

AN EXTENSION OF PLANARIAN BEHAVIORAL MODEL: CANNABINOID
PHYSICAL DEPENDENCE AND WITHDRAWAL

A Dissertation
Submitted to
the Temple University Graduate Board

In Partial Fulfillment
of the Requirements for the Degree
MASTER OF SCIENCE

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July 2016

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ABSTRACT

Background: Planarians have mammalian-like neurotransmitter systems and have been established as a novel *in vivo* model for neuropharmacology. In previous research, planarians exposed to the cannabinoid receptor (CB-R) agonist WIN 55,212-2 (10 $\mu\text{mol/L}$) for 1 h displayed a significant ($p < 0.05$) decrease in spontaneous locomotor velocity (pLMV) when subsequently tested in drug-free, but not in drug-containing, water. This demonstrated abstinence-induced withdrawal from a CB-R agonist as a manifestation of the development of physical dependence.

Purpose: The purpose of the present study was to extend previous work and to further establish a cannabinoid behavioral model with planarians. Specifically, the goals included (i) confirm the work with WIN 55,212-2 and extend to a second agonist (ii) interfere with agonist-induced physical dependence using several CB-R antagonists, (iii) demonstrate antagonist-induced precipitated withdrawal behavior, and (iv) try to induce withdrawal behavior from CB-R agonists using UV light.

Methods: Two CB agonists (WIN 55,212-2 and JWH251) and four CB antagonists (AM251, AM281, SLV319 and SR144528) were used. Planarians were placed individually in CB-R agonist or agonist + antagonist mixtures for 20 and 30 min of exposure (with or without UV radiation), and withdrawal was quantified by measuring pLMV in drug-free vs drug-containing water (with or without UV light irradiation).

Results: (i) Four different CB1-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). (ii) None of the same four antagonists

(AM251, AM281, SLV319 and SR144528) precipitated withdrawal. (iii) Short wavelength (254 nm), but not long wavelength (366 nm), UV light attenuated abstinence-induced withdrawal from WIN 55,212-2, while short wavelength UV light induced moderate withdrawal behavior.

Conclusions: 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 model is more rapid and sensitive than the usual rodent models. The effect of UV irradiation adds to the supposition 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.

ACKNOWLEDGEMENTS

I would like to express my gratitude to my major advisor Dr. Robert B. Raffa, for leading me to the world of planarians, for encouraging and guiding me with great patience and for giving me suggestions both in academia and career goals. I thank Dr. Scott Rawls and Dr. Ellen Walker for serving as committee members and supporting me to make my project better. Also, I am grateful to Dr. Daniel Canney for providing me an opportunity to pursuing my master degree at Temple University School of Pharmacy.

Secondly, I would also like to thank Hemang Patel (PharmD candidate) who helped me with my experiments as a volunteer. Also, I appreciate my friends in Temple University. You helped me with every little thing these years. You made my life colorful and made me feel warm all the time.

Finally, my special thanks go to my beloved parents (Yu Sheng and Hua Xu), to my grandparents (Xiulin Ding and Jianting Xu), my boyfriend (Zhuangzhuang Geng) and to my best friends (Huimin Yu, Yaqian Di, Yuanbo Wang, Lanni Luo and Juyi Hou), for trusting me and loving me all the time. I am so glad that you are accompanying with me. I cannot imagine how my life will be without you.

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CHAPTER 1

TERMINOLOGY AND PURPOSE

1.1. Physical Dependence

Dependence in substance use disorder is defined as an adaptive state that develops during long-term administration of almost any drug (drugs of abuse and not abuse) (Halbach 2009; Malenka, Nestler et al. 2009). It is likely related to compensatory physiological responses to chronic drug stimulus. Physical dependence is a state of physiological need (Halbach 2009). Somatic symptoms (e.g., fatigue, hypertension and sleep disturbance in humans) occur upon the abrupt stopping of drug administration (Malenka, Nestler et al. 2009).

1.2. Withdrawal

Withdrawal in substance use disorder is defined as symptoms (e.g., vomiting, anxiety, fatigue, and sweating in humans) that occur upon the acute cessation or decrease of drug administration. Withdrawal symptoms are usually opposite to the effects produced by drugs. (Malenka, Nestler et al. 2009)

Abstinence-induced withdrawal is the spontaneous symptoms that occur after rapid cessation or decrease of drug use (also known as spontaneous withdrawal or withdrawal) (False 2004). Precipitated withdrawal symptoms are caused by external interruption of drug effects (False 2004). The commonly used interruption is an antagonist that targets the same receptors as the administered drug (this is also called ‘antagonist-induced withdrawal’). Precipitated withdrawal symptoms are similar to

abstinence-induced withdrawal symptoms, although precipitated withdrawal can start suddenly and more intense (False 2004).

1.3. Purpose

Previously, the laboratories of Raffa and Rawls developed a quantifiable planarian withdrawal model (introduced in Chapter 3). The present study is an extension of the planarian withdrawal model to the cannabinoid receptor-neurotransmitter system. Specifically, we would optimize previous cannabinoid withdrawal methodology on the pretreatment time course, and would extend the study to two cannabinoid agonists (WIN 55,212-2 and JWH251). The improved model would serve as a prototype of subsequent experiments. Second, we would expand previous work by interrupting cannabinoid agonist-induced physical dependence using multiple (four) cannabinoid receptor antagonists (AM251, AM281, SLV319 and SR144528). Third, we would try to develop a cannabinoid antagonist-induced precipitated withdrawal model using the same antagonists. Fourth, we would add UV light as an alternative interruption of agonist-produced effects, and try to induce CB-R withdrawal behavior and interfere with CB-R agonist-induced effects.

CHAPTER 2

INTRODUCTION: *PLANARIA* AS A PHARMACOLOGICAL MODEL

2.1. Planarians and Their Neuro System

‘Planarian’ is the common name for the genus *Planaria*. They are free-living flatworms that are widely distributed in water and land. They are generally 1-30 mm in length and have ‘auticles’ (ear-like parts for the location of food at the base of the triangular head), ‘ocelli’ (two eyespots sensitive to light located in the head), digestive system (a mouth, a pharynx and a gastrovascular cavity) and excretory system (multiple tubes and excretory pores) (Pagėan and MyiLibrary 2014). Planarians do not have circulatory or respiratory systems; the body wall is responsible for oxygen entering and carbon dioxide leaving (Pagėan and MyiLibrary 2014). Planarians move by muscle contraction or sliding with cilia. For reproduction, both asexual and sexual mechanisms are available to planarians. The capacity to regenerate is another character of planarians. They are able to regenerate their missing part to recreate a new animal from tiny fragments about in one week (Oviedo, Nicolas et al. 2008). Because of that, planarians are used as a model to study stem cells, tissue regeneration, and aging (Oviedo, Nicolas et al. 2008; Raffa and Rawls 2009).

Planarians have a simple but well-organized centralized nervous system made of the ‘brain’ and the ventral nerve cords (VNCs) (Raffa and Rawls 2009). Under the eyespots are two ganglia (‘brain’), which are the originating points of two nerve cords (‘longitudinal nerves’). The nerve cords extend from head to tail with many ‘transverse nerves’ connected, giving a ladder-like nerve system. Molecular studies, see (Raffa and Rawls 2009), (expressed sequence tags and cDNA microarray techniques) revealed the

details of the planarian nerve system. A wide variety of nervous system-related genes were found, and at least seven functional regions were detected indicating planarians have a well-organized central nerve system (CNS) allowing the performance of complex process. Moreover, planarians have neurotransmitters similar (analogous to) higher organisms, such as dopamine, acetylcholine, norepinephrine, and serotonin (Sarnat and Netsky 1985). Although the CNS of planarians is recognized as a primitive simple brain, the facts support the notion that the planarian CNS is potentially the ancestor of the vertebrate brain. Because of those advantages, planarians are used as a model for memory, learning and behavior (Oviedo, Nicolas et al. 2008).

2.2. A Neuro-pharmacological Model for Drug Action and Substance Abuse

Planarians have mammal-like neuro systems, because several neurotransmitter-receptor systems (serotonin, catecholamine, GABA, opioid, dopamine) were identified by neurochemical and immunohistochemical methods (Buttarelli, Pellicano et al. 2008). With this advantage, scientists use planarians as a useful model for studying processes related to nervous system.

Palladini's laboratory did several studies with planarians to develop qualitative description of the changed behaviors induced by dopaminergic and cholinergic drugs (Carolei, Margotta et al. 1975; Palladini, Margotta et al. 1980). The results showed that stimulation of dopaminergic transmission produced stereotyped behaviors of drug-induced hyperkinesia while stimulation of cholinergic transmission produced hypokinesia, and these effects were blocked by receptor-specific antagonists. In summary, the balance between dopaminergic and cholinergic stimulation regulates the physiological motility in planarians. In the following years, the group of Palladini further

studied how opioid and cannabinoid systems contributed to planarian motor activity (Passarelli, Merante et al. 1999). ‘Screw-like hyperkinesia’ and ‘C-like’ position were identified as qualitative stereotyped behaviors for kappa-opioid receptor agonist-treated planarians. In 2002, Buttarelli et al. reported cannabinoid receptor agonist produced, dose-dependent hyperkinesia in planarians. This hyperkinesia was characterized with indistinguishable stereotyped behaviors from kappa-opioid receptor agonist-induced behaviors. Moreover, these cannabinoid agonist-produced behaviors were attenuated by co-exposure to either a cannabinoid receptor antagonist, dopamine receptor antagonists, or an opioid receptor antagonist in planarians. That is to say, the behavioral consequences they observed are partially mediated by the activation of endogenous dopamine and opioid systems, which are consistent with the mechanisms reported in mammals. The experiments mentioned above are introduced in details in Buttarelli et al.’s review (Buttarelli, Pellicano et al. 2008).

The planarian models involve qualitative evaluation. In 2000, Raffa et al. (Raffa, Holland et al. 2001) advanced a quantitative method of planarian locomotor activity. Planarians are individually placed into a petri dish over graph paper having gridlines. Planarian locomotor velocity (pLMV) is measured by counting the lines crossed or re-crossed in a certain observation period (5-10 min). The group of Raffa reported the decreased pLMV of planarians in dopamine antagonist solution (Raffa, Holland et al. 2001) and increased pLMV of planarians in amphetamine solution (Raffa and Martley 2005). Based on this convenient metric, planarians were further used for studying drug abuse by pretreating them with test substances (Raffa and Valdez 2001; Raffa, Stagliano et al. 2003; Raffa, Cavallo et al. 2007). Using the same model, Rawls investigated the

roles of nitric oxide pathway (Rawls, Rodriguez et al. 2006) and excitatory amino acid-mediated pathway (Rawls, Gomez et al. 2007) in the development of the withdrawal behaviors.

CHAPTER 3

DEVELOPMENT OF A CANNABINOID ABSTINENCE-INDUCED WITHDRAWAL BEHAVIORAL MODEL

3.1. Introduction

3.1.1. *Cannabinoid Receptors in Human Central Nervous System (CNS)*

The endocannabinoid system includes cannabinoid receptors (CBrs), endocannabinoid ligands, and their biosynthesizing and metabolizing enzymes. There are at least two cannabinoid receptors, type 1 (CB1) receptors and type 2 (CB2) receptors. CB1 and CB2 receptors are differentiated in their amino acid sequence (48% identity), tissue distribution (CB1 receptors are mainly located in CNS while CB2 receptors are mainly found in peripheral tissues.), mediated biological effects (e.g. analgesic effects of CB1 receptors versus immune effects of CB2 receptors) and the sensitivity to potent selective cannabinoids (Howlett, Barth et al. 2002). They are both belong to GPCR (G-protein coupled receptor) superfamily. Since types are coupled with G_i or G_o protein, activation of CB receptors inhibits adenylyl cyclase, activates MAP (Mitogen-activated protein) kinase, and attenuates the synthesis of cAMP (cyclic adenosine monophosphate). CB receptors maintain a high level of constitutive basal activity in the absence of high concentrations of agonists. (Svíženská, Dubový et al. 2008; Mustonen 2010)

CB1 receptors are mainly distributed in the central nervous system, especially in the hippocampus, cerebellum and basal ganglia. A Small amount of CB1 receptors are also found in the peripheral axonal branches and vertebrate organs, such as the reproductive and cardiovascular systems (Svíženská, Dubový et al. 2008). Previous reports indicate that cannabinoids play a role in inhibiting the release of neurotransmitter,

such as glutamate (Shen, Piser et al. 1996), acetylcholine (Gifford, Samiian et al. 1997), and noradrenaline (Schlicker, Timm et al. 1997). In the ventral tegmental area (VTA), CB1 receptors are found in presynaptic glutamatergic and GABAergic neurons. Activating CB1 receptors indirectly activates reward pathways by inhibiting GABA release, which reduces the inhibitory effects of GABA on VTA dopaminergic neurons (Maldonado, Valverde et al. 2006) .

In contrast to CB1 receptors, only a small number of CB2 receptors are located in CNS, such as the anterior olfactory nucleus and amygdala nuclei. Higher amounts of CB2 receptors are found in peripheral tissues, especially in immune tissues (Svíženská, Dubový et al. 2008). However, the presence of CB2 receptors in the CNS suggests a possible additional central site of action of cannabinoids, and potential interaction with CB1 receptor-mediated effects in the CNS (Maldonado, Valverde et al. 2006).

3.1.2. Pharmacological Effects and Withdrawal Symptoms of Cannabinoids

Cannabinoids produce characteristic pharmacological effects including euphoria, increased appetite, pain suppression, disruption of short-term memory, attentional impairment, improved body awareness, amotivational syndrome, anxiety, and sleepiness. (Svíženská, Dubový et al. 2008)

During chronic administration of cannabinoids, humans and other mammals display tolerance and dependence, and withdrawal syndromes upon cessation of administration ('abstinence-induced withdrawal') or administration of CB antagonists ('antagonists-induced withdrawal'). The common withdrawal symptoms in humans include dysphoria (restlessness, depression, anxiety and irritability), sleep disturbance, gastrointestinal symptoms and decreased appetite (Svíženská, Dubový et al. 2008).

Typical withdrawal signs in rats include increased paw tremors and head shakes.

Conditional place preference and self-administration models are helpful animal models to study cannabinoid motivational and reinforcing properties (Maldonado and de Fonseca 2002).

It can be relatively difficult to demonstrate abstinence-induced withdrawal from cannabinoids in animal models (rodents, dogs, and monkeys) even in high doses. Aceto et al. (Aceto, Scates et al. 2001) were the first to report spontaneous withdrawal signs (wet-dog shakes and facial rubs) 24 h after stopping chronic cannabinoid receptor agonist (WIN 55,212-2) treatment (medium dose for 4 days) in rats. Differences pharmacokinetic properties were suggested to explain why the interruption of THC usage failed to show apparent abstinence-induced withdrawal. The result of this report is one of the reasons why we choose WIN 55,212-2 as the first compound to start with.

Cannabinoid antagonist-induced precipitated withdrawal is more easy to obtain in laboratory animals: the administration of a cannabinoid receptor antagonist (SR141716A) precipitates profound withdrawal syndromes in mice after chronic Δ^9 -THC treatment (Diana, Melis et al. 1998). SR141716A also induced withdrawal signs in WIN 55,212-2-treated rats (Aceto, Scates et al. 2001).

3.1.3. *Cannabinoid Receptors in Planarians*

The existence of cannabinoid receptors in planarians was inferred by Buttarelli et al., who reported that the dose-dependent abnormal motor behaviors (“snake-like” movements and “screw-like” hyperkinesia) displayed by planarians (species *Dugesia gonocephala*) when exposed to WIN 55,212-2 were attenuated by co-exposure to the cannabinoid receptor antagonist SR141716A (Buttarelli, Pontieri et al. 2002). Moreover,

Rawls et al. (Rawls, Rodriguez et al. 2006) demonstrated abstinence-induced withdrawal behavior from WIN 55,212-2 in planarians by using the spontaneous locomotor velocity (pLMV) model described below. This study was the first report of cannabinoid withdrawal in planarians. In addition to behavioral pharmacological evidences, Mustonen explored the endocannabinoid system in planarians (*D. dorocephala*) in 2010 (Mustonen 2010) and reported 2-Arachidonoylglycerol (2-AG), *N*-arachidonylethanolamide, Anandamide (AEA) and selected *N*-acylethanolamines (NAEs) detected, and the basal concentrations of these endocannabinoids were not beyond the range of values of other animal organisms.

Although we still lack direct evidences at the molecular level to prove the existence of cannabinoid receptors in planarians, there is strong pharmacological evidences to conclude that cannabinoid receptors, at least CB1 receptors exist planarians.

3.1.4. Physical Dependence and Withdrawal in Planarians

Animal behavioral models are important to study drug craving and substance disorder. Planarians are a simple and convenient model to quantifiably study physical dependence and withdrawal. Moreover, in terms of substances displaying mild withdrawal in mammals, planarians are often more easy to display withdrawal symptoms than are mammals (Raffa and Rawls 2009). Needleman (Needleman 1967) developed one of the earliest planarian dependence and withdrawal models. Planarians were exposed to morphine for 43 days. Then he recorded the time the planarians took to swim to the perimeter of the observation chamber in drug-free water. The increased time period, i.e. decreased movement, was defined as dependence.

Raffa and Rawls established a quantifiable withdrawal behavioral model in planarians in 2001 (Raffa, Holland et al. 2001). Planarians were pretreated individually in drug solutions for 1 h, and then placed individually into a petri dish containing drug-free water for observation. The petri dish was put over graphing paper having gridlines, and planarian locomotor velocity (pLMV) was measured as the number of gridlines crossed or re-crossed by planarians during 5-10 min of observation period. Abstinence-induced withdrawal was obtained by testing pLMV in drug-free water, and the decreased pLMV was recognized as withdrawal symptom. Antagonist-induced precipitated withdrawal was obtained by pretreating planarians with agonist solution to develop physical dependence, and then measuring pLMV in agonist-antagonist mix solution to induce withdrawal. Similarly, decreased pLMV was the endpoint of withdrawal. This model was successfully utilized in several drug categories: opioids (U-50,488H, *nor*-BNI) (Raffa, Stagliano et al. 2003), cocaine (Raffa and Valdez 2001), benzodiazepines (clorazepate, zolpidem, midazolam) (Raffa, Cavallo et al. 2007), cannabinoid (WIN 55, 212-2) (Rawls, Rodriguez et al. 2006; Rawls, Gomez et al. 2007), and amphetamines (amphetamine, methamphetamine) (Rawls, Cavallo et al. 2008).

During withdrawal, planarians display withdrawal 'signs' including 'HeadBop', 'Squirming', 'Clinging', 'Head Swing', 'TailTwist' and 'Corkscrew' (Raffa and Desai 2005) (Figure 1).

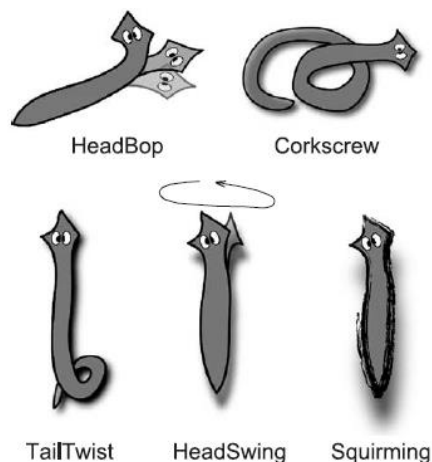


Figure 1 Withdrawal signs in planarians (Raffa and Desai 2005)

Although the planarian models cannot substitute for humans or rodents for more complex study, it does provide some advantages over more complicated models. First, it is a much simpler and time-saving model. Second, it is robust and sensitive (it reveals more apparent withdrawal when mammals only display mild withdrawal in high doses). Third, planarians have fewer pharmacokinetic complications compared with mammals, e.g. planarians do not have a blood-brain barrier, which is better to study mechanism fewer pharmacokinetic complications. In our case, spontaneous withdrawal from CB1 agents is difficult to observe in rodents, whereas pLMV is a simple, quantifiable model for us to do basic research. Thus, we decided to use planarians as our animal model to study cannabinoid physical dependence and withdrawal.

3.1.5. Aim 1

In order to study cannabinoid physical dependence and withdrawal behavior, we first wanted to further develop a standard CB withdrawal behavioral model in planarians. We had two purposes here: one, to establish the baseline of planarian locomotor activity

following vehicle pretreatment, two, to measure the time course of WIN 55, 212-2-induced physical dependence as measured by abstinence-induced withdrawal.

3.2. Methods

3.2.1. Animal and Drugs

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

(+)-WIN 55,212-2 (mesylate) ([*(3R)*-2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo [1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone, monomethanesulfonate) was purchased from Cayman Chemical. Cremophor was a gift from Dr. Rawls. AmQuel® water conditioner (5 mL per 40 liters of water) was added to tap water at least 12 hours before use (subsequently abbreviated “water”). For (+)-WIN 55,212-2, a stock solution (1 mM) was prepared fresh every two days in 10/90% cremophor/water, and the treatment solution (10 µM) was diluted with water. 0.1% cremophor solution was made with water as vehicle control solution.

3.2.2. Behavior Measurements

Planarian locomotor velocity (pLMV) (Raffa, Holland et al. 2001) was used to measure planarians behavior. Planarians were placed individually into a clear plastic petri dish (14-cm diameter) containing room-temperature (21°C) water or WIN 55,212-2 (10 µM) solution. The dish was placed over graph paper with gridlines spaced 0.5 cm apart. pLMV was measured by counting the number of gridlines that a planarian crossed or re-

crossed in every minute over a 10-minutes observation period. Each planarian was used only once. pLMV was plotted as the mean (\pm S.E.M.) of the cumulative number of gridlines crossed by individual planarian per minute. Before measurements, planarians were pretreated with either 0.1% cremophor solution or WIN 55,212-2 (10 μ M) solution. Three pretreatment periods were tested: 10 min, 20 min, and 30 min. The groups are shown as Table 1.

Table 1 Groups Design for Cannabinoid Abstinence-Induced Withdrawal Behavioral Model

Pretreating Solution	Pretreating Period	Test Solution	N
(+)-WIN 55,212-2 (10 μ M)	10 min	Water	7
(+)-WIN 55,212-2 (10 μ M)	20 min	Water	12
(+)-WIN 55,212-2 (10 μ M)	30 min	Water	10
(+)-WIN 55,212-2 (10 μ M)	20 min	(+)-WIN 55,212-2 (10 μ M)	6
(+)-WIN 55,212-2 (10 μ M)	30 min	(+)-WIN 55,212-2 (10 μ M)	6
Water	20 min	(+)-WIN 55,212-2 (10 μ M)	6
0.1 % Cremophor	30 min	Water	10

3.2.3. Statistical Analysis

Minitab 17 Statistical Software and Excel 2013 were used to perform the statistical analyses. 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 $p < 0.05$.

3.3. Results

3.3.1. Baseline of pLMV

In 0.1% cremophor, planarians displayed a stable locomotor velocity of approximately 12-16 gridlines/min when measured in drug-free water. The planarians attained a cumulative mean (\pm S.E.M) of 147.4 (\pm 7.5) crossed gridlines in 10 min, as shown in Figure 2. As a result, planarians provided a stable baseline behavior in vehicle control, 0.1% cremophor solution.

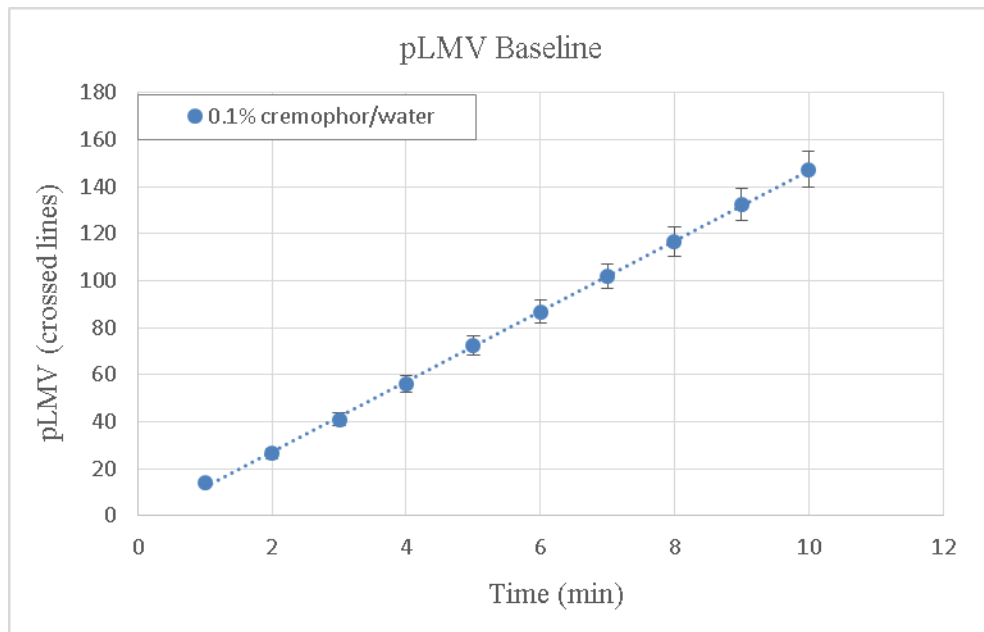


Figure 2 Baseline of pLMV

3.3.2. *Pretreatment Periods of WIN 55,212-2 to Cause Abstinence-Induced Withdrawal*

As shown in Figure 3 and Table 2, planarians pretreated in (+)-WIN 55,212-2 (10 μ M) for both 20 min and 30 min then placed into drug-free water displayed a significantly decreased ($p < 0.05$) pLMV compared with control groups. The cumulative means (\pm S.E.M) of pLMV were 30.7 (\pm 8.5) and 15.9 (\pm 10.3) respectively. However, planarians exposed to (+)-WIN 55,212-2 (10 μ M) for 10 min then tested in drug-free water did not show a significant difference ($p > 0.05$) of pLMV.

The pLMV of planarians pretreated with (+)-WIN 55,212-2 (10 μ M) for 20 min or 30 min then tested in (+)-WIN 55,212-2 water showed no significant difference ($p > 0.05$) from (+)-WIN 55,212-2-naïve planarians tested in drug-free water. Also as a negative control, planarians pretreated with water for 20 min then tested in (+)-WIN 55,212-2 (10 μ M) did not display significant difference ($p > 0.05$) in pLMV.

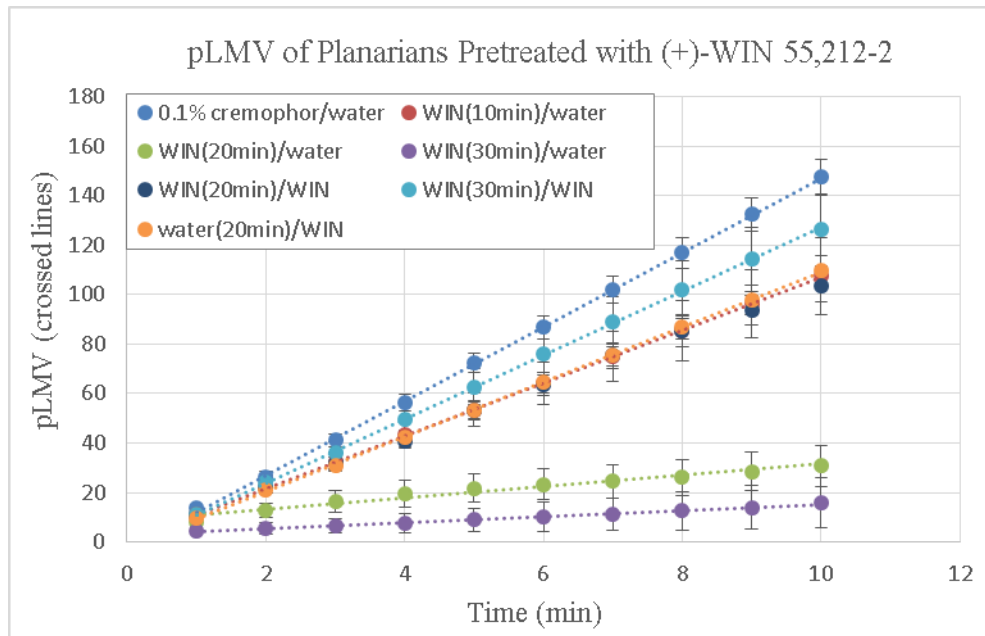


Figure 3 Cumulative pLMV of planarians pretreated with (+)-WIN 55,212-2 (10 μ M) or control in different pretreating periods

Table 2 Tukey's Pairwise Comparisons: Cumulative pLMV of planarians during a 10-min observation period (Test) after pretreatment ($p < 0.05$)*

Pretreatment	Test	N	Cumulative pLMV	Groupin g
0.1% Cremophor	Water	10	147.40 ± 7.5	A
(+)-WIN 55,212-2 (30 min)	(+)-WIN 55,212-2	6	126.3 ± 14.5	A
Water (20 min)	(+)-WIN 55,212-2	6	109.67 ± 6.0	A
(+)-WIN 55,212-2 (10 min)	Water	7	107.4 ± 15.8	A
(+)-WIN 55,212-2 (20 min)	(+)-WIN 55,212-2	6	103.50 ± 6.7	A
(+)-WIN 55,212-2 (20 min)	Water	12	30.67 ± 8.4	B
(+)-WIN 55,212-2 (30 min)	Water	10	15.2 ± 10.3	B

*Means which do not share the same letter have a significant difference.

3.4. Discussion and Conclusion

In previous reports (Rawls, Rodriguez et al. 2006; Rawls, Gomez et al. 2007), abstinence-induced withdrawal from (+)-WIN 55,212-2 (10 μ M) followed 1 h of pretreatment. Now we report that after 20-min (+)-WIN 55,212-2 (10 μ M) pretreatment, planarians showed decreased cumulative pLMV in water (i.e. abstinence-induced withdrawal), which indicates that 20-min exposure of (+)-WIN 55,212-2 (10 μ M) is sufficient to develop cannabinoid agonist physical dependence in planarians. Planarians in the 20-min and 30-min (+)-WIN 55,212-2 pretreatment groups displayed the head bob,

head swing and tail twist behaviors (results are not shown) that are characteristic of withdrawal signs in planarians (Raffa and Desai 2005).

After 0.1% cremophor exposure, planarians showed stable locomotor velocity of 147.4 (± 7.48), which is consistent with the value of vehicle control groups in prior work (Rawls, Rodriguez et al. 2006; Rawls, Gomez et al. 2007). In addition, other negative controls (i.e., pretreated and tested in (+)-WIN 55,212-2, pretreated in water, and tested in (+)-WIN 55,212-2 showed normal cumulative pLMV. This indicates that the decreased pLMV in 20-min and 30-min observed in the cannabinoid agonist pretreatment groups was caused by the removal of the cannabinoid agonist, rather than non-receptor related effects. Likewise, even the longest exposure of 30 min is likely too short to cause gene expression changes. Thus, the concentration of 10 μ M and the pretreating period of 20 min were used for designing the subsequent experiments.

In summary, planarians displayed abstinence-induced withdrawal from (+)-WIN 55,212-2 (10 μ M) following 20 min of pretreatment.

CHAPTER 4

THE USE OF CB RECEPTOR ANTAGONISTS IN CB RECEPTOR AGONIST- PRETREATED PLANARIANS

4.1. Introduction

4.1.1. Cannabinoid Receptor Ligands: Agonists

50 years ago, the principal psychoactive component of marijuana, Δ^9 -tetrahydrocannabinol (Δ^9 -THC) was isolated and synthesized (Mechoulam, Braun et al. 1967). It stimulated scientists to synthesize novel and potent CB receptor ligands and to study cannabinoid receptor pharmacology. At present, CB receptor agonists are primarily subdivided into 3 groups based on their chemical structures: dibenzopyran derivatives (classical cannabinoids), bicyclic and tricyclic analogues of Δ^9 -THC (nonclassical cannabinoids), and aminoalkylindoles. (Howlett, Barth et al. 2002).

Dibenzopyran derivatives include natural cannabis and synthetic their analogues. They are the first classical cannabinoids, and they display poor selectivity between CB1 and CB2 receptors. Δ^9 -THC (structure shown in Figure 4a), Δ^8 -THC and 11-hydroxy- Δ^8 -THC-dimethylheptyl (HU-210) are the most investigated classical cannabinoids. Of these, Δ^9 -THC and Δ^8 -THC behave as partial agonists, whereas HU-210 is a relatively potent cannabinoid receptor agonist (Howlett, Barth et al. 2002).

The lead compound of nonclassical cannabinoids is (-)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexan-1-ol (CP47497) synthesized at Pfizer by removing the dihydropyran ring of THC. (1*R*,3*R*,4*R*)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol (CP55940)

was then synthesized and served as prototype cannabinoid agonists. It is less lipophilic and more potent than THC and lack CB1/CB2 selectivity. [³H]CP55940 has been the most commonly used radiolabeled cannabinoid agonist (Howlett, Barth et al. 2002).

Aminoalkylindoles was the first group that was not structurally derived from THC. [(3*R*)-2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo [1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone, monomethanesulfonate ((+)-WIN 55,212-2) (structure shown in Figure 4b) is the most studied compound in this series. For both CB1 and CB2 receptors, it displays high affinity and potency with mild selectivity to CB2 receptors (Howlett, Barth et al. 2002). In order to further study the structure activity relationship, researcher replaced the *N*-aminoalkyl group with an alkyl group. Both *in vitro* and *in vivo* assays revealed high affinity and efficacy. The first identifies compound in this group was [naphthalen-1-yl-(1-pentylindol-3-yl)methanone (JWH018). 1-pentyl-3-(2-methylphenylacetyl)indoles (JWH251) (structure shown in Figure 4c) and 1-pentyl-3-(3-methoxyphenylacetyl)indole (JWH302) displayed CB1 selectivity with high affinity for the CB1 receptor (Huffman, Szklennik et al. 2005).

Classical cannabinoids show poor solubility and relatively moderate potency at CB1 receptors. Furthermore, it was reported to be difficult to induce spontaneous withdrawal in Δ^9 -THC-pretreated rats (Aceto, Scates et al. 2001). Thus, we precluded using Δ^9 -THC in planarians. On the other hand, (+)-WIN 55,212-2 shows sufficient affinity and efficacy at cannabinoid receptors. It is the first cannabinoid agonist that showed abstinence-induced withdrawal in rats after cessation of administration (Aceto, Scates et al. 2001). In addition, previous works (Rawls, Rodriguez et al. 2006; Rawls, Gomez et al. 2007) showed that planarians displays abstinence-induced withdrawal from

(+)-WIN 55,212-2. Thus, (+)-WIN 55,212-2 is the prototypical cannabinoid agonist we chose for our project. JWH251 belongs to the same series as (+)-WIN 55,212-2. It shows moderate selectivity at CB1 receptors while (+)-WIN 55,212-2 shows moderate selectivity at CB2 receptors. Therefore, JWH251 is the second cannabinoid agonist we chose for our experiments. Table 3 shows the affinity of cannabinoid agonists.

4.1.2. *Cannabinoid Receptor Ligands: Antagonist*

Cannabinoid antagonists are structurally subdivided into 2 groups: diarylpyrazoles and others. The most studied compounds in diarylpyrazole derivatives are *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride (SR141716A) and 5-(4-chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-*N*-[(1*S*,2*S*,4*R*)-1,3,3-trimethylbicyclo[2.2.1]hept-2-yl]-1*H*-pyrazole-3-carboxamide (SR144528) (structure shown in Figure 4g). SR141716A is a potent CB1 selective antagonist while SR144528 is CB2 selective. Many reports state they, by themselves, can produce effects opposite of effects produced by cannabinoid receptor agonists, which indicates that cannabinoid receptors exhibit constitutive activity. *N*-(piperidin-1-yl)-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (AM251) (structure shown in Figure 4d) and *N*-(morpholin-4-yl)-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (AM281) (structure shown in Figure 4e) are two analogs of SR141716A with selectivity for CB1 receptors. Similar to SR141716A, AM281 was reported to be an inverse agonist when used by itself (Cosenza, Gifford et al. 2000).

In 2004, Lange, et al. (Lange, Coolen et al. 2004; Lange, van Stuivenberg et al. 2005) reported novel 3,4-diarylpyrazolines as CB1 receptor selective antagonists. The

prototypical member is 4*S*-(-)-3-(4-chlorophenyl)-*N*-methyl-*N'*-[(4-chlorophenyl)-sulfonyl]-4-phenyl-4,5-dihydro-1*H*-pyrazole-1-carboxamide (SLV319) (structure is shown in Figure 4f). It is usually used to explore obesity treatment (Srivastava, Joharapurkar et al. 2007). The progressive characteristic of this series is its improved water solubility.

In addition to diarylpyrazoles, there are several other notable cannabinoid antagonists, such as 4-[6-methoxy-2-(4-methoxyphenyl)-1-benzofuran-3-carbonyl]benzotrile (LY320135) and 1-[2-(morpholin-4-yl)ethyl]-2-methyl-3-(4-methoxybenzoyl)-6-iodoindole (AM630). LY320135 behaves much higher affinity (but lower than SR141716A) to CB1 than CB2 receptors. However, it also binds to 5-HT₂ receptors at concentrations in micromolar level. AM630 is recognized as a selective CB2 inverse agonist and weak partial CB1 agonist.

AM251 displays selectivity to CB1 receptor with high potency. In addition, it is often used in research to study substance disorder (Yamamoto, Anggadiredja et al. 2004; Riebe, Lee et al. 2010), so it is the first CB antagonist we chose for our project. In order to obtain consistent and convincing results, we chose AM281, which belongs to the same series as AM251, as well. SLV319 shows better water solubility than traditional cannabinoid antagonists. Moreover, SLV319 was reported to be orally active in cannabinoid pharmacological *in vivo* models (Howlett, Barth et al. 2002). Therefore SLV319 was chosen as well. We also chose one classic CB2 selective antagonist, SR144528, in order to observe the effects caused by CB2-prefering antagonist. The affinity of the compounds are listed in Table 3.

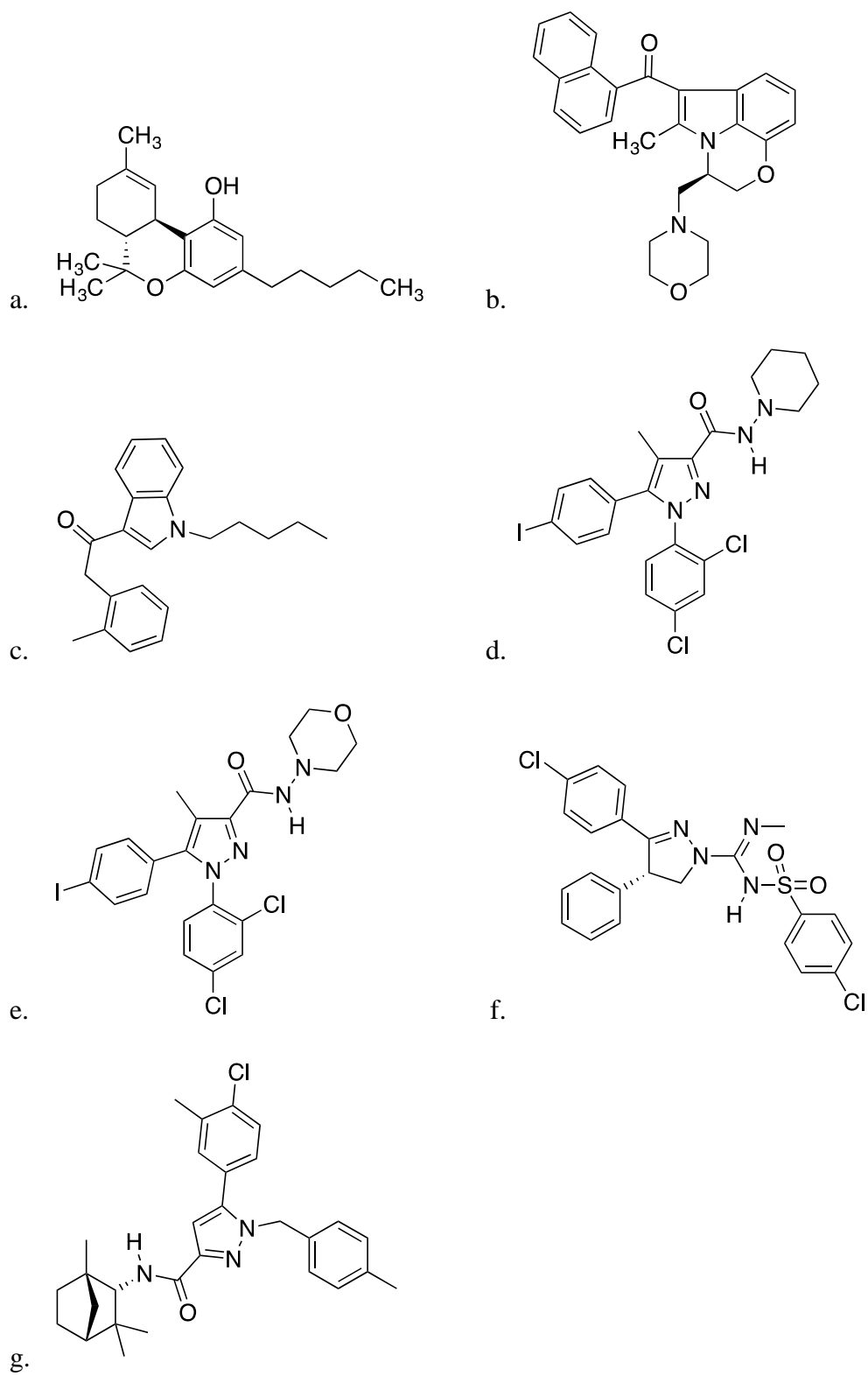


Figure 4 Structures of cannabinoid agonists and antagonists. a. Δ^9 -THC,
 b. WIN 55,212-2, c. JWH251, d. AM251, e. AM281, f. SLV319, g. SR144528

Table 3 Affinity of Cannabinoid Ligands*

	CB1 K _i (nM)	CB2 K _i (nM)	Reference
Agonists			
Δ ⁹ -Tetrahydrocannabinol	35-53	4.9-75	(Howlett, Barth et al. 2002)
(+)-WIN 55,212-2	1.9-123	0.3-39	(Howlett, Barth et al. 2002)
JWH251	29	143	(Wiley, Marusich et al. 2012)
Antagonists			
AM251	7.5	2290	(Howlett, Barth et al. 2002)
AM281	12	4200	(Howlett, Barth et al. 2002)
SLV319	7.8	7493	(Lange, van Stuijvenberg et al. 2005)
SR144528	437	0.60	(Howlett, Barth et al. 2002)

* The K_i values of CB1 and CB2 ligands were measured by the *in vitro* displacement of [³H]CP55940, [³H]R-(+)-WIN55212, or [³H]HU-243 from CB1 and CB2 specific binding sites.

4.1.3. Aim 2

The goals were establishing cannabinoid antagonist-induced precipitated withdrawal model in planarians and exploring the effects of CB antagonists on the development of CB agonist-induced physical dependence. In order to do that, we had three purposes here: one, establish an abstinence-induced withdrawal planarian model by using CB agonist JWH251; two, using different pairs (7 pairs in total) of agonist plus antagonist from 2 agonists ((+)-WIN 55,212-2 and JWH251) and 4 antagonists (AM251,

AM281, SLV319 and SR144528) to study the effects of antagonists on the agonist-developed physical dependence; three, using the same pairs to study the ability of CB antagonist-precipitated withdrawal in planarians.

4.2. Methods

4.2.1. Animal and Drugs

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

(+)-WIN 55,212-2 (mesylate), JWH251, AM251, AM281, SLV319, SR144528 were purchased from Cayman Chemical. Cremophor was a gift from Dr. Rawls. AmQuel® water conditioner (5 mL per 40 liters of water) was added to tap water at least 12 hours before use (subsequently abbreviated “water”). (+)-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 directly dissolved with water into 0.1 mM stock solution. Agonist test solutions ((+)-WIN 55,212-2, 10 µM; JWH251, 10 µM) were diluted with water. Agonist-antagonist solutions were prepared by mixing two stock solutions with a certain ratio (1:2, 1:3, 1:4, or 1:5), and then diluting with water. Cremophor solutions (0.01%, 0.07% and 0.42%) was made with water as vehicle control solution.

4.2.2. Behavior Measurements

As described in Chapter 3 (page 13), we used pLMV to measure the behavior of planarians. Planarians were pretreated with CB agonists (WIN 55,212-2 and JWH251), combinations of CB agonist and antagonist (WIN 55,212-2 + AM251, WIN 55,212-2 + SLV319, WIN 55,212-2 + AM281 and WIN 55,212-2 + SR144528, JWH251 + AM251, JWH251 + SLV319 and JWH251 + AM281) or vehicle controls. After that, planarians were placed individually into a clear plastic petri dish (14-cm diameter) containing room-temperature (21°C) water, CB agonist-antagonist mix solutions (WIN 55,212-2 + AM251, WIN 55,212-2 + SLV319, WIN 55,212-2 + AM281 and WIN 55,212-2 + SR144528, JWH251 + AM251, JWH251 + SLV319 and JWH251 + AM281) or vehicle control. The dish was placed over paper that has gridlines spaced 0.5 cm apart. pLMV was measured by counting the number of gridlines that planarian crossed or re-crossed in every minute over a 10-minute observation period. Each planarian was used only once. pLMV was plotted as the mean (\pm S.E.M.) of the cumulative number of gridlines crossed by individual planarian per minute. The groups are shown in *Table 4, Table 5, Table 6, Table 7, Table 8, Table 9, Table 10, and Table 11.*

Table 4 Groups Design for Vehicle Controls and Developing JWH251 Withdrawal Model

Pretreating Solution	Pretreating Period	Test Solution	N
		JWH251 (10 μ M)	6
(+)-WIN 55,212-2 (10 μ M)	20 min	JWH251 (10 μ M)	5
JWH251 (10 μ M)	30 min	JWH251 (10 μ M)	6
JWH251 (10 μ M)	20 min	Water	4
JWH251 (10 μ M)	30 min	Water	6
0.1 % Cremophor	20 min	Water	10
0.07 % Cremophor	20 min	Water	4
0.42 % Cremophor	20 min	0.1 % Cremophor	6

Table 5 Groups Design for Interrupting (+)-WIN 55,212-2 (10 μ M)-Induced Physical Dependence with SLV319

Pretreating Solution	Pretreating Period	Test Solution	N
(+)-WIN 55,212-2 (10 μ M)	20 min	Water	12
0.42% Cremophor	20 min	Water	6
0.07% Cremophor	20 min	0.42% Cremophor	4
WIN + SLV319 (20 μ M)	20 min	Water	3
WIN + SLV319 (30 μ M)	20 min	Water	8
WIN + SLV319 (40 μ M)	20 min	Water	8
(+)-WIN 55,212-2 (10 μ M)	20 min	WIN + SLV319 (10 μ M)	4
(+)-WIN 55,212-2 (10 μ M)	20 min	WIN + SLV319 (20 μ M)	4
(+)-WIN 55,212-2 (10 μ M)	20 min	WIN + SLV319 (30 μ M)	8
(+)-WIN 55,212-2 (10 μ M)	20 min	WIN + SLV319 (40 μ M)	8
(+)-WIN 55,212-2 (10 μ M)	20 min	WIN + SLV319 (50 μ M)	6

Table 6 Groups Design for Interrupting (+)-WIN 55,212-2 (10 μ M)-Induced Physical Dependence with AM251

Pretreatment	Pretreating Period	Test Solution	N
0.42% Cremophor	20 min	Water	6
0.07% Cremophor	20 min	0.42% Cremophor	4
(+)-WIN 55,212-2	20 min	Water	12
WIN+AM251 (20 μ M)	20 min	Water	6
WIN+AM251 (30 μ M)	20 min	Water	6
(+)-WIN 55,212-2	20 min	WIN+AM251(20 μ M)	5
(+)-WIN 55,212-2	20 min	WIN+AM251(30 μ M)	9
(+)-WIN 55,212-2	20 min	WIN+AM251(40 μ M)	5
(+)-WIN 55,212-2	20 min	WIN+AM251(50 μ M)	7

Table 7 Groups Design for Interrupting (+)-WIN 55,212-2 (10 μ M)-Induced Physical Dependence with AM281

Pretreatment	Pretreating Period	Test	N
0.42% Cremophor	20 min	Water	6
0.07% Cremophor	20 min	0.42% Cremophor	4
(+)-WIN 55,212-2	20 min	Water	12
WIN+AM281 (20 μ M)	20 min	Water	6
WIN+AM281 (30 μ M)	20 min	Water	6
WIN+AM281 (40 μ M)	20 min	Water	6
WIN+AM281 (50 μ M)	20 min	Water	6
(+)-WIN 55,212-2	20 min	WIN+AM281 (30 μ M)	6
(+)-WIN 55,212-2	20 min	WIN+ AM281 (40 μ M)	6
(+)-WIN 55,212-2	20 min	WIN+AM281 (50 μ M)	6

Table 8 Groups Design for Interrupting (+)-WIN 55,212-2 (10 μ M)-Induced Physical Dependence with SR144528

Pretreatment	Pretreating Period	Test	N
0.42% Cremophor	20 min	Water	6
0.07% Cremophor	20 min	0.42% Cremophor	4
(+)-WIN 55,212-2	20 min	Water	12
WIN+SR144528 (20 μ M)	20 min	Water	7
WIN+SR144528 (30 μ M)	20 min	Water	6
WIN+SR144528 (40 μ M)	20 min	Water	6
(+)-WIN 55,212-2	20 min	WIN+SR144528 (30 μ M)	6
(+)-WIN 55,212-2	20 min	WIN+SR144528 (40 μ M)	7

Table 9 Groups Design for Interrupting JWH251 (10 μ M)*-Induced Physical
Dependence with AM251

Pretreatment	Pretreating Period	Test	N
0.42% Cremophor	20 min	Water	6
0.07% Cremophor	20 min	0.42% Cremophor	4
JWH251	30 min	Water	6
JWH251 + AM251 (30 μ M)	30 min	Water	5
JWH251 + AM251 (40 μ M)	30 min	Water	5
JWH251 + AM251 (50 μ M)	30 min	Water	6
JWH251	30 min	JWH251 + AM251 (40 μ M)	4
JWH251	30 min	JWH251 + AM251 (50 μ M)	6

Table 10 Groups Design for Interrupting JWH251 (10 μ M)-Induced Physical
Dependence with SLV319

Pretreatment	Pretreating Period	Test	N
0.42% Cremophor	20 min	Water	6
0.07% Cremophor	20 min	0.42% Cremophor	4
JWH251	30 min	Water	6
JWH251 + SLV319 (40 μ M)	30 min	Water	7
JWH251 + SLV319 (50 μ M)	30 min	Water	6
JWH251	30 min	JWH251 + SLV319 (40 μ M)	6
JWH251	30 min	JWH251 + SLV319 (50 μ M)	6

Table 11 Groups Design for Interrupting JWH251 (10 μ M)-Induced Physical Dependence with AM281

Pretreatment	Pretreating Period	Test	N
0.42% Cremophor	20 min	Water	6
0.07% Cremophor	20 min	0.42% Cremophor	4
JWH251	30 min	Water	6
JWH251 + AM281 (20 μ M)	30 min	Water	6
JWH251 + AM281 (30 μ M)	30 min	Water	6
JWH251 + AM281 (40 μ M)	30 min	Water	7
JWH251 + AM281 (50 μ M)	30 min	Water	6
JWH251	30 min	JWH251 + AM281 (20 μ M)	6
JWH251	30 min	JWH251 + AM281 (30 μ M)	6
JWH251	30 min	JWH251 + AM281 (40 μ M)	5
JWH251	30 min	JWH251 + AM281 (50 μ M)	6

4.2.3. Statistical Analysis

Minitab 17 Statistical Software and Excel 2013 were used to perform the statistical analyses. 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 $p < 0.05$.

4.3. Results:

4.3.1. *JWH251-Induced Dependence and Withdrawal Model*

The results of JWH251 by itself are shown in Figure 5 and Table 12. Planarians pretreated in JWH251 (10 μ M) for 30 min then placed in drug-free water is the only group that displayed a significantly decreased ($p < 0.05$) pLMV (58.5 ± 17.2) compared with negative control groups (0.1% Cremophor / water and JWH251 / JWH251). If the pretreatment period was reduced to 20 min, we obtained mildly, but not significantly ($p > 0.05$) decreased pLMV (101.5 ± 38.7) compared with negative controls.

When planarians were observed directly in JWH251 (10 μ M) solution, there was no difference from vehicle control group (0.1% Cremophor), with constant pLMV of 166.67 ± 7.1 . Also, after pretreatment in (+)-WIN 55,212-2 (10 μ M), planarians did not show a significant ($p > 0.05$) difference of pLMV measured in JWH251 (10 μ M) solution. 30 min of pretreating period and 10 μ M of concentration were set as the standard to develop JWH251-induced physical dependence in subsequent experiments.

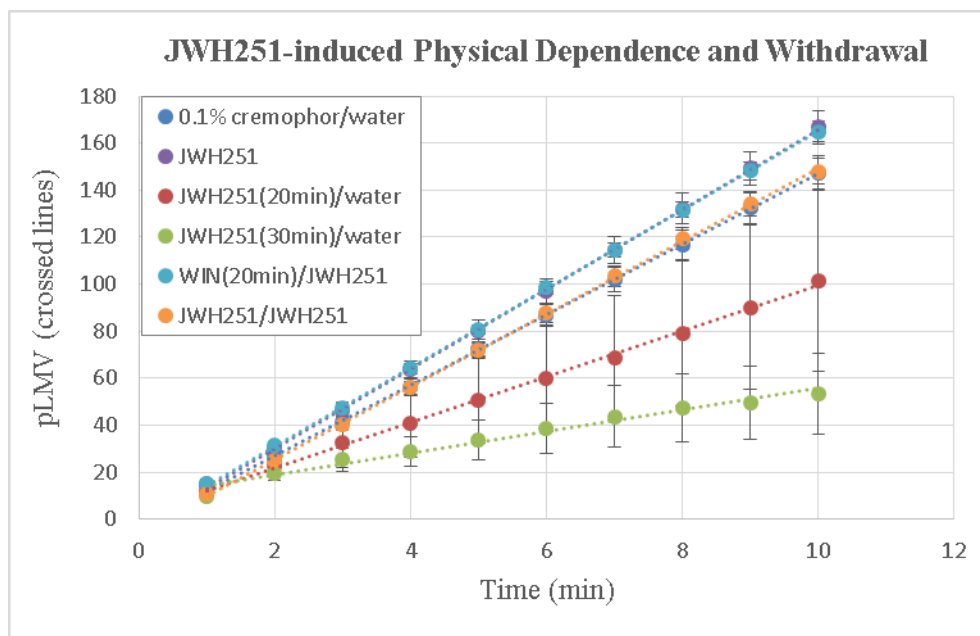


Figure 5 JWH251-induced physical dependence and withdrawal: JWH251 and (+)-WIN 55,212-2 were in the concentration of 10 μ M. WIN: (+)-WIN 55,212-2.

Table 12 Tukey's Pairwise Comparisons: Cumulative pLMV of Planarians During A 10-Min Observation Period (Test) After Pretreatment in JWH251 or Cremophor ($p < 0.05$)*

Pretreatment	Test	N	Cumulative pLMV	Grouping
	JWH251	6	166.67 ± 7.1	A
(+)-WIN 55,212-2 (20 min)	JWH251	5	165.00 ± 4.5	A
JWH251 (30 min)	JWH251	6	148.00 ± 5.4	A
0.1% Cremophor (20 min)	Water	10	147.40 ± 7.5	A
JWH251 (20 min)	Water	4	101.5 ± 38.7	AB
JWH251 (30 min)	Water	6	58.5 ± 17.2	B

*Means which do not share the same letter have a significant difference.

4.3.2. Vehicle Controls

Before using CB receptor antagonists to influence CB agonist effect, we did vehicle controls with the highest cremophor concentration we would use in CB agonist-antagonist mixed solutions. 0.42% cremophor corresponds to the cremophor concentration in the mixed solution that contains 50 μM antagonist and 10 μM agonist. 0.07% cremophor represents the cremophor concentration in CB agonist (10 μM) solution. Three control groups showed no significant difference ($p > 0.05$) (Figure 6 and Table 13) indicating that any results we obtained in this chapter were not caused by cremophor.

Thus, in the following experiments, pretreatment in 0.42% cremophor and then testing in water was chosen to be the negative control of the experiments that would

study the effects of CB antagonists on developing physical dependence with CB agonists. And pretreatment in 0.07% cremophor and then testing in 0.41% cremophor would be the control group of experiments that would study the effects of CB antagonists on precipitating withdrawal behavior.

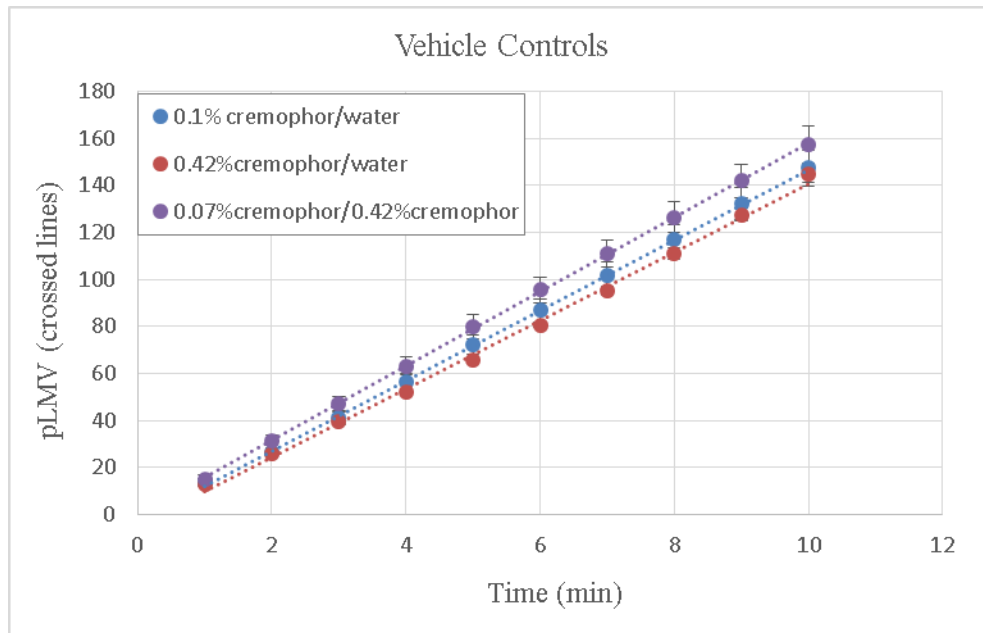


Figure 6 Cumulative pLMV of vehicle control groups

Table 13 Tukey's Pairwise Comparisons: Cumulative pLMV of Vehicle Control Groups

($p < 0.05$)*

Pretreatment	Test	N	Cumulative pLMV	Grouping
0.07% Cremophor	0.42% Cremophor	4	157.50 ± 7.9	A
0.1% Cremophor	Water	10	147.40 ± 7.5	A
0.42% Cremophor	Water	6	144.67 ± 3.6	A

*Means which do not share the same letter have a significant difference.

4.3.3. *The Use of CB1 Receptor Antagonist SLV319 and CB Receptor Agonist WIN 55,212-2*

As shown in Figure 7, planarians co-pretreated in WIN 55,212-2 and SLV319 (30 and 40 μM) then placed into drug-free water showed greater pLMV than pLMV of positive control (WIN (20min) / water: 30.67 ± 8.4). Moreover, pLMV was increased with the increase of the concentration of SLV319. The cumulative pLMV means (\pm S.E.M) of planarians co-pretreated with SLV319 in different concentrations were 21.7 ± 16.2 for 20 μM , 76.60 ± 24.4 for 30 μM , and 123.00 ± 13.6 for 40 μM . However, only the pLMV mean of 40 μM SLV319 group showed significant difference ($p < 0.05$) compared with vehicle control group (Figure 7 and Table 14).

None of the planarians pretreated in WIN 55,212-2 then placed into combinations of WIN 55,212-2 (10 μM) and SLV319 (10, 20, 30, 40 and 50 μM) showed significantly decreased pLMV ($p > 0.05$) compared with negative control group (Figure 8 and Table 15).

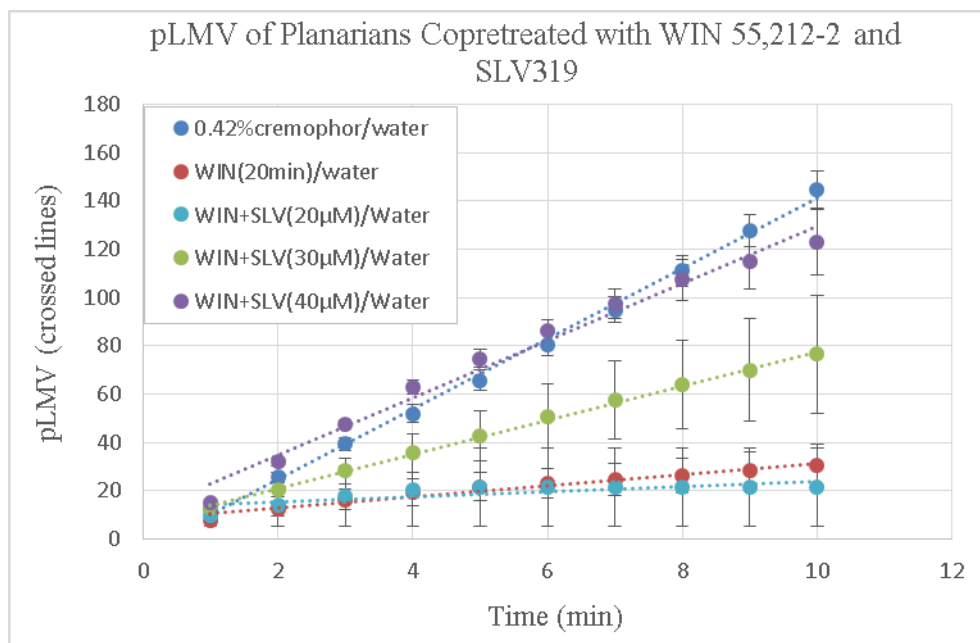


Figure 7 pLMV of Planarians co-pretreated with WIN 55,212-2 and SLV319: WIN: (+)-WIN 55,212-2 (10 µM), SLV: SLV319

Table 14 Tukey's Pairwise Comparisons: Cumulative pLMV of Planarians Co-pretreated with WIN 55,212-2 and SLV319 ($p < 0.05$)*

Pretreatment	Test	N	Cumulative pLMV	Grouping
0.42% Cremophor	Water	6	144.67 ± 3.6	A
WIN+SLV319 (40 µM)	Water	8	123.00 ± 13.6	AB
WIN+SLV319 (30 µM)	Water	8	76.60 ± 24.4	BC
(+)-WIN 55,212-2	Water	12	30.67 ± 8.4	C
WIN+SLV319 (20 µM)	Water	3	21.7 ± 16.2	C

*Means which do not share the same letter have a significant difference.

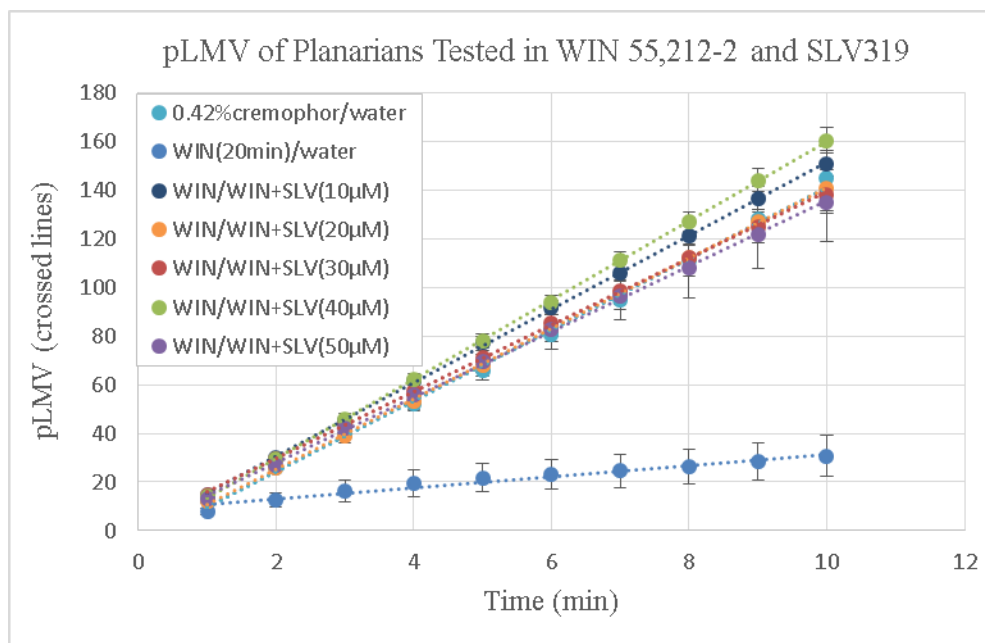


Figure 8 pLMV of planarians pretreated in WIN 55,212-2 and tested in combinations of WIN 55,212-2 and SLV319. WIN: (+)-WIN 55,212-2 (10 µM), SLV: SLV319

Table 15 Tukey's Pairwise Comparisons: Cumulative pLMV of Planarians Pretreated in WIN 55,212-2 and Tested in Combinations of WIN 55,212-2 and SLV319 ($p < 0.05$)*

Pretreatment	Test	N	Cumulative pLMV	Grouping
(+)-WIN 55,212-2	WIN+SLV319(40 μ M)	8	160.25 \pm 5.4	A
0.07% Cremophor	0.42% Cremophor	4	157.50 \pm 7.9	A
(+)-WIN 55,212-2	WIN+SLV319(10 μ M)	4	150.75 \pm 5.4	A
(+)-WIN 55,212-2	WIN+SLV319(20 μ M)	4	140.75 \pm 9.4	A
(+)-WIN 55,212-2	WIN+SLV319(30 μ M)	8	138.00 \pm 7.8	A
(+)-WIN 55,212-2	WIN+SLV319(50 μ M)	6	134.8 \pm 16.1	A
(+)-WIN 55,212-2	Water	12	30.67 \pm 8.4	B

*Means which do not share the same letter have a significant difference.

4.3.4. The Use of CB1 Receptor Antagonist AM251 and CB Receptor Agonist

WIN 55,212-2

The effects of AM251 (20 and 30 μ M) on the development of physical dependence induced by WIN 55212-2 (10 μ M) are shown in Figure 9 and Table 16. After exposure to (+)-WIN 55,212-2 and AM251 mixed solutions, planarians displayed significantly ($p < 0.05$) increased pLMV compared with positive control group (WIN (20min) / water: 30.67 \pm 8.4), and showed no difference ($p > 0.05$) with vehicle control (0.42% cremophor / water: 144.67 \pm 3.6). The two different concentrations showed no difference ($p > 0.05$) with each other.

pLMV of Planarians pretreated in WIN 55,212-2 then placed into combinations of WIN 55,212-2 (10 μ M) and AM251 (20, 30, 40 and 50 μ M) showed no statistical difference ($p > 0.05$) compared with negative control group. Among them, the group of 50 μ M showed lowest pLMV. In addition, different concentrations of AM251 displayed no difference ($p > 0.05$) in pLMV (Figure 10 and Table 17).

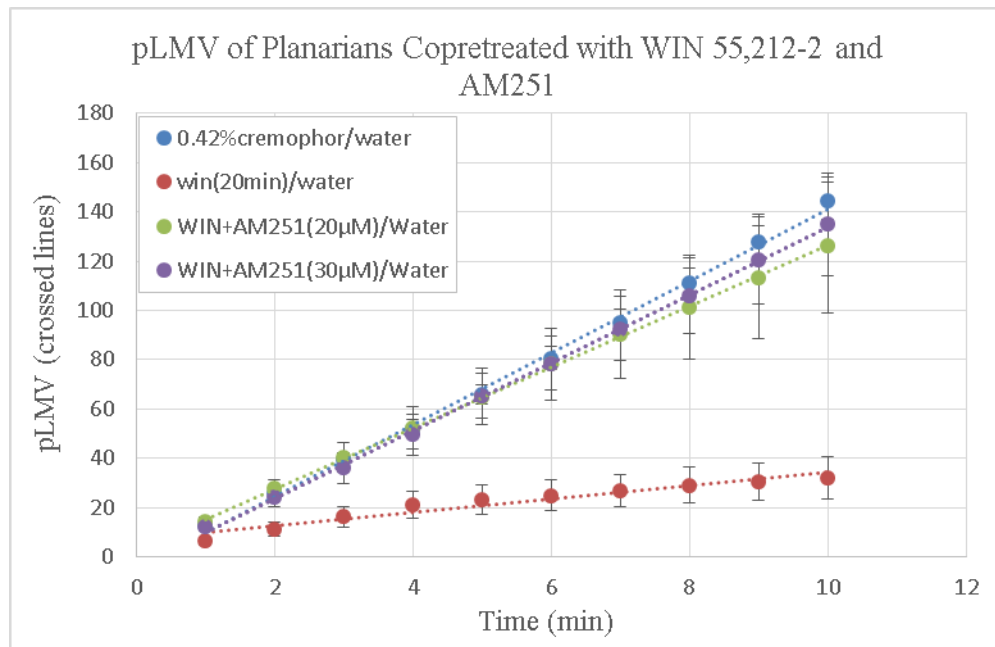


Figure 9 pLMV of planarians co-pretreated with WIN 55,212-2 and AM251, WIN: (+)-WIN 55,212-2 (10 μ M)

Table 16 Tukey's Pairwise Comparisons: Cumulative pLMV of Planarians Co-pretreated in WIN 55,212-2 and AM251 ($p < 0.05$) *

Pretreatment	Test	N	Cumulative pLMV	Grouping
0.42% Cremophor	Water	6	144.67 ± 3.6	A
WIN+AM251 (30 μ M)	Water	6	135.00 ± 20.9	A
WIN+AM251 (20 μ M)	Water	6	126.50 ± 27.7	A
(+)-WIN 55,212-2	Water	12	30.67 ± 8.4	B

*Means which do not share the same letter have a significant difference.

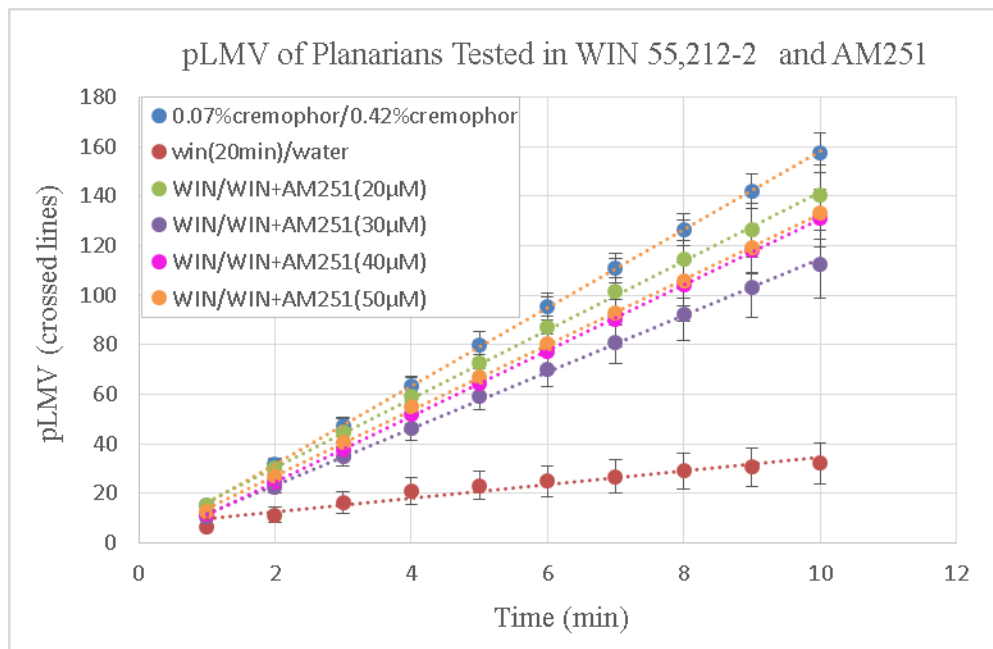


Figure 10 pLMV of planarians pretreated in WIN 55,212-2 and tested in combinations of WIN 55,212-2 and AM251. WIN: (+)-WIN 55,212-2 (10 μ M)

Table 17 Tukey's Pairwise Comparisons: Cumulative pLMV of Planarians Pretreated in WIN 55,212-2 and Tested in Combinations of WIN 55,212-2 and AM251 ($p < 0.05$)*

Pretreatment	Test	N	Cumulative pLMV	Grouping
0.07% Cremophor	0.42% Cremophor	4	157.50 ± 7.9	A
(+)-WIN 55,212-2	WIN+AM251 (20 µM)	5	140.60 ± 18.1	A
(+)-WIN 55,212-2	WIN+AM251 (50 µM)	7	133.00 ± 19.4	A
(+)-WIN 55,212-2	WIN+AM251 (40 µM)	5	131.20 ± 11.7	A
(+)-WIN 55,212-2	WIN+AM251 (30 µM)	9	112.8 ± 13.6	A
(+)-WIN 55,212-2	Water	12	30.67 ± 8.4	B

*Means which do not share the same letter have a significant difference.

4.3.5. The Use of CB1 Receptor Antagonist AM281 and CB Receptor Agonist

WIN 55,212-2

Among the groups of 4 concentrations of AM281, planarians co-pretreated with (+)-WIN 55,212-2 (10 µM) and the lowest two concentrations (20 and 30 µM) of AM281 then tested in water showed significantly ($p < 0.05$) decreased pLMV (71.50 ± 17.6 and 54.70 ± 22.1 respectively) compared with vehicle control, while the pLMV of the highest two concentration groups (40 and 50 µM) showed no difference ($p > 0.05$) with negative control (Figure 11 and Table 18).

As shown in Figure 12 and Table 19, although planarians displayed decreased pLMV when they were pretreated in (+)-WIN 55,212-2 (10 µM) and tested in AM281

(20, 30, 40 and 50 μM), neither of the groups obtained statistical difference ($p > 0.05$) compared with vehicle control. Moreover, different groups have no difference ($p > 0.05$) compared with each other.

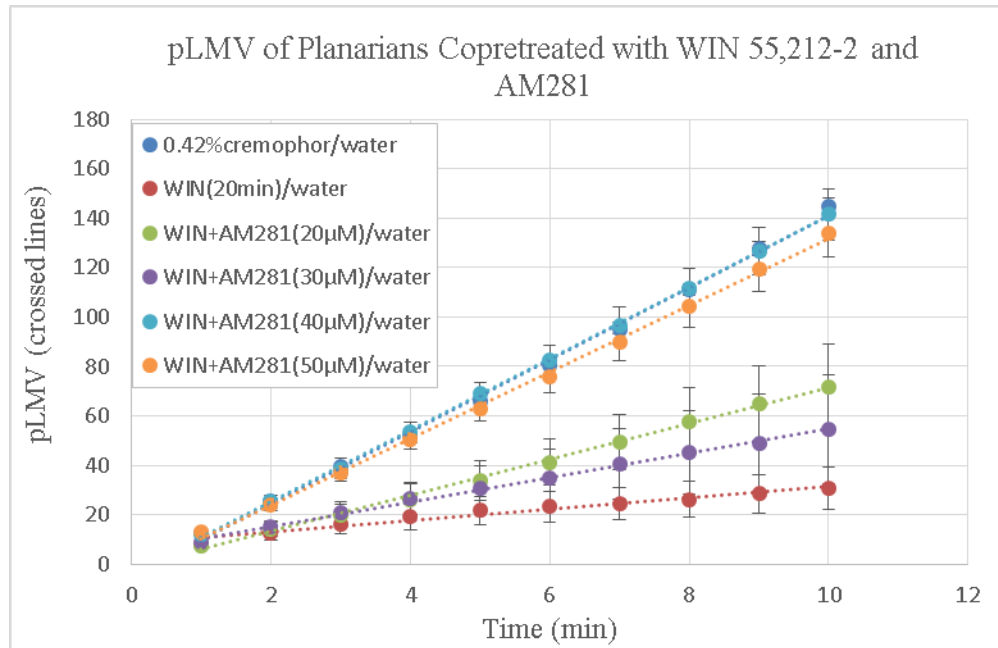


Figure 11 pLMV of planarians co-pretreated with WIN 55,212-2 and AM281, WIN: (+)-WIN 55,212-2 (10 μM)

Table 18 Tukey's Pairwise Comparisons: Cumulative pLMV of Planarians Co-pretreated in WIN 55,212-2 and AM281 ($p < 0.05$) *

Pretreatment	Test	N	Cumulative pLMV	Grouping
0.42% Cremophor	Water	6	144.67 ± 3.6	A
WIN+AM281 (40 µM)	Water	6	141.70 ± 10.4	A
WIN+AM281 (50 µM)	Water	6	134.00 ± 9.6	A
WIN+AM281 (20 µM)	Water	6	71.50 ± 17.6	B
WIN+AM281 (30 µM)	Water	6	54.70 ± 22.1	B
(+)-WIN 55,212-2	Water	12	30.67 ± 8.4	B

*Means which do not share the same letter have a significant difference.

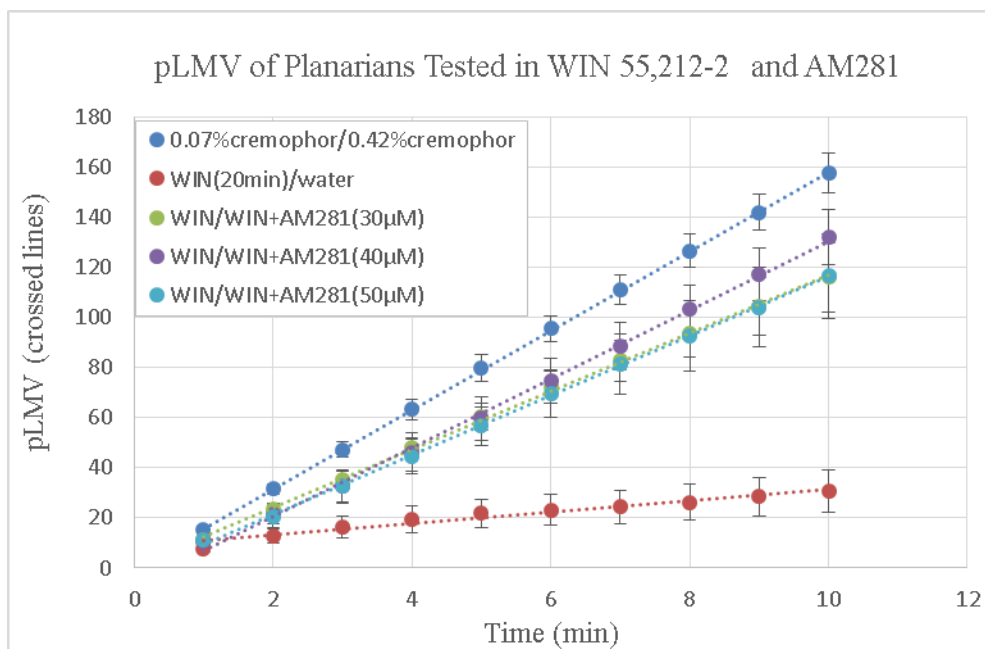


Figure 12 pLMV of planarians pretreated in WIN 55,212-2 and tested in combinations of WIN 55,212-2 and AM281. WIN: (+)-WIN 55,212-2 (10 µM)

Table 19 Tukey's Pairwise Comparisons: Cumulative pLMV of Planarians Pretreated in WIN 55,212-2 and tested in combinations of WIN 55,212-2 and AM281 ($p < 0.05$)*

Pretreatment	Test	N	Cumulative pLMV	Grouping
0.07% Cremophor	0.42% Cremophor	4	157.50 ± 7.9	A
(+)-WIN 55,212-2	WIN+ AM281 (40 µM)	6	132.0 ± 11.0	A
(+)-WIN 55,212-2	WIN+AM281 (50 µM)	6	116.70 ± 16.9	A
(+)-WIN 55,212-2	WIN+AM281 (30 µM)	6	116.00 ± 13.9	A
(+)-WIN 55,212-2	Water	12	30.67 ± 8.4	B

*Means which do not share the same letter have a significant difference.

4.3.6. *The Use of CB2 Receptor Antagonist SR144528 and CB Receptor Agonist WIN 55,212-2*

Figure 13 and Table 20 show the effects of SR144528 (20, 30, and 40 μM) on the development of physical dependence caused by WIN 55,212-2 (10 μM). Planarians pretreated in combinations of WIN 55,212-2 (10 μM) and SR144528 (20, 30, and 40 μM) then placed into drug-free water displayed significantly ($p < 0.05$) greater pLMV than planarians only pretreated in WIN 55,212-2 then tested in water. Among different dose levels, the 30 μM SR144528 group displayed the highest cumulative pLMV (121.67 ± 8.3) which is the only group having no difference ($p > 0.05$) with negative control group. Planarians pretreated with the combinations of WIN 55,212-2 (10 μM) and both 20 and 40 μM of SR144528 then tested in drug-free water showed significantly ($p < 0.05$) lower pLMV (96.43 ± 9.2 and 95.00 ± 14.1 , respectively) than negative control. No statistical difference ($p > 0.05$) was obtained among three groups with different SR144528 concentrations.

Planarians pretreated in WIN 55,212-2 (10 μM) then tested in combinations of WIN 55,212-2 (10 μM) and SR144528 (30 and 40 μM) showed lower pLMV than negative control group. However, no statistical difference ($p > 0.05$) was obtained. (Figure 14 and Table 21)

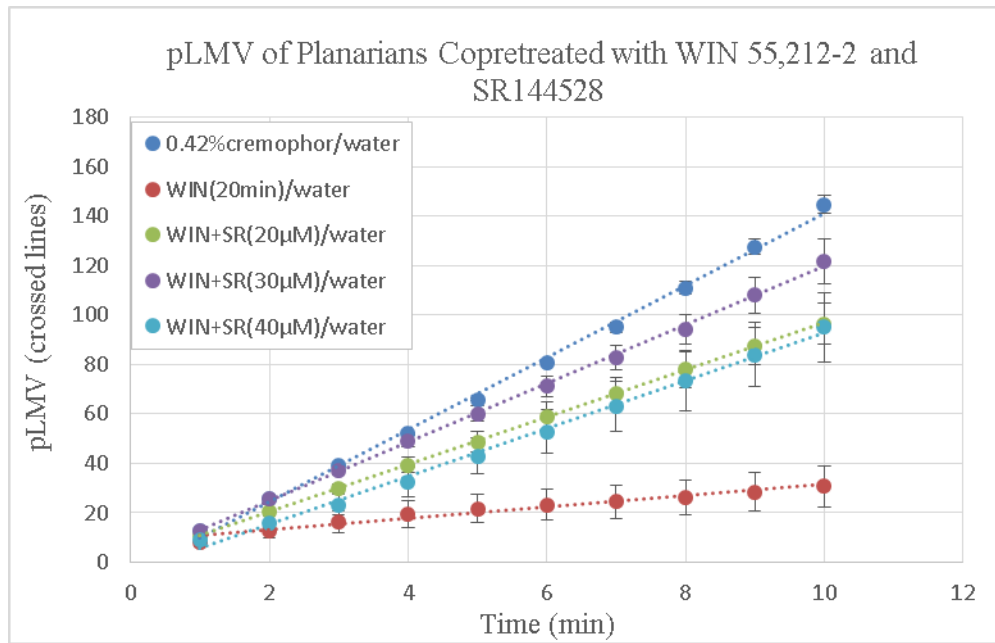


Figure 13 pLMV of planarians co-pretreated with WIN 55,212-2 and SR144528, WIN: (+)-WIN 55,212-2 (10 µM)

Table 20 Tukey's Pairwise Comparisons: Cumulative pLMV of Planarians Co-pretreated in WIN 55,212-2 and SR144528 ($p < 0.05$) *

Pretreatment	Test	N	Cumulative pLMV	Grouping
0.42% Cremophor	Water	6	144.67 ± 3.6	A
WIN+SR144528 (30 μ M)	Water	6	121.67 ± 8.3	AB
WIN+SR144528 (20 μ M)	Water	7	96.43 ± 9.2	B
WIN+SR144528 (40 μ M)	Water	6	95.00 ± 14.1	B
(+)-WIN 55,212-2	Water	12	30.67 ± 8.4	C

*Means which do not share the same letter have a significant difference.

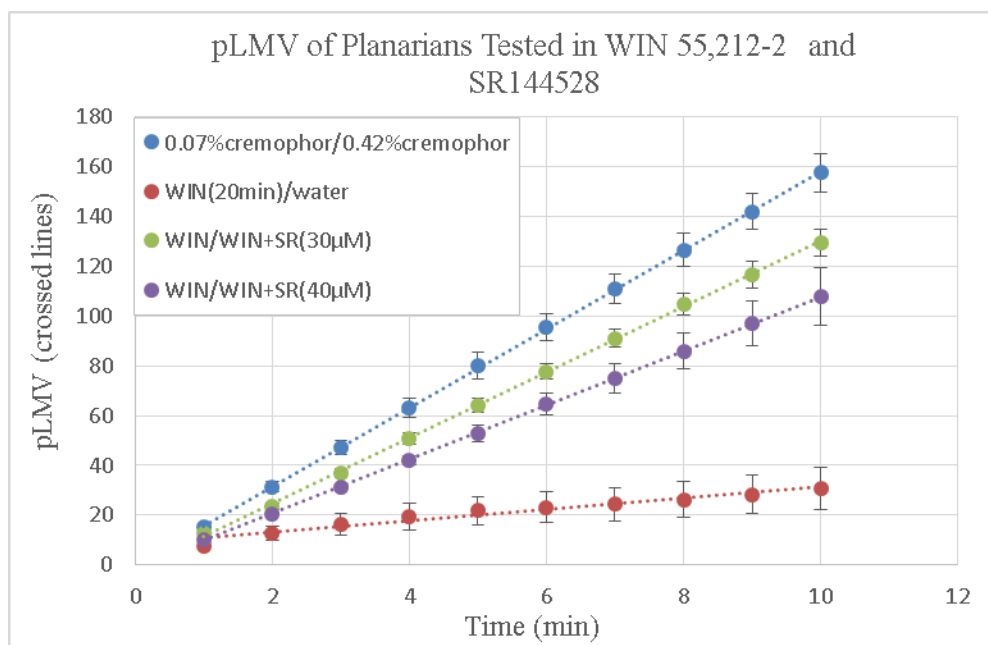


Figure 14 pLMV of planarians pretreated in WIN 55,212-2 and tested in combinations of WIN 55,212-2 and SR144528. WIN: (+)-WIN 55,212-2 (10 µM)

Table 21 Tukey's Pairwise Comparisons: Cumulative pLMV of Planarians Pretreated in WIN 55,212-2 and Tested in Combinations of WIN 55,212-2 and SR144528 ($p < 0.05$)*

Pretreatment	Test	N	Cumulative pLMV	Grouping
0.07% Cremophor	0.42% Cremophor	4	157.50 ± 7.9	A
(+)-WIN 55,212-2	WIN+SR144528 (30 µM)	6	129.33 ± 5.6	A
(+)-WIN 55,212-2	WIN+SR144528 (40 µM)	7	107.70 ± 11.6	A
(+)-WIN 55,212-2	Water	12	30.67 ± 8.4	B

*Means which do not share the same letter have a significant difference.

4.3.7. *The Use of CB1 Receptor Antagonist AM251 and CB Receptor Agonist JWH251*

The effects of AM251 (30, 40, and 50 μM) on the development of physical dependence caused by JWH251 (10 μM) are shown in Figure 15 and Table 22. None of the groups pretreated with JWH251 (10 μM) and AM251 (50 μM) then placed into drug-free water showed statistical difference ($p > 0.05$) compared with positive control group (JWH251/water: 58.5 ± 17.22). Even though, the highest concentration of AM251 attenuated JWH251-induced physical dependence, which is indicated by the greater pLMV (117.70 ± 14.4) than positive control group (JWH251/water: 58.5 ± 17.22) and by no statistical difference ($p > 0.05$) with negative control group.

pLMV of Planarians pretreated in JWH251 then placed into combinations of JWH251 (10 μM) and AM251 (40 and 50 μM) showed no statistical difference ($p > 0.05$) compared with negative control group. In addition, different concentrations of AM251 displayed no difference in pLMV (Figure 16 and Table 23).

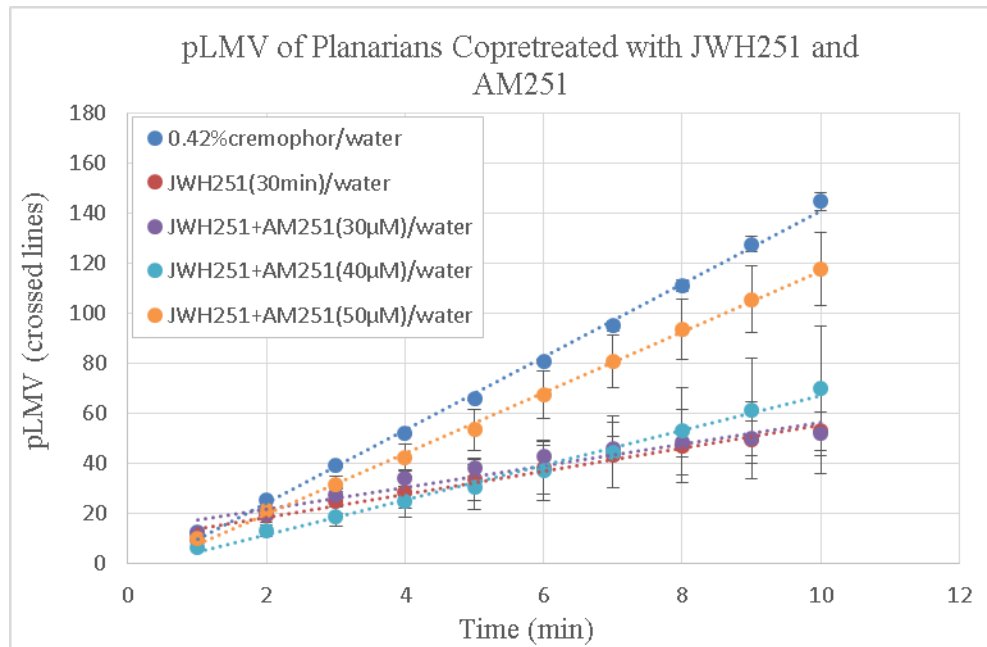


Figure 15 pLMV of planarians co-pretreated with JWH251 and AM251. JWH251 (10 µM)

Table 22 Tukey's Pairwise Comparisons: Cumulative pLMV of Planarians Co-pretreated in JWH251 and AM251 ($p < 0.05$) *

Pretreatment	Test	N	Cumulative pLMV	Grouping
0.42% Cremophor	Water	6	144.67 ± 3.6	A
JWH251 + AM251 (50 μ M)	Water	6	117.70 ± 14.4	AB
JWH251 + AM251 (40 μ M)	Water	5	70.20 ± 24.9	B
JWH251	Water	6	58.5 ± 17.22	B
JWH251 + AM251 (30 μ M)	Water	5	52.00 ± 8.75	B

*Means which do not share the same letter have a significant difference.

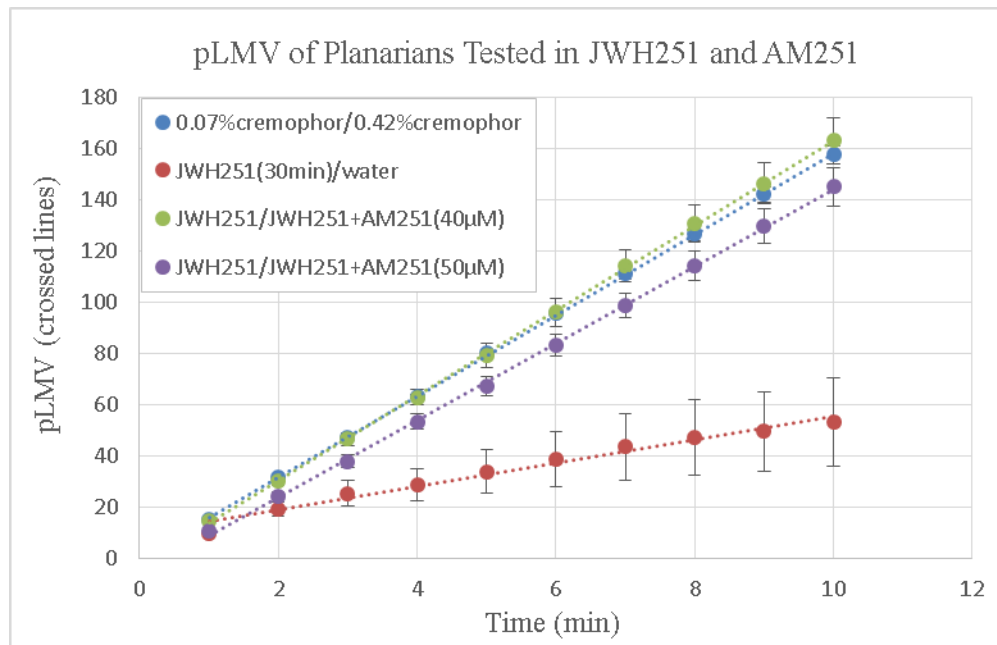


Figure 16 pLMV of planarians pretreated in JWH251 and tested in combinations of JWH251 and AM251. JWH251 (10 μ M)

Table 23 Tukey's Pairwise Comparisons: Cumulative pLMV of Planarians Pretreated in JWH251 and Tested in Combinations of JWH251 and AM251 ($p < 0.05$)*

Pretreatment	Test	N	Cumulative pLMV	Grouping
JWH251	JWH251 + AM251 (40 μ M)	4	163.00 \pm 9.2	A
0.07% Cremophor	0.42% Cremophor	4	157.50 \pm 7.9	A
JWH251	JWH251 + AM251 (50 μ M)	6	145.00 \pm 7.4	A
JWH251	Water	6	58.5 \pm 17.22	B

*Means which do not share the same letter have a significant difference.

4.3.8. The Use of CB1 Receptor Antagonist SLV319 and CB Receptor Agonist JWH251

As shown in Figure 17, planarians co-pretreated in combinations of JWH251 (10 μ M) and SLV319 (40 and 50 μ M) then tested in drug-free water showed increased pLMV (100.30 \pm 14.4 and 129.80 \pm 13.9, respectively) compared with positive control group. However, only the group of 50 μ M SLV319 showed significant difference ($p < 0.05$) compared with positive control (Table 24).

Figure 18 and Table 25 elucidate the effects of SLV319 (40 and 50 μ M) on antagonist-induced withdrawal from JWH251 (10 μ M). Planarians pretreated with JWH251 then tested in combinations of JWH251 (10 μ M) and SLV319 (40 and 50 μ M) did not show significantly ($p > 0.05$) different pLMV compared with negative control

group. In addition, there was no difference ($p > 0.05$) in terms of pLMV among different concentrations of SLV319.

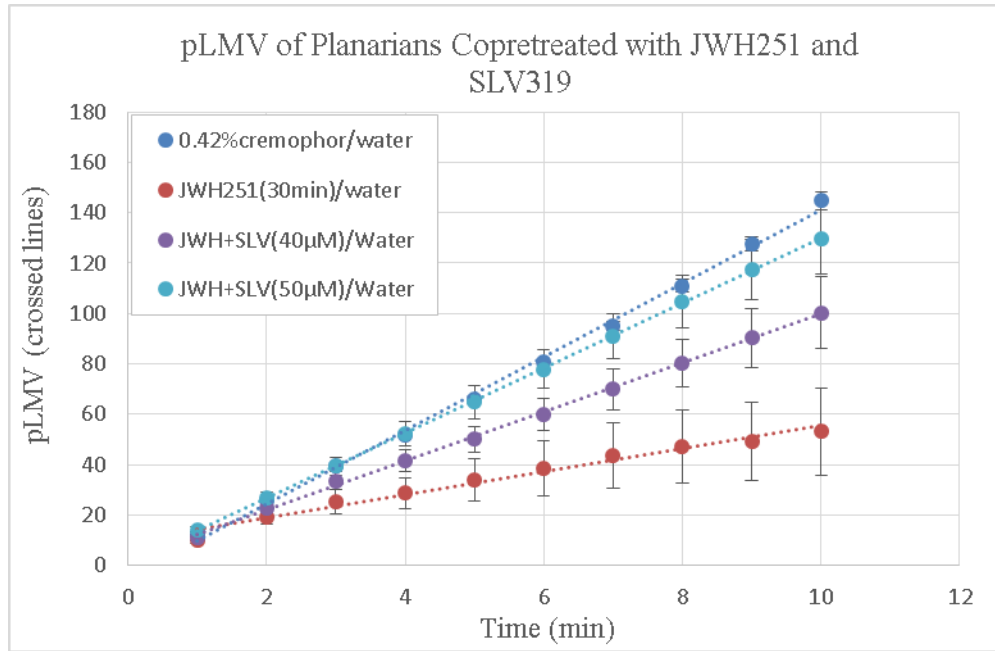


Figure 17 pLMV of planarians co-pretreated with JWH251 and SLV319. JWH251 (10 μ M)

Table 24 Tukey's Pairwise Comparisons: Cumulative pLMV of Planarians Co-pretreated in JWH251 and SLV319 ($p < 0.05$) *

Pretreatment	Test	N	Cumulative pLMV	Grouping
0.42% Cremophor	Water	6	144.67 ± 3.6	A
JWH251 + SLV319 (50 μ M)	Water	6	129.80 ± 13.9	A
JWH251 + SLV319 (40 μ M)	Water	7	100.30 ± 14.4	AB
JWH251	Water	6	58.5 ± 17.22	B

*Means which do not share the same letter have a significant difference.

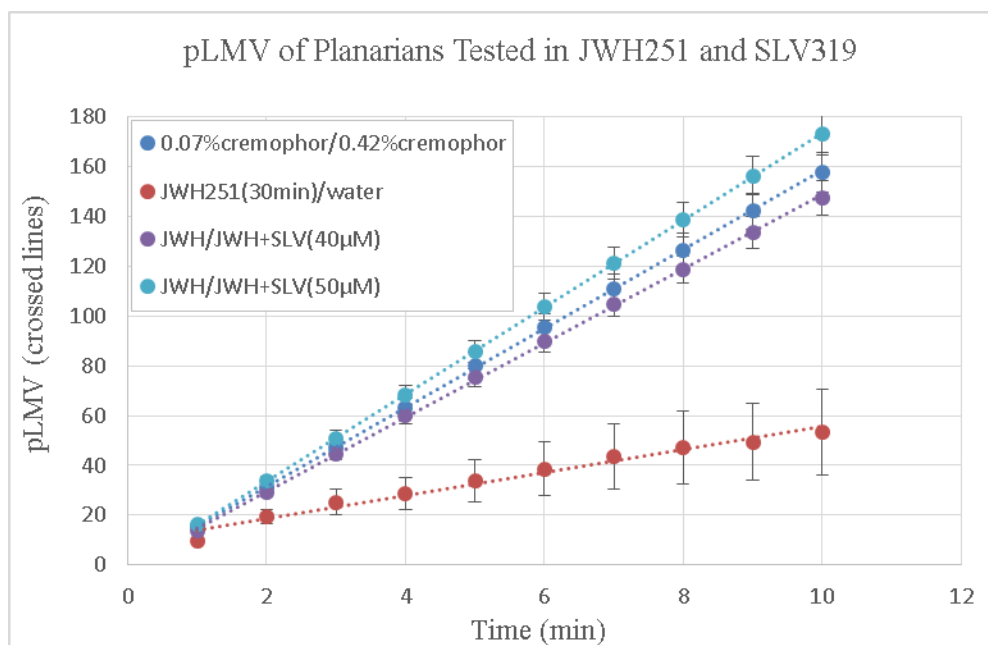


Figure 18 pLMV of planarians pretreated in JWH251 and tested in combinations of JWH251 and SLV319. JWH251 (10 μ M)

Table 25 Tukey's Pairwise Comparisons: Cumulative pLMV of Planarians Pretreated in JWH251 and Tested in Combinations of JWH251 and SLV319 ($p < 0.05$)*

Pretreatment	Test	N	Cumulative pLMV	Grouping
JWH251	JWH251 + SLV319 (50 μ M)	6	173.00 \pm 8.4	A
0.07% Cremophor	0.42% Cremophor	4	157.50 \pm 7.9	A
JWH251	JWH251 + SLV319 (40 μ M)	6	147.50 \pm 6.9	A
JWH251	Water	6	58.5 \pm 17.22	B

*Means which do not share the same letter have a significant difference.

4.3.9. The Use of CB1 Receptor Antagonist AM281 and CB Receptor Agonist

JWH251

As shown in Figure 19, planarians co-pretreated in JWH251 (10 μ M) and AM281 (20, 30, 40, and 50 μ M) then placed into drug-free water showed greater pLMV compared with planarians only pretreated in JWH251 (10 μ M). The group having highest concentration of AM281 (50 μ M) showed the highest pLMV (126.20 \pm 11.3). However, there was no significant difference ($p > 0.05$) among all the groups except between negative control and positive control (Table 26).

Figure 20 and

Table 27 elucidate the effects of AM281 on precipitating withdrawal from JWH251. Planarians pretreated in JWH251 (10 μ M) then tested in combinations of JWH251 (10 μ M) and AM281 (20, 30, 40, and 50 μ M) displayed similar pLMV with vehicle control group, and all the groups showed significant differences ($p < 0.05$) with positive control.

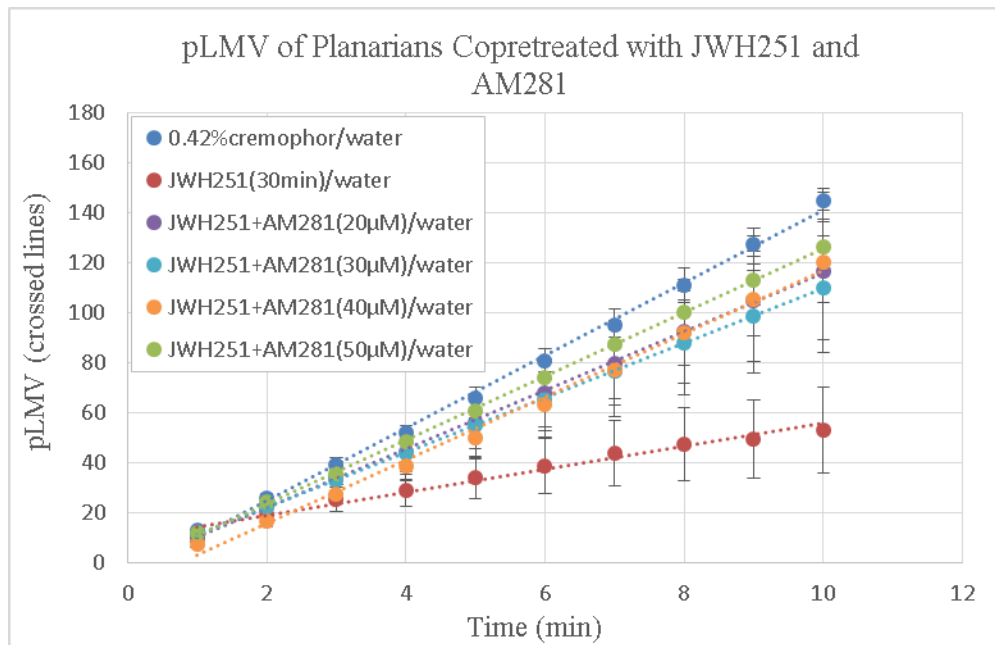


Figure 19 pLMV of planarians co-pretreated with JWH251 and AM281

Table 26 Tukey's Pairwise Comparisons: Cumulative pLMV of Planarians Co-pretreated in JWH251 and AM281 ($p < 0.05$) *

Pretreatment	Test	N	Cumulative pLMV	Grouping
0.42% Cremophor	Water	6	144.67 \pm 3.6	A
JWH251 + AM281 (50 μ M)	Water	6	126.20 \pm 11.3	AB
JWH251 + AM281 (40 μ M)	Water	7	120.30 \pm 16.2	AB

JWH251 + AM281 (20 μ M)	Water	6	116.8 \pm 32.9	AB
JWH251 + AM281 (30 μ M)	Water	6	110.0 \pm 20.7	AB
JWH251	Water	6	58.5 \pm 17.22	B

*Means which do not share the same letter have a significant difference.

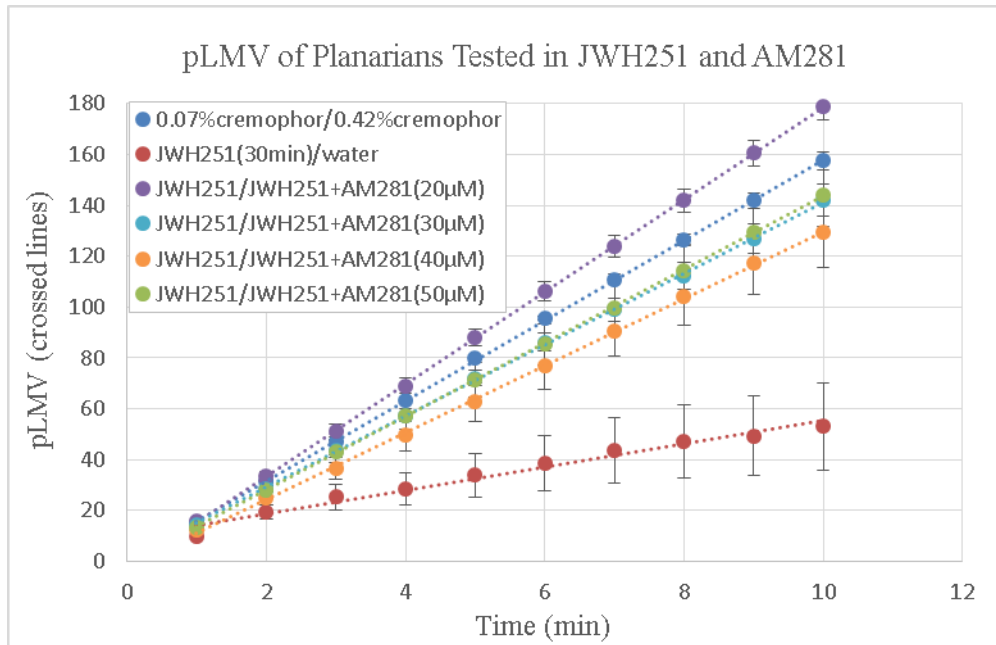


Figure 20 pLMV of planarians pretreated in JWH251 and tested in combinations of JWH251 and AM281. JWH251 (10 μ M)

Table 27 Tukey's Pairwise Comparisons: Cumulative pLMV of Planarians Pretreated in JWH251 and Tested in Combinations of JWH251 and AM251 ($p < 0.05$)*

Pretreatment	Test	N	Cumulative pLMV	Grouping
JWH251	JWH251 + AM281 (20 μ M)	6	179.00 \pm 5.4	A
0.07% Cremophor	0.42% Cremophor	4	156.70 \pm 7.9	A
JWH251	JWH251 + AM281 (50 μ M)	6	144.30 \pm 12.5	A
JWH251	JWH251 + AM281 (30 μ M)	6	142.00 \pm 6.2	A
JWH251	JWH251 + AM281 (40 μ M)	5	129.20 \pm 13.6	A
JWH251	Water	6	58.5 \pm 17.22	B

*Means which do not share the same letter have a significant difference.

4.4. Discussion and conclusion

4.4.1. Effects of JWH251

According to Figure 5 and Table 12, JWH251 by itself showed no effect on planarian spontaneous movement. Also, JWH251 did not display CB receptor antagonist-like effect, since planarians pretreated with (+)-WIN 55,212-2 showed normal pLMV when placed in JWH251 solution. These are similar with the results of (+)-WIN 55,212-2

shown in Pretreatment Periods of WIN 55,212-2 to Cause Abstinence-Induced Withdrawal in Chapter 3.

Abstinence-induced withdrawal from JWH251 (10 μ M) was obtained, which was shown as decreased locomotor activity after replacing JWH251 solution with drug-free water. JWH251 was reported as a CB receptor agonist in the GTP γ S binding assay (Huffman, Szklennik et al. 2005) and mice behavior model (Huffman, Szklennik et al. 2005). Now we report JWH251 (10 μ M) also showed CB receptor agonist effect in planarians by causing physical dependence after 30 min of exposure.

Compared with (+)-WIN 55,212-2, JWH251 showed less capability, or less potency, to develop physical dependence, in terms of the severity of withdrawal behavior in planarians. When the pretreatment process was set as the same as (+)-WIN 55,212-2 (20 min time course and 10 μ M agonist solution), only 2 of 4 planarians pretreated in JWH251 then placed into drug-free water showed obvious decreased pLMV. 30-min pretreating time is necessary for JWH251 (10 μ M) to stably produce physical dependence, although the standard error is larger (± 17.2) and the cumulative mean pLMV (58.5) is greater than that of (+)-WIN 55,212-2-exposed planarians (30.67 ± 8.4). JWH251 has lower affinity and lower efficacy in the GTP γ S binding assay (Compton, Rice et al. 1993; Huffman, Szklennik et al. 2005), which can be the reason to explain the weaker withdrawal behavior than (+)-WIN 55,212-2.

4.4.2. Effects of CB Receptor Antagonists

In this project, we explored the effects of 7 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), which are 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. Among them, pLMV of

	(+)-WIN 55,212-2		JWH251	
	Physical Dependence*	Precipitated Withdrawal [#]	Physical Dependence	Precipitated Withdrawal
AM251	Y; Y	N;N	Y; N	N; N
AM281	Y; Y	N;N	Y; N	N; N
SLV319	Y; Y	N;N	Y; Y	N; N
SR144528	Y; Y	N;N	Unknown	Unknown

WIN 55,212-2 + AM281, WIN 55,212-2 + AM251, WIN 55,212-2 + SLV319, JWH251 + SLV319 showed significant increase ($p < 0.05$) at least in one concentration group compared with positive controls, while other combinations showed no difference ($p > 0.05$) with either negative control or positive controls (

Table 28). Similar studies were done on the opioid receptor system (Raffa, Stagliano et al. 2003) and benzodiazepine receptor system (Raffa, Cavallo et al. 2007) in planarians. Both an opioid receptor antagonist and a benzodiazepine receptor antagonist attenuated abstinence-induced withdrawal from certain receptor-specific agonists. Together, this suggests that pharmacological effects we obtained in this project are receptor-mediated.

On the other hand, 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. Thus none of the four antagonists

(AM251, AM281, SLV319 and SR144528) precipitated withdrawal. The results surprisingly-but consistently-indicate we separated the development of CB agonist-induced physical dependence from CB antagonist-induced precipitated withdrawal in planarians. The summarized results are shown in

	(+) -WIN 55,212-2		JWH251	
	Physical Dependence*	Precipitated Withdrawal [#]	Physical Dependence	Precipitated Withdrawal
AM251	Y; Y	N;N	Y; N	N; N
AM281	Y; Y	N;N	Y; N	N; N
SLV319	Y; Y	N;N	Y; Y	N; N
SR144528	Y; Y	N;N	Unknown	Unknown

Table 28. The potential explanation could be the existence of different CB

receptor-mediated pathways for the development of physical dependence and antagonist-induced precipitated withdrawal in planarians. Another possibility is that the separation of physical dependence and precipitated withdrawal in planarians is related, despite the precautions taken, to the pharmacological properties (i.e., possibility of partial agonist effects) of the available commercial CB antagonists. Different hypothesizes are discussed in Chapter 6.

SR144528	Y; Y	N;N	Unknown	Unknown
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Table 28 Effects of CB Antagonists

*Results are expressed as Increased pLMV; Significant difference ($p < 0.05$) with positive control.

Results are expressed as Decreased pLMV; Significant difference ($p < 0.05$) with negative

	(+)-WIN 55,212-2	JWH251
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control.

We also found AM251 (Figure 9) and SLV319 (Figure 7) dose-relatedly antagonized the development of WIN 55212-2-induced physical dependence (Figure 9). AM251 (Figure 15) and SLV319 (Figure 17) dose-relatedly blocked the development of JWH251-induced physical dependence. AM281 showed mild dose-response relationship when blocking the development of physical dependence caused by WIN 55212-2 and JWH251: The two highest concentrations (40 and 50 μ M) of AM281 showed stronger potency to attenuate CB agonist-induced physical dependence (Figure 11 and Figure 19). More apparent dose-dependent effects might be obtained if we uses more dose levels and repeat trials with more planarians.

As shown in

	Physical Dependence*	Precipitated Withdrawal [#]	Physical Dependence	Precipitated Withdrawal
AM251	Y; Y	N;N	Y; N	N; N
AM281	Y; Y	N;N	Y; N	N; N
SLV319	Y; Y	N;N	Y; Y	N; N
SR144528	Y; Y	N;N	Unknown	Unknown

Table 28, both AM251 and AM281 prevented the development of JWH251-caused physical dependence in planarians, which is expressed as no significant increase ($p > 0.05$) of pLMV compared with positive control group (JWH251 / water: 58.5 ± 17.2). However, the highest accumulative means of pLMV of these groups are 117.70 ± 14.4 and 126.20 ± 11.3 respectively, which showed no difference ($p > 0.05$) with the result of negative control (144.67 ± 3.6). Thus, the relatively large standard error of the pLMV of planarians pretreated in JWH251 alone then measured in water (as mentioned in page 63) can be the reason why we did not obtain statistical differences with positive control.

A special antagonist/inverse agonist we used is SR144528. It is the first potent and selective CB2 antagonist with the affinity of $K_i = 0.6$ nM and $K_i = 400$ nM for CB2 and CB1 receptors, respectively, in rat spleen and cloned human membrane (Rinaldi-Carmona, Barth et al. 1998). CB2 receptor selective ligands have shown clinical potential in neuroprotection (Sagredo, González et al. 2009) and antinociception (Landry, Martinez et al. 2012). However, even highly selective CB2 ligands have the potential to occupy a certain amount CB1 receptors and cause CB1-mediated effects. In our project, we chose a CB2 receptor antagonist in order to study whether 700-fold lower affinity towards CB1 receptor was still sufficient to influence CB agonist effects. The results (Figure 13, Table

20) showed that SR144528 in 30 μM is the only concentration group that fully prevented WIN 55212-2 from developing physical dependence. These results indicate the concentrations of SR144528 we set are sufficient to occupy CB1 receptors in planarians, although the high selectivity towards CB2 receptor led to weaker ability to inhibit CB1 receptor-involved effects.

In our project, we failed to precipitate antagonist-induced withdrawal behavior with any antagonist in planarians. However, it does not mean the phenomena we observed were not receptor-mediated effects. The relatively higher standard errors also indicate CB antagonists affected the behavior of planarians although we did not obtain significant difference. Specifically, after the pretreatment of WIN 55212-2 (10 μM), 4 of 9 planarians displayed decreased pLMV (74.75 ± 8.9) in the combination of WIN 55212-2 and 30 μM of AM251, 2 of 7 planarians displayed decreased pLMV (64 ± 16) in the combination of WIN 55212-2 and 50 μM of AM251, 2 of 6 planarians displayed decreased pLMV (85.5 ± 0.5) in the combination of WIN 55212-2 and 50 μM SLV319, and 2 of 7 planarians displayed decreased pLMV (66.5 ± 2.5) in the combination of WIN 55212-2 and 40 μM SR144528 (Table 29). These data suggest that CB antagonists we used have the ability to precipitate antagonist-induced withdrawal, although in the concentrations that inhibited the development of physical dependence, these antagonists cannot precipitate stable withdrawal behavior in planarians. That is, precipitating antagonist-induced withdrawal is more difficult than preventing agonist-induced physical dependence using the same antagonist in planarians. This phenomenon in planarians differs than mammals. CB receptor antagonist-induced withdrawal is generally easier to

precipitate in other animal models, while abstinence-induced-withdrawal from CB receptor agonist is difficult to observe (Diana, Melis et al. 1998). In contrast, planarians displayed apparent, direct and stable abstinence-induced withdrawal behavior from CB agonist, whereas CB antagonists failed to precipitate overt withdrawal signs. It suggests that the planarian model is suited for studying differences in spontaneous withdrawal vs. antagonist-induced withdrawal.

Table 29 Data of Planarians Showed Antagonist-Precipitated Withdrawal

	1	2	3	4	5	6	7	8	9	10
WIN/WIN+AM251(30 μ M) Cumulative Mean (\pm S.E.M.) at 10 min: 74.75 \pm 8.9	9	19	29	34	43	47	52	61	72	82
	8	22	26	33	41	47	56	63	65	65
	8	18	28	35	47	58	66	73	83	96
	5	13	25	38	51	56	56	56	56	56
WIN/WIN+AM251(50 μ M) Cumulative Mean (\pm S.E.M.) at 10 min: 64 \pm 16	5	9	16	25	29	30	31	31	41	48
	10	22	30	38	43	49	57	66	69	60
WIN/WIN+SLV(50 μ M) Cumulative Mean (\pm S.E.M.) at 10 min: 85.5 \pm 0.5	9	19	30	36	44	55	64	68	78	85
	8	17	27	40	49	62	71	75	81	86
WIN/WIN+SR(40 μ M) Cumulative Mean (\pm S.E.M.) at 10 min: 66.5 \pm 2.5	11	19	26	33	38	45	51	57	63	64
	15	24	31	41	47	59	64	69	69	69

4.4.3. Conclusion

CB receptor antagonists (AM251, AM281, SLV319 and SR144528) and 2 CB receptor agonists (WIN 55212-2 and JWH251) were used to study planarian cannabinoid withdrawal model. We proved that co-exposure with cannabinoid antagonists attenuated cannabinoid agonist-induced physical dependence. All of the antagonists attenuated CB agonists-induced physical dependence at least in one dose level in planarians. However, none of the same antagonists precipitated withdrawal from the same agonists even in the highest concentration. It indicates we separated the development of CB receptor-involved physical dependence and antagonist-induced precipitated withdrawal in planarians.

CHAPTER 5

THE USE OF ULTRA VIOLET LIGHT IN CB-R AGONIST-PRETREATED PLANARIANS

5.1. Introduction:

5.1.1. *History of UV light on Drug-receptor Theory*

Furchgott et al. (Furchgott, Ehrreich et al. 1961) first reported that near ultraviolet radiation rapidly induced photorelaxation of contracted smooth muscle of phenylephrine-treated rabbit aorta in the absence of a photosensitizing agent. Furchgott et al. explained their results as the existence of some endogenous photosensitive material that was activated by the radiation and then caused the inhibition of the production of contraction. In 1975, Tallarida et al. (Tallarida, Sevy et al. 1975) used the similar model to study the affinity of norepinephrine for the α -adrenergic receptor in rabbit aorta. They found the results (exponential return to equilibrium, concentration-related relaxation and the inversely related time course and agonist concentration) were in favor of another interpretation that the effect of UV light on contracted aorta was caused by the disruption of drug-receptor binding. In the following decade, several studies (Tallarida, Laskin et al. 1976; Jacob and Tallarida 1977; Raffa, Robinson et al. 1985) were done to further support the hypothesis *in vitro*. Although people do not know the precise mechanism of UV-induced smooth muscle relaxation, there is strong evidences indicating that UV radiation disrupts the drug-receptor equilibrium.

Raffa et al. first studied the effect of UV light on dopamine receptor activity with an *in vivo* model, planarian (Raffa, Valdez et al. 2000). The results showed high-energy (254 nm) UV light attenuated dopamine D2 receptor antagonist (sulpiride)-caused decreased planarian locomotor velocity, which supported the hypothesis that UV light disrupts drug-receptor bonds *in vivo*. This hypothesis can only be true when UV light is able to disrupt various endpoints and disrupt effects caused by drugs targeting different receptors. Therefore, Raffa et al. repeated this experiment in different planarian models with different drugs, such as cocaine and acetylcholine receptor agonist (pilocarpine) in planarian seizure-like activity (Raffa, Tallarida et al. 2012), cocaine in planarian place preference model (Raffa, Dasrath et al. 2003), amphetamine in planarian locomotor activity (Raffa and Martley 2005), and opioid-kappa receptor agonist U-50,488H in abstinence-induced withdrawal model (Raffa, Tallarida et al. 2012). All of them showed that UV light attenuated drug-induced effects in planarians.

Thus, we chose UV light as an alternative of cannabinoid antagonist in our project.

5.1.2. Aim 3

Our goal was studying the differences between the development of cannabinoid physical dependence and withdrawal in planarian. As mentioned, UV light would be used as a tool to disrupt the action produced by cannabinoid agonist. In detail, we had two purposes here: one, study how UV light radiation influences cannabinoid agonist-induced physical dependence; two, study whether UV light would precipitate withdrawal from a cannabinoid agonist.

5.2. Methods

5.2.1. *Animal and Materials*

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

(+)-WIN 55,212-2 (mesylate) were purchased from Cayman Chemical.

Cremophor was a gift from Dr. Rawls. AmQuel® water conditioner (5 mL per 40 liters of water) was added to tap water at least 12 hours before use (subsequently abbreviated “water”). (+)-WIN 55,212-2 stock solutions (1 mM) were prepared fresh every two days in 7%/93% cremophor/water. Test solutions ((+)-WIN 55,212-2, 10 µM) were diluted with water. 0.01% cremophor solutions was made with water as vehicle control solution. MINERALIGHT® LAMP was used as ultra violet light source. It contains two choices of wavelength: long wavelength (366 nm) and short wavelength (254 nm).

5.2.2. *Behavior Measurements*

Similar with Chapter 3 (page 13) and Chapter 4 (page 27), planarian locomotor velocity (pLMV) (Raffa, Holland et al. 2001) was used to measure planarians behavior. Planarians were placed individually into a clear plastic petri dish (14-cm diameter) containing room-temperature (21°C) water, 0.1% cremophor solution or WIN 55,212-2 (10 µM) solution with or without UV light (254 nm) placed 10 cm above the petri dish. The dish was placed over graph paper with gridlines spaced 0.5 cm apart. pLMV was measured by counting the number of gridlines that a planarian crossed or re-crossed in every minute over a 10-minutes observation period. Each planarian was used only once.

pLMV was plotted as the mean (\pm S.E.M.) of the cumulative number of gridlines crossed by individual planarian per minute. Before measurements, planarians were pretreated with water, 0.1% cremophor solution or WIN 55,212-2 (10 μ M) solution for 20 min with or without UV light (254 nm and 366 nm). Groups were designed as Table 30 and Table 31.

Table 30 Groups Design for Planarians Pretreated with WIN 55,212-2 under UV Light

Pretreating Solution	Pretreating Period	Test Solution	Wavelength of UV Light	N
WIN 55,212-2*	20 min	Water		12
Water + UV	20 min	Water	254 nm	6
Cremophor + UV	20 min	Water	254 nm	6
WIN 55,212-2 + UV	20 min	Water	254 nm	6
Water + UV	20 min	Water	366 nm	6
Cremophor + UV	20 min	Water	366 nm	6
WIN 55,212-2 + UV	20 min	Water	366 nm	6

*Concentration of (+)-WIN 55,212-2: 10 μ M

Table 31 Groups Design for Planarians Pretreated with WIN 55,212-2 then Tested under UV Light

Pretreating Solution	Pretreating Period	Test Solution	Wavelength of UV Light	N
WIN 55,212-2*	20 min	Water		12
Water	20 min	Water + UV	254 nm	6
Cremophor	20 min	Cremophor + UV	254 nm	7
WIN 55,212-2	20 min	WIN 55,212-2 + UV	254 nm	6

*Concentration of (+)-WIN 55,212-2: 10 μ M

5.2.3. Statistical Analysis

Minitab 17 Statistical Software and Excel 2013 were used to perform the statistical analyses. 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 $p < 0.05$.

5.3. Results:

5.3.1. Effects of UV Light on Cannabinoid Agonist-induced Physical

Dependence

As negative control groups (illuminated in Figure 21 and Figure 22), pLMV of planarians pretreated with water and 0.1% cremophor under the radiation of UV light (254 nm and 366 nm) then tested in drug-free water were about 105.33 to 113.67, which is little lower but still acceptable compared with pLMV baseline (147.4 ± 7.5) shown in page 15.

As shown in Figure 21 and Table 32, planarians exposed to WIN 55,212-2 (10 μ M) for 20 min 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 (10 μ M) 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 rather than short wavelength UV light, pLMV (29.0 ± 12.0) of planarians pretreated with WIN 55,212-2 (10 μ M) under UV light (366 nm) then tested in water showed no difference ($p > 0.05$) compared with pLMV of planarians pretreated with WIN 55,212-2 (10 μ M) alone, but showed significant decrease ($p < 0.05$) compared with negative control groups. Results are shown in Figure 22 and Table 33.

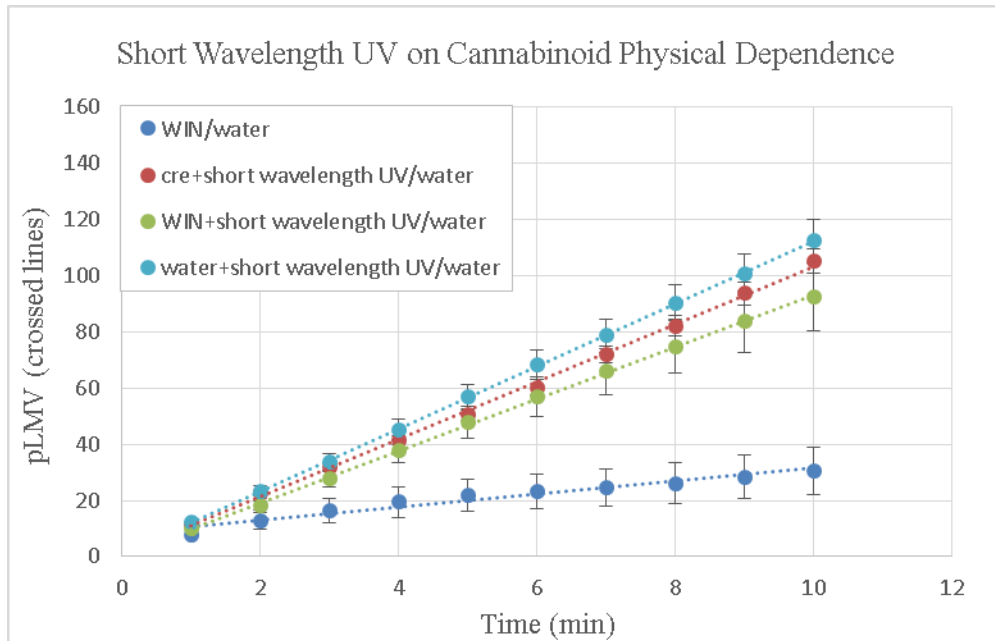


Figure 21 Effects of short wavelength (254 nm) UV light on cannabinoid physical dependence

Table 32 Tukey's Pairwise Comparisons: Cumulative pLMV of Planarians Pretreated in WIN 55,212-2 Under Wavelength (λ) UV (254 nm) Light ($p < 0.05$) *

Pretreatment	Test	N	Cumulative pLMV	Grouping
Water + UV (short λ)	Water	6	112.33 \pm 7.6	A
Cremophor + UV (short λ)	Water	6	105.33 \pm 4.4	A
WIN 55,212-2 + UV (short λ)	Water	6	92.3 \pm 12.0	A
WIN 55,212-2	Water	12	30.67 \pm 17.22	B

*Means which do not share the same letter have a significant difference.

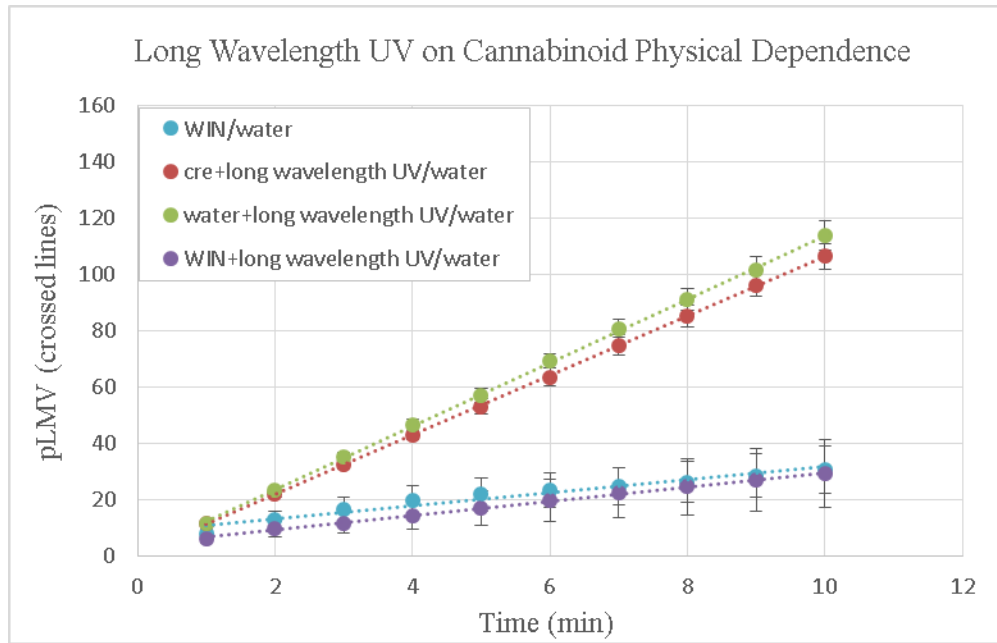


Figure 22 Effects of long wavelength (366 nm) UV light on cannabinoid physical dependence

Table 33 Tukey's Pairwise Comparisons: Cumulative pLMV of Planarians Pretreated in WIN 55,212-2 Under Long Wavelength (λ) UV (366 nm) Light ($p < 0.05$) *

Pretreatment	Test	N	Cumulative pLMV	Grouping
Water + UV (long λ)	Water	6	113.67 \pm 5.2	A
Cremophor + UV (long λ)	Water	6	106.33 \pm 4.5	A
WIN 55,212-2	Water	12	30.67 \pm 17.22	B
WIN 55,212-2 + UV (long λ)	Water	6	29.0 \pm 12.0	B

*Means which do not share the same letter have a significant difference.

5.3.2. Effects of UV Light on Precipitating Withdrawal

Figure 23 and Table 34 showed the influence of UV light (254 nm) on planarians pretreated with WIN 55,212-2 (10 μM). As negative controls, the cumulative pLMV of planarians pretreated with water or 0.1% cremophor then tested under the radiation of 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 (μM) then measured under the radiation of 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 positive control (30.67 ± 17.22).

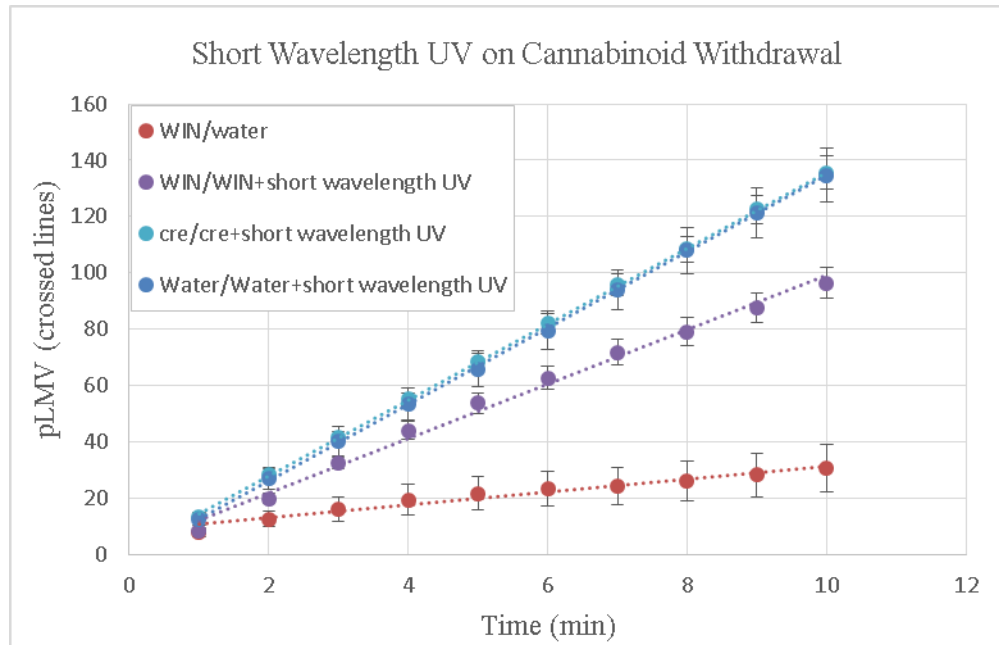


Figure 23 Effects of short wavelength (254 nm) UV light on precipitating withdrawal

Table 34 Tukey's Pairwise Comparisons: Cumulative pLMV of Planarians Pretreated in WIN 55,212-2 then tasted Under Short Wavelength (λ) UV (254 nm) Light ($p < 0.05$) *

Pretreatment	Test	N	Cumulative pLMV	Grouping
Cremophor	Cremophor + UV (short λ)	7	135.57 \pm 5.9	A
Water	Water + UV (short λ)	6	134.67 \pm 9.5	A
WIN 55,212-2	WIN 55,212-2 + UV (short λ)	6	96.38 \pm 5.4	B
WIN 55,212-2	Water	12	30.67 \pm 17.22	C

*Means which do not share the same letter have a significant difference.

5.4. Discussion and conclusion

Similar to previous reports with non-cannabinoid receptor agonists (Raffa, Dasrath et al. 2003; Raffa, Tallarida et al. 2012), short wavelength, i.e. high energy UV light (254 nm), attenuated abstinence-induced withdrawal behavior from a cannabinoid agonist WIN 55,212-2. Whereas the long wavelength, i.e. low energy UV light (266 nm) showed no effect on the behavior of planarians (Figure 21, Figure 22). The energy-related results are likely due to the wavelengths of light that proteins absorb. It was reported (Beaven and Holiday 1952) 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. This is also the reason why we used UV at 254 nm to precipitate withdrawal in the subsequent experiments.

We tested UV light (254 nm) on planarians pretreated with cannabinoid agonist WIN 55,212-2 trying to precipitate withdrawal. Planarians showed significantly decreased ($p < 0.05$) pLMV (96.38 ± 5.4) compared with negative control. However, it also showed significant difference ($p < 0.05$) with pLMV (30.67 ± 17.22) of planarians pretreated with WIN 55,212-2 then tested in water. Thus, UV light at 254 nm precipitated moderate withdrawal behavior in planarians.

These results are consistent with our results in Chapter 4. Cannabinoid antagonists/UV light dose/energy-relatedly prevented cannabinoid agonists from developing physical dependence. However, the same antagonists/UV light precipitated no/weak withdrawal behavior in planarians. It further proved our hypothesis that different cannabinoid receptor-mediated mechanisms of developing physical dependence and – precipitated withdrawal exist in planarians.

To our knowledge, this is the first report that cannabinoid receptor-mediated abstinence-induced withdrawal was attenuated by UV light (254 nm) radiation. It indicates the suppressive effects caused by UV is non-specific to different drugs targeting on different receptors. In addition, the maximal UV exposing time course is 20 min that is not long enough to cause any genetic changes. Thus, the results we obtained were caused by the interruption of cannabinoid receptor-mediated effects with high possibility. This is also the first time we know to precipitate withdrawal through UV light shining. UV light was proven again as a useful tool to study drug actions.

In summary, the effects of UV light (254 nm and 366 nm) were studied in the ability of preventing the development of cannabinoid agonist-produced physical

dependence (WIN 55212-2) and precipitating withdrawal behavior in planarians. The short wavelength (254 nm) UV light fully attenuated WIN 55212-2-induced physical dependence in planarians. However, the same UV light only precipitated moderate withdrawal from the same agonist. Consistent with results in Chapter 4 (page 20), it indicates we separated the development of CB receptor-involved physical dependence and precipitated withdrawal in planarians.

CHAPTER 6

DISCUSSION

6.1. Withdrawal

Although cannabinoid spontaneous withdrawal occurs in human users, they are difficult to develop in mammalian models (Diana, Melis et al. 1998). Planarians were chosen as a convenient yet quantifiable alternative model to study cannabinoid physical dependence and abstinence-induced withdrawal. Our project intended to optimize and extend the previous planarian withdrawal model, and establish an antagonist-induced precipitated withdrawal model. Similar to previous reports (Rawls, Rodriguez et al. 2006; Rawls, Gomez et al. 2007), cannabinoid agonists (WIN 55212-2 and JWH251)-treated planarians displayed abstinence-induced withdrawal behaviors (represented by decreased planarian locomotor velocity during drug cessation). In order to develop an antagonist-induced precipitated withdrawal model, we studied the effects of cannabinoid antagonists (AM251, AM281, SLV319 and SR144528) in the physical dependence and precipitated withdrawal. The antagonists dose-relatedly attenuated CB agonist-induced physical dependence at least in one dose level in planarians. However, to our surprise, none of the antagonists precipitated withdrawal from the same agonists even in the highest usable concentration. To further study our results, we used UV light as an alternative tool of cannabinoid antagonism. 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, inconsistent with simple drug-receptor theory, cannabinoid antagonists

that blocked the agonist effect in one format (attenuating agonist-induced physical dependence) failed to block the agonist effect in another format (inducing withdrawal symptoms). The results provided a hint that it might be possible to separate development of CB receptor-mediated physical dependence and antagonist-induced precipitated withdrawal in planarians.

According to previous report (Umeda, Stagliano et al. 2004), our observed effects were not caused by factors such as osmolality, pH, etc. The time course of every trial was no longer than 40 min, which is presumably not long enough to induce genetic changes. As discussed in previous chapters, control groups excluded the possibility of receptor desensitization and downregulation. Therefore, our observed results were receptor-mediated effects from the viewpoint of behavioral pharmacology.

I now would like to discuss potential explanations why cannabinoid antagonists and UV light failed to precipitate the expected withdrawal in planarians. First, did we use the correct method? Raffa et al. reported (Raffa, Stagliano et al. 2003) opioid receptor antagonist, naloxone-precipitated withdrawal from kappa-opioid agonist, U-50,488H in planarians. Naloxone (1.0, 5.0 and 10.0 μM) dose-relatedly precipitated withdrawal from U-50,488H (1 μM) in planarians, while 10 μM naloxone also prevented the development of U-50,488H (1 μM)-induced physical dependence. We used the same endpoint and methodology in our project, which excluded the possibility of inappropriate method.

Second, did we use a reasonable and high enough concentrations of antagonists? I tried to find references of the similar experiments in mammals in order to compare the dose of antagonists used to prevent dependence development with the dose to precipitate withdrawal. However, since different studies focused on different purposes, it is difficult

to summarize the paired reports using same animals, models (behavior signs, measurement, etc.), drugs, agonist dose regimen, etc. According to two referable reports done with rats, 3 mg/kg of CB1 receptor antagonist SR141716A was used to prevent the physical dependence produced by CB agonist WIN 55212-2 (Fattore, Cossu et al. 2001), whereas 10 mg/kg of SR141716A was used to precipitate withdrawal from WIN 55212-2 (Moranta, Esteban et al. 2009). Although these two studies were done in separate laboratories, it still gave a suggestion: in cannabinoid receptor-neurotransmitter system, precipitating withdrawal may need higher concentration of cannabinoid antagonists than blocking the development of physical dependence. As discussed previously, 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 whole data. It indicates cannabinoid antagonists have the ability to precipitate withdrawal, but for some reason, they fail to cause the obvious effects. Therefore, higher concentrations of antagonists may precipitate withdrawal in planarians. However, the concentrations of AM251, AM281 and SR144528 are limited by the concentration of cremophor. Because of the poor solubility, we had to use cremophor to make drug solution, and cremophor showed toxicity to planarians with the concentration of 0.48% in planarians (data not shown). SLV319 is the only compound we dissolved with water because of its comparatively better solubility. However the concentration of SLV319 is still limited by its own poor solubility (we used the saturated solution in our project). Thus, we were unable to adjust our drug concentrations. 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 level in planarians.

Third, do species differences explain our unique results? Both in mice and rats, cannabinoid antagonists completely prevented CB induced self-administration (Tanda and Goldberg 2003). Also, an apparent withdrawal syndrome was precipitated by administration of cannabinoid antagonist in cannabinoids-treated mice and rats (Tanda and Goldberg 2003). 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.

Fourth, while i.p. is the commonly used administration method in mammals, planarians absorb drug molecules through their body wall, which causes different pharmacokinetics profile between mammals and planarians. Could it be the reason of difficulty in planarian antagonist-precipitated withdrawal? As mentioned above, the counter example is the successfully developed opioid receptor antagonist-induced withdrawal in planarians (Raffa, Stagliano et al. 2003).

Fifth, another hypothesis is that the separation of physical dependence and precipitated withdrawal in planarians is 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 shown in Table 35. After pretreatment in JWH251, 2 of 5 planarians showed normal pLMV (> 100) when placed in cannabinoid antagonist, SLV319, alone. It suggests the high concentration (40 μ M) of SLV319 somehow maintained the activation of cannabinoid

receptors in those two planarians. In addition, after pretreatment only in SLV319, 3 of 6 planarians showed low pLMV (≤ 100) when placed into drug free water, which means high concentration (40 μ M) of SLV319 developed physical dependence in those 3 planarians. These data indicate SLV319 showed moderate “partial cannabinoid receptor agonist” effects. Exploring the mechanism is not our primary purpose. More experiments are needed to obtain a convincing conclusion, due to the large error bar of our current data.

Table 35 Potential Partial Agonist Effects of SLV319

	1	2	3	4	5	6	7	8	9	10
JWH251/SLV319(40 μ M) Cumulative Mean (\pm S.E.M.) at 10 min: 92.8 \pm 28.5	13	25	33	33	33	33	33	33	33	33
	12	26	37	46	56	62	73	81	90	100
	11	23	36	51	63	77	93	109	123	138
	8	15	21	24	24	24	24	24	24	24
	14	27	43	62	82	100	117	135	152	169
SLV319 (40 μ M)(20min)/Water Cumulative Mean (\pm S.E.M.) at 10 min: 83.5 \pm 26.9	8	14	22	29	33	42	50	56	60	62
	6	8	8	8	8	8	8	8	8	8
	14	24	41	56	72	81	97	113	130	145
	9	19	32	46	58	71	81	86	101	116
	13	27	44	61	78	91	107	124	141	158
	9	11	12	12	12	12	12	12	12	12

6.2. Implications

The implications of the present results in planarians to humans is unknown, since we did not expect to obtain the current results. However, our project proved again that planarians are a useful model to study physical dependence and withdrawal. At least 4 days of cannabinoids administration is needed to develop physical dependence in rats (Diana, Melis et al. 1998; Aceto, Scates et al. 2001). More than 40 days are needed when complex study includes surgery, agonist treatment and antagonist treatment in rats (Fattore, Cossu et al. 2001). Planarians only take 20 min to 30 min for developing physical dependence, which is much more time-saving compared with mammalian models. More importantly, as mentioned before, planarians show direct and obvious spontaneous withdrawal behavior while mammals are difficult to display abstinence-induced withdrawal syndromes (Diana, Melis et al. 1998). If the potential explanations of our results are further studied, the finding of “separated physical dependence and withdrawal” can be one hint when scientists consider to choose planarians as a cannabinoid drug abuse model. Even more, the potential explanations may suggest unknown cannabinoid receptor-mediated actions that can be useful for clinical practice.

6.3. Limitations

No doubt, our experiments retain some limitations. First, we only did *in vivo* behavioral study. We lack data about the biochemical changes in planarians during physical dependence and withdrawal. Changes of biochemical indexes are usually tested in rats and mice in studies of cannabinoid drug abuse. For example, the basal dopamine levels were dose-dependently decreased with the acute administration of cannabinoid antagonist (SR141716A) in THC pretreated rats (Tanda, Loddo et al. 1999). Increases in

adenyl-cyclase activity and reduction of dopamine neurons are also commonly measured in animals in cannabinoid withdrawal (Tanda and Goldberg 2003). Second, we did not find a commercially available compound with sufficient CB1 or CB2 receptor selectivity and good water solubility when we started this project. With such a compound, we might be able to reach a high enough dose without off-site effects to strengthen our findings. Third, we used pLMV as our only endpoint because our initial purpose was extending pLMV model in cannabinoid withdrawal study. However, several other drug abuse models are also available in planarians, such as conditional place preference model (Kusayama and Watanabe 2000) and light/dark preference model (Raffa, Dasrath et al. 2003). Testing with other planarian models would help confirm our results.

6.4. Future Plans

Firstly, looking for pure CB1 receptor antagonists with acceptable water solubility is the next step in order to exclude off-target effects. Also, to see whether it is possible to precipitate cannabinoid withdrawal in planarians, repeat the experiments of precipitating withdrawal with antagonists using higher dose of cannabinoid antagonists. Still, choosing compounds with good solubility or designing another planarian-friendly vehicle are the precondition to do this.

Secondly, it would be interesting to use planarian conditional place preference model and planarian light/dark preference model to confirm our results from different endpoints. It would also be interesting to analyze and compare the change of biochemical indexes (e.g. the amount of dopaminergic neurons, basal dopamine level, etc.) during the development of cannabinoid physical dependence, abstinence-induced withdrawal and precipitated withdrawal in planarians.

Thirdly, it would be interesting to repeat these experiments in different drug categories targeting different receptors in order to explore whether the “separated” physical dependence and cannabinoid withdrawal behavior is specific to cannabinoid receptor-neurotransmitter system or not. To our knowledge, the only report (Raffa, Stagliano et al. 2003) about precipitated withdrawal in planarians was done with kappa-opioid receptor agonist, U-50,488H and antagonist. This report suggests that the results of current study could be cannabinoid-receptor specific, and repeating experiments with drugs targeting other receptors would be necessary to confirm it.

6.5. Summary

In summary, we optimized a planarian cannabinoid physical dependence and abstinence-induced withdrawal model based on previous work. Using this model, we studied the effects of four cannabinoid receptor antagonists (AM251, AM281, SLV319 and SR144528) on antagonizing agonist-induced physical dependence. We proved that co-exposure with cannabinoid antagonists attenuated cannabinoid agonist-induced physical dependence. The four cannabinoid antagonists dose-dependently attenuated CB agonists (WIN 55212-2 and JWH251)-induced physical dependence at least in one concentration in planarians. We also attempted to extend our model into an antagonist-induced precipitated withdrawal model. However, we failed to establish a cannabinoid antagonist-induced precipitated withdrawal model. 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. However, it only precipitated moderate withdrawal behavior in planarians when we added UV light in the test step. These results gave us a hint that we separated the development of CB receptor-involved physical dependence and antagonist-induced precipitated withdrawal in planarians.

LEGEND OF ABBREVIATIONS

Abbreviation	Term
D9-THC	D9-tetrahydrocannabinol
2-AG	2-Arachidonoylglycerol
AEA	Anandamide
AM251	<i>N</i> -(piperidin-1-yl)-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-1 <i>H</i> -pyrazole-3-carboxamide
AM281	<i>N</i> -(morpholin-4-yl)-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-1 <i>H</i> -pyrazole-3-carboxamide
ANOVA	Analysis of variance
cAMP	Cyclic adenosine monophosphate
CB	Cannabinoid
CNS	Central nervous system
CP47497	(-)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexan-1-ol
CP55940	(1 <i>R</i> ,3 <i>R</i> ,4 <i>R</i>)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol
GABA	γ -Aminobutyric acid
GPCR	G-protein coupled receptor
GTP γ S	Guanosine 5'-O-[gamma-thio]triphosphate
HU-210	11-hydroxy- Δ 8-THC-dimethylheptyl
JWH018	Naphthalen-1-yl-(1-pentylindol-3-yl)methanone
JWH251	2-(2-methylphenyl)-1-(1-pentyl-1 <i>H</i> -indol-3-yl)ethanone

MAP	Mitogen-activated protein
NAEs	<i>N</i> -acylethanolamines
pLMV	Planarian locomotor velocity
SR141716A	<i>N</i> -(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1 <i>H</i> -pyrazole-3-carboxamide hydrochloride
SR144528	5-(4-chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]- <i>N</i> -[(1 <i>S</i> ,2 <i>S</i> ,4 <i>R</i>)-1,3,3-trimethylbicyclo[2.2.1]hept-2-yl]-1 <i>H</i> -pyrazole-3-carboxamide
Slv319	3-(4-Chlorophenyl)- <i>N</i> -[(4-Chlorophenyl)sulfonyl]-4,5-dihydro- <i>N</i> ¹ -methyl-4-phenyl-1 <i>H</i> -pyrazole-1-carboximidamide
U-50,488H	488H trans-(-)-3,4-Dichloro- <i>N</i> -methyl- <i>N</i> -[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide hydrochloride
UV	Ultra violet
VNCs	Ventral nerve cords
VTA	Ventral tegmental area
WIN 55,212-2	(<i>R</i>)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo [1,2,3- <i>de</i>]-1,4-benzoxazin-6-yl]-1-napthalenylmethanone

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