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An NMDA antagonist (LY 235959) attenuates abstinence-induced withdrawal of planarians following acute exposure to a cannabinoid agonist (WIN 52212-2)

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Abstract

The mechanisms that facilitate the development and expression of cannabinoid physical dependence in humans and other mammals are poorly understood. The present experiments used a planarian model to provide evidence that pharmacological antagonism of NMDA receptors significantly attenuates the development of cannabinoid physical dependence. Abstinence-induced withdrawal from the cannabinoid agonist WIN 55212-2 (10 μM) was manifested as a significant ($P<0.05$) decrease in the rate of planarian spontaneous locomotor velocity (pLMV) when WIN 55212-2 (10 μM)-exposed planarians were placed into drug-free water. No change in pLMV occurred when WIN 55212-2 (10 μM)-exposed planarians were placed into water containing WIN 55212-2 (10 μM). WIN 55212-2 (10 μM)-exposed planarians placed into water containing LY 235959 (1 or 10 μM) did not display withdrawal (no significant difference, $P>0.05$, in pLMV). In addition, withdrawal was not observed (no significant difference, $P>0.05$, in pLMV) in planarians that were co-exposed to a solution containing WIN 55212-2 (10 μM) and LY 235959 (10 μM). The present results reveal that NMDA receptor activation mediates the development of cannabinoid physical dependence and the expression of cannabinoid withdrawal in planarians.

Keywords: WIN 55212-2; Cannabinoid; Planaria; Glutamate; NMDA; LY 235959; Dependence; Withdrawal

1. Introduction

Discontinuation of chronic marijuana consumption causes spontaneous withdrawal in humans (Crowley et al., 1998; Kouri et al., 1999). The cannabinoid withdrawal signs resulting from marijuana abstinence are milder compared to those signs caused by opioid withdrawal and are not prevalent in all individuals (Pertwee, 1999, 2006; Perkonigg et al., 1999). The situation in animals remains inconclusive (Lichtman and Martin, 2002; Gonzalez et al., 2005). A number of investigators demonstrate that the administration of a cannabinoid receptor antagonist, SR 141716A (rimonobant), to rats receiving chronic Δ^9-tetrahydrocannabinol (Δ^9-THC) precipitates a withdrawal syndrome (Aceto et al., 1995, 1996; Tsou et al., 1995; Beardsley and Martin, 2000; Rubino et al., 1998). However, spontaneous withdrawal from Δ^9-THC does not cause any withdrawal signs in rats. Yet, rats spontaneously withdrawn from a synthetic cannabinoid agonist with higher pharmacological potency or significantly different pharmacokinetics than Δ^9-THC do display withdrawal signs (Aceto et al., 2001).

It is clear that some cannabinoids can produce physical dependence in mammals, but the complexity of mammalian models and the pharmacokinetic properties (i.e., late onset, greater duration, slow metabolic clearance, etc.) of the cannabinoids themselves (Justinova et al., 2005; Pertwee, 1997; Gonzalez et al., 2005) is most likely responsible for the inconsistent results cited above. An attractive alternative to a mammalian model of drug withdrawal is a planarian model (Raffa and Valdez, 2001). Planarians are free-living, fresh-water flatworms that are considered to be the most primitive extant animals having bilateral symmetrical nerve processes consisting

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of cephalic ganglia and peripheral nerve cords (Sarnat and Netsky, 1985). Planarians provide a useful and convenient model for the study of nervous system function and drug-induced effects (Algeri et al., 1983; Agata, 2002; Agata and Watanabe, 1999; Carolei et al., 1975; Newmark et al., 2003; Newmark and Sanchez Alvarado, 2002; Palladini et al., 1996; Passarelli et al., 1999; Venturini et al., 1989). Our laboratory has developed and utilized a metric-change in spontaneous locomotor velocity that quantifies withdrawal behavior in planarians following exposure to cocaine, amphetamine, or opioids (Raffa and Desai, 2005, Raffa et al., 2001, 2000, 2003, Raffa and Martley, 2005, Raffa and Valdez, 2001, Umeda et al., 2005, 2004). The effect is not due to factors such as osmolarity or pH (Umeda et al., 2004). Most recently, we have demonstrated that cannabinoid-exposed, but not naïve, planarians undergo an abstinence-induced decrease in spontaneous locomotor velocity when placed into cannabinoid-free, but not cannabinoid-containing, water (Rawls et al., 2006a,b).

A series of molecular and neurochemical events accompany the withdrawal signs precipitated by rimonobant in rats exposed chronically to Δ^9-THC. These included decreased dopamine release, *c-fos* induction, changes in adenylate cyclase/cAMP signaling, and increased corticotrophin releasing factor (Hutcheson et al., 1998; Tzavara et al., 2000; Diana et al., 1998). Another neurotransmitter that may be involved is glutamate, the major excitatory transmitter in the mammalian brain. For example, it is known that blockade of glutamatergic transmission at NMDA receptors abolishes withdrawal signs precipitated by naloxone in rats exposed to chronic morphine (Tanganelli et al., 1991; Koyuncuoglu et al., 1990; Rasmussen et al., 1991; Tokuyama et al., 1996). In the case of opioids, these data indicate that NMDA receptor activation contributes to the development and/or expression of physical dependence in rats. The role of NMDA receptors in cannabinoid dependence is not yet known. Because planarians contain endogenous glutamate, express the genes for glutamate receptors and undergo abstinence-induced withdrawal from a cannabinoid agonist, they are a desirable model for investigating a role for NMDA receptors in cannabinoid physical dependence and withdrawal (Rawls et al., 2006a,b; Cebria et al., 2002).

The present study used planarians to test the hypothesis that NMDA receptor antagonism decreases: (1) development of physical dependence to a cannabinoid agonist and (2) expression of abstinence-induced withdrawal from a cannabinoid agonist. Actual experiments revealed that the NMDA receptor antagonist LY 235959 blocked the development of physical dependence to WIN 55212-2 and the expression of withdrawal following abstinence from WIN 55212-2. These results reveal that an increase in glutamatergic transmission at NMDA receptors mediates cannabinoid physical dependence and withdrawal in planarians.

2. Materials and methods

2.1. Animals and drugs

Planarians (*Dugesia dorotocephala*) were purchased from Carolina Biological Supply Company (Burlington, NC). Planarians were acclimated to temperature-controlled room temperature (21 °C) and tested within 72 h. Each planarian was used only once. WIN 55212-2 [4,5-dihydro-2-methyl-4(4-morpholinylmethyl)-1-(1-naphthalenylcarbonyl)-6*H*-pyrrolo [3,2,1ij]quinolin-6-one] and LY 235959 [(−)-6-[phosphonomethyl-1,2,3,4,4a,5,6,7,8,8a-decahydro-isoquinoline-2-carboxylate] were purchased from Tocris-Cookson (St. Louis, MO). LY 235959 was dissolved in room-temperature (21 °C) tap water containing AmQuel® water conditioner. A stock solution of 1 mM WIN 55212-2 was prepared in 10/90% cremophor/water (Rawls et al., 2006a,b). Treatment solutions were diluted with tap water containing AmQuel® water conditioner.

2.2. Behavioral measurements

Planarian locomotor velocity (pLMV) was quantified by placing individual planarians into a clear plastic petri dish (Raffa et al., 2001) containing room-temperature (21 °C) tap water containing AmQuel® water conditioner. The dish was placed over paper with gridlines spaced 0.5 cm apart. pLMV was quantified as the number of gridlines crossed or re-crossed per min over a 5-min observation period. pLMV was expressed as the mean (± S.E.M.) of the cumulative number of gridlines crossed by each planarian per min. Prior to behavioral observations, each planarian was placed into individual 0.5 ml vials containing room-temperature vehicle or test drug(s) for 60 min. In the first set of experiments, planarians were exposed to either water or LY 235959 (10 μM) for 60 min and then tested individually for pLMV in either water or LY 235959 (10 μM). In a second set of experiments, planarians were exposed to either water or WIN 55212-2 (10 μM) and then tested individually for pLMV in one of the following: water, WIN 55212-2 (10 μM), LY 235959 (0.1 μM), LY 235959 (1 μM), or LY 235959 (10 μM). In a final experimental set, planarians were exposed for 60 min to water, WIN 55212-2 (10 μM), LY 235959 (1 μM), or WIN 55212-2 (10 μM) plus LY 235959 (1 μM) and then tested individually for pLMV in water.

2.3. Statistical analysis

Comparisons of the cumulative group means at 5 min were evaluated by a one-way ANOVA followed by a Tukey's post-hoc analysis. Values of $P<0.05$ were considered to be statistically significant.

3. Results

3.1. LY 235959 does not affect pLMV

The results with LY 235959 by itself are shown in Fig. 1. Drug-naïve planarians displayed a nearly constant pLMV of approximately 15–18 gridlines/min when tested in drug-free water (Raffa and Valdez, 2001; Raffa et al., 2001, 2003; Rawls et al., 2006a,b). Planarians that were tested for 60 min in LY 235959 (10 μM) and then tested in drug-free water or water containing the same concentration of LY 235959 displayed pLMV that was not significantly different ($P>0.05$) than LY

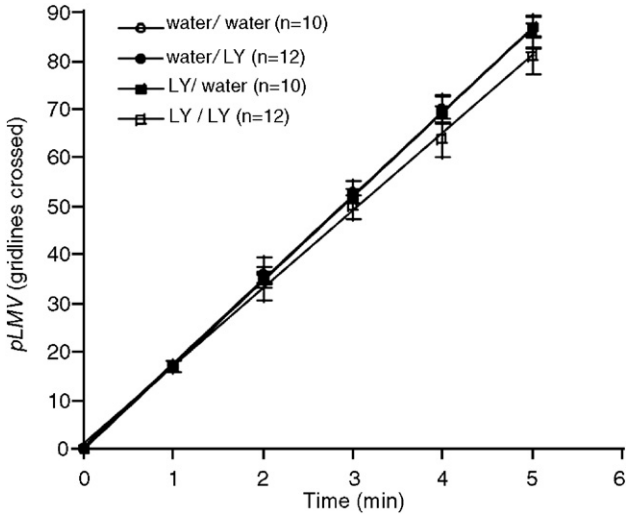

Fig. 1. pLMV is not affected by LY 235959 (LY) alone. Planarians were pre-treated in water or LY (10 μM) for 60 min and then tested in either water or LY (10 μM) for 5 min. pLMV was quantified as the number of gridlines crossed or re-crossed per minute over a 5-min interval and expressed as the mean±S.E.M. of the cumulative number of gridlines crossed by each planarian per min.

235959-naïve planarians tested in drug-free water. Similarly, LY 235959-naïve planarians that were tested in LY 235959 (10 μM) displayed pLMV that was not significantly different ($P>0.05$) than LY 235959-naïve planarians tested in drug-free water.

3.2. LY 235959 antagonizes abstinence-induced withdrawal from WIN 55212-2

The effects of LY 235959 (0.1, 1, and 10 μM) on the withdrawal caused by WIN 55212-2 (10 μM) are shown in Fig. 2. Planarians that were exposed to WIN 55212-2 (10 μM) for 60 min and then tested in water containing the same concentration of

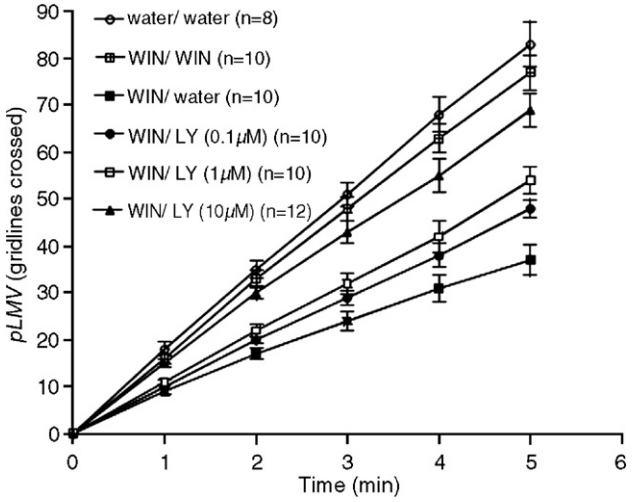

Fig. 2. LY 235959 (LY) blocks abstinence-induced withdrawal from WIN 55212-2 (WIN). Planarians were pre-treated in water or WIN (10 μM) for 60 min and then tested in one of the following: water, WIN (10 μM), or LY (0.1, 1 or 10 μM) for 5 min. pLMV was quantified as the number of gridlines crossed or re-crossed per minute over a 5-min interval and expressed as the mean±S.E.M. of the cumulative number of gridlines crossed by each planarian per min.

Table 1
LY 235959 (LY) blocks abstinence-induced withdrawal from WIN 55212-2 (WIN)

Group	Pretreatment	Test	pLMV±S.E.M.	*P*
A	Water	Water	83±5	–
B	WIN	WIN	77±4	n.s. vs. A
C	WIN	Water	37±3	<0.001 vs. A
D	Water	WIN	78±5	n.s. vs. A
E	WIN	LY (0.1 μM)	48±2	<0.001 vs. A; n.s. vs. C
F	WIN	LY (1 μM)	54±3	<0.001 vs. A; <0.05 vs. C
G	WIN	LY (10 μM)	69±5	n.s. vs. A; <0.001 vs. C

Cumulative pLMV of planarians during a 5-min observation period (test) following a 60-min exposure (pretreatment) to drug or control (water) condition ($F_{5,\ 54}=26.14$, $P<0.0001$). $N=8$–12 planarians per group. n.s. = not significant.

WIN 55212-2 displayed pLMV that was not significantly different ($P>0.05$) from WIN 55212-2-naïve planarians tested in drug-free water. In contrast, planarians that were exposed to WIN 55212-2 (10 μM) for 60 min and then tested in drug-free water displayed a significantly reduced ($P<0.05$) pLMV compared to WIN 55212-2-naïve planarians tested in drug-free water. Buttarelli et al. (2002) investigated several concentrations of WIN 52212-2 on planarian locomotor activity using methodology similar to ours (for example, Raffa et al., 2001). Although an increase in locomotor activity was observed at concentrations of WIN 52212-2 of 50 μg and higher, no increase was observed at 20 μg, which corresponds to 12.54 μM. We used a concentration of 10 μM WIN 55212-2 in our experiments. No increase in locomotor activity was observed in our study during a 5-min or 65-min exposure to 10 μM WIN 55212-2 (Table 1).

Planarians exposed to WIN 55212-2 (10 μM) and placed into water containing LY 235959 (0.1–10 μM) displayed a dose-

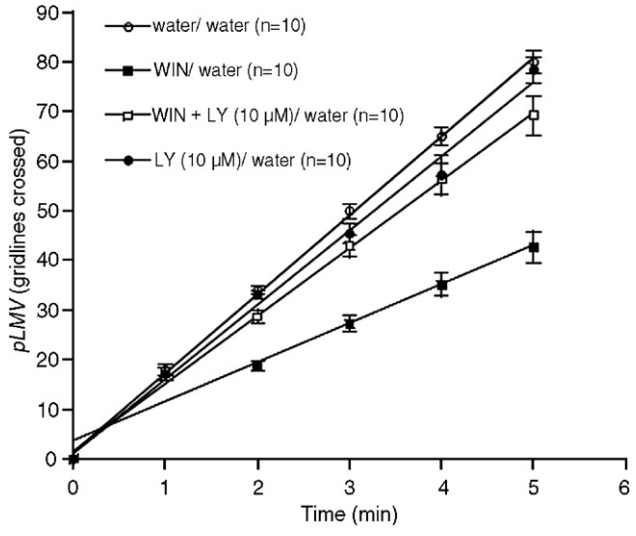

Fig. 3. LY 235959 (LY) blocks the development of physical dependence to WIN 55212-2 (WIN). Planarians were pre-treated in one of the following: water; WIN (10 μM); LY (10 μM); or a combination of WIN (10 μM) and LY(10 μM) for 60 min. Planarians were then tested in water for 5 min. pLMV was quantified as the number of gridlines crossed or re-crossed per minute over a 5-min interval and expressed as the mean±S.E.M. of the cumulative number of gridlines crossed by each planarian per min.

Table 2
LY 235959 (LY) (10 μM) blocks the development of physical dependence to WIN 55212-2 (WIN) (10 μM)

Group	Pretreatment	Test	pLMV±S.E.M.	*P*
A	Water	Water	80±3	–
B	WIN	Water	43±4	<0.001 vs. A
C	WIN+LY	Water	69±4	n.s. vs. A; <0.001 vs. B
D	Water	LY	78±3	n.s. vs. A; n.s. vs. B

Cumulative pLMV of planarians during a 5-min observation period (test) following a 60-min exposure (pretreatment) to drug or control (water) condition ($F_{3,\ 36}$=30.98, P<0.0001). N=10 planarians per group. n.s. = not significant.

related attenuation of withdrawal. These data indicate that LY 235959 attenuates abstinence-induced withdrawal from WIN 55212-2 in planarians.

3.3. LY 235959 antagonizes the development of WIN 55212-2 physical dependence

The effects of LY 235959 (10 μM) on the development of physical dependence caused by WIN 55212-2 (10 μM) are shown in Fig. 3 and Table 2. Planarians that were exposed to WIN 55212-2 (10 μM) for 60 min and then tested in drug-free water displayed a significantly reduced (P<0.05) pLMV compared to WIN 55212-2-naïve planarians tested in drug-free water. Planarians pre-treated with a combination of WIN 55212-2 (10 μM) and LY 235959 (10 μM) displayed significantly greater pLMV when tested in water than when planarians exposed only to WIN 55212-2 (10 μM) were tested in water (P<0.05). These data reveal that LY 235959 attenuates the development of physical dependence to WIN 55212-2 in planarians (Table 2).

4. Discussion

Although known to occur in humans, cannabinoid withdrawal or physical dependence is difficult to demonstrate in mammalian models. Consistent with prior work (Rawls et al., 2006a,b), WIN 55212-2-exposed, but not naïve, planarians underwent abstinence-induced withdrawal when placed into drug-free water. These data demonstrate that discontinuation of cannabinoid exposure produces a quantifiable withdrawal syndrome in planarians. Our results suggest that cannabinoid withdrawal is easier to demonstrate in planarians than in rodents, where the majority of studies have shown that a withdrawal syndrome is prevalent after precipitated, but not spontaneous withdrawal from repeated cannabinoid administration (Aceto et al., 1995, 1996; Tsou et al., 1995; Beardsley and Martin, 2000; Rubino et al., 1998). Two studies have demonstrated a significant withdrawal syndrome following the discontinuation of cannabinoid treatment in rodents (Aceto et al., 2001). Similar to the present study, both of those studies used synthetic cannabinoid agonists, rather than Δ^9-THC. It is possible that synthetic agonists of higher pharmacological potency and significantly different pharmacokinetics than herbal cannabinoids cause physical dependence which is not entirely the same as that which develops in response to Δ^9-THC. The poor water solubility of Δ^9-THC, and the highly lipophilic vehicles required to dissolve it, preclude testing Δ^9-THC in planarians.

Only two studies, to our knowledge, have previously investigated the effect of cannabinoids on a planaria model. The first (Buttarelli et al., 2002) found that the cannabinoid agonist WIN 52212-2 produced a dose-related, SR 141716A (cannabinoid receptor antagonist) sensitive, stimulation of motor behavior and, at higher doses, stereotyped activities like those produced in planarians by (kappa) opioid agonists (Passarelli et al., 1999). Both the motor-enhancing and the stereotyped activities produced by WIN 52212-2 were dose-relatedly attenuated by the opioid receptor antagonist naloxone (Buttarelli et al., 2002), suggesting the involvement of an opioid-dependent pathway. In the second study (Rawls et al., 2006a,b), planarians displayed an abstinence-induced dose-related withdrawal following exposure to WIN 52212-2. The selective nitric oxide (NO) inhibitor L-NAME (L-nitro-arginine methyl ester) attenuated the withdrawal from WIN 52212-2, as well as from cocaine, suggesting a common NO-dependent pathway.

In the present study, the usual abstinence-induced withdrawal from WIN 52212-2 did not occur when cannabinoid-exposed planarians were placed into a solution containing a NMDA antagonist (LY 235959). Similarly, planarians co-exposed to a drug combination of a cannabinoid agonist and NMDA antagonist did not display the usual abstinence-induced withdrawal when placed into a solution of water. The most obvious interpretation of these data is that NMDA receptor activation is required for the development of cannabinoid physical dependence and the expression of withdrawal in cannabinoid-dependent planarians. LY 235959 is a selective NMDA antagonist that competitively blocks the glutamate recognition site at the NMDA receptor complex (Rasmussen et al., 1991). The mechanism by which LY 235959 acts to inhibit cannabinoid physical dependence is unclear. One explanation is that an increase in glutamatergic transmission at NMDA receptors occurs in planarians that are chronically exposed to WIN 55212-2, and that this augmentation in glutamatergic transmission is required for the development of physical dependence to WIN 55212-2. When planarians are co-exposed to a drug combination of LY 235959 and WIN 55212-2, glutamatergic transmission is blocked at NMDA receptors. In the presence of LY 235959, the development of cannabinoid physical dependence is disrupted, resulting in a decrease in the subsequent withdrawal syndrome. The involvement of glutamate in cannabinoid physical dependence is supported by evidence that planarians utilize endogenous glutamate (Rawls et al., 2006a,b) and express the genes for at least two types of ionotropic glutamate receptors which share high sequence similarity to neural specific genes isolated from humans and mice (Cebria et al., 2002).

The involvement of glutamate in cannabinoid physical dependence in planarians is consistent with the overall premise that NMDA receptors mediate the common adaptive processes which cause the development, maintenance, and expression of drug addiction. This hypothesis is especially pertinent to opioid addiction. NMDA receptor antagonists such as ketamine, dextromethorphan, MK-801 (dizocilpine), and memantine attenuate the expression of withdrawal signs (including jumping, teeth-

chattering, diarrhea, ptosis, etc.) in morphine-dependent mice, rats, and guinea pigs when administered immediately before naloxone-precipitated morphine withdrawal (Tanganelli et al., 1991; Koyuncuoglu et al., 1990; Rasmussen et al., 1991; Tokuyama et al., 1996). Mammalian models have also demonstrated that NMDA antagonists decrease the withdrawal syndrome resulting from discontinuation of alcohol, diazepam, or barbiturate exposure (Fidecka and Langwinski, 1989; McCaslin and Morgan, 1987; Rabbani et al., 1994; Morrisett et al., 1990; Steppuhn and Turski, 1993). Surprisingly, the effects of NMDA antagonists on cannabinoid physical dependence and withdrawal in mammals have not been reported in the literature. Perhaps this lack of evidence in mammalian models is because spontaneous withdrawal from Δ^9-THC does not produce a withdrawal syndrome in rodents. It is clear from our data that planarians represent a model in which a withdrawal syndrome results following spontaneous discontinuation of cannabinoid exposure, and that the withdrawal behavior is decreased by NMDA receptor antagonism.

Although conjectural, NMDA receptor activation may facilitate the development of cannabinoid physical dependence in planarians by increasing nitric oxide (NO) production. It is well established that NO is one of the intracellular second messengers important in NMDA receptor activation. The activation of NMDA receptors increases calcium influx into neurons, which leads to the activation of nitric oxide synthase (NOS), an enzyme responsible for nitric oxide production (MacDermott et al., 1986; Garthwaite et al., 1989). As a result, NMDA receptor activation is accompanied by an increase in NO production (Garthwaite, 1991). NO then activates a number of second messenger systems, including the guanylyl cyclase-cyclic GMP system (Arnold et al., 1977). An increase in NO production contributes to cannabinoid-induced withdrawal in planarians (Rawls et al., 2006a,b) and opioid withdrawal in rats and mice (Adams et al., 1993; Kimes et al., 1993; Cappendijk et al., 1995).

In summary, the present results reveal that pharmacological antagonism of NMDA receptors prevents both the development of cannabinoid physical dependence and the expression of cannabinoid withdrawal in planarians. The extent to which acute withdrawal in planarians corresponds or models withdrawal from chronic drug use in humans is unclear. However, these data suggest that planarians represent a sensitive model of cannabinoid withdrawal, and that cannabinoid dependence and withdrawal is mediated, in part, by an increase in glutamatergic transmission at NMDA receptors.

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