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Surprising Separation of Cannabinoid Physical Dependence and Withdrawal in an Invertebrate Model

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Abstract

Planarians have mammalian-like neurotransmitter systems and have been established as a novel *in vivo* model for neuropharmacology. In previous research, planarians that have been exposed to the cannabinoid receptor (CB-R) agonist WIN 55,212-2 for 1 h displayed abstinence-induced withdrawal when tested in drug-free, but not in drug-containing, water. The goals of the present study were to extend previous work and to further establish a cannabinoid behavioral model with planarians. The results showed 1) four different CB-R antagonists (AM251, AM281, SLV319 and SR144528) dose-relatedly blocked development of physical dependence induced by two different CB-R agonists (WIN 55,212-2 and JWH251); 2) none of the same four antagonists (AM251, AM281, SLV319 or SR144528) precipitated withdrawal; 3) short wavelength (254 nm), but not long wavelength (366 nm), ultraviolet (UV) light attenuated abstinence-induced withdrawal from WIN 55,212-2, while short wavelength UV light induced moderate withdrawal behavior. The results confirm the use of a planarian model as a simple yet robust way to study development of physical dependence to cannabinoid agonists. The effect of UV irradiation adds to the evidence that the results are receptor-related. The results also give rise to the surprising suggestion, within the limitations of the methodology, that development of cannabinoid physical dependence and antagonist-induced precipitated withdrawal might be separable phenomena in planarians.

Keywords

Cannabinoid, Physical Dependence, Withdrawal, Planarians, UV Light

1. Introduction

1.1. Planarians as a Cannabinoid Model

Planarians have a simple but well-organized centralized, mammalian-like nervous system made of a "brain" (bi-lobed cerebral ganglia) and spinal cord (ventral nerve cords). Planarians also have neurotransmitter systems similar (or analogous) to higher organisms [1]. Although planarians are established as an invertebrate model in regeneration, pharmacologists have used the model to study behavioral changes elicited by abused compounds [2] [3] [4] [5] [6]. Raffa and colleagues developed a quantitative method for measuring planarian locomotor activity [7] and applying the metric to phenomena related to drug use/abuse such as the development of physical dependence and withdrawal [8] [9] [10]. Planarian pharmacological behavior model is robust and sensitive, and offers unique advantages compared to rodents.

Cannabinoids are some of the most frequently used recreational drugs in the United States. Cannabinoids indirectly cause reinforcing effects by inhibiting GABA (γ-aminobutyric acid) release, which reduces the inhibitory effects of GABA on VTA (Ventral Tegmental Area) dopaminergic neurons [11]. However, it can be relatively difficult to demonstrate the development of physical dependence to cannabinoids in mammalian animal models even in high doses, although precipitated withdrawal is easier to show [12]. The complex pharmacokinetics of some CB-R ligands has been speculated to be the potential reason [13].

The presence of cannabinoid receptors in planarians was inferred by Buttarelli *et al.*, based on the dose-dependent abnormal motor behaviors ("snake-like" movements and "screw-like" hyperkinesia) elicited in planarians by the CB-R agonist WIN 55,212-2 [14]. Rawls *et al.* [15] first demonstrated abstinence-induced withdrawal behavior from WIN 55,212-2 in planarians by using the spontaneous locomotor velocity (pLMV) model. Withdrawal behavior manifested as decreased locomotor velocity in drug-free vehicle, but not the same concentration of agonist to which the planarians had been exposed. The effect is dose-related to the concentration of both. The fact that planarians have fewer pharmacokinetic complications compared with mammals, provides further advantage as a model to study cannabinoid physical dependence and withdrawal.

The present study is an extension of the planarian withdrawal model to the cannabinoid receptor-neurotransmitter system. Specifically, we examined the pretreatment time course, and extended the model to two CB-R agonists (WIN 55,212-2 and JWH251). Further, we used multiple (four) cannabinoid receptor antagonists (AM251, AM281, SLV319 and SR144528) in order to further develop the cannabinoid precipitated withdrawal model. Among the chosen CB-R compounds, AM251, AM281 and SLV319 are CB1-R selective antagonists [16]; SLV319 has better water solubility than traditional cannabinoid antagonists. SLV319 was reported to be orally active in cannabinoid pharmacological models *in vivo* [17]; and SR144528 is a more CB2-prefering antagonist. The two agonists

(WIN 55,212-2 and JWH251) have little preference for either CB-R subtype [17].

1.2. Ultraviolet (UV) Light as a Biological Tool

Furchgott *et al.* [18] first reported that near ultraviolet radiation (250 mμ) induced photorelaxation of contracted smooth muscle of phenylephrine-treated rabbit aorta. In 1975, Tallarida *et al.* [19] used the same model and postulated that the effect of UV light on contracted aorta was caused by the disruption of drug-receptor binding. Applying UV light in the planarian model, Raffa *et al.* [20] first reported that high-energy (254 nm) UV light attenuated dopamine D2 receptor antagonist (sulpiride)-induced decreased planarian locomotor velocity, which supported the hypothesis that UV light disrupts drug-receptor bonds in planarians. In the present study, we chose UV light in addition to CB-R antagonists as a second means to disrupt the action of a cannabinoid agonist at CB-R.

2. Materials and Methods

2.1. Planarians

Planarians (*Dugesia dorotocephala*) were purchased from Carolina Biological Supply Co. (Burlington, NC) and kept at temperature-controlled room temperature (21˚C). They were allowed to acclimate to laboratory conditions for at least one hour before experiments and were tested within three days.

2.2. Chemicals and UV

Two CB-R agonists, (+)-WIN 55,212-2 (mesylate) (*R*)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-napthalenylmethanone) and JWH251 (2-(2-Methylphenyl)-1-(1-pentyl-1H-indol-3-yl)ethanone), and four CB-R antagonists, AM251 (*N*-(Piperidin-1-yl)-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-1H-pyrazole-3-carboxamide), AM281 (*N*-(Morpholin-4-yl)-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-1H-pyrazole-3-carboxamide), SLV319 (3-(4-Chlorophenyl)-N-[(4-Chlorophenyl) sulfonyl]-4,5-dihydro-*N*'-methyl-4-phenyl-1*H*-pyrazole-1-carboxi-midamide), and SR144528 (5-(4-Chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-N-[(*1S*,*2S*,*4R*)-1,3,3-trimethylbicyclo[2.2.1]hept-2-yl]-1*H*-pyrazole-3-carboxamide), were purchased from Cayman Chemical. Cremophor (PEG-35) was a gift from Dr. Rawls (Temple University School of Medicine, Philadelphia, PA). (+)-WIN 55,212-2, JWH251, AM251, AM281, SR144528 stock solutions (1 mM) were prepared fresh every two days in 7%/93% cremophor/water. SLV319 was dissolved with water into 0.1 mM stock solution. Agonist test solutions were diluted with water. Agonist-antagonist solutions were prepared by mixing two stock solutions at the desired ratio (1:2, 1:3, 1:4, or 1:5), and then diluting with water. Cremophor solutions (0.1%, 0.07% and 0.42%) were made with water as vehicle control solutions, based on the corresponding amount in the drug solutions.

MINERALIGHT® LAMP (UVP, model UVGL-58, Upland CA) was used as

ultraviolet light source. It provides two choices of wavelength: long wavelength (366 nm) and short wavelength (254 nm).

2.3. Behavioral Measurement

Planarians were pretreated with CB agonists (WIN 55,212-2 or JWH251), fixed-ratio combinations (1:2, 1:3, 1:4, or 1:5) of CB agonist and antagonist (WIN 55,212-2 + AM251, WIN 55,212-2 + SLV319, WIN 55,212-2 + AM281, WIN 55,212-2 + SR144528, JWH251 + AM251, JWH251 + SLV319 or JWH251 + AM281), or vehicle controls for 20 min (pretreated for 30 min when JWH 251 was used). Planarians were then placed individually into a clear plastic petri dish (14-cm diameter) containing room-temperature (21˚C) water, fixed-ratio combinations (1:2, 1:3, 1:4, or 1:5) of CB agonist and antagonist (WIN 55,212-2 + AM251, WIN 55,212-2 + SLV319, WIN 55,212-2 + AM281, WIN 55,212-2 + SR144528, JWH251 + AM251, JWH251 + SLV319 or JWH251 + AM281), or vehicle control. The transparent dish was placed over paper with gridlines spaced 0.5 cm apart. pLMV was measured by counting the number of gridlines that each individual planarian crossed or re-crossed per minute over a 10-minute observation period. Each planarian was used only once. pLMV was plotted as the mean (±S.E.M.) of the cumulative number of gridlines crossed by an individual planarian per minute.

UV light (254 nm or 366 nm) was placed 10 cm above the planarian-containing petri dish during pretreatment or test step. The experimental design is summarized in Table 1.

2.4. Statistical Analysis

Comparison of the group means at 10 min were analyzed by one-way ANOVA followed by Tukey's post-hoc test with the significance level of $p < 0.05$.

3. Results

3.1. Abstinence-Induced Cannabinoid Withdrawal

In 0.1% cremophor (negative control group), planarians displayed a stable locomotor velocity of approximately 12 - 16 gridlines/min when measured in drug-free water. The planarians attained a cumulative mean (±S.E.M) of 147.4 (±7.5) crossed gridlines in 10 min, which served as the baseline of subsequent experiments. Planarians pretreated in (+)-WIN 55,212-2 (10 μM) for 20 min then placed in drug-free water displayed a cum-ulative mean (±S.E.M) pLMV of 30.7 (±8.5), significantly decreased ($p < 0.05$) pLMV compared to the baseline. Similarly, after pretreatment in JWH251 (10 μM) for 30 min, planarians showed a significantly decreased ($p < 0.05$) pLMV (58.5 ± 17.2) compared with negative control groups. When the planarians were pretreated with (+)-WIN 55,212-2 (10 μM) or JWH251 (10 μM) then tested in same drug/concentration solutions, there was no significant difference ($p > 0.05$) in the 10-min cumulative pLMV compared to planarians pretreated in cremophor and tested in drug-free water.

Thus, abstinence-induced withdrawal from WIN 55,212-2 (20 min) and JWH251 (30 min) was demonstrated. The results are displayed in Figure 1.

3.2. CB-R Antagonists to Block Agonist-Induced Physical Dependence

Figure 2 shows the pLMV of planarians co-pretreated with fixed-ratio (1:2, 1:3, 1:4, or 1:5) combinations of cannabinoid agonists and antagonists then tested in drug-free water. As shown in Figures 2(a)-(c), when planarians were co-incubated with the combination of one CB1-R antagonist (AM251, AM281 or SLV319) and one CB-R agonist (WIN 55,212-2 or JWH251) then tested in water, planarians displayed no difference ($p > 0.05$) compared to vehicle control (0.42%

Table 1. Experimental design. The concentration of WIN 55,212-2 and JWH251 = 10 μM. N is the number of planarians for each concentration of antagonist.

Pretreatment	Test	N
WIN 55,212-2 + AM281 (20, 30, 40, 50 μM)	Water	6, 6, 6, 6,
WIN 55,212-2 + SR144528 (20, 30, 40 μM)	Water	7, 6, 6
JWH251 + AM251 (30, 40, 50 μM)	Water	5, 5, 6
JWH251 + SLV319 (40, 50 μM)	Water	7, 6
JWH251 + AM251 (20, 30, 40, 50 μM)	Water	6, 6, 7, 6
WIN 55,212-2 + UV (254, 366 nm)	Water	6, 6
WIN 55,212-2	WIN 55,212-2 + AM251 (20, 30, 40, 50 μM)	5, 9, 5, 7
WIN 55,212-2	WIN 55,212-2 + AM281 (30, 40, 50 μM)	6, 6, 6
WIN 55,212-2	WIN 55,212-2 + SLV319 (20, 30, 40, 50 μM)	4, 8, 8, 6
WIN 55,212-2	WIN 55,212-2 + SR144528 (30, 40 μM)	6, 7
JWH251	JWH251 + AM251 (40, 50 μM)	4, 6
JWH251	JWH251 + SLV319 (40, 50 μM)	6, 6
JWH251	JWH251 + AM251 (20, 30, 40, 50 μM)	6, 6, 5, 6
WIN 55,212-2	WIN 55,212-2 + UV (254 nm)	6

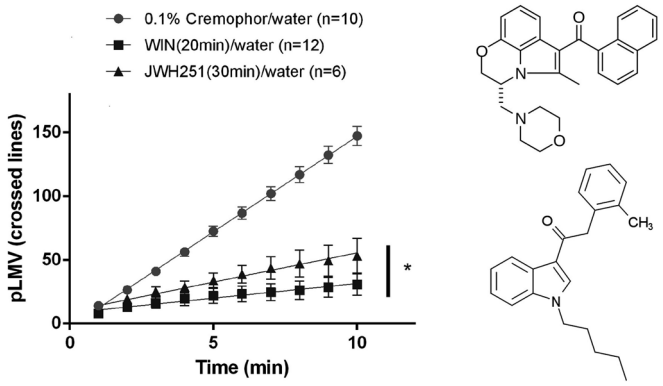

Figure 1. pLMV of planarians pretreated in 0.1% cremophor, WIN 55,212-2 (10 μM) (inset top) or JWH251 (10 μM) (inset bottom) then tested in drug-free water. *P < 0.05 compared to cremophor/water.

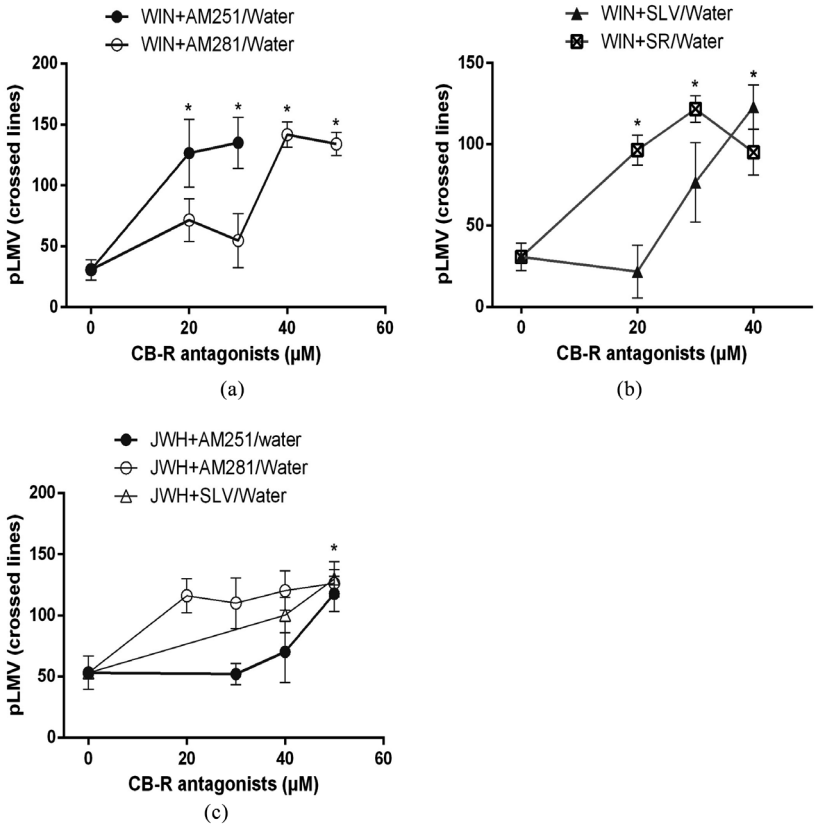

Figure 2. (a)-(c) show dose-response relationships of fixed-ratio (1:2, 1:3, 1:4, or 1:5) combinations of CB agonists and CB antagonists verses cumulative pLMV at 10 min. pLMV was tested in drug-free water after the pretreatment of cocktail solution. (a) Planarians were pretreated in 10 μM WIN 55,212-2 with either AM251 or AM281. (b) Planarians were pretreated in 10 μM WIN 55,212-2 with either SLV319 or SR144528. (c) Planarians were pretreated in 10 μM JWH 251 with either AM251, AM281 or SLV319. *P < 0.05 compared to WIN/water or JWH251/water.

cremophor/water: 144.67 ± 3.6) at least in one of the fixed-ratio of each combination. Moreover, most of these groups displayed significantly increased (p <

post-hoc Tukey test: $P < 0.05$) pLMV compared with positive control groups. The results indicate that these CB-R antagonists blocked the development of CB-R agonist-induced physical dependence (shown as attenuated withdrawal behavior). Specifically, the 1:2 and 1:3 ratios of WIN 55,212-2 and AM251 combinations (126.50 ± 27.7 and 135.00 ± 20.9 respectively), the 1:4 and 1:5 ratios of WIN 55,212-2 and AM281 combinations (141.70 ± 10.4 and 134.00 ± 9.6, respectively), the 1:4 ratio of WIN 55,212-2 and SLV319 combination (123.00 ± 13.6), and the 1:5 ratio of JWH251 and SLV319 combination (129.80 ± 13.9) differed significantly ($p < 0.05$) from positive control (WIN 55,212-2/water: 30.67 ± 8.4 or JWH251/water: 58.5 ± 17.22). The experiments were repeated with the CB2-R selective antagonist SR144528 (result shown in **Figure 2(b)**). Among different ratios of combinations of WIN 55,212-2 and SR144528, planarians pretreated in 1:2, 1:3 and 1:4 ratios of combinations then tested in water (96.43 ± 9.2, 121.67 ± 8.3 and 95.00 ± 14.1, respectively) all showed significantly greater pLMV ($p < 0.05$) than positive control group (WIN 55,212-2/water: 30.67 ± 8.4). However, only the 1:3 ratio of combination showed no significant ($p > 0.05$) difference compared with negative control.

3.3. CB-R Antagonist-Precipitated Withdrawal

Figures 3(a)-3(c) display the pLMV of planarians pretreated in WIN 55,212-2 or JWH251 and tested in fixed-ratio (1:2, 1:3, 1:4, or 1:5) combinations of cannabinoid agonists (WIN 55,212-2 or JWH251) and antagonists (AM251, AM281, SLV319, SR144528). No matter using the CB1 selective (AM251, AM281 and SLV319) or CB2 selective (SR144528) antagonist, all showed no statistical difference was obtained among group means. Consistently, post-hoc Tukey test showed no difference of each group from negative control (0.07% cremophor/0.42% cremophor: 157.50 ± 7.9). These results indicate that none of the same CB-R antagonists that inhibited the development of physical dependence precipitated antagonist-induced withdrawal in planarians.

3.4. UV Light and Cannabinoid Physical Dependence and Withdrawal

As negative control groups (**Figure 4**), pLMV of planarians pretreated with water and 0.1% cremophor under UV light (254 nm and 366 nm) then tested in drug-free water displayed slightly lower pLMV (105 to 114), but still comparable with pLMV baseline (147.4 ± 7.5) shown in **Figure 1**. As shown in **Figure 4**, planarians exposed to WIN 55,212-2 with short wavelength (254 nm) UV light then placed into drug-free water displayed significantly greater ($p < 0.05$) pLMV (92.3 ± 12.0) compared with pLMV (30.67 ± 17.22) of planarians pretreated in WIN 55,212-2 without UV light. In addition, the pLMV of agonist plus UV light (254 nm)-pretreated planarians showed no differences ($p > 0.05$) from negative control groups. On the other hand, when we repeated the same trials with long wavelength (366 nm) UV light radiation, pLMV (29.0 ± 12.0) showed no difference ($p > 0.05$) compared with pLMV of planarians pretreated with WIN 55,212

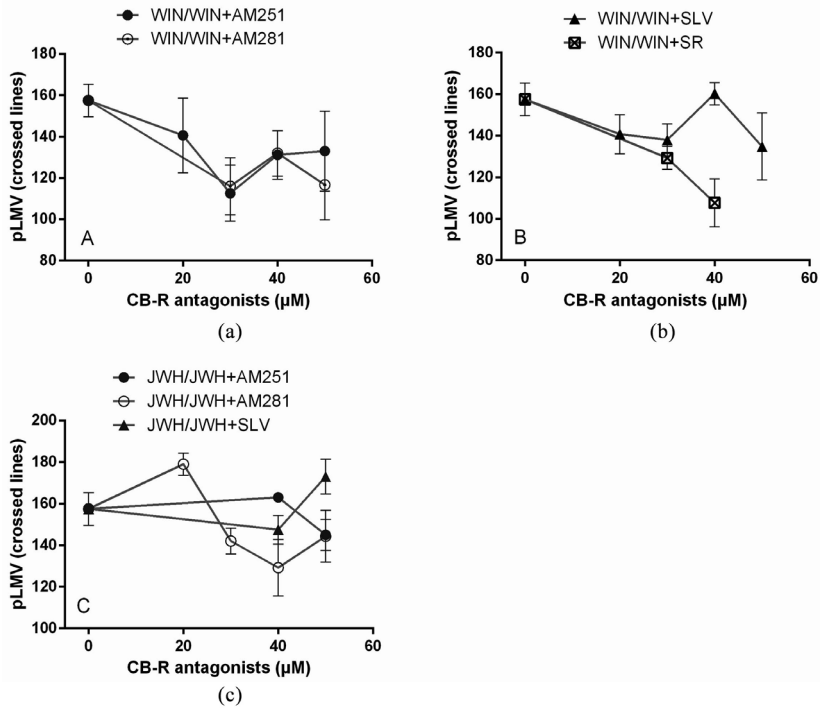

Figure 3. (a)-(c) show dose-response relationships of fixed-ratio (1:2, 1:3, 1:4, or 1:5) combinations of CB agonists and CB antagonists verses cumulative pLMV at 10 min. pLMV was tested in cocktail solution after the pretreatment in CB agonist. (a) Planarians were pretreated in WIN 55,212-2 (10 μM) then tested in either WIN 55,212-2 and AM251 or WIN 55,212-2 and AM281. (b) Planarians were pretreated in WIN 55,212-2 (10 μM) then tested in either WIN 55,212-2 and SLV319 or WIN 55,212-2 and SR144528. (c) Planarians were pretreated in JWH251 (10 μM) then tested in either JWH25 and AM251, JWH251 and AM281, or JWH251 and SLV319. No significant differences among means according to ANOVA (P = 0.7591).

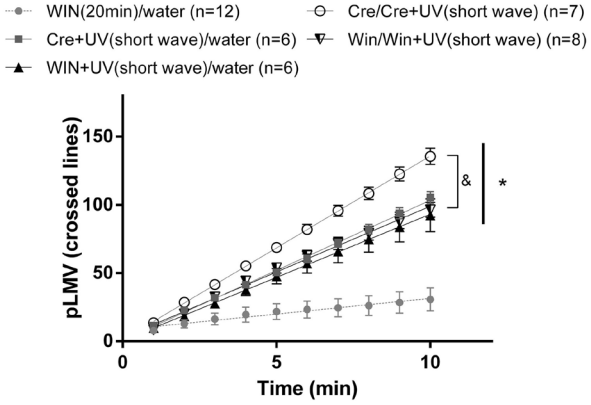

Figure 4. Also shows the influence of UV light (254 nm) on planarians pretreated with WIN 55,212-2. As negative controls, the cumulative pLMV of planarians pretreated with water or 0.1% cremophor then tested under UV light (254 nm) were 134.67 ± 9.5 and 135.57 ± 5.9 respectively, which are close to pLMV baseline (147.4 ± 7.5). As our test group, planarians pretreated with WIN 55,212-2 then measured under UV light (254 nm) showed significantly decreased ($p < 0.05$) pLMV (96.38 ± 5.4) compared with negative controls, although it displayed significantly higher ($p < 0.05$) pLMV than the positive control (30.67 ± 17.22).

alone, but showed significant decrease ($p < 0.05$) compared with negative control groups (not shown in figure). Thus, high-energy (254 nm) UV light interrupted CB agonist-developed physical dependence.

4. Discussion

In previous reports [15] [21], abstinence-induced withdrawal from (+)-WIN 55,212-2 (10 μM) was demonstrated following 1 h of pretreatment. We report here that 20-min (+)-WIN 55,212-2 (10 μM) pretreatment significantly decreased ($p < 0.5$) cumulative LMV in water (*i.e.* abstinence-induced withdrawal), indicating that 20-min exposure to (+)-WIN 55,212-2 (10 μM) is sufficient to develop cannabinoid agonist physical dependence in planarians. Likewise, Abstinence-induced withdrawal from JWH251 (10 μM) was obtained, which was shown as decreased locomotor activity in water after 30-min pretreatment. The results are shown in **Figure 1**.

Although cannabinoid spontaneous withdrawal occurs in human users, it is difficult to demonstrate in mammalian models [12]. Therefore, planarians were chosen as a convenient yet quantifiable alternative model to study cannabinoid physical dependence and abstinence-induced withdrawal. We explored the effects of seven pairs of CB agonists plus antagonists. All the antagonists (AM251, AM281, SLV319 and SR144528) showed certain ability to prevent the development of physical dependence induced by two agonists (WIN 55,212-2 and JWH251), expressed as the increased pLMV of planarians pretreated in combinations of agonists and antagonists then placed in drug-free water compared with planarians pretreated with agonists alone then tested in water (**Figure 2**). Similar studies were done on the opioid receptor system [9] [22] and benzodiazepine receptor system [10] [23] in planarians, wherein an opioid receptor antagonist and a benzodiazepine receptor antagonist attenuated abstinence-induced withdrawal from receptor-specific agonists. We also found that AM251 and SLV319 (**Figure 2(a)**) dose-relatedly antagonized the development of WIN 55212-2-induced physical dependence. AM251 and SLV319 (**Figure 2(c)**) dose-relatedly blocked the development of JWH251-induced physical dependence. AM281 showed a mild dose-response relationship for attenuating the development of physical dependence caused by WIN 55212-2 or JWH251: the two highest concentrations (40 and 50 μM) of AM281 showed stronger potency to attenuate CB agonist-induced physical dependence (**Figure 2**). More apparent dose-dependent effects might be obtained using more dose levels and planarians. We also used R144528, the first potent and selective CB2 antagonist [24]. CB2 receptor selective ligands have shown clinical potential in neuroprotection [25] and antinociception [26]. In this study, SR144528 showed certain ability to prevent CB1-R-mediated effects, *i.e.* the development of physical dependence.

PLMV of planarians pretreated in CB agonists (WIN 55,212-2 and JWH251) then tested in antagonist-containing agonist solutions failed to show statistical difference ($p > 0.05$) compared with vehicle control (shown as **Figure 3**). Thus,

none of the four antagonists (AM251, AM281, SLV319 or SR144528) precipitated withdrawal. The results indicate we "separated" the development of CB receptor-mediated physical dependence and precipitated withdrawal in planarians (discussed below).

Similar to previous reports with non-cannabinoid receptor agonists [27] [28], short wavelength (high energy) UV light (254 nm), attenuated abstinence-induced withdrawal behavior from a cannabinoid agonist (WIN 55,212-2) the long wavelength (low energy) UV light (366 nm) showed no effect on the behavior of planarians. To our knowledge, this is the first report that cannabinoid receptor-mediated abstinence-induced withdrawal is attenuated by UV light (254 nm). It indicates that the suppressive effects caused by UV light generalizes to ligands targeting CB-R and suggests that the results we obtained were caused by the interruption of cannabinoid receptor-mediated effects. The energy-dependent results are likely related to the wavelengths of light that proteins absorb. It was reported [29] that peptide bonds usually absorb light at 200 nm. Depending on structure (the aromatic amino acids: tryptophan, tyrosine and phenylalanine), the range of wavelength that proteins absorb is about 250 nm to 320 nm, which includes the short wavelength (254 nm) we used. We tested UV light (254 nm) on planarians pretreated with the cannabinoid agonist WIN 55,212-2 trying to precipitate withdrawal (**Figure 4**). Planarians showed significantly decreased ($p < 0.05$) pLMV (96.38 ± 5.4) compared with negative control, indicating UV light at 254 nm precipitated moderate withdrawal behavior in planarians. These results are consistent with our results with the use of CB-R antagonists. Cannabinoid antagonists/UV light dose/energy-relatedly prevented cannabinoid agonists from developing physical dependence. However, the same antagonists/UV light precipitated no, or only weak, withdrawal behavior in planarians.

The present study sought to optimize and extend the planarian withdrawal model, and establish an antagonist-induced precipitated withdrawal model. All four CB-R antagonists (AM251, AM281, SLV319 and SR144528) attenuated CB-R agonists-produced physical dependence. However, to our surprise, none of the antagonists precipitated withdrawal from the same agonists even at the highest usable antagonist concentration. Similarly, short wavelength UV light (254 nm) fully blocked cannabinoid agonist-induced physical dependence, but it only precipitated moderate withdrawal behavior. Compared to mammalian models, planarians seem to be easier to demonstrate abstinence-induced withdrawal than to precipitate antagonist-induced withdrawal. Also surprisingly, cannabinoid antagonists that blocked the agonist effect in one format (attenuating agonist-induced physical dependence) failed to block agonist effect in another format (inducing withdrawal behavior). The results suggest that it might be possible to separate development of CB receptor-mediated physical dependence and antagonist-induced precipitated withdrawal in planarians.

These results raise the question: what are the potential explanations for the "separated development of physical dependence and precipitated withdrawal"?

First, method sensitivity. Raffa *et al.* reported [9] that naloxone (1.0, 5.0 and 10.0 μM) dose-relatedly precipitated withdrawal from U-50,488H (1 μM) in planarians, and 10 μM naloxone also prevented the development of U-50,488H (1 μM)-induced physical dependence. We used the same endpoint and methodology in the present study. Second, high enough concentrations of antagonists? We did find that several planarians pretreated in cannabinoid agonist solution then placed into combination of agonist and antagonist solution showed withdrawal behaviors, although no statistical differences were obtained when we analyzed the mean data. This indicates that cannabinoid antagonists have the ability to precipitate withdrawal in some planarians, but for some reason, not all. Therefore, higher concentrations of antagonists might precipitate withdrawal in a greater number of planarians. However, because of the limitation of the concentration of cremophor and the solubility of compounds, we were unable to adjust our drug concentrations to such a high range. The important point is, even if a higher concentration of antagonist does precipitate withdrawal, we still separated the development of cannabinoid physical dependence and precipitated withdrawal in terms of antagonist dose. Third, the separation of physical dependence and precipitated withdrawal in planarians may be related, despite the precautions taken, to the pharmacological properties of the available CB antagonists (*i.e.*, possibility of partial agonist effects). In fact, we did two groups of experiments to study this hypothesis. After pretreatment in JWH251, two of five planarians showed normal pLMV (>100) when placed in cannabinoid antagonist, SLV319, alone. This suggests that the high concentration (40 μM) of SLV319 somehow maintained the activation of cannabinoid receptors in the two planarians. In addition, after pretreatment only in SLV319, three of six planarians showed low pLMV (≤100) when placed into drug free water, which means that high concentration (40 μM) of SLV319 developed physical dependence in the three planarians. These data suggest that SLV319 displayed moderate "partial cannabinoid receptor agonist" effects. However, exploring the mechanism was not our primary purpose so more experiments are needed to obtain a convincing conclusion.

Eliminating the methodological concerns, the other explanation is that our results reflect differences in planarians compared to mammals. In rodents, CB-R antagonists usually easily precipitate withdrawal syndromes [12]. Although we do not have sufficient knowledge about the biological mechanism of cannabinoid receptor-mediated pathways in planarians, it is possible that different cannabinoid receptor-mediated mechanisms are involved in the development of physical dependence and precipitated withdrawal in planarians. Mammals could have evolved different CB neurotransmitter-receptor systems.

5. Conclusion

In summary, we extended a planarian cannabinoid physical dependence and abstinence-induced withdrawal model. Using this model, we studied the effects of

four cannabinoid receptor antagonists (AM251, AM281, SLV319 and SR144528) on agonist-induced physical dependence and showed that co-exposure with cannabinoid antagonists attenuates cannabinoid agonist-induced physical dependence. The four cannabinoid antagonists dose-dependently attenuated CB agonist (WIN 55212-2 and JWH251)-induced physical dependence in planarians. Surprisingly, however, none of the same antagonists that inhibited development of physical dependence precipitated withdrawal from the same agonists even in the highest testable concentration. To further confirm our results, we used UV light as a tool to interrupt the agonist-receptor bonds. When UV light was added in the pretreatment step, short wavelength UV light (254 nm) attenuated abstinence-induced withdrawal from cannabinoid agonist in planarians, but it only precipitated moderate withdrawal behavior when UV light was added in the test step. Together these results suggest possible separation of the development of CB receptor-involved physical dependence and antagonist-induced precipitated withdrawal in planarians.

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Conflicts of Interest

The authors have no potential financial, professional or personal conflict of interest related to this manuscript.

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