999)? In substantia nigra pars reticulata, the presence of CB1-immunoreactive fibers (Tsou et al., 1998) suggests that disinhibition of GABAergic afferents participates to the net excitatory actions of cannabinoids. In contrast, CB1 receptors are absent from the ventral tegmental area (VTA) (Herkenham et al., 1991; Tsou et al., 1998) and the present demonstration of CB1 receptors at the glutamatergic synapses in the NAc provides another means for cannabinoids to excite dopaminergic neurons. The glutamatergic afferents to the NAc control the firing of the NAc GABAergic neurons, which in turn inhibit the dopaminergic neurons of the VTA. Via the reduction of excitatory transmission in the NAc, cannabinoids could disinhibit dopamine cells of the VTA, increase their firing rate, and trigger the release of dopamine in the nucleus accumbens.

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