

## Inhibition of Striatal Dopamine Release by CB1 Receptor Activation Requires Nonsynaptic Communication Involving GABA, H<sub>2</sub>O<sub>2</sub>, and K<sub>ATP</sub> Channels

By: Zsuzsanna Sidló, Patricia H. Reggio, and Margaret E. Rice

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### **Abstract:**

The main psychoactive component of marijuana,  $\Delta^9$ -tetrahydrocannabinol (THC), acts in the CNS via type 1 cannabinoid receptors (CB1Rs). The behavioral consequences of THC or synthetic CB1R agonists include suppression of motor activity. One explanation for movement suppression might be inhibition of striatal dopamine (DA) release by CB1Rs, which are densely localized in motor striatum; however, data from previous studies are inconclusive. Here we examined the effect of CB1R activation on locally evoked DA release monitored with carbon-fiber microelectrodes and fast-scan cyclic voltammetry in striatal slices. Consistent with previous reports, DA release evoked by a single stimulus pulse was unaffected by WIN55,212-2, a cannabinoid receptor agonist. However, when DA release was evoked by a train of stimuli, WIN55,212-2 caused a significant decrease in evoked extracellular DA concentration ( $[DA]_o$ ), implicating the involvement of local striatal circuitry, with similar suppression seen in guinea pig, rat, and mouse striatum. Pulse-train evoked  $[DA]_o$  was not altered by either AM251, an inverse CB1R agonist, or VCHSR1, a neutral antagonist, indicating the absence of DA release regulation by endogenous cannabinoids with the stimulation protocol used. However, both CB1R antagonists prevented and reversed suppression of evoked  $[DA]_o$  by WIN55,212-2. The effect of WIN55,212-2 was also prevented by picrotoxin, a GABA<sub>A</sub> receptor antagonist, and by catalase, a metabolizing enzyme for hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Furthermore, blockade of ATP-sensitive K<sup>+</sup> (K<sub>ATP</sub>) channels by tolbutamide or glibenclamide prevented the effect of WIN55,212-2 on DA release. Together, these data indicate that suppression of DA release by CB1R activation within striatum occurs via a novel nonsynaptic mechanism that involves GABA release inhibition, increased generation of the diffusible messenger H<sub>2</sub>O<sub>2</sub>, and activation of K<sub>ATP</sub> channels to inhibit DA release. In addition, the findings suggest a possible physiological substrate for the motor effects of cannabinoid agonist administration.

### **Article:**

The main psychoactive component of marijuana,  $\Delta^9$ -tetrahydrocannabinol (THC), exerts its CNS effects via activation of type 1 cannabinoid receptors (CB1Rs), which are highly expressed in the brain, including the basal ganglia (Herkenham et al., 1990, Herkenham et al., 1991a, Herkenham et al., 1991b and Mailleux and Vanderhaeghen, 1992). Consistent with effects on basal-ganglia function, CB1R activation by THC or other agonists alters motor performance, with dose-dependent effects ranging from increased activity (Sulcova et al., 1998 and Sañudo-Peña et al.,

2000) to inhibition of spontaneous activity, including decreased and/or irregular locomotion (Gough and Olley, 1978, Navarro et al., 1993, Onaivi et al., 1996, Sañudo-Peña et al., 2000 and Shi et al., 2005), and ultimately immobility or catalepsy (Gough and Olley, 1978, Onaivi et al., 1996 and Sulcova et al., 1998). Moreover, other studies suggest that THC or other cannabinoid agonists also show promise as therapeutic agents to ameliorate hyperkinetic movement, including the tics of Tourette's syndrome (Muller-Vahl et al., 2003) and DOPA-induced dyskinesia that can accompany the treatment of Parkinson's disease (Sieradzan et al., 2001, Segovia et al., 2003, Ferrer et al., 2003 and Venderova et al., 2004; but see Carroll et al., 2004). Given the key role of the nigrostriatal dopamine (DA) system in basal-ganglia mediated movement (Albin et al., 1989 and Olanow and Tatton, 1999), a likely contributing factor to these motor effects is decreased DA release in the dorsolateral striatum.

Although CB1R activation has been shown to inhibit release of striatal transmitters, including GABA and glutamate (e.g., Szabo et al., 1998, Gerdeman and Lovinger, 2001, Huang et al., 2001, Gerdeman et al., 2002, Ronesi et al., 2004, Kőfalvi et al., 2005, Freiman et al., 2006, Kreitzer and Malenka, 2005 and Kreitzer and Malenka, 2007), few studies have addressed consequences of CB1R activation on DA release. Using fast-scan cyclic voltammetry to monitor evoked extracellular DA concentration ( $[DA]_o$ ) in rat striatal slices, Szabo et al. (1999) found no effect of CB1R agonists or antagonists on single-pulse evoked  $[DA]_o$  in either dorsal striatum or ventral striatum (nucleus accumbens). In other *in vitro* studies, in which prolonged electrical stimulation (3-min stimulation at 0.5–10 Hz) was used to elicit release of [ $^3H$ ]DA from striatal slices, cannabinoid agonist application produced either a decrease or no effect on DA release (Cadogan et al., 1997 and Kőfalvi et al., 2005). *In vivo* voltammetric data from the nucleus accumbens indicate that systemic administration of the cannabinoid agonist WIN55,212-2 leads to inhibition of  $[DA]_o$  evoked by pulse-train stimulation of the median forebrain bundle (Cheer et al., 2004), although the frequency of spontaneous  $[DA]_o$  transients increased. However, whether these observations reflect a direct effect within the nucleus accumbens or the involvement of long feedback pathways between the accumbens and midbrain dopamine neurons in the ventral tegmental area was not addressed.

It is important to note that modulation of single-pulse evoked  $[DA]_o$  by a given receptor agonist implies a direct effect on receptors located on DA axons, e.g., D2 autoreceptors (Palij et al., 1990 and Benoit-Marand et al., 2001) or nicotinic acetylcholine (ACh) receptors (Zhou et al., 2001, Rice and Cragg, 2004 and Zhang and Sulzer, 2004). On the other hand, pulse-train stimulation can reveal indirect effects of striatal microcircuitry and concurrently released transmitters on DA release, including the release of GABA and glutamate (Avshalumov et al., 2003 and Avshalumov et al., 2007). The lack of effect of CB1R agonists on DA release elicited by a single stimulus in previous studies (Szabo et al., 1999), therefore, suggests the absence of direct presynaptic modulation of axonal DA release. This implies that any effect of CB1Rs on striatal DA release must be indirect.

The aim of the present work was to resolve whether CB1R activation within striatum modulates axonal DA release. Using striatal slices to eliminate confounding effects of long feedback pathways, we compared the effect of WIN55,212-2 on endogenous  $[DA]_o$  evoked by single-pulse or pulse-train stimulation to assess direct *versus* indirect effects of CB1R activation on DA release. Evoked  $[DA]_o$  was monitored in real-time using fast-scan cyclic voltammetry and

carbon-fiber microelectrodes. Pharmacological agents were used as appropriate to determine the mechanism of DA release suppression seen during pulse-train stimulation.

## METHODS

### *Brain slice preparation*

For most studies, coronal forebrain slices were prepared from young adult male guinea pigs; however, in some experiments, slices were prepared from adult rats (male, Wistar) or mice (male, C57/BL6). All animals were deeply anesthetized with 40 mg/kg pentobarbital (intraperitoneal) and decapitated. After removal, the brain was cooled for 1–2 min in ice-cold HEPES-buffered ACSF containing (in mM), 120 NaCl; 5 KCl; 20 NaHCO<sub>3</sub>; 6.7 HEPES acid; 3.3 HEPES salt; 2 CaCl<sub>2</sub>; 2 MgSO<sub>4</sub>; and 10 glucose, saturated with 95% O<sub>2</sub>/5% CO<sub>2</sub> (Rice et al., 1997). Striatum was blocked and coronal slices (400 μm thickness for guinea-pig slices; 350 μm for rat and mouse) were cut on a Vibratome; slices were maintained for at least 1 h at room temperature in HEPES-buffered ACSF. For recording, slices were transferred to a submersion chamber at 32 °C and superfused at 1.2 mL/min with recording ACSF containing the following (in mM): 124 NaCl; 3.7 KCl; 26 NaHCO<sub>3</sub>; 2.4 CaCl<sub>2</sub>; 1.3 MgSO<sub>4</sub>; 1.3 KH<sub>2</sub>PO<sub>4</sub>; and 10 glucose, equilibrated with 95% O<sub>2</sub>/5% CO<sub>2</sub> (Rice et al., 1997).

### *Voltammetric recording*

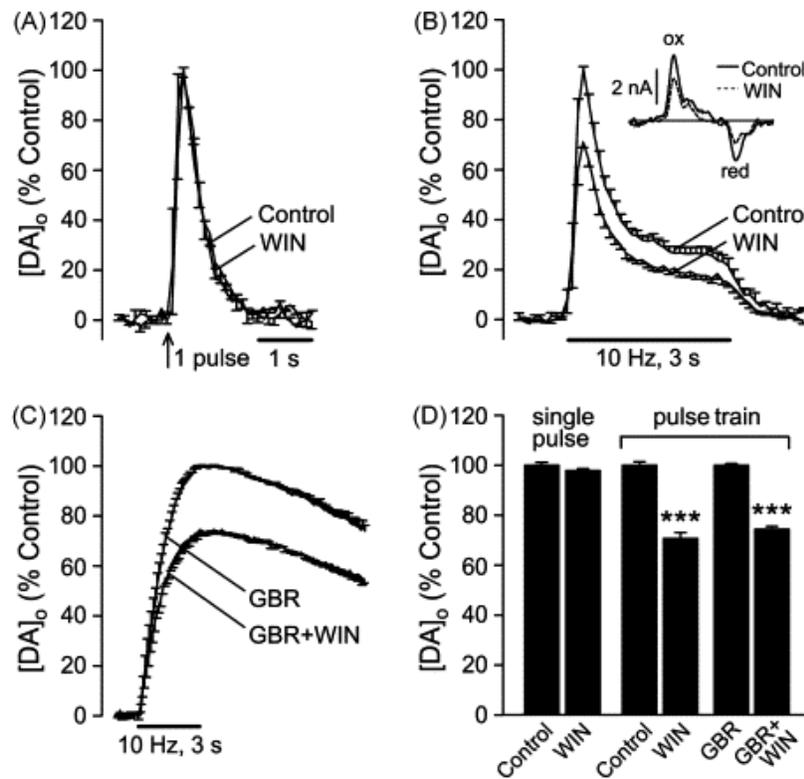
Axon-terminal DA release was elicited in dorsolateral striatum using a surface concentric bipolar stimulating electrode and detected at a carbon-fiber microelectrode positioned ~100 μm away. Single-pulse or pulse-train (10 Hz, 30 pulses) stimulation was used with pulse duration of 100 μs and amplitude of 0.6–0.8 mA; DA release under these conditions is tetrodotoxin-sensitive and Ca<sup>2+</sup>-dependent (Chen and Rice, 2001). Evoked [DA]<sub>o</sub> was monitored using fast-scan cyclic voltammetry (Millar Voltammeter; Dr. Julian Millar, Queen Mary, University of London, UK) with 8-μm carbon-fiber microelectrodes that were made in-house using methods modified from Millar and Pelling (2001) or obtained commercially (MPB Electrodes, London, UK; or WPI, Sarasota, FL, USA). Scan rate was 800 V/s, voltage range was –0.7 to +1.3 V *versus* Ag/AgCl, and sampling interval was 100 ms. Data acquisition and analysis were as described previously (Chen and Rice, 2001). Released DA was identified by characteristic oxidation and reduction peak potentials (e.g., Fig. 1B, inset); [DA]<sub>o</sub> was calculated from post-experiment electrode calibration in the recording chamber at 32 °C in all media used in a given experiment.

### *Chemicals and statistical analysis*

The non-selective cannabinoid agonist WIN55,212-2 (*R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]1,4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate), the CB1R inverse agonist AM251 (1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-1-piperidinyl-1*H*-pyrazole-3-carboxamide), and the DA uptake inhibitor GBR-12909 were from Tocris (Ellisville, MO, USA). The neutral antagonist VCHSR1 (5-(4-chlorophenyl)-3-[(*E*)-2-cyclohexylethenyl]-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole) was synthesized in the Reggio Laboratory (Hurst et al., 2002). PicROTOXIN, glybenclamide (glyburide, glibenclamide), tolbutamide, dimethylsulfoxide (DMSO), and components of ACSF were from Sigma (St. Louis, MO, USA). Catalase (bovine liver) was from Calbiochem (La Jolla, CA, USA). Individual stock solutions of WIN55,212-2, AM251, and VCHSR1 were made in DMSO. Final DMSO level was 0.01% in ACSF, which was used to obtain control data for these agents; 0.01% DMSO alone had no effect on evoked [DA]<sub>o</sub>. For studies with the effect of WIN55,212-2 on DA uptake, slices

were preincubated in GBR-12909 (2  $\mu\text{M}$ ) in HEPES-buffered ACSF for at least 1 h before experimentation; GBR-12909 was also included in the recording ACSF. For studies with catalase (500 U/mL), active or heat-inactivated enzyme was included in the recording ACSF throughout the experiment; catalase was inactivated as described previously (Avshalumov et al., 2003). In all studies, at least three consistent evoked  $[\text{DA}]_o$  records were obtained to provide control data before drug application. To minimize the effect of inter-slice variation, data were normalized, with the average of the final 2–3 control records taken as 100%; the response in the presence of the drug(s) was evaluated as % same-site control. Data are given as means  $\pm$  S.E.M.,  $n$  = number of slices, with 1–2 slices per animal. Statistical evaluation was performed using paired Student's  $t$ -test or one-way ANOVA with appropriate post hoc analysis.

**Figure 1:**



Effect of CB1R activation on DA release in dorsolateral striatum. (A) Averaged DA release records after single-pulse stimulation under control conditions and in the presence of WIN55,212-2 (WIN; 5  $\mu\text{M}$ ) indicate no effect of WIN on either the amplitude or the time-course of evoked  $[\text{DA}]_o$  ( $n = 3$ ). (B) Effect of WIN (1–5  $\mu\text{M}$ ; see Section 2.1) on averaged  $[\text{DA}]_o$  evoked by pulse-train stimulation (30 pulses, 10 Hz) ( $n = 13$ ). Inset, representative cyclic voltammograms for peak evoked  $[\text{DA}]_o$  at a given site under control conditions and in the presence of WIN; DA was identified by characteristic oxidation (ox) and reduction (red) peak potentials (typically +600 and -200 mV vs. Ag/AgCl). (C) The effect of WIN (5  $\mu\text{M}$ ) persisted when the DA transporter (DAT) was inhibited by GBR-12909 (GBR; 2  $\mu\text{M}$ ) ( $n = 3$ ). (D) Activation of CB1Rs by WIN had no effect on single-pulse evoked  $[\text{DA}]_o$  ( $p > 0.05$  WIN vs. same-site control;  $n = 3$ ), but suppressed evoked  $[\text{DA}]_o$  during pulse-train stimulation (\*\*\*)  $p < 0.001$  WIN vs. same-site control;  $n = 13$ ) in ACSF alone, as well as when the DAT was inhibited by GBR (\*\*\*)  $p < 0.001$  GBR + WIN vs. GBR alone;  $n = 3$ ).

## RESULTS

### *Suppression of pulse-train evoked DA release by CB1R activation*

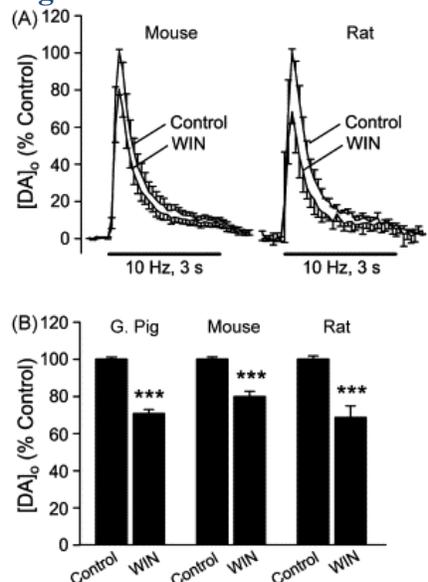
We first tested the effect of the CB1R agonist WIN55,212-2 on single-pulse evoked  $[\text{DA}]_o$  in dorsolateral striatum. Mean peak  $[\text{DA}]_o$  was  $1.3 \pm 0.4 \mu\text{M}$  ( $n = 3$ ). In agreement with previous studies (Szabo et al., 1999), WIN55,212-2 (5  $\mu\text{M}$ ) had no effect on single-pulse evoked  $[\text{DA}]_o$

( $p > 0.05$ ;  $n = 3$ ) (Fig. 1A and D). We then examined DA release evoked by pulse-train stimulation (10 Hz, 30 pulses); mean peak evoked  $[DA]_o$  was  $1.4 \pm 0.1 \mu\text{M}$  ( $n = 13$ ). In contrast to single-pulse evoked  $[DA]_o$ , pulse-train evoked  $[DA]_o$  was suppressed by  $\sim 30\%$  in the presence of WIN55,212-2 (Fig. 1B and D) ( $p < 0.001$ ;  $n = 13$ ). Maximal release suppression was achieved with 1–5  $\mu\text{M}$  WIN, depending on the batch, consistent with the range of effective concentrations reported previously for *in vitro* brain-slice studies (Cadogan et al., 1997, Szabo et al., 1998, Szabo et al., 1999, Hájos and Freund, 2002, Kőfalvi et al., 2005, Mátyás et al., 2006, Kreitzer and Malenka, 2005 and Kreitzer and Malenka, 2007). In the present studies, the minimal effective concentration was determined and used for each new batch of drug.

Monitored  $[DA]_o$  reflects the net effect of release and uptake, such that a decrease in evoked  $[DA]_o$  could reflect either decreased DA release or increased DA uptake via the DA transporter (DAT). The complete lack of effect of WIN55,212-2 on the time course of single-pulse evoked  $[DA]_o$  (Fig. 1A) argues against an effect of this drug on DA uptake. We then examined whether the effect of WIN55,212-2 (5  $\mu\text{M}$ ) on pulse-train evoked  $[DA]_o$  was altered when the DAT was inhibited by GBR-12909 (2  $\mu\text{M}$ ). Inhibition of the DAT increased and prolonged evoked  $[DA]_o$ , as reported previously (Chen and Rice, 2001 and Avshalumov et al., 2003). However, the  $\sim 30\%$  WIN-induced decrease in evoked  $[DA]_o$  (from  $4.6 \pm 0.4 \mu\text{M}$  to  $3.3 \pm 0.5 \mu\text{M}$ ;  $p < 0.001$ ;  $n = 3$ ) persisted in GBR-12909 (Fig. 1C and D), confirming that WIN55,212-2 alters DA release rather than uptake.

To ascertain that the effect of WIN55,212-2 was species-independent, we examined the effect of CB1 receptor activation in striatal slices from adult mouse (male, CD57/BL6) and adult rat (male, Wistar). WIN55,212-2 (5  $\mu\text{M}$ ) suppressed pulse-train evoked  $[DA]_o$  in dorsal striatum in both species, with a decrease of  $\sim 20\%$  in mouse striatum ( $n = 8$ ,  $p < 0.001$ ), and  $\sim 30\%$  in rat striatum ( $n = 4$ ,  $p < 0.001$ ) (Fig. 2).

**Figure 2:**

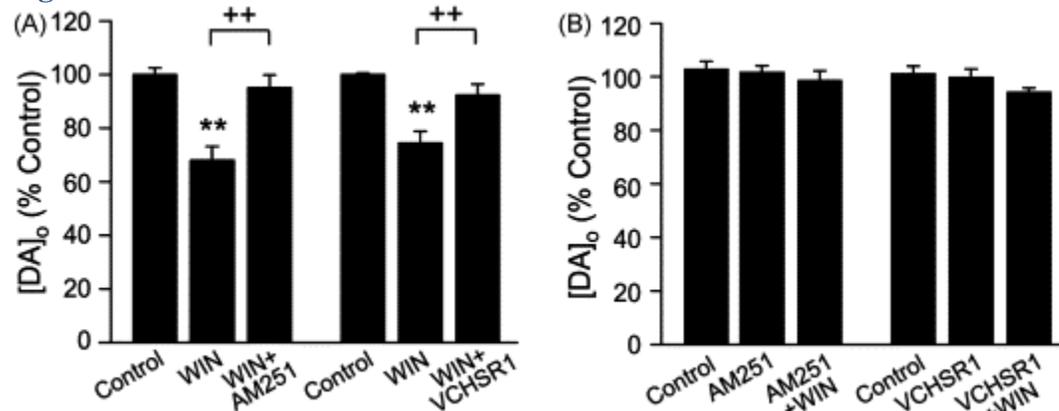


The effect of WIN55,212-2 on DA release is species independent. (A) Activation of CB1Rs by WIN55,212-2 (WIN; 5  $\mu\text{M}$ ) caused a similar decrease in pulse-train evoked  $[DA]_o$  in the dorsolateral striatum of rats and mice to that seen in guinea pig striatum (Fig. 1B). (B) Comparison of the effect of WIN on average peak evoked  $[DA]_o$  in guinea pig (\*\*\*)  $p < 0.001$  WIN vs. same-site control;  $n = 13$ ), mouse (\*\*\*)  $p < 0.001$ ;  $n = 8$ ), and rat (\*\*\*)  $p < 0.001$ ;  $n = 4$ ), striatum.

### Effect of CB1R antagonists

To confirm that the effect of WIN55,212-2 on DA release was CB1R-mediated, we examined whether release suppression could be reversed by CB1R antagonists. Although CB2 receptors have recently been identified in the CNS, these are found at much lower levels than CB1Rs ([Van Sickle et al., 2005] and [Gong et al., 2006]), so that CB2R involvement is unlikely. We examined the effect of a CB1R inverse agonist, AM251 (1–5  $\mu$ M; [Hájos and Freund, 2002], [Köfalvi et al., 2005], [Brown et al., 2003], [Ronesi and Lovinger, 2005], [Slanina et al., 2005], [Kreitzer and Malenka, 2005] and [Kreitzer and Malenka, 2007]) and a neutral antagonist, VCHSR1 (2  $\mu$ M; Hurst et al., 2002). Consistent with action at CB1Rs, the effect of WIN55,212-2 (1–5  $\mu$ M) was reversed by AM251 (1  $\mu$ M) in the continued presence of WIN ( $p > 0.05$  versus control;  $n = 4$ ) (Fig. 3A). The neutral antagonist VCHSR1 similarly reversed DA release suppression by WIN ( $p > 0.05$ ;  $n = 3$ ) (Fig. 3A), confirming that under the conditions tested, AM251 acted only as a CB1R antagonist.

**Figure 3:**



CB1R antagonists prevent and reverse the effect of WIN55,212-2 on DA release, but have no effect alone. (A) The effect of WIN55,212-2 (WIN; 1–5  $\mu$ M) on pulse-train evoked [DA]<sub>o</sub> was reversed by AM251 (1  $\mu$ M) (\*\* $p < 0.01$  WIN vs. control; \*\* $p < 0.01$  WIN vs. WIN + AM251;  $n = 4$ ) and VCHSR1 (2  $\mu$ M) (\*\* $p < 0.01$  WIN vs. control; \*\* $p < 0.01$  WIN vs. WIN + VCHSR1;  $n = 3$ ). (B) Neither AM251 (1–5  $\mu$ M) nor VCHSR1 (2  $\mu$ M) altered pulse-train evoked [DA]<sub>o</sub>, indicating the absence of endocannabinoid regulation of DA release under these conditions. AM251 and VCHSR1 also prevented the effect of WIN (1–5  $\mu$ M) (\* $p > 0.05$  for all comparisons;  $n = 3$ –4 per mean).

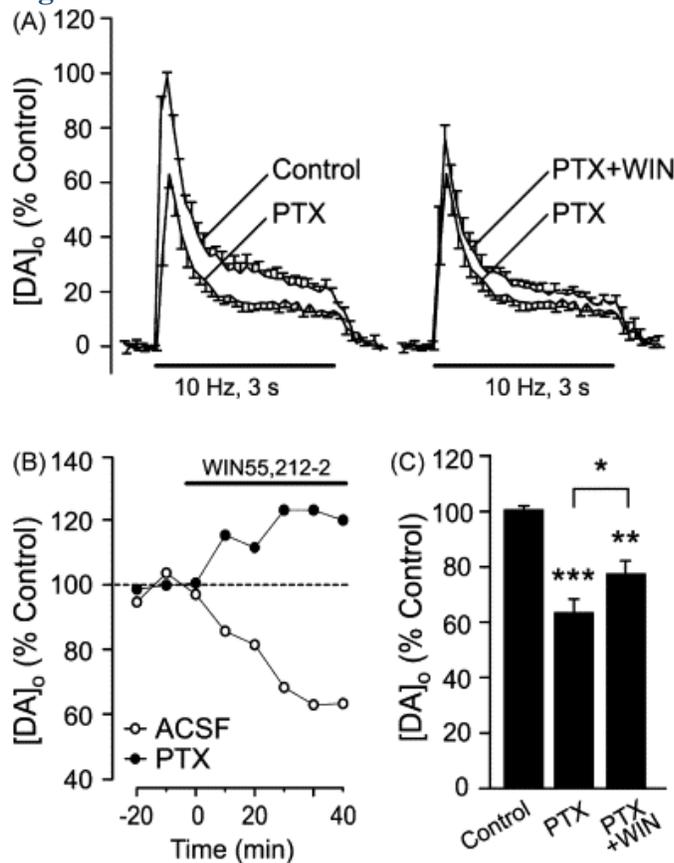
To test whether endogenous cannabinoid release contributed to DA release regulation during local pulse-train stimulation, we examined the effect of CB1R blockade on pulse-train evoked [DA]<sub>o</sub>. Under the stimulation conditions examined (10 Hz for 3 s), neither AM251 (1–5  $\mu$ M) ( $p > 0.05$  versus control;  $n = 4$ ) nor VCHSR1 (2  $\mu$ M) ( $p > 0.05$ ;  $n = 3$ ) altered peak evoked [DA]<sub>o</sub> (Fig. 3B), suggesting that modulatory endocannabinoids are not generated by this stimulation protocol. Lastly, WIN55,212-2 had no effect in the continued presence of these CB1R antagonists. Overall, therefore, these results show that the WIN55,212-2-induced suppression of evoked [DA]<sub>o</sub> is mediated by CB1Rs.

### CB1R activation suppresses DA release by inhibiting striatal GABA release

Local pulse-train stimulation elicits release not only of DA, but also of other transmitters, including GABA and glutamate. The effect of these concurrently released transmitters on evoked [DA]<sub>o</sub> can be revealed using receptor antagonists (Avshalumov et al., 2003). We noted that the suppression of pulse-train evoked [DA]<sub>o</sub> by WIN55,212-2 was similar to that seen when GABA<sub>A</sub> receptors (GABA<sub>A</sub>Rs) are blocked by picrotoxin (Avshalumov et al., 2003), which suggested that the effect on DA release might reflect inhibition of GABA release. To test this, we

examined the effect of WIN55,212-2 (5  $\mu$ M) when GABA<sub>A</sub>Rs were blocked by picrotoxin (100  $\mu$ M; Avshalumov et al., 2003). As reported previously, picrotoxin alone decreased evoked [DA]<sub>o</sub> by  $37 \pm 5\%$  ( $p < 0.001$  versus control;  $n = 5$ , Fig. 4A and C). In the continued presence of picrotoxin, the suppressing effect of WIN55,212-2 on evoked DA release was prevented, indicating a key role for GABA in mediating the effect of CB1R activation on DA release (Fig. 4). Interestingly, under these conditions, WIN caused a small but significant increase in evoked [DA]<sub>o</sub>, in contrast to the usual decrease (Fig. 4B and C) ( $p < 0.05$ , PTX versus PTX + WIN;  $n = 5$ ). This suggests that WIN55,212-2 also alters the release of at least one other modulatory transmitter, the influence of which was masked by the effect of GABA acting via GABA<sub>A</sub>Rs. Overall, these data demonstrate that the predominant effect of CB1R activation on striatal DA release is a consequence of GABA release inhibition.

**Figure 4:**



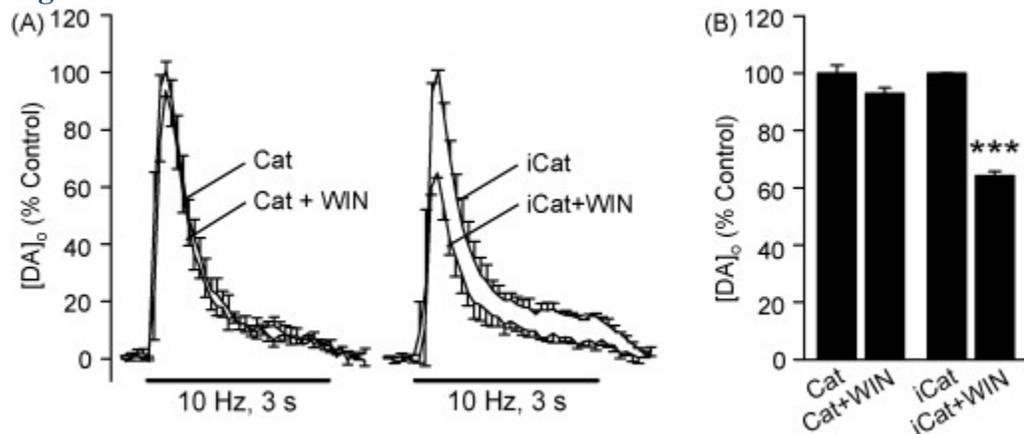
CB1R-dependent suppression of DA release is mediated by GABA release inhibition. (A) Averaged evoked [DA]<sub>o</sub> records showing a significant decrease in pulse-train evoked [DA]<sub>o</sub> when GABA<sub>A</sub>Rs are blocked by picrotoxin (PTX, 100  $\mu$ M,  $n = 5$ ) (left panel). The usual effect of WIN55,212-2 was prevented in the continued presence of PTX (right panel) ( $n = 5$ ). (B) Representative peak evoked [DA]<sub>o</sub> vs. time profiles showing the effect of WIN55,212-2 (WIN; 5  $\mu$ M) alone (open circles) and WIN applied in the presence of PTX (100  $\mu$ M) (filled circles) unmasking an increase in evoked [DA]<sub>o</sub> with WIN when GABA<sub>A</sub>Rs were blocked. Baseline data were normalized to respective control conditions (ACSF or PTX; see Section 2.3). (C) Comparison of mean peak evoked [DA]<sub>o</sub> in the presence of PTX vs. PTX + WIN confirmed a slight, but significant increase in evoked [DA]<sub>o</sub> with WIN application in the presence of PTX (\*\* $p < 0.001$  control vs. PTX; \* $p < 0.05$  PTX vs. PTX + WIN;  $n = 5$ ); means are normalized to control (ACSF alone).

### *H2O2 and ATP-sensitive K<sup>+</sup> channels mediate CB1R-dependent suppression of DA release*

Previous studies have shown that GABA<sub>A</sub>-receptor blockade increases activity-dependent production of the diffusible second messenger H<sub>2</sub>O<sub>2</sub>, which suppresses axonal DA release in striatum via opening of ATP-sensitive K<sup>+</sup> (K<sub>ATP</sub>) channels (Avshalumov et al.,

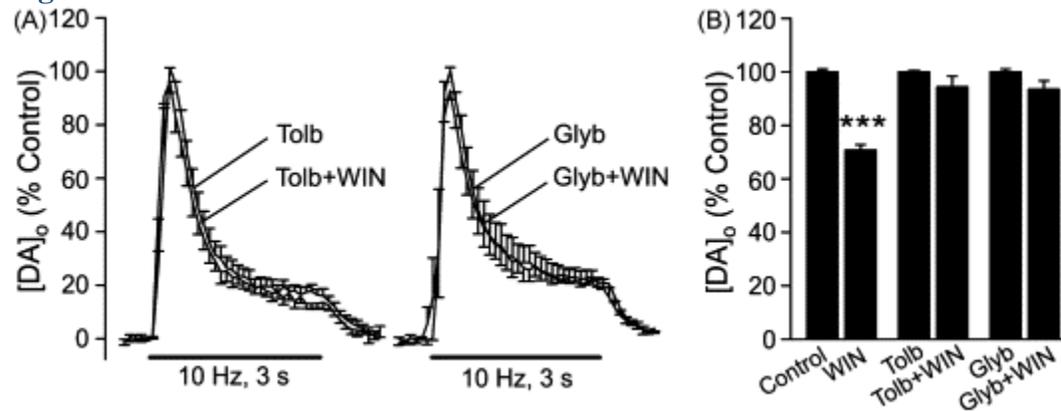
2003 and Avshalumov and Rice, 2003). To evaluate whether  $H_2O_2$  is required for the suppression of evoked  $[DA]_o$  by CB1R activation, we applied WIN55,212-2 (2  $\mu M$ ) in the presence of the  $H_2O_2$  metabolizing enzyme catalase (500 U/mL). Catalase prevented the effect of WIN55,212-2 ( $p > 0.05$ ;  $n = 4$ ) (Fig. 5), indicating that the diffusible messenger  $H_2O_2$  is essential for the effects of CB1R activation on DA release. In the presence of heat-inactivated catalase, WIN had its usual effect ( $p < 0.001$ ;  $n = 3$ ) (Fig. 5). We then tested whether  $K_{ATP}$  channels play a role in the mediation of CB1R-dependent suppression of DA release. When  $K_{ATP}$  channels were blocked either with tolbutamide (200  $\mu M$ ,  $n = 4$ ) or glybenclamide (10  $\mu M$ ,  $n = 5$ ), the effect of WIN55,212,2 (up to 5  $\mu M$ ) on evoked  $[DA]_o$  was prevented (Fig. 6).

**Figure 5:**



The effect of CB1R activation on evoked  $[DA]_o$  is prevented by the  $H_2O_2$ -metabolizing enzyme catalase. (A) Averaged pulse-train evoked  $[DA]_o$  records in the presence of catalase (Cat; 500 U/mL) and in catalase + WIN55,212-2 (WIN; 2  $\mu M$ ), and in heat-inactivated catalase (iCat) and iCat + WIN. (B) Catalase prevented the effect of WIN ( $p > 0.05$  Cat vs. Cat + WIN;  $n = 4$ ), whereas the usual WIN-induced suppression of evoked  $[DA]_o$  was seen in iCat (\*\*\*) ( $p < 0.001$  vs. same-site control;  $n = 3$ ).

**Figure 6:**



$K_{ATP}$  channel activation is required for CB1R-dependent suppression of DA release. (A) Averaged pulse-train evoked  $[DA]_o$  records in the presence of a  $K_{ATP}$  channel blocker tolbutamide (Tolb, 200  $\mu M$ ,  $n = 4$ ), or glybenclamide (Glyb, 10  $\mu M$ ,  $n = 5$ ) and in Tolb or Glyb + WIN55,212-2 (up to 5  $\mu M$ );  $K_{ATP}$  channel blockade prevented the effect of WIN on evoked  $[DA]_o$ . (B) Comparison of the effect of WIN on average peak evoked  $[DA]_o$  under control conditions (\*\*\*) ( $p < 0.001$ ;  $n = 13$ ), in tolbutamide ( $p > 0.05$ ;  $n = 4$ ), and in glybenclamide ( $p > 0.05$ ;  $n = 5$ ).

## DISCUSSION

The data presented here demonstrate that activation of CB1Rs within the dorsolateral striatum leads to inhibition of axonal DA release, independent of any effects in DA cell body regions. The

lack of effect of WIN55,212-2 on single-pulse evoked DA release is consistent with the absence of CB1Rs on DA axons. This further implies that suppression of  $[DA]_o$  evoked by pulse-train stimulation must be indirect. Indeed, the data indicate that modulation of striatal DA release is mediated predominantly by inhibition of GABA release, which leads to enhanced generation of the diffusible messenger  $H_2O_2$ , and consequent activation of  $K_{ATP}$  channels. This study therefore reveals that inhibition of DA release by CB1R activation in striatum involves a nonsynaptic mechanism, in which direct CB1R-agonist-mediated inhibition of one transmitter, GABA, regulates release of another, DA, via generation of a diffusible messenger,  $H_2O_2$ . This is analogous to the mechanism of DA release regulation involving glutamate receptor activation and consequent increase in  $[DA]_o$  via diffusible nitric oxide (NO), described previously by Kiss, Vizi, and colleagues (for review see Kiss and Vizi, 2001 and Kiss et al., 2004).

### ***CB1R activation inhibits axonal DA release in the striatum***

Increasing evidence indicates that CB1R activation can inhibit transmitter release, including GABA release in the striatum (Szabo et al., 1998, Kőfalvi et al., 2005 and Freiman et al., 2006). Inhibition of GABA release is mediated directly by presynaptic CB1Rs, with localization of CB1R protein on GABAergic terminals in rodent striatum (Julian et al., 2003, Fusco et al., 2004, Kőfalvi et al., 2005, Mátyás et al., 2006 and Uchigashima et al., 2007) and coexpression of mRNAs for CB1Rs and the GABAergic markers parvalbumin (Marsicano and Lutz, 1999) and GAD67 (Hohman and Herkenham, 2000). By contrast, CB1R localization on DA axons remains controversial. Although there is some evidence for a low level of CB1R expression on DA axons from double immunohistochemical labeling for CB1Rs and tyrosine hydroxylase (TH), the rate-limiting enzyme in DA synthesis (Wenger et al., 2003 and Kőfalvi et al., 2005), other studies found no colocalization of CB1Rs and DAT protein (Uchigashima et al., 2007) or CB1Rs and TH mRNAs (Julian et al., 2003). Voltammetric studies showing a lack of effect of WIN55,212-2 on single-pulse evoked  $[DA]_o$  in striatal slices, including data reported here, further support the absence of CB1Rs on DA axons in dorsal striatum (Szabo et al., 1999 and Fig. 1A).

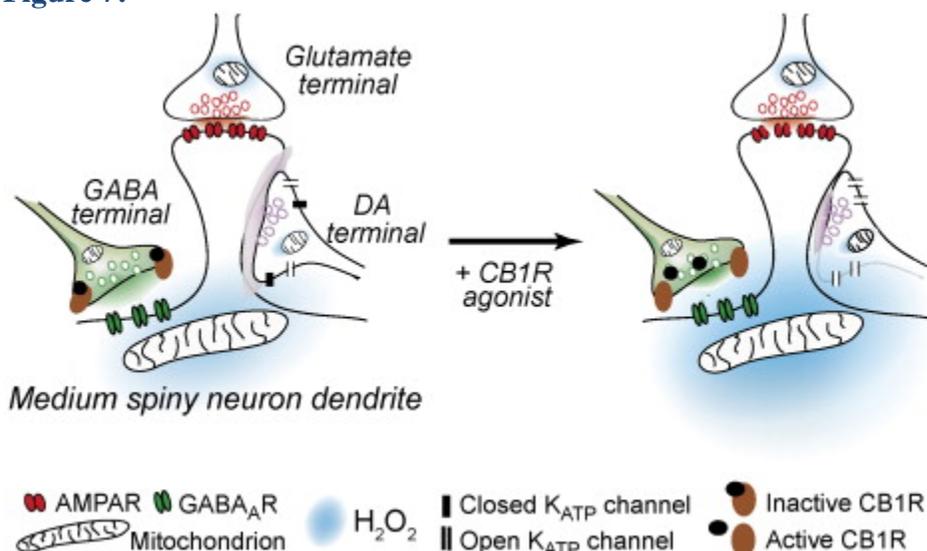
As noted above, a change in evoked  $[DA]_o$  can reflect a change in either DA release or in DAT-mediated DA uptake. Indeed, previous studies by Kiss et al. (1999) showed that NO increases basal  $[DA]_o$  in striatum monitored using *in vivo* microdialysis. Importantly, this effect was prevented by a DAT inhibitor, showing that NO acted via DA uptake, rather than release. We used a similar rationale here to test whether CB1R activation inhibited DA release or enhanced DA uptake, by applying the WIN55,212-2 in the presence of the DAT inhibitor GBR 12909. DAT blockade did not alter the inhibitory effect of WIN (Fig. 1C and D), which is consistent with the lack of effect on DA uptake observed *in vivo* (Cheer et al., 2004) and *in vitro* (Kőfalvi et al., 2005). Although a recent chronoamperometric study suggested that WIN might decrease striatal DAT activity *in vivo*, this was not altered by CB1R blockade, indicating a non-receptor mediated effect (Price et al., 2007). We found no evidence for such an effect in the present studies, given that the time course of single-pulse evoked  $[DA]_o$  was unaltered by WIN55,212-2 (Fig. 1A). Overall, therefore, our data confirm that CB1R activation in the present studies indeed modulates DA release, not uptake.

### ***CB1R-dependent inhibition of DA release requires GABA, $H_2O_2$ and $K_{ATP}$ channels***

How then does CB1R activation lead to DA release inhibition? We have reported previously that locally released glutamate and GABA in dorsolateral striatum can modulate DA release by

modulating levels of the diffusible messenger  $H_2O_2$  (Avshalumov et al., 2003 and Avshalumov et al., 2007). Based on the apparent absence of ionotropic glutamate or GABA receptors on DA axons, but abundant expression of these receptors on striatal medium spiny neurons (MSNs) (Bernard and Bolam, 1998, Chen et al., 1998 and Fujiyama et al., 2000), we have proposed that modulatory  $H_2O_2$  is generated in MSNs. Our working model involves a triad of DA, glutamate, and GABA synapses converging on MSN dendritic spines (Fig. 7, left panel) (Avshalumov et al., 2007). In this model, glutamatergic corticostriatal input activates MSNs via AMPA receptors, which leads to increased production of mitochondrial  $H_2O_2$ ;  $H_2O_2$  then diffuses to adjacent DA axons and activates  $K_{ATP}$  channels that inhibit DA release. Importantly, GABA acting at  $GABA_A$ Rs opposes glutamate-dependent excitation of MSNs and consequent  $H_2O_2$  generation (Fig. 7, left panel). When  $GABA_A$ Rs are blocked by picrotoxin, therefore,  $H_2O_2$  generation is enhanced, which causes a further inhibition of DA release that is prevented by catalase (Avshalumov et al., 2003).

**Figure 7:**



Schematic diagram showing the proposed mechanism for the effect of CB1R-dependent suppression of evoked DA release. Glutamate, GABA, and DA synapses converging on a medium spiny neuron (MSN) dendrite. Left panel: AMPA receptor activation by glutamate on a dendritic spine results in increased mitochondrial  $H_2O_2$  production, which diffuses to dopaminergic terminals to open  $K_{ATP}$  channels and thereby decrease DA release. This process is attenuated by activation of  $GABA_A$ Rs. The amount of  $H_2O_2$  produced is therefore determined by the net effect of glutamatergic excitation and GABAergic inhibition. Right panel: activation of CB1Rs on GABAergic terminals by the agonist WIN55,212-2 results in the suppression of GABA release, and a consequent enhancement of  $H_2O_2$  production. As a result, more  $K_{ATP}$  channels will be opened on dopaminergic terminals, causing suppression of evoked DA release.

Based on this model, one would predict that if activation of presynaptic CB1Rs on GABA terminals inhibits GABA release, this would mimic the effects of  $GABA_A$ R blockade and lead to increased  $H_2O_2$  generation and decreased DA release (Fig. 7, right panel). A decrease in local pulse-train evoked DA release was in fact seen when CB1Rs were activated by WIN55,212-2 (Fig. 1B and D). In further consonance with this model, the effect of WIN55,212-2 was prevented by catalase and by the  $K_{ATP}$  channel blockers tolbutamide and glybenclamide, confirming a key role for  $H_2O_2$  and  $K_{ATP}$  channel activation in DA release regulation by CB1Rs.

In addition to the predominant role of GABA and  $GABA_A$ Rs in mediating effects of CB1R activation on striatal DA release, the increase in evoked  $[DA]_o$  seen with WIN55,212-2 in the presence of the  $GABA_A$ R antagonist picrotoxin (Fig. 4A) revealed the involvement of at least

one other neurotransmitter system. The most likely candidates are ACh and glutamate, both of which are known to modulate striatal DA release. For example, tonically released ACh strongly regulates striatal DA release, such that a decrease in cholinergic tone markedly suppresses single-pulse evoked  $[DA]_o$  (Zhou et al., 2001, Rice and Cragg, 2004 and Zhang and Sulzer, 2004). Arguing against a significant influence of CB1R activation on ACh release in the present studies, however, is the lack of effect of WIN55,212-2 on single-pulse evoked  $[DA]_o$ , which would be predicted to cause a marked decrease in evoked  $[DA]_o$ . A lack of ACh involvement is further supported by previous studies showing no effect of WIN on evoked  $[^3H]ACh$  release from striatal slices (Cadogan et al., 1997). Moreover, anatomical data suggest that localization of CB1Rs on striatal cholinergic interneurons is minimal. Although Fusco et al. (2004) reported low levels of colocalization of CB1Rs and choline acetyl-transferase (ChAT), Uchigashima et al. (2007) found no CB1Rs on terminals labeled with vesicular acetylcholine transporter (VACHT) and Hohman and Herkenham (2000) found no colocalization of mRNAs for CB1R and the cholinergic markers VACHT or ChAT in striatum.

Glutamate also modulates striatal DA release: voltammetric studies show that blockade of glutamatergic AMPA receptors causes an increase in pulse-train evoked  $[DA]_o$  in striatal slices via diffusible  $H_2O_2$  (Avshalumov et al., 2003) (see Fig. 7, left panel). Importantly, anatomical studies have confirmed the presence of CB1Rs on glutamatergic terminals in striatum (Köfalvi et al., 2005 and Uchigashima et al., 2007). Moreover, WIN55,212-2 has been shown to suppress evoked  $[^3H]glutamate$  from striatal slices (Köfalvi et al., 2005) and to inhibit glutamate-dependent EPSCs in MSNs (e.g., Gerdeman and Lovinger, 2001, Huang et al., 2001, Kreitzer and Malenka, 2005 and Kreitzer and Malenka, 2007). Regardless of whether glutamate or yet another transmitter might also influence striatal DA release during CB1R activation, the present studies show that the primary effect of CB1R activation on DA release is mediated by GABA.

## IMPLICATIONS

The data reported here demonstrate that CB1R activation can indeed suppress DA release within striatum, independent of long feedback pathways. Dose-dependent variation in motor responses after administration of THC or synthetic CB1R agonists that range from increased motor activity to catalepsy (Gough and Olley, 1978, Navarro et al., 1993, Onaivi et al., 1996, Sañudo-Peña et al., 2000 and Shi et al., 2005) might reflect in part the competing consequences of DA release suppression on the efficacy of the direct and indirect motor pathways, as well as CB1R-mediated effects in other basal ganglia structures (Albin et al., 1989). Furthermore, the present findings suggest a physiological substrate for the clinically relevant observations that CB1R agonists can attenuate motor tics in Tourette's syndrome (Muller-Vahl et al., 2003) and DOPA-induced dyskinesia in a Parkinson's disease model (Sieradzan et al., 2001, Ferrer et al., 2003, Segovia et al., 2003 and Venderova et al., 2004).

More generally, our findings reveal a mechanism by which CB1R activation can inhibit transmitter release from sites that lack presynaptic CB1Rs, thereby mediating nonsynaptic communication (see Fuxe and Agnati, 1991 and Vizi, 2000 for reviews). Consequences of CB1R activation extend beyond synaptic boundaries by regulating GABA release, which in turn enhances production of the diffusible messenger  $H_2O_2$ , to inhibit DA release from adjacent axonal release sites. It is likely that endogenous cannabinoids can also act through these same pathways to inhibit DA release via suppression of GABA release and subsequent activation of

H<sub>2</sub>O<sub>2</sub>-sensitive K<sub>ATP</sub> channels. Although we found no evidence for endocannabinoid release with the stimulation protocol used in the present studies, previous work has shown that higher frequency stimulation paradigms do generate endocannabinoids, which induce synaptic plasticity in MSNs (Gerdeman et al., 2002, Ronesi et al., 2004, Kreitzer and Malenka, 2005 and Kreitzer and Malenka, 2007). Appreciation of the indirect consequences of CB1R activation via GABA release inhibition and diffusible H<sub>2</sub>O<sub>2</sub> may be important for interpretation of data from such studies.

Overall, our findings from motor striatum should help clarify the emerging roles of cannabinoid agents in the regulation of movement by the basal ganglia. Additionally, given the dorsoventral continuum of striatal circuitry (Voorn et al., 2004), the findings may also have implications for understanding regulation of DA release by CB1R activation in the nucleus accumbens, which receives mesolimbic DA input from the ventral tegmental area (van der Stelt and Di Marzo, 2003).

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