ผลของมิทราไกนีนต่อการเสพติดมอร์ฟีนและเมทแอมเฟทามีนในแบบจำลองการติดยาของสัตว์

นายฤทธิณรงค์ มีพงษ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเภสัชศาสตร์ชีวภาพ ภาควิชาชีวเคมีและจุลชีววิทยา
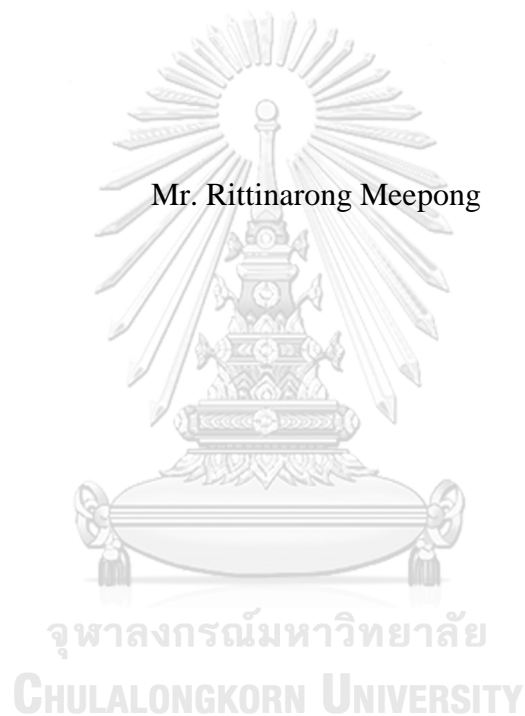คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2560

# EFFECTS OF MITRAGYNINE ON MORPHINE AND METHAMPHETAMINE ADDICTION IN ANIMAL MODELS OF ADDICTION

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ฤทธิณรงค์ มีพงษ์ : ผลของมิทราไกนีนต่อการเสพติดมอร์ฟีนและเมทแอมเฟทามีนใน แบบจำลองการติดยาของสัตว์ (EFFECTS OF MITRAGYNINE ON MORPHINE AND METHAMPHETAMINE ADDICTION IN ANIMAL MODELS OF ADDICTION) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ภก. ดร. ธงชัย สุขเศวต, 81 หน้า.

กระท่อม (*Mitragyna speciosa* Korth.) เป็นพืชที่มีการใช้ประโยชน์ในการแพทย์ พื้นบ้านในเอเชียตะวันออกเฉียงใต้มาเป็นเวลานาน เพื่อใช้บรรเทาปวด และใช้เพื่อบรรเทาอาการ ขาดยาในกลุ่มฝิ่น สารอัลคาลอยด์สำคัญที่พบในใบของพืชกระท่อมคือ มิทราไกนีน โดยออกฤทธิ์ ผ่านการกระตุ้นตัวรับโอปิออยด์ และตัวรับชนิดอื่นในระบบประสาทส่วนกลาง การศึกษานี้มี จุดมุ่งหมายเพื่อศึกษาฤทธิ์เสพติดและความเป็นไปได้ในการใช้สารมิทราไกนีนเพื่อบำบัดการเสพ ติดสารในกลุ่มโอปิออยด์ และสารกระตุ้นประเภทแอมเฟทามีน การศึกษาฤทธิ์เสพติดของมิทรา ไกนีนด้วยวิธีแยกแยะยา (drug discrimination) การชอบสถานที่อย่างมีเงื่อนไข (conditioned place preference) และการขาดยาจากการให้สารนาล็อกโซนในสัตว์ฟันแทะ พบว่ามิทราไกนีน สามารถทดแทนเมทแอมเฟทามีนในสัตว์ทดลองที่ถูกฝึกให้แยกแยะเมทแอมเฟทามีนได้ นอกจากนั้นแล้ว มิทราไกนีน สามารถเหนี่ยวนำให้เกิดการชอบสถานที่อย่างมีเงื่อนไขได้ในขนาด สูง ในแบบจำลองการขาดยาด้วยการให้นาล็อกโซน พบว่ามิทราไกนีนขนาดสูงทั้งแบบเฉียบพลัน และเรื้อรังจะแสดงพฤติกรรมกระโดดซ้ำๆ และ มีหางชี้งอ เช่นเดียวกับสัตว์ทดลองที่ได้รับมอร์ฟีน มิทราไกนีนยับยั้งการเกิดการชอบสถานที่อย่างมีเงื่อนไข และสามารถลดการแสดงออกการชอบ สถานที่อย่างมีเงื่อนไขของมอร์ฟีนได้ เมื่อให้มิทราไกนีนร่วมกับมอร์ฟีน ยังสามารถลดพฤติกรรม การกระโดด และ การชี้งอของหางที่เกิดจากการได้รับนาล็อกโซนได้ การให้มิทราไกนีน สามารถ ลดผลของการให้เมทแอมเฟทามีนในสัตว์ทดลองที่ฝึกให้แยกแยะเมทแอมเฟทามีน และลดการชอบ สถานที่อย่างมีเงื่อนไขที่เหนี่ยวนำด้วยเมทแอมเฟทามีนได้ โดยสรุปมิทราไกนีนมีฤทธิ์เสพติดต่ำ กว่ามอร์ฟีนและเมทแอมเฟทามีน และสามารถลดฤทธิ์เสพติดของมอร์ฟีนและเมทแอมเฟทามีนได้ ซึ่งผลการศึกษานี้สนับสนุนการใช้พืชกระท่อมในแพทย์พื้นบ้านเพื่อบำบัดการเสพติดยาในกลุ่ม โอปิออยด์และเมทแอมเฟทามีน

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| ภาควิชา | ชีวเคมีและจุลชีววิทยา | ลายมือชื่อนิสิต ................................. |
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# # 5276957833 : MAJOR BIOPHARMACEUTICAL SCIENCES

KEYWORDS: KRATOM / MITRAGYNA SPECIOSA / MITRAGYNINE / MORPHINE / CONDITIONED PLACE PREFERENCE / WITHDRAWAL / ADDICTION

RITTINARONG MEEPONG: EFFECTS OF MITRAGYNINE ON MORPHINE AND METHAMPHETAMINE ADDICTION IN ANIMAL MODELS OF ADDICTION. ADVISOR: ASSOC. PROF. THONGCHAI SOOKSAWATE, Ph.D., 81 pp.

Kratom (*Mitragyna speciosa* Korth) has long been used in folklore medicine in Southeast Asian countries. It has been used for its analgesic effects and treatment of opioid withdrawal. Mitragynine is a major alkaloid found in the leaves of kratom plant that exerts its effects via activation of opioid receptors and other receptors in the central nervous system. This study aimed to provide evaluation of abuse liability and potential of mitragynine in the treatment for opioids and amphetamine type stimulants addictions. Abuse liability of mitragynine and its effects on morphine and methamphetamine addiction were evaluated by drug discrimination, conditioned place preference and naloxone precipitated withdrawal models in rodents. Mitragynine substituted methamphetamine in drug discrimination model. It also induced conditioned place preference at high doses. Acute mitragynine withdrawal precipitated by naloxone showed significant repeated jumping behavior in both acute and chronic withdrawal. Straub tail reaction was observable in mitragynine treated group the same as in morphine-treated group. Mitragynine attenuated acquisition and expression of morphine conditioned place preference. When given with morphine, mitragynine reduced jumping behavior and Straub tail reaction. Pretreatment of mitragynine weakened methamphetamine discrimination and attenuated methamphetamine induced conditioned place preference. In conclusions, mitragynine had low abuse liability, when compared with morphine and methamphetamine, and could attenuate rewarding effect of morphine and methamphetamine. These results support not only the use of kratom in traditional medicine and self-medication in the treatment for opioid addiction and withdrawal but also in methamphetamine addiction.

| Department: | Biochemistry and | Student's Signature | .................................... |
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| | |
|---|---|
| °C | Degree Celsius |
| % | Percent |
| δ | Delta |
| κ | Kappa |
| μ | Mu |
| 5-HT | 5-hydroxytryptamine, serotonin |
| 6-OHDA | 6-hydroxydopamine |
| AMPA | α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| ANOVA | Analysis of variance |
| ATS | Amphetamine type stimulant |
| B.E. | Buddhism Era |
| BLA | Basolateral amygdala |
| BRET | Bioluminescent resonance energy transfer |
| BW | Body weight |
| $Ca^{2+}$ | Calcium ion |
| cAMP | Cyclic adenosine monophosphate |
| CeA | Central amygdala |
| cm | Centimeter |
| CNS | Central nervous system |
| CPA | Conditioned place aversion |
| CPP | Conditioned place preference |
| CRF | Corticotropin releasing factor |
| CYP | Cytochrome P450 isozyme |
| DAT | Dopamine transporter |
| DEA | Drug Enforcement Administration |
| DOR | Delta opioid receptor |
| FR | Fixed ratio |
| g | Gram |

| | |
|---|---|
| GABA | Gamma aminobutyric acid |
| GRK2 | G-protein-coupled receptor kinase 2 |
| HEK | Human embryonic kidney cells |
| HPA | Hypothalamic-pituitary-adrenal |
| hrs | Hours |
| i.p. | Intraperitoneally |
| ICR | Institute of Cancer research |
| kg | Kilogram |
| KOR | Kappa opioid receptor |
| lBNST | Lateral portion of bed nucleus of stria terminalis |
| LC | Locus coeruleus |
| MDMA | 3,4-methylenedioxy-metamphetamine |
| MET | Methamphetamine |
| MG | Mitragynine |
| min | Minute |
| mm | Millimeter |
| mPFC | Medial prefrontal cortex |
| mRNA | Messenger Ribonucleic Acid |
| MSN | Medium spiny neuron |
| NE | Norepinephrine |
| NMDA | N-methyl-d-aspartic acid |
| MOR | Mu opioid receptor |
| NAcc | Nucleus Accumbens |
| $pA_2$ | Negative logarithm of the concentration of antagonist needed to shift the curve by a factor of 2 |
| PAG | Periaqueductal gray |
| PET | Positron emission tomography |
| PVT | Paraventricular nucleus of thalamus |
| TO | Time-out |
| UK | United Kingdom |
| US | United States |
| V | Volt |

| VP | Ventral pallidum |
| VMAT2 | Vesicular monoamine transporter 2 |
| VTA | Ventral tegmental area |

# Chapter 1
# Introduction

Addiction is a brain disorder involving compulsive use of drug despite negative or harmful consequences to quality of life. United Nations Office for Drug Control and Crime (2017) reported that about 5 percent of adult used drug at least once in a lifetime in 2015. Drug use caused many problems, such as premature death and disability. Among many types of abuse substances, opioids is the most harmful. It is accountable to 70 percent of global burden of disease attributable to drug use disorders. Amphetamine type stimulants (ATSs) is the second most harmful drug after opioids. The use of methamphetamine, one of ATSs, is increasing in many parts of the world.

Kratom plant (*Mitragyna speciosa* Korth.) has been used traditionally for its pharmacological effects and its narcotic effects in Southeast Asian nations, especially in Thailand and Malaysia. People use kratom plant to treat pain, cough, diarrhea, and intestinal infection, for examples (Suwanlert, 1975; Chan et al., 2005). Laborers and farmers use kratom to increase endurance, reduce fatigue, and suppress appetite so they are able to work longer hours in unfavorable conditions (Thongpradichote et al., 1998). Kratom is also used as a substitution for heroin and morphine when access to drug is prevented and as a treatment for drug withdrawal (Beckett et al., 1965; Rätsch, 2005). Plantation and consumption of kratom plants are now made illegal in many countries for the concerns of its abusive use. In Thailand, kratom plant is placed in Schedule V of Narcotic Act B.E. 2522, the same category to marijuana, which prohibits planting and possessing of kratom plant and its parts. Recently, there was an attempt to relax the ban on kratom plantation and use in folklore medicine in Thailand. Kratom became illegal in Malaysia in 2003 and in Australia in 2005 (Vicknasingam et al., 2010). In the United States, kratom is not controlled under Federal Controlled Substances Act. However, possession and use of kratom are regulated and prohibited in some states. It is also on the Drug and Chemical of Concern List by US Drug Enforcement Administration (United States Drug Enforcement Administration, 2015) and considered that it had no legitimate medical

use making advertising kratom for any medical use illegal in the US (Prozialeck et al., 2012). In 2016, the US Drug Enforcement Administration had published a notice of intention to place active materials in kratom plant, mitragynine and 7-hydroxymitragynine into Schedule I of Controlled Substance Act for public safety before withdrew it later in the same year. European countries such as Denmark, Latvia, Lithuania, Romania, and Sweden put kratom and its derivatives as controlled drugs while they are still legal, but under surveillance, in the United Kingdom and Germany(Suhaimi et al., 2016). Kratom is one of the top 25 seller "legal high" in United Kingdom's online market study (Schmidt et al., 2011).

Mitragynine is an alkaloid believed to contribute to the effects of kratom. More than 40 alkaloids from kratom have been isolated and characterized. The leaves of kratom plant contain from 0.5 to 1.5 % of alkaloid content (Hassan et al., 2013). Mitragynine is responsible for 66% of total alkaloid content (Takayama, 2004; Brown et al., 2017). It is able to bind to all three types of opioid receptors with highest affinity to κ opioid receptor (KOR) (Taufik Hidayat et al., 2010). Mitragynine acts as partial agonist on human μ opioid receptor (MOR) and δ opioid receptor (DOR) while it acts as competitive antagonist on human KOR (Kruegel et al., 2016). In addition, *in vivo* test showed that antinociceptive effects of mitragynine is mediated by supraspinal MOR and DOR (Prozialeck et al., 2012). Antinociceptive effect of mitragynine can be blocked by administration of MOR and DOR antagonists, naloxone and natrindole, respectively (Shamima et al., 2012). Mitragynine can also bind to many receptors such as adenosine $A_{2a}$ receptor, dopamine $D_2$ receptor, serotonin receptors 5-$HT_{2C}$ and 5-$HT_7$, and $\alpha_2$ adrenergic receptor (Matsumoto et al., 1996; Boyer et al., 2008). Abuse liability of mitragynine has been tested by several studies. Mitragynine served as discriminative stimulus and was able to fully substitute morphine in drug discrimination model in higher dose (Harun et al., 2015). In conditioned place preference model, mitragynine could induce conditioned place preference at 10 mg/kg. Acquisition of mitragynine induced place preference could be prevented by administration of naloxone pretreatment. However, naloxone was not able to block expression of mitragynine induced place preference (Yusoff et al., 2017). More recently, it is found that baclofen was able to block both acquisition and expression of mitragynine-induced conditioned place preference in rats (Yusoff et al.,

2018). Together, involvement of opioidergic and gamma aminobutyric acid (GABA) -
ergic pathway are suggested in mitragynine rewarding effects.

Since it is still necessary to find better alternative drugs for agonist-
replacement therapy for drug addiction and kratom leaves are used traditionally for
opioid use disorder, the present study aimed to evaluate abuse liability of mitragynine,
the major alkaloid in kratom leaves, and its effects on morphine-induced conditioned
place preference, morphine withdrawal, methamphetamine-induced conditioned place
preference, and methamphetamine discrimination.

**Objectives**

1. To study abuse liability of mitragynine
2. To study the effect of mitragynine on reinforcing and withdrawal effects of
   morphine and methamphetamine

**Hypothesis**

1. Mitragynine has low reinforcing and withdrawal effects.

2. Mitragynine can attenuate reinforcing and withdrawal effects of morphine
   and methamphetamine in animal models of drug addiction.

**Expected benefits**

The results obtained will reveal abuse potential of mitragynine and its
potential use in treatment of morphine and methamphetamine addiction.

# Chapter 2
# Literature Review

## Addiction

Addiction is defined as chronic relapsing disorder of the brain including compulsion to drug seeking and taking, control loss over drug taking with escalating use despite emergence of negative consequences such as irritability, discomfort, and anxiety (Pierce and Kumaresan, 2006; Goldstein and Volkow, 2011; Koob, 2013)

Kalivas and Volkow (2005) divided addiction into three temporal stages which are 1) acute drug effects, 2) transition to addiction, and 3) end-stage addiction. Each stage is related to involving brain regions and neurotransmitters. Similarly, Koob (2013) proposed a three-stage cycle of addiction – binge/intoxication, withdrawal/negative effects, and preoccupation/anticipation. The first stage explain the early exposure to the drug of abuse. Acute effects of the drug drive user into impulsive use of drug before entering the second stage where initial effects of the drug are diminish with occurrence of negative consequence that user compulsively seek and use drugs in order to alleviate discomfort of drug abstinence. The third stage is when the user is vulnerable to relapse. Repeated drug use causes allosteric changes in either cell level or brain circuitry level and produces tolerance and dependence (Hyman et al., 2006).

Body adjusts its response to drug by changes in reward and stress system neurocircuitries which are key motivational systems (Koob and Kreek, 2007; Koob, 2013). It is hypothesized that addiction is a progressive disorder from impulsivity in binge/intoxication stage to compulsivity in withdrawal stage. In short, allostasis in neural elements decreases reward system functions while increases stress system functions.

**Reward systems**

Olds and Milner (1954) demonstrated that rats would repeatedly perform lever pressing for electrical stimulation at certain brain areas. Some rats pressed the lever repeatedly for 24 hours without rest. The rate of lever depression could go up more than 5000 times per hour. As a consequence of their findings, the pleasure center in the brain was proposed. The most sensitive areas included medial forebrain bundle (Olds, 1962; Koob, 2013). Nowadays, we have learned that these structures connect ventral forebrain (nucleus accumbens (NAcc), olfactory tubercle and septal area) and ventral midbrain (vental tegmental area, VTA). Dopamine neurons in VTA projecting to NAcc is important in the processing of reward-related stimuli (Volkow and Morales, 2015). Basal forebrain includes NAcc, olfactory tubercle, septum, diagonal band nuclei, bed nucleus of stria terminalis, substantia innominata, olfactory cortex, hippocampus formation and amygdala (Napier et al., 1991). Projections from VTA release dopamine. This pathway is a part of mesocorticolimbic dopamine pathway where neurons located in VTA send their projection to limbic system and prefrontal cortex (Kauer and Malenka, 2007). This dopamine connection is important in processing of reward-related stimuli and motivation (Volkow and Morales, 2015). Acute effect of drug of abuse, regardless of type, is to increase dopamine in the shell of NAcc (Di Chiara, 2002; Pierce and Kumaresan, 2006). In addition to dopamine role in the effect of drug of abuse. Dopamine independent pathways are also observed in opioid and alcohol rewards (Pierce and Kumaresan, 2006).

NAcc is an area related to reward prediction, adaptive behaviors and expression learning (Reid and Lingford-Hughes, 2006). In addition to reward, this area of the brain is also involved in aversive response (Russo and Nestler, 2013). Major population of neurons in NAcc is GABA-ergic neurons. Most of them are medium spiny neurons (MSNs) expressing dopamine receptors. They can also be divided into 2 subpopulations by dopamine receptor type that expressed. Dopamine $D_1$ receptors containing MSNs and dopamine $D_2$ receptors containing MSNs (Scofield et al., 2016). Dopamine $D_1$ receptors expressed MSNs signal through direct striatal pathway innervate directly at VTA. This pathway is associated with reward. Dopamine $D_2$ receptors containing MSNs signal through indirect striatal pathway that

innervate at ventral pallidum (VP). Activation of dopamine $D_2$ receptors in MSNs in indirect pathway inhibit GABA release from MSNs. This pathway is associated with punishment (Marcellino et al., 2008; Kravitz et al., 2012). About 5-10 % of the population are interneurons; GABA-ergic and cholinergic. The rest are glia cells, especially astrocytes that regulate glutamate levels at the synapses (Kalivas et al., 1993). In addition to dopaminergic pathway from VTA, NAcc also receives innervations from many areas such as cortex, hippocampus, thalamus and amygdala.

Upon acute exposure to drugs of abuse, rewarding effect occurs. This is described by pleasurable feeling. Drugs of abuse works to increase the level of dopamine in mesocorticolimbic dopaminergic system in the dorsal and ventral striatum mimicking high frequency phasic VTA dopaminergic neuron firing despite different mechanisms of action (Hyman et al., 2006; Volkow et al., 2012). Dopamine plays important roles in reinforcing effects of drugs. However, it does not work exclusively. Many researches indicated the roles of other pathways rather than dopamine in reinforcing effects of the drug. Berridge and Robinson (1998) performed an experiment by making lesion in the brain to see whether the reinforcing effects of drug were diminished. The results from this experiment showed that the effect of the drug was not reduced by the lack of dopamine. In agreement with brain lesion, depletion of dopamine production by genetically inactivation of tyrosine hydroxylase enzyme did not prevent the animal in showing preference in drugs. In addition, dopamine is involving in reward prediction. Dopaminergic neurons firing rates increased upon presentation of novel reward and also cue associated with reward presentation. Firing frequency was increase if the reward was present and met expectation but was ceased if there was no reward presented (Schultz, 2002).

Euphoric effects of drugs encourage repeated use which may then develop from impulsive use to compulsive use. After repeated exposure to drugs, individuals may exhibit withdrawal symptoms, persistent vulnerability to relapse and alterations in decision making and cognitive function (Gould, 2010). This involves disruption of neurochemical systems and neurocircuits in both positive reinforcing effect of the drug and in the system other than the system mediate positive reinforcing effect of the drug such as stress system. Changes in response to drug exposure in the reward system is called within-system neuroadaptation process where changes in the other

systems rather than the reward system is called between-system neuroadaptation process (Koob and Le Moal, 2008). Brain reward threshold was found to increase during acute withdrawal by all drugs of abuse (Koob and Le Moal, 2005). Increase in reward threshold means decrease rewarding effect of the drug. This increase in reward threshold is correlate to increase in drug taking with extended access (Ahmed et al., 2002). Mesocorticolimbic dopamine system activity is decrease during acute withdrawal as well as opioid peptide, GABA, serotonin, and glutamate in NAcc and amygdala (Weiss et al., 1996; Koob and Le Moal, 2008). Repeat drug exposure is found to associate to downregulaion of dopamine $D_2$ receptor in striatum including NAcc (Volkow et al., 2001a; Nader et al., 2006). In alcohol self-administration model, overexpression of dopamine $D_2$ receptor reduced alcohol intake and preference (Thanos et al., 2001). This reduction in dopamine $D_2$ receptor in striatum resulted in reduction of dopamine inhibition of ventral striatal indirect pathway that is associated with punishment. It also resulted in reduction of thalamo-cortical stimulation that leads to reduction of prefrontal cortex activity (Black et al., 2010). Prefrontal cortex is known to involve in many processes, especially self-control, emotion regulation, and decision making (Goldstein and Volkow, 2011). Disruption in prefrontal cortex could lead to loss of self-control which drive individual into compulsive drug use despite negative consequences to health and social life (Volkow and Fowler, 2000)

In between system neuroadaptation, brain stress and emotional systems are recruited. The hypothalamic-pituitary-adrenal axis and extrahypothalamic stress systems are dysregulated after chronic administration of drugs of abuse (Koob, 2013). Evidences showed that levels of adrenocorticotropic hormone, corticosterone, and corticotropin releasing factor in extended amygdala were elevated during acute withdrawal (Koob, 2009). In addition, administration of corticotropin releasing factor (CRF) receptor antagonists could reverse irritability-like effect produced by opioid and alcohol withdrawal (Baldwin et al., 1991; Navarro-Zaragoza et al., 2010). Other neurotransmitter systems localized to the extended amygdala such as norepinephrine (NE) and dynorphin were also activated during drug withdrawal (Koob et al., 2014b). Taken together, the brain reward system becomes compromised where the brain stress systems are activated. These changes are responsible for negative emotional state that motivates drug seeking behaviors leads to compulsive use of drugs and addiction.

**Methamphetamine (MA)**

Methamphetamine is an analogue of amphetamine. It belongs to amphetamine type stimulants (ATSs). The acute effect of methamphetamine when used at recreational dose are increased stimulation, improved coordination, increased strength and endurance, increased activeness both mentally and physically, increased feeling of boldness, elation and friendliness and decrease of appetite . Methamphetamine is an indirect agonist at dopamine receptor by facilitating dopamine release from dopamine neurons (Nestler, 2001). The mechanism of action of methamphetamine is similar to other drugs in ATSs. It causes elevation of extracellular monoamine dopamine, NE, and serotonin via plasmalemmal uptake transporters, particularly the dopamine transporter (DAT), the NE transporter (NET), and the serotonin transporter (SET) (Rothman and Baumann, 2003; Hart et al., 2012). Methamphetamine releases NE most efficiently followed by dopamine and serotonin (Rothman et al., 2001). It works by blocking vesicular monoamine transporter 2 (VMAT2) activity thus inhibit storage of monoamine into the vesicles. Methamphetamine inhibits monoamine oxidase which is the enzyme that breaks down monoamines. It also decreases expression of DAT onto cell surface.  It can increase the activity and the expression of tyrosine hydroxylase enzyme which is a rate limiting enzyme in catecholamine synthesis (Karila et al., 2010; Daubner et al., 2011). Repeated exposure to methamphetamine can cause loss of DAT in cortex and caudate-putamen (Volkow et al., 2001b; Sekine et al., 2003). Decrease in dopamine and its metabolites are also observed in methamphetamine abuser in the same brain region (Wilson et al., 1996). Serotonin transporters density in the midbrain, thalamus, caudate, putamen, cerebral cortex, and cerebellum was lower in methamphetamine abuser compared to non-abuser controls using positron emission tomography (PET). The extent of reduction was inversely correlated to duration of methamphetamine use (Sekine et al., 2006). Methamphetamine neurotoxicity could arise from generation of reactive oxygen species in the brain as well. Metabolism of dopamine by monoamine oxidase generates reactive oxygen species. Methamphetamine alters balance of reactive oxygen species and antioxidase enzymes that scavenge reactive oxygen species via

upregulation of monoamine oxidase enzyme and interference of glutathione system (Harold et al., 2000)

**Opiates**

Opiates refers to natural or synthetic substances that mimic the effects of endogenous opioids (enkephalins, dynorphins, endomorphins) by binding to opioid receptors. Examples of opiates are morphine, codeine, hydrocodone, and methadone (Christie and Morgan, 2015). They are used medically as analgesics, cough suppressants, antidiarrheal drug. However, opiates are addictive.

There are three subtypes of classical opioids receptors; MOR, DOR, and KOR regarding to The International Union of Basic and Clinical Pharmacology nomenclature. They are G-protein coupled receptors coupled with inhibitory G-proteins. Activation of opioid receptors causing inhibitory effects in the neurons. These effects are, but not limited to, opening of inwardly rectifying potassium channels allowing potassium efflux, inhibition of calcium channels, and inhibition of adenylyl cyclase. Taken together, activation of opioid receptors induces cell hyperpolarization, reduction of neurotransmitters release, and reduction of intracellular cAMP (Al-Hasani and Bruchas, 2011).

Opioid analgesics and drugs of abuse work exclusively on MORs. It is thought that MORs and DORs, in less extent, are responsible for positive reinforcement of drugs where KORs activation cause negative reinforcing effects (Ting and van der Kooy, 2012). MORs are expressed along reward circuit in the brain mediating positive reinforcing effect of many drugs of abuse acting both directly, such as morphine and heroine, and indirectly, such as alcohol, nicotine, and cannabinoids. MORs are expressed on GABA neurons in the VP, rostromedial tegmental nucleus and within the VTA that innervate dopamine neuron at VTA. Activation of MORs on these neurons causes disinhibition of dopamine neurons and increases firing rate of dopamine neurons in VTA (Johnson and North, 1992; Jalabert et al., 2011). However, this disinhibition from GABA neuron requires VTA glutamatergic transmission as application of intra-VTA infusion of both AMPA and NMDA receptor antagonists prevented morphine-induced excitation of VTA-dopamine neurons (Jalabert et al., 2011). Acute opioid administration elevated dopamine level in NAcc (Di Chiara and

Imperato, 1988). However, dopamine is not sole responsible to opioid rewarding effect as rats could still perform heroine self-administration despite administration of dopamine receptor antagonist or lesion of dopamine neurons by application of 6-hydroxydopamine (6-OHDA) (Pettit et al., 1984; Gerrits and Van Ree, 1996). NAcc is another area rich in MORs (Svingos et al., 1996). Olds (1982) demonstrated that rats performed morphine self-administration directly into their NAcc.

In contrast to MORs, KORs mediates dysphoria. Direct injection of KORs agonists into VTA or NAcc produced conditioned place aversion (Bals-Kubik et al., 1993). A more recent study showed that administration of KOR agonist failed to induce conditioned place aversion in KOR knockout mice with KORs deleted in dopamine neurons in VTA (Chefer et al., 2013) suggesting the role of KORs in negative modulation of the mesolimbic dopamine system. The roles of KORs become dominant in drug withdrawal and tolerance.

Tolerance to opioid develop after acute use of opioid. It is defined as the need for higher dose in order to achieve desirable effects of the drugs for responsiveness is reduced (Christie, 2008). Opioid tolerance could occur in every stage from receptor level to neutral system level. At receptor level, expression of MOR is decreased. The receptors are uncoupled with effector proteins such as inward rectifying potassium channels. Receptor internalization and desensitization is also developed. Agonist activation induces MORs phosphorylation by G-protein-coupled receptor kinase 2 (GRK2) is then increases the affinity of interaction of the receptors for beta arrestin-2. The binding of beta arrestin-2 uncouples the receptor from G-protein signaling and desensitizing the receptors. This process also initiates receptor sequestration and internalization through beta arrestin-2- and dynamin-dependent mechanism (Bohn et al., 2000; Williams et al., 2001; Koch et al., 2005). Adenylyl cyclase is superactivated after chronic activation of opioid receptors activation in order to compensate to depressed level of cAMP that is the result of receptor activation (Avidor-Reiss et al., 1996). This superactivation upregulates adenylyl cyclase signaling system and opposes the inhibitory effects of opioid receptors activation (Walwyn et al., 2010). A study by Bobeck et al. (2014) showed that repeated morphine or adenylyl cyclase activator administration into ventrolateral periaqueductal gray decreased morphine nociception where administration of adenylyl cyclase could inhibit development and

expression of tolerance to morphine, indicating the role of adenylyl cyclase in opioid tolerance.

VTA dopamine neurons sends their projections to many structure such as NAcc, basolateral amygdala (BLA), central amygdala (CeA), and lateral portion of bed nucleus of stria terminalis (lBNST). These structures involve in drug withdrawal (Hasue and Shammah-Lagnado, 2002; Meloni et al., 2006). Administration of opioid antagonist into these areas can precipitate withdrawal symptoms, both somatic and motivational, in morphine dependence animals (Berger and Whistler, 2010).

cAMP upregulation are found in many brain areas, such as locus coeruleus (LC), periaqueductal gray (PAG), NAcc, and VTA (Berger and Whistler, 2010). Recently, Zhu et al. (2016) demonstrated that chronic morphine could potentiate excitatory transmission between paraventricular nucleus of thalamus (PVT) and medium spiny neuron expressing dopamine $D_2$ receptor. In the same study, bilateral optogenetic silencing of pathway between PVT and NAcc could significantly reduce withdrawal symptoms (jumping, rearing, and tremor) that is induced by administration of opioid receptor antagonist, naloxone.

Exposure to opioid could activate several pathways in the brain. Acute effect of opioid in addiction is euphoric effect that is the result from binding of opioid to MORs causing disinhibition of GABA interneurons in the VTA that suppresses dopamine neurons that sends their innervation to several sites in the brain, especially NAcc, medial prefrontal cortex, hippocampus, and amygdala. Apart from dopamine neurons, GABA neurons and glutamate neurons are also found in the VTA (Fields and Margolis, 2015). Repeated exposure to opioid induces changes in brain reward pathways in many levels from receptor level to system level. MORs are desensitized and internalized while superactivation of adenylyl cyclase occurs that magnify downstream effects of such activation. Tolerance and withdrawal symptoms develop over time, forcing the subject to drug seeking and increase the amount of drug consumed in order to reach desirable effects (Christie, 2009).

**Animal models of drugs addiction**

Abuse liability assessment is required for drug expected to acts on central nervous system. Many guidelines documents specify models to be tested. These models are self-administration, drug discrimination and physical dependence/withdrawal assay, for examples (Swedberg, 2016; Gauvin et al., 2017) to ensure safety of the drugs.

Drug discrimination

Drug discrimination is a model which subject learns to distinguish the different between two drugs or treatments effects and work in order to obtain reward, usually food or water. The procedures are based on state-dependent learning and the repeated pairing of a drug effect with an operant behavioral response, which results in presentation of a reinforcer of that behavior. The subjects perform particular behavior at higher frequency (Lynch et al., 2010).

The apparatus for this model is usually a chamber with two levers located on the left and right sides on the same wall with food receptacle located between two levers. Other reinforcement such as water dispenser may also be equipped. Sometimes additional equipment such as shock generator providing electrical stimulus or cue light are added as experimenter needed. This technique is able to identify relative reinforcing effects of drugs. The animals are first trained to press one lever (for example, right lever) in order to obtain reward (such as food or water) in drug-induced state or the other lever (the left lever) to obtain reward during placebo-induced state or drug-free state. The effects of the drugs produce interoceptive cues that serve as conditioned stimuli which direct the animals to exhibit appropriate response or behavior in order to obtain reward (Koob et al., 2014a). The animals are trained until they are able to exhibit stable response to particular treatment. The animals are tested with doses of training drug for specificity of discrimination before doses of testing drugs are introduced. This is called substitution test which the test compound is given as substitute for training drug. On the test day, both lever are made active. Animals are given test drug and put into the apparatus so they would press the

lever for food. The choice of response provides information about similarity of the drugs given as interoceptive cues compared with control drugs. For example, it is predictable that the rat trained with amphetamine will press on the lever paired with amphetamine when it is given methamphetamine. Thus, should subjects respond on the drug paired lever could be considered that the compound given shares some discriminative stimulus effect or it produces subjective effects like training drug. In the other hand, if the subjects respond on the placebo or vehicle paired lever, it is interpret as the compound given does not share discriminative stimulus effect as training drug. Generally, drugs with same mechanism of action or similar pharmacological effects tend to substitute or cross substitute for each other in this model. (Harun et al., 2015).

Conditioned place preference (CPP)

CPP is a model to evaluate abuse liability of a drug regarding rewarding effect of the drug. In this model, an animal receives drug or vehicle and then placed into one of 2 compartment in an apparatus with distinct environments. Each compartment has different visual cues such as wall pattern, usually vertical vs. horizontal stripes, and tactile cues such as smooth floor vs. grid floor. Other cues could also be implemented for example, olfactory cues could be introduced by placing different essential oils into each compartment. The animal are always place into the compartment paired with drug/vehicle given so they could learn association between the effects of the drug with the environment. This pairing is performed repeatedly for several days to strengthen the association. Finally, the animal, usually in drug free state, is place into the apparatus where the doors between each compartment are remove and allow them to roam freely within the apparatus. The amount of time spent in each compartment is recorded. The preference for one environment over the other confers information regarding the motivational state created by the drug. The animal should spends more time in the compartment paired with the drug that has 'rewarding effect' compared with its initial amount of time before pairing process. In the other hand, if the animal spend significantly less time in the compartment paired with drug administration, the drug is considered to have 'aversive effect' to the animal. Thus, this is so called

'conditioned place aversion' (CPA) (Koob et al., 2014a). Conditioned place preference is different from drug self-administration that it is more under control of the experimenter than the subject itself (Myers and Carlezon, 2010).

In general, conditioned place preference protocol contains three to five phases depending on the purpose of the study which are

1) Preconditioning phase (Phase I)

In this phase, the animals are allowed to explore and acclimate to the apparatus and testing environment and, more importantly, to determine pre-existing preference of one compartment over another one. Each animal will be put within the apparatus and allow to roam freely in the apparatus. Amount of time spent in each compartment is collected to evaluate the pre-existing preference and such preference will be used to assign which compartment is to be paired with drug and vehicle. Usually, the drug is paired with less preferred compartment to avoid misinterpretation of the result.

2) Conditioning phase (phase II)

In this phase, the animal is placed inside the compartment paired with the treatment for defined amount of time. It is placed inside another compartment paired with vehicle (or different treatment) for the same amount of time in alternate sessions. Number of session may vary from two to twelve. The pairings are counterbalanced. For example, in eight session procedure, the animal receives 4 of the treatment and another 4 of another treatment, alternatively. Time spent in the compartment is partly defined by half-life of the drug. The length of session is longer in drug with longer half-life such as morphine. There are two approaches in assigning the animal to one compartment. one approach is 'bias approach' where the least preferred compartment is paired with testing drug and another compartment is paired with vehicle. Another approach is 'unbias approach' where the animals are assigned to receive either testing drug or vehicle in either of the two chambers. In short, pairing of the treatment and compartment is done regardless of pre-existing preference (Prus et al., 2009).

3) Testing phase (Phase III)

This phase is conducted like phase I. the animal is placed in the apparatus where it can roam and explore freely. The doors separating each compartments are removed. Time spent in each compartment is recorded for analysis.

4) Extinction phase (phase IV)

CPP is sometimes used to study extinction of conditioning. The animal would show decline in conditioned response in term of frequency or intensity when conditioned stimulus (drug paired compartment or the apparatus, for example) is present without the unconditioned stimulus (the drug). The procedure is that the animal to be in the apparatus repeatedly like in testing phase without receiving the drug. Other procedure repeat conditioning phase but the drug is replaced by vehicle. After several session, the animal is then put into testing phase where the time spent in each compartment is recorded and compared with testing phase before the drug is taken away. Reduction in time spent in drug paired compartment indicate extinction of conditioned place preference. Other criterion are also used to determination of extinction.

5) Reinstatement (phase V)

Conditioned place preference can also be used to study reinstatement of drug addiction. Reinstatement is defined as the relapse of conditioned response after extinction. In this phase, the animal is exposed to the drug before it is then place into the apparatus like in testing phase. However, not only drug itself could bring up reinstatement of conditioned response, but also exposure of the animal to stressor or other drugs, for example.

Physical dependence

Cessation from either acute or chronic drug taking could lead to negative state called withdrawal that compel the subject into drug seeking in order to alleviate the negative states. Withdrawal could be either physical or psychological (Evans and Cahill, 2016).

To study the effect of morphine withdrawal, the procedures require the subjects to become dependent to a known drug which is morphine in this scenario by means of continuous administration or repeated injection. After the subjects become dependent to the drug, antagonist is given to precipitate withdrawal symptoms. Naloxone is given to subjects to abruptly precipitate physical withdrawal symptoms. After administration of naloxone, subject's behavior and symptoms are observed for a period of time. In mouse, most studies performed observation of jumping. However, many other behaviors such as rearing, piloerection, tremor, Straub tail, urination and diarrhea (Matsumoto et al., 2005a; Hajhashemi and Abed-Natanzi, 2011; Wu et al., 2012; Zhu et al., 2016) are also observed alongside jumping. The dose of naloxone given varies from 1 mg/kg (Cichewicz and Welch, 2003) to 50 mg/kg (Wu et al., 2012) and route of administration could be subcutaneous or intraperitoneal injection.

Many compounds with different mechanisms of action attenuate withdrawal signs in animal subjects. For examples, SR 141716A (cannabinoid receptor 1 antagonist), phenothiazine-type anti psychotics, SU-6656a (selective inhibitor of src family kinase), and carbamazepine (anticonvulsant), could attenuate precipitated withdrawal signs (Mas-Nieto et al., 2001; Hajhashemi and Abed-Natanzi, 2011; Rehni and Singh, 2011; Wu et al., 2012) suggesting several molecular substrate mediating exhibition of withdrawal signs.

**Kratom plant (*Mitragyna speciosa*) and mitragynine**

Also known as ketum, biak-biak and many names, kratom (*Mitragyna speciose* Korth.) is an indigenous plant of Thailand, Malaysia and other countries in Southeast Asia. It is a plant belongs to Rubiaceae family. Leaves of kratom are harvested and used for both folklore medications and narcotic effects. Many preparation can be made from the leaves. Fresh leaves are chewed alone or with betel

nut (*Areca catechu*) (Vicknasingam et al., 2010). Dried leaves can be smoked. Powdered dried leaves are brewed with hot water and drunk as tea. It can also be mixed with other ingredients in order to mask kratom's bitterness or to obtain greater effects (Suhaimi et al., 2016). In addition, kratom leaves can be boiled into syrup which is then mixed with chopped palm leaves and rolled into pills called "Madat" and smoked with bamboo pipe. In United States and European countries, kratom products are sold as tea, capsules containing pulverized or powdered dried leaves and compressed tablets of dried leaves or resins (Prozialeck et al., 2012).

In folklore medication, kratom is used for treatment of muscle pain, diarrhea, intestinal infection, and cough (Suwanlert, 1975; Chan et al., 2005). It is also used to enhance working capacity and endurance to fatigue. Kratom consumption produces euphoric effects. It can alleviate opioid withdrawal symptoms. Therefore, people use it as substitution for opioids and methamphetamine when access to drugs is prevented or as substitution treatment (Beckett et al., 1965; Rätsch, 2005). Kratom is categorized as drug of abuse in many countries due to its stimulating effect at low to moderate dose and sedative effect at high dose (Prozialeck et al., 2012). In Thailand, kratom is classified into Category V of Narcotic Act B.E. 2522, the same category to marijuana, which prohibits planting and possessing of kratom plant and its parts. Kratom became illegal in Malaysia in 2003 and in Australia in 2005 (Vicknasingam et al., 2010). In the United States, kratom is not controlled under Federal Controlled Substances Act. However, possession and use of kratom are regulated and prohibited in some states. It is also on the Drug and Chemical of Concern List by United States Drug Enforcement Administration (2015) and considered no legitimate medical use making advertising kratom for any medical use illegal in the US (Prozialeck et al., 2012). European countries such as Denmark, Latvia, Lithuania, Romania and Sweden put kratom and its derivatives as controlled drugs while they are still legal, but under surveillance, in the United Kingdom and Germany (Suhaimi et al., 2016). Kratom is one of the top 25 seller "legal high" in United Kingdom's online market study (Schmidt et al., 2011).

Suwanlert et al. (1975) reported kratom addiction from chronic administration of kratom. Users exhibited symptoms of aggregation, inability to work, muscle and bone ache, limb jerks, anorexia, weight loss and insomnia. A more recent study showed that kratom poisoning symptoms are palpitation followed by seizure where

common kratom withdrawal symptoms are myalgia, insomnia, fatigue, and chest discomfort (Trakulsrichai et al., 2013). In Thailand, people in the southern part combine the leaves of kratom with cola drinks and codeine or diphenhydramine containing cough syrups making a homemade cocktail drug called "4 x 100" which sometimes leads to fatality due to multidrug actions (Tungtananuwat and Lawanprasert, 2010). Another kratom containing herbal blend "Krypton" was reported to cause unintentional fatal intoxication (Kronstrand et al., 2011). Other kratom toxicity such as intrahepatic cholestasis following kratom abuse and seizures was also reported (Nelsen et al., 2010; Kapp et al., 2011). Methanolic extract was able to block several cytochrome P450 (CYP) isozymes CYP2C9 CYP3A4, CYP2D6 and CYP1A2 in vitro with highest potency for CYP2D6 (Kong et al., 2011). Findings suggested interactions of kratom with CYP substrate such as opioids, codeine, dextromethorphan, and diphenhydramine that lead to potentiation of drugs in many cocktail preparations combining kratom with other ingredients.

More than 40 alkaloids from kratom have been isolated and characterized. Some of them are mitragynine, speciogynine, paynantheine, speciociliatine and 7-hydroxy mitragynine for examples. The leaves of kratom plant contain from 0.5 to 1.5 % of alkaloid content (Hassan et al., 2013). Mitragynine is a major alkaloid constituent in kratom leaves. It is responsible for 66% of total alkaloid content (Takayama, 2004). . It has molecular formula of $C_{23}H_{30}N_2O_4$ (IUPAC name: methyl (E)-2-[(2S,3S,12bS)-3-ethyl-8-methoxy-1,2,3,4,6,7,12,12b-octahydroindolo[2,3-a]quinolizin-2-yl]-3-methoxyprop-2-enoate). Structure of mitragynine is shown in **Figure 1**. Mitragynine is white amorphous powder soluble in alcohol, acetic acid and chloroform (Suhaimi et al., 2016). Mitragynine is an indole alkaloid structurally related to yohimbine, an $\alpha_2$ adrenergic receptor agonist. Competitive binding assay revealed that mitragynine is able to bind to all three subtypes of opioid receptors with greatest affinity to KOR followed by MOR and DOR (Taufik Hidayat et al., 2010). More recent study showed that mitragynine and 7-hydroxymitragynine are partial agonist of MOR in human opioid receptors expressed on HEK cells (Kruegel et al., 2016). In addition to opioid receptors, mitragynine can also bind to many other receptors, such as adenosine receptor, $\alpha_2$ adrenergic receptor, dopamine $D_2$ receptor,

serotonin 5-HT$_{2C}$ receptor and serotonin 5-HT$_7$ receptor, suggesting other mechanism of action regardless of opioid receptor activation or inhibition (Boyer et al., 2008).
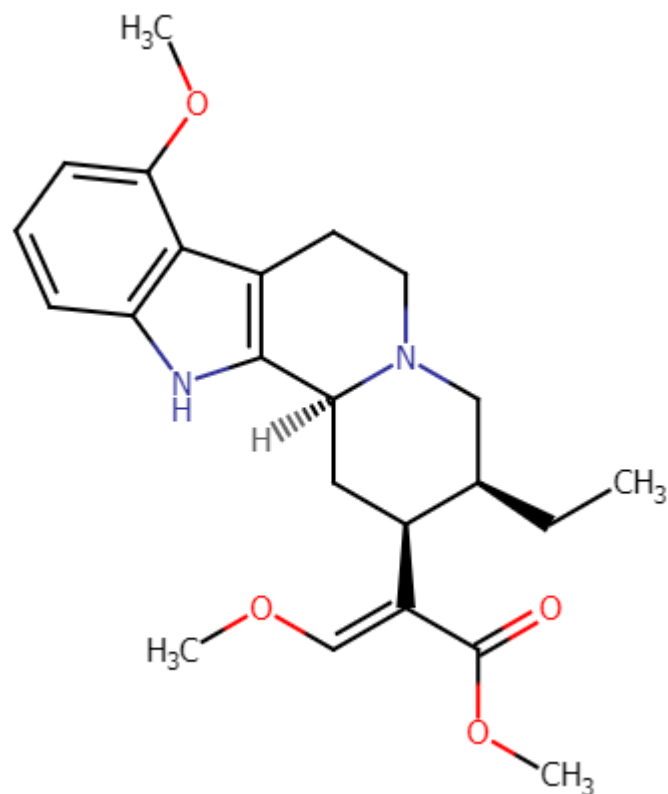


**Figure 1** Structure of mitragynine

Mitragynine can also block neuronal $Ca^{2+}$ channels which probably leads to inhibition of neurotransmitter release from the neuron innervating vas deferens and this is believed to mediate other physiological effects of mitragynine as well (Matsumoto et al., 2005b).

Mitragynine exerts many pharmacological effects. Antinociceptive property is one of the most interested activity of mitragynine due to traditional use and its ability to bind to opioid receptors. Many antinociception studies suggested that kratom possessed antinociceptive activity, primarily due to opioid receptor activation as its effect could be blocked by administration of naloxone (Suhaimi et al., 2016). A study by carpenter et al. (2016) showed that mitragynine has antinociceptive activity in thermal nociception assay in rats when given either intraperitoneally or orally.

Kratom extract and mitragynine also have antidepressant activity. Kratom extract could reduce immobility time in rat forced swimming test (Kumarnsit et al., 2007b). Mitragynine itself also exhibited antidepressant activity by reduction of corticosterone level in mice exposed to forced swimming test and tail suspension test without significant effect on locomotor activity tested by open field test (Idayu et al., 2011). Mitragynine also has anxiolytic effect in open field and elevated plus maze. This anxiolytic effect is thought to mediate by opiodergic, GABAergic and/or dopaminergic system since it could significantly be blocked by naloxone, flumazenil and sulpiride (Hazim et al., 2014). Another study by Yusoff et al. (2016) supported anxiolytic effect of mitragynine in elevated plus maze and light/dark box models.

Kratom possesses several other pharmacological effects those in agreement of its traditional uses. Gastrointestinally, the effects of kratom include, but not limited to, reduction of defecation frequency and fecal weight, inhibition of gastrointestinal transit induced by castor oil (Chittrakarn et al., 2008), and inhibition of gastric acid secretion stimulated by 2-deoxy-D-glucose (Tsuchiya et al., 2002). Anorexigenic effect was observed in animal studies. Rats acutely received alkaloid extract of kratom showed reduction of food and water intake. When given chronically, food and water intake reduction was prolonged which led to weight gain suppression (Kumarnsit et al., 2007a).

There are concerns about abuse liability of kratom and its constituents (United States Drug Enforcement Administration, 2015; Suhaimi et al., 2016). As mentioned

earlier, kratom has been traditionally used as narcotics (Suwanlert, 1975). It has stimulant effect at low dose and opioid sedative effect at high dose. Users reported that they feel more active, more appetite, and more sexual desire using kratom products (Vicknasingam et al., 2010).

Yusoff et al. (2016) demonstrated that mitragynine induced drug conditioned place preference at dose of 10 mg/kg and 30 mg/kg. More recent study suggested the role of GABA$_B$ receptor in the rewarding effect of mitragynine. Administration of baclofen at 2.5 and 5 mg/kg was able to block acquisition and expression of mitragynine conditioned place preference (Yusoff et al., 2018).

Rats exposed to mitragynine for 28 days showed withdrawal signs at day 1 and 14 after drug abstinence (Sabetghadam et al., 2013) The presence of physical dependence was relatively lower in all mitragynine treated groups on day 14 compared with day 1 suggesting reversibility and recovery. Alkaloid rich extract of kratom plant could attenuate morphine precipitated withdrawal jumping in mice by naloxone in dose dependent manner. However, mitragynine from 90 to 120 mg/kg showed some reduction in the number of jumping in the same experimental settings but it was not statistically significant (Cheaha et al., 2017). The result from this study is partly in agreement with a study by Khor et al. (2011). Mitragynine could attenuate withdrawal syndromes in morphine treated zebrafish in the study. In the same study the whole body cortisol level and mRNA levels of CRF-R1, CRF-R2 and prodynorphin were lower in mitragynine treated group compared with control group. Taken together with conventional use of kratom plant, this suggests potential of mitragynine in treatment of drug addiction.

## Chapter 3
## Materials and Methods

**Animals**

Male ICR mice weighing 18-25 g and Male Wistar rats weighing 180-200 and 250-300 g were used in the experiments. Male ICR mice were used in naloxone precipitated withdrawal model. Male Wistar rats weighing 180-200 g were used in conditioned place preference model while rats weighing 250-300 g were used in drug discrimination model. The animals were obtained from National Laboratory Animal Center, Mahidol University, Salaya, Nakhonpathom, Thailand. They were allowed to acclimate for 7 days in the Laboratory Animal Research Building, Faculty of Pharmaceutical Sciences, Chulalongkorn University before experiments. Housing room environment was controlled with $24 \pm 2$ °C, 40-60% relative humidity and standard 12 hours light/dark cycle. Food and water were available *ad libitum* except in drug discrimination model. All behavioral experiments were conducted between 08:00 – 18:00. Research proposal was reviewed and approved by Institution Animal Care and Use Committee (IACUC), Faculty of Pharmaceutical Science, Chulalongkorn University (Protocol No. 1333009). All experimental procedures were performed according to the Ethical Principles and Guidelines for the Procedures on Animals for Scientific Purposes, National Research Council of Thailand.

**Chemicals**

- Methamphetamine HCl (98.22%, analyzed by Department of Medical Science, Ministry of Public Health, Thailand)

- Mitragynine was extracted and isolated from the leaves of *Mitragyna speciosa* Korth. using the method described by Ponglux et al. (2007)

- Morphine Sulfate (Temed Co.,Teharan, Iran)

- Naloxone (Sigma-Aldrich, St. Louis, MO, USA)

- Glacial acetic acid (J.T. Baker, Center Valley, PA, USA)

- Dichloromethane (J.T. Baker, Center Valley, PA, USA)

- Normal saline solution (Thai Nakorn Pattana, Nonthaburi, Thailand)

- Tween 20 (Ajax Finechem, NSW, Australia)

Morphine sulfate, methamphetamine HCl, and naloxone hydrochloride were dissolved in saline. Mitragynine was dissolved in 20% tween 80 in saline solution. All the drug preparations were freshly prepared and injected intraperitoneally (i.p.) into the animals. Morphine, methamphetamine, and mitragynine were used with permission from The Ministry of Public Health. Mitragynine doses are based on its solubility and previous studies (Shamima et al., 2012)

**Experimental apparatus**

Operant conditioning chamber

Operant conditioning chambers (**Figure 2**, ENV-001; Med Associates, St Albans, VT, USA) connected to Med Associates Interface Model SG-503 with MED-IV software. Each chamber is placed within a sound attenuated chamber. The front and back walls of the chamber are made of Plexiglas. The front wall is served as a door into the chamber. The other two walls are aluminum. The floor consisted of 18 stainless steel rods (4.8 mm diameter) placed 1.6 cm apart from each other. On the top of one aluminum wall located a 28-V with house light 20 cm centered above the floor. Another aluminum wall has two levers with white cue light 6 cm above each lever. Food receptacle is located between two levers. Food receptacle is connected to food magazine attached to the chamber.
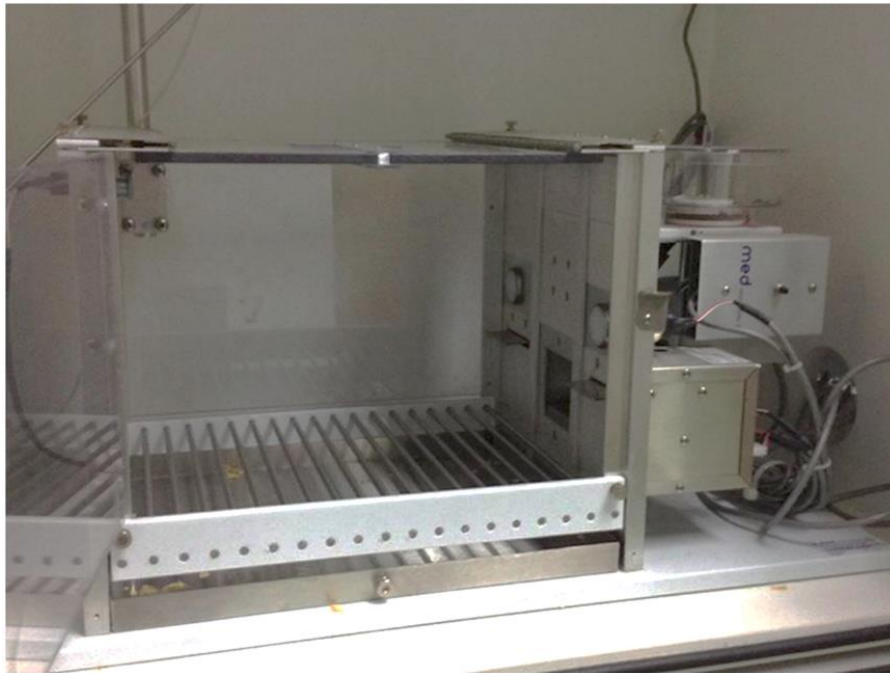
**Figure 2** Operant conditioning chambers

Conditioned place preference apparatus

A rectangular chamber is consisted of three compartments that is isolated by solid removable vertical screens. One central chamber connecting to two lateral compartments has gray walls. One lateral compartment has black and white vertical stripes walls with black textured floor. The other lateral compartment has black and white horizontal stripes walls with black smooth floor.

Close circuit camera system connecting to personal computer is positioned above the chamber for behavior observation. Subjects' behaviors are then analyzed by VideoMot2 software (TSE Systems, Bad Homburg, Germany)

**Methods**

**Part 1: Abuse liability of mitragynine**

**Experiment 1: mitragynine discrimination in rats trained with methamphetamine**

      **Animals**

      Rats were used in this experiment according to protocol by Solinas et al. (2006). They were under food restriction to gradually reach 85 percent of free feeding weight. They were fed following completion of training or testing session. The experiment was conducted on Monday to Friday from 7:00 to 18:00 hrs. Food was given *ad libitum* after session completion on Friday until 10:00 of Sunday. Water was given *ad libitum* except during training or test session.

      **Procedure**

      **Training phase**

      Rats were acclimated to small food pellet used in the experiment in their home cages. After they were able to consume the food pellets. They were trained to discriminate saline (1 ml/kg i.p.) from methamphetamine (1 mg/kg i.p., equivolume to saline). Rats were injected with either saline or methamphetamine 10 minutes before start of the training session. Right lever was made active lever for methamphetamine where left lever was made active lever for saline for half of that rats. Left lever was active lever for methamphetamine and right lever was active for saline for another half of the rats. This was counterbalanced to neutralized lever bias in rats. The schedule of reinforcement began at fixed ratio 1 (FR1) with timeout period of 10 seconds (TO10). House light was on at the beginning of the session. Every completion of response required on the schedule resulted in delivery of a 45 mg food pellet following by timeout period in which all the lights in the chamber are off and any lever depression, both correct and incorrect lever, was recorded but yield no consequence. Depression of incorrect lever during the trial was recorded. It also reset the number of pressing required on correct lever. House light was turned on again after completion of timeout period for the next trial. Methamphetamine and saline were given in double alternate schedule. For example, a two week schedule was MMSSM MSSMM where M represented methamphetamine and S represented saline.

The schedule of reinforcement and timeout were gradually increase until the rats could reliably response to FR10 and TO45. Each session ended when 20 trials were completed or 30 minutes time elapses, whichever came first. Training continued until rats could complete 1) 90% or more response occurred on the correct lever and 2) not more than 4 responses occurred on the incorrect lever on the first trial for 8 consecutive sessions before the testing phase begins.

**Testing phase**

In this phase, different doses of methamphetamine, morphine and mitragynine were given to trained rats. Test session was similar to training session, except that both levers were made active and 10 consecutive pressing was required on the same lever for food pellet delivery. Test sessions were conducted in between methamphetamine training and saline training session. A two-week schedule was MTSMT STMST (T= test).Test sessions were conducted only when that rats were still able to maintain 1) 90% accuracy on the correct lever and 2) not more than 4 responses on incorrect lever in the first trail in the two preceding training sessions. Data were expressed as percentage of response on the correct lever.

**Experiment 1.1: Dose effect curve of methamphetamine**

Methamphetamine at several doses (0, 0.1, 0.3, 0.6 and 1.0 mg/kg i.p.) were given on the test sessions to obtain dose effect curve.

**Experiment 1.2 Mitragynine generalize test**

Mitragynine at 0, 1, 5, and 10 mg/kg and morphine 1 and 2 mg/kg were given 30 minutes before test session begins. Percentage response on methamphetamine paired lever was obtain to see generalization effect of mitragynine and morphine.

**Experiment 2: Mitragynine conditioned place preference**

      **Procedure**

          Conditioned place preference method was modified from Dias et al. (2012) and Mizoguchi et al. (2004). It was used to evaluate reinforcing effect of drugs (Huston et al., 2013). Upon finishing preconditioning phase (day 1-3), rats were randomly divided into 6 groups. Time spent in each compartment was recorded on day 3 to determine which lateral compartment was preferred by the rats. The preferred compartment was then paired with saline administration (saline paired compartment) and the other lateral compartment was paired with drug administration (drug paired compartment). Each group received different doses of mitragynine (5, 10, 30, and 90 mg/kg) on day 4, 6, 8, and 10 before being confined in drug paired compartment of conditioned place preference apparatus for 30 minutes. Rats in control groups received either saline or morphine (5 mg/kg), on the same days. On day 5, 7, 9, and 11, rats received saline before being confined in saline paired compartment for 30 minutes. Treatments were given intraperitoneally. Injection volume was 1 ml/kg. On day 12 each rat , in drug free state, was confined in central compartment of the apparatus for 5 minutes before the doors connecting to both lateral compartments were removed allowing the rat to freely explore the apparatus for 15 minutes. Time spent in each compartment was collected. Difference in time spent in drug-paired compartment was reported and used for statistical analysis.

**Experiment 3: Physical withdrawal effect of mitragynine**

**Experiment 3.1: Naloxone precipitated withdrawal symptoms of acute mitragynine treatment**

**Procedure**

To determine abuse liability of mitragynine. This experiment evaluated physical withdrawal symptoms after acute mitragynine treatment. Mice were randomly divided into 6 groups. Each group received different doses of mitragynine (5, 10 and 30 mg/kg). Positive control group receives morphine (100 mg/kg). Negative control group receives saline (10 ml/kg). Two hours after treatment, each mouse receives naloxone (3 mg/kg) and is placed into a glass cylinder for 30 minutes to observe withdrawal symptoms. Withdrawal symptoms observed are jumping, rearing, grooming, wet dog shake, Straub tail reaction, and C shaped tail (Matsumoto et al., 2005a).

**Experiment 3.2: Naloxone precipitated withdrawal symptoms of chronic mitragynine treatment**

**Procedure**

To determine abuse liability of chronic mitragynine treatment, mice were divided into 4 groups. Treatment groups received mitragynine (10, and 30 mg/kg) twice daily for 7 consecutive days (09:00 and 19:00 hrs.), except that only one dose was given on day seventh. Saline control received normal saline solution (10 ml/kg) twice daily. Morphine control group received increasing doses of morphine. Doses of morphine from first day to seventh day were 10, 20, 30, 40, 50, 60, and 70 mg/kg, respectively. Two hours after treatment of seventh day, mouse was injected with naloxone (3 mg/kg) to precipitate withdrawal symptoms and immediately was placed into the glass cylinder for behavioral observation for 30 minutes (Matsumoto et al., 2005a).

**Part 2: Effects of mitragynine on morphine addiction**

**Experiment 4: Effects of mitragynine on acquisition of morphine induced place preference**

**Procedure**

To determine the effects of mitragynine on acquisition of morphine conditioned place preference. After preconditioning phase, rats were divided into 5 groups. On day 4, 6, 8, and 10, each rat received two saline treatments with 30 minutes interval before being placed into preferred compartment determined from preconditioning phase. On day 5, 7, 9, and 11, rat received either saline or mitragynine (5, 10, and 30 mg/kg) 30 minutes before morphine (5 mg/kg) treatment. Control group received two saline treatments with 30 minutes interval before being placed into less preferred compartment. Rat was confined in assigned compartment for 30 minutes before being returned to its home cage.

On day 12 (post conditioning phase), each rat, in drug free state, was placed into central compartment of the apparatus before the doors connecting to both lateral compartments were open. Time spent in each compartment was recorded and compared with the time they spent in matched compartment during preconditioning phase.

**Experiment 5: Effect of mitragynine on expression of morphine induced conditioned place preference.**

**Procedure**

In conditioning phase, rats were treated with saline on day 4, 6, 8, and 10 and they were treated with either morphine (5 mg/kg) on day 5, 7, 9, and 11 before being confined in assigned compartment for 30 minutes. On day 12, they were treated with saline or mitragynine (10 or 30 mg/kg). 30 minutes after drug administration, they were put into the central compartment for 5 minutes before the doors connecting to both lateral compartments were open. Rats were allowed to explore freely for 15 minutes. Time spent in each compartment was collected. Calculations were performed as described in Part 1.

**Experiment 6: Effect of mitragynine pretreatment on chronic morphine physical withdrawal symptoms precipitated by naloxone**

**Procedure**

Mice were divided into 4 groups. Morphine was administrated twice daily (07:00-19:00) to each mouse to induce morphine dependence except only single dose on day seven. Doses of morphine was escalated each day from day1 to day7 as follows; 10, 20, 30, 40, 50, 60, and 70 mg/kg. 30 minutes before each morphine administration, each mouse received pretreatment of either saline or mitragynine (10 or 30 mg/kg). Control group received saline solution instead of morphine and mitragynine. On day 7, mouse received naloxone (3 mg/kg) 2 hours after treatment and was immediately placed into glass cylinder for withdrawal behaviors observation for 30 minutes.

**Part 3: Effects of mitragynine on methamphetamine addiction**

**Experiment 7 Effect of mitragynine on methamphetamine discrimination**

   **Procedure**

   This experiment was perform in rats trained to discriminate 1 mg/kg methamphetamine from saline from experiment 1. Mitragynine at 0, 1 and 5 mg/kg were given 30 minutes before methamphetamine (1 mg/kg) administration. Rats were put into the chamber immediately after methamphetamine treatment. Percentage response on methamphetamine paired lever was obtained to see the effect of mitragynine pretreatment on methamphetamine discrimination.

**Experiment 8: Effect of mitragynine on acquisition of methamphetamine induced conditioned place preference**

   **Procedure**

   This experiment was performed similar to experiment 4. It was to determine the effect of mitragynine on acquisition of methamphetamine induced place preference. On conditioning phase, rats received two saline injections with 30 minutes interval before being placed into saline paired compartment determined from preconditioning phase on day 4, 6, 8, and 10. On day 5, 7, 9, and 11, they received different doses of mitragynine (0, 5, 10, and 30 mg/kg) 30 minutes before methamphetamine (2 mg/kg) injection and being placed into drug paired compartment. They were confined in such compartment for 30 minutes before being returned to their home cages. Post conditioning phase was performed on day 12. Rats in drug free state were placed into the central compartment for 5 minutes before the doors connecting each compartment were open. Time spent in each compartment was record for analysis as previously described.

**Data analysis**

Data were expressed as mean ± standard error of the mean and analyzed by one way analysis of variance (ANOVA). Difference between control and test groups was revealed by Bonferroni's test as *pos hoc* analysis. Statistical significance was set at $p < 0.05$.

# Chapter 4
# Results

**Mitragynine discrimination in rats trained with methamphetamine**

In rats trained to discriminate methamphetamine (1 mg/kg) from saline, the response on methamphetamine appropriate lever increased in dose dependent manner from 0.1 to 1.0 mg/kg with mean maximal response for 0.6 and 1.0 mg/kg at $82.54 \pm 10.43\%$ and $91.95 \pm 4.971\%$, respectively (**Figure 3**, n=6). $ED_{50}$ was 0.34 mg/kg (0.1691-0.6754; 95% confidence interval). Mitragynine could substitute methamphetamine in dose dependent manner. It could fully substitute methamphetamine at the dose of 10 mg/kg with mean maximal response at $80.88 \pm 10.37\%$ (n=6). Morphine could also fully substitute methamphetamine at the dose of 2 mg/kg with mean maximal response at $86.09 \pm 6.592\%$ (n=4).
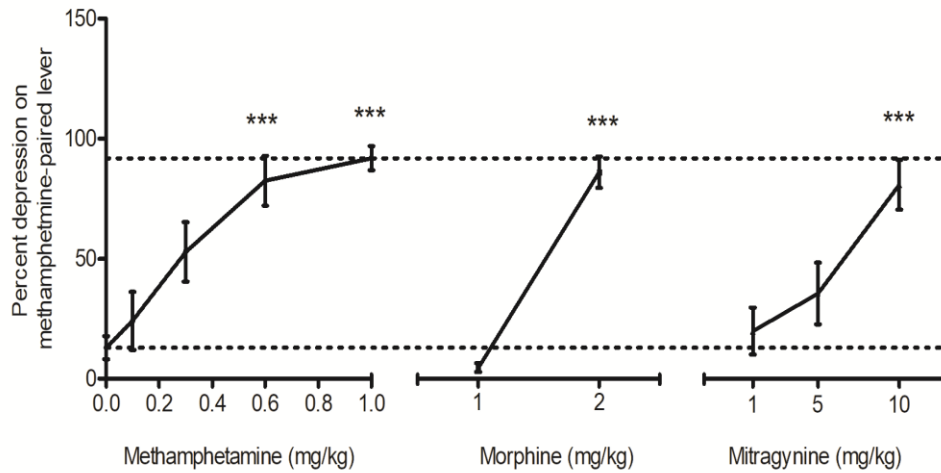
**Figure 3** Effect of methaphetamine, morphine (1-2 mg/kg), and mitragynine (1, 5, and 10 mg/kg) in rats trained to discriminate 1 mg/kg methamphetamine from saline. Under fixed ratio 10 schedule, percent response on methamphetamine paired lever for each treatment is shown. Each data point represent the average of response in 4 - 6 rats. The above dotted line represent average percent response at 1 mg/kg methamphetamine and below dotted line represent average percent response of saline treatment. (***p<0.001 versus saline-treated group; n = 4-6)

**Mitragynine-induced conditioned place preference**

Rats showed preference to lateral compartment with vertical stripes wall and perforated floor over horizontal stripes wall and smooth floor in preconditioning phase. Thus, horizontal stripes wall compartment was assigned as drug paired compartment. After 4 sessions conditioning with saline and 4 sessions conditioning with drugs, changes in time spent in drug paired compartment in 5 mg/kg morphine treated groups was $207.4 \pm 29.02$ s (n=7) compared with saline treated group, $8.8 \pm 18.80$ s (n=10) (**Figure 4**). The time changes increased in dose dependent manner in mitragynine treated groups where they were statistically significant from saline-treated group but not statistically significant from morphine-treated group at the doses of 30 and 60 mg/kg, $143.4 \pm 28.29$ s (n=8) and $143.0 \pm 33.69$ s (n=12), respectively.
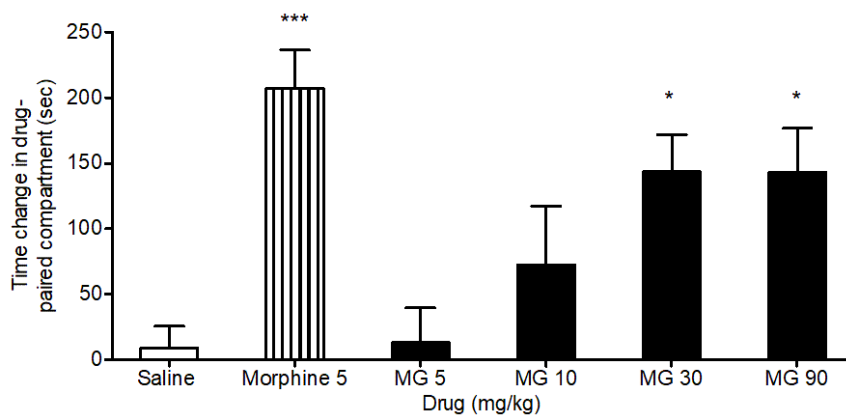


**Figure 4** Conditioned place preference of mitragynine. The effects of mitragynine (MG; 5-90 mg/kg) and morphine (5 mg/kg) on conditioned place preference in rats. Data are expressed as mean $\pm$ SEM of time changes in drug paired compartment between postconditioning and preconditioning phases. (*p $<$ 0.05 and ***p$<$0.001 versus saline-treated group; n $=$ 7-12)

**Mitragynine withdrawal**

Acute withdrawal

Naloxone injection induced repeated jumping in both mice treated with morphine and mitragynine (**Figure 5A**). The numbers of jumping in the 30 minute period were statistically significant in mice given single dose of 100 mg/kg morphine and 60 mg/kg mitragynine, 49.17 ± 11.08 times (n=12) and 43.25 ± 8.37 times (n=8), respectively, compared with saline control, 0.88 ± 0.64 times (n=8). Straub tail reaction observed in morphine and mitragynine treated mice except in 5 mg/kg mitragynine treated group. Percent of mice exhibiting Straub tail reaction was 83.33% in morphine group while in mitragynine treated group were 0, 8.33, 75.00, and 75.00% in 5, 10, 30, and 60 mg/kg mitragynine groups, respectively. No Straub tail reaction could be observed in saline treated group (**Figure 5B**).

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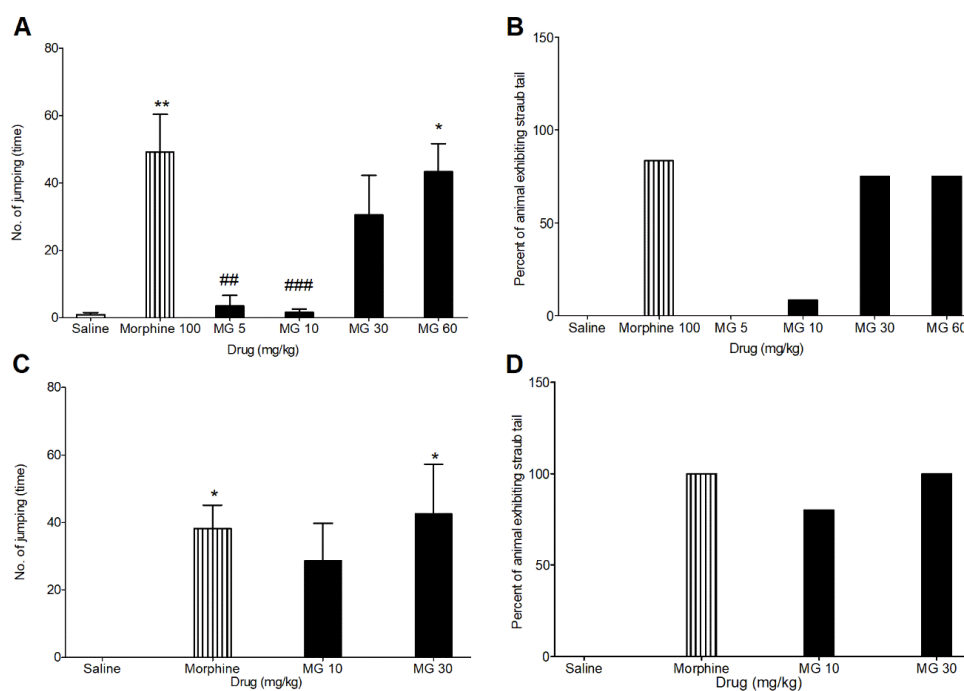
**Figure 5** Mitragynine withdrawal precipitated with naloxone. Upper panel (A, B) shows acute mitragynine (5, 10, 30, and 60 mg/kg) withdrawal (A) number of repeated jumping behavior and (B) Straub tail reaction. Lower panel (C, D) shows chronic mitragynine (10 and 30 mg/kg) withdrawal (C) number of repeated jumping behavior and (D) Straub tail reaction. (A) and (C) Bars represent mean frequency of jumping in the 30 minute period after naloxone injection $\pm$ SEM. (B) and (D) bars represent percent of animal exhibited Straub tail behavior. (*$p < 0.05$ and **$p < 0.01$ versus saline-treated group; ## $p < 0.01$, and ###$p < 0.001$ versus morphine treated group; n = 8-12)

Chronic withdrawal

After chronic treatment of either 10-70 mg/kg morphine or 10-30 mg/kg mitragynine for 7 days, naloxone injection could precipitate repeated jumping behaviour in mice. The numbers of jumping in 30 minute period in morphine and 30 mg/kg mitragynine group were statistically higher than of saline treated group, 38.25 ± 6.86 times (n=8) and 42.50 ± 14.68 times (n=6), respectively (**Figure 5C**). The number of jumping in 30 minute period in 10 mg/kg mitragynine group was 28.60 ± 11.08 times (n=5). All mice (100%) in morphine and 30 mg/kg mitragynine treated groups showed Straub tail reaction while 80% of the mice in 10 mg/kg mitragynine group exhibit such reaction (**Figure 5D**).

**Effects of mitragynine on acquisition of morphine-induced conditioned place preference**

Injection of mitragynine prior to morphine could suppress acquisition of morphine-induced conditioned place preference. The level of suppression was dependent on the dose given. Changes in time spent in morphine paired compartment in saline/5 mg/kg morphine and 5 mg/kg mitragynine/ 5 mg/kg morphine treated groups were significantly higher than of saline/saline control group, 219.6 ± 31.11 s (n=7), 124.0 ± 28.21 s (n=9) and 24.83 ± 13.22 s (n=6), respectively (**Figure 6A**).
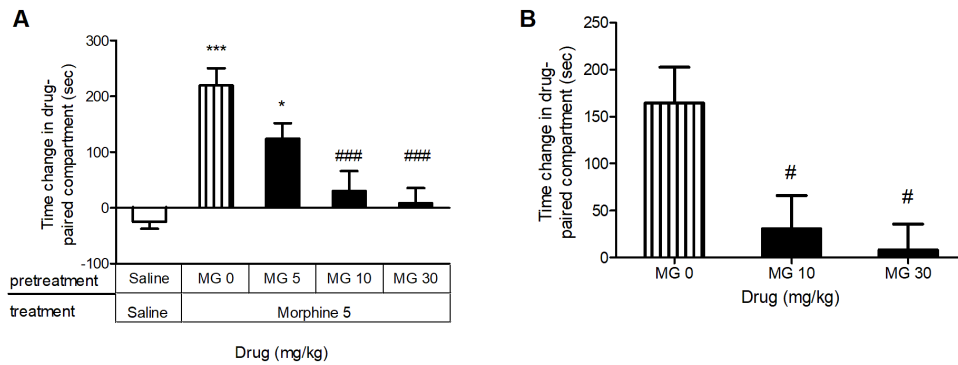
**Figure 6** Effect of mitragynine on morphine-induced conditioned place preference. (A) the effects of mitragynine (MG; 0, 5, 10, and 30 mg/kg) on morphine induced conditioned place preference acquisition. (B) the effects of mitragynine 10 and 30 mg/kg on expression of morphine induced conditioned place preference. Data are expressed as mean time changes in morphine paired compartment between postconditioning and preconditioning phases ± SEM. (*p< 0.05 and ***p<0.001 versus saline-treated group; # p<0.05 and ###p<0.001 versus MG 0 group; n = 6-9)

**Effect of mitragynine on expression of morphine induced conditioned place preference**

Mitragynine was able to attenuate expression of morphine induced conditioned place preference. This attenuation was statistically significant at dose of 10 and 30 mg/kg mitragynine compared with the saline group, $30.38 \pm 35.72$ s (n=8), $8.38 \pm 27.30$ s (n=8) and $164.30 \pm 38.10$ s (n=8), respectively (**Figure 6B**).

**Effect of mitragynine on chronic morphine withdrawal**

**Figure 7A** showed that only mice pretreated with saline and 30 mg/kg mitragynine followed by chronic morphine treatment for 7 days showed significantly higher numbers of jumping in 30 minute time period. The number of jumping in 10 mg/kg mitragynine pretreated group was lower than saline pretreated group, $38.25 \pm 6.86$ times (n=8) and $19.83 \pm 2.09$ time (n=6), respectively, to the same level as chronic treatment of 10 mg/kg mitragynine alone ($28.60 \pm 11.08$ times, n=5). Straub tail reaction was found in all groups with mice treated with morphine. The percent of mice showing Straub tail reaction were 83.33% and 83.33% in mice pretreated with saline and 10 mg/kg mitragynine, respectively (**Figure 7B**). At the dose of 30 mg/kg mitragynine reduced the Straub tail reaction down to 50.00%.
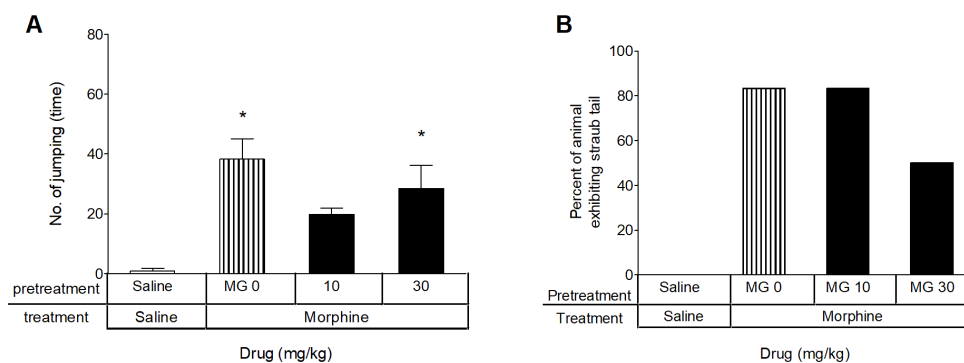
**Figure 7** Effect of mitragynine 0, 10, and 30 mg/kg pretreatment on chronic morphine withdrawal precipitated with naloxone. (A) Mean frequency of repeated jumping behavior in the 30 minute period after naloxone injection ± SEM. (B) Percent of animal exhibited Straub tail behavior. (*p < 0.05 versus saline-treated group; n = 6-8)

**Effect of mitragynine on methamphetamine discrimination**

Mitragynine pretreatment showed tendency to weaken discriminative stimulus effect of 1 mg/kg methamphetamine at 1 and 5 mg/kg (n=6). Mean percent depression on methamphetamine paired lever for saline, 1 mg/kg and 5 mg/kg mitragynine pretreated groups were 79.00±10.80, 72.72±10.50, and 66.19±19.57 percent, respectively (**Figure 8**).

Y-axis: Percent depression on methamphetmine-paired lever

X-axis: Mitragynine (mg/kg)
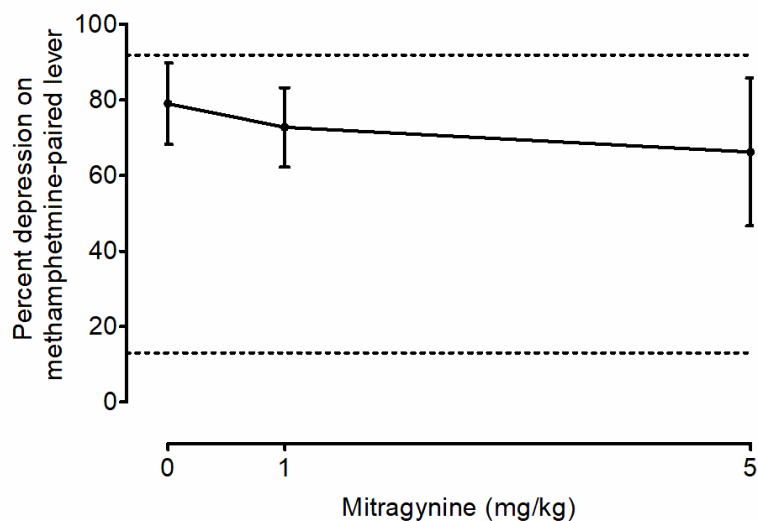
**Figure 8** Effects of mitragynine pretreatment at doses of 0, 1, and 5 mg/kg in rats trained to discriminate methamphetamine from saline, shown as percent depression on methamphetamine paired lever.

## Effects of mitragynine on acquisition of methamphetamine-induced conditioned place preference

Changes in time spent in drug paired compartment in 1 and 2 mg/kg methamphetamine treated groups were, $161.0 \pm 23.08$ s (n=9) and, $189.2 \pm 27.00$ s (n=9), respectively, compared with saline treated group, $8.8 \pm 16.80$ s (n=10) (**Figure 9**). The time changes increased in dose dependent manner in mitragynine treated groups where they were statistically significant from saline-treated group.

Injection of 30 mg/kg mitragynine prior to methamphetamine could suppress acquisition of methamphetamine -induced conditioned place preference. Changes in time spent in methamphetamine paired compartment in saline/5 mg/kg methamphetamine, and 30 mg/kg mitragynine/ 5 mg/kg methamphetamine treated groups were significantly different, $261.40 \pm 24.91$ s (n=8) and $-83.50 \pm 24.05$ s (n=6), respectively (**Figure 10**).
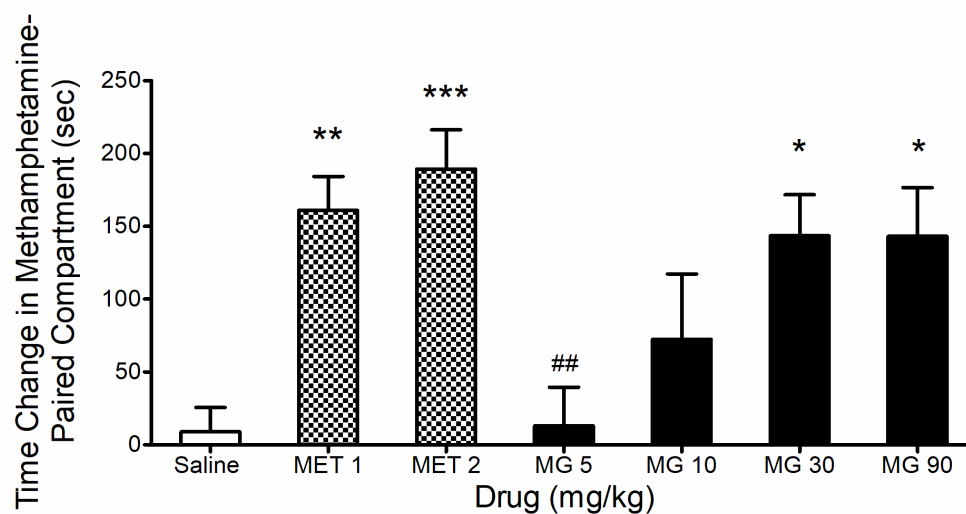
**Figure 9** Conditioned place preference of mitragynine and methamphetamine. The effects of mitragynine (MG) and methamphetamine (MET) on conditioned place preference in rats. Data are expressed as mean ± SEM of time changes in drug paired compartment between postconditioning and preconditioning phases. (*$p < 0.05$, **$p<0.01$ and ***$p<0.001$ versus saline-treated group; ## $p<0.01$ versus 2 mg/kg methamphetamine-treated group, n = 7-12)
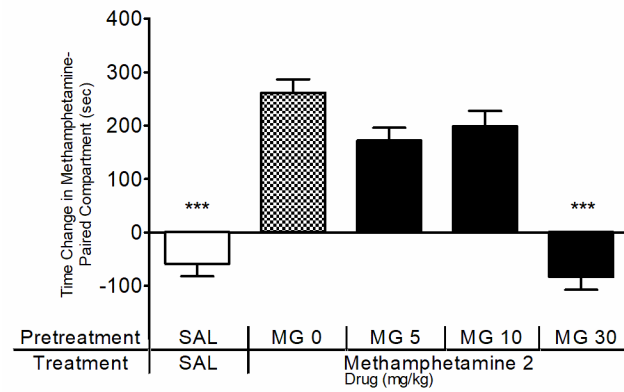
**Figure 10** Effect of mitragynine on methamphetamine conditioned place preference acquisition. The effects of mitragynine (MG; 0-30 mg/kg) on methamphetamine conditioned place preference acquisition in rats. Data are expressed as mean ± SEM of time changes in methamphetamine paired compartment between postconditioning and preconditioning phases. (***p<0.001 versus MG 0/METH 5 group, n = 6-10)

## Chapter 5

## Discussion and Conclusions

Abuse liability of mitragynine had been access by drug discrimination, conditioned place preference and physical withdrawal in the present study. In drug discrimination model, 10 mg/kg mitragynine was able to substitute methamphetamine in rats trained to discriminate 1 mg/kg methamphetamine from saline despite different mechanisms of action. This was confirmed by administration of morphine at 1 and 2 mg/kg. It showed that morphine could also fully substitute methamphetamine. A study by Harun et al. (2015) demonstrated that cocaine could only partially substitute mitragynine in rats trained to discriminate 15 mg/kg mitragynine from vehicle. This showed that mitragynine and stimulants share at least some similar interoceptive stimulus properties. In the same study, morphine fully substituted mitragynine at 5 mg/kg where mitragynine was able to fully substitute 5 mg/kg morphine at dose of 15 mg/kg. Dose greater than 15 mg/kg of mitragynine could partially substitute morphine (Harun et al., 2015).

In conditioned place preference model, the present study demonstrated that only relatively high doses of mitragynine (30 and 90 mg/kg) could produce conditioned place preference (**Figure 4**). Although 10 mg/kg mitragynine also showed the tendency to induce conditioned place preference, no significantly difference from control was found. Other studies suggested lower doses of 10 and 15 mg/kg were also able to produce conditioned place preference as well (Yusoff et al., 2016; Yusoff et al., 2018). We speculated that there was some influence from intraspecies difference (Wistar rats versus Sprague-Dawley rats). One study indicated that the dose of morphine used for induction of conditioned place reference in Wistar rats was significantly higher than the one used to induced Sprague-Dawley rats (Shoaib et al., 1995).

In withdrawal models, naloxone successfully precipitated repeated jumping behavior in mice given both acute and chronic morphine. The numbers of jumping in 30 minute time for mitragynine treated groups were significant only at relatively high

doses (30 and 60 mg/kg in acute treatments and 30 mg/kg in chronic treatments). Plus, the numbers of jumping reaction was dose dependent in mitragynine treated mice in both acute and chronic treatments. Activation of opioid receptors by morphine caused many brain adaptations, calcium flux was one of the phenomena found to be altered by administration of morphine (Bhargava, 1978; Seth et al., 2011). Many calcium channels blockers such as verapamil, diltiazem, and nifedipine were successfully able to attenuate opioid withdrawal symptoms (Vitcheva and Mitcheva, 2004; Seth et al., 2011). Morphine dependence was independent of beta arrestin pathway as beta arrestin knockout mice still developed morphine physical withdrawal and showing jumping reaction following naloxone precipitation comparable to those observed in wild type mice (Bohn et al., 2000). Intracellular signaling regarding opioid receptors activation by mitragynine biased toward G proteins over beta arrestin (Kruegel et al., 2016). Taken from these studies, it was not surprising that application of naloxone would precipitate jumping behavior in mice received mitragynine in this study.

Other behavior observed in this experiment was Straub tail reaction. Straub tail reaction is defined by rigidity and erection of tail across the back of the animal in an S-shaped curve due to contraction of acrococcygeus muscle (Bilbey et al., 1960). The mechanisms of this reaction were mediated by activation of μ opioid subtype 2 receptors (MOR2) and serotonin subtype 2 receptor ($5\text{-HT}_2$) (Nath et al., 1994; Zarrindast et al., 2001). Straub tail reactions were observed in mice given morphine and mitragynine in both acute and chronic treatment after injection of naloxone. Considering with receptor binding assay, it is suggested that mitragynine physical dependence might be due to activation of MOR2 and/or $5\text{-HT}_2$ receptor.

This study is the first to demonstrate that mitragynine attenuated both acquisition and expression of morphine induced conditioned place preference (**Figure 6**). Mitragynine was a partial agonist on MOR and competitive antagonist at KOR (Kruegel et al., 2016). The affinity of mitragynine at DOR was low (Stolt et al., 2013; Kruegel et al., 2016). In addition, Guanosine 5′-(gamma-thio)triphosphate (^{35}S-GTPγS) binding assay showed that mitragynine had less specific effect to MOR compared with [D-Ala[2], N-Me-Phe[4], Gly-ol[5]]-enkephalin (DAMGO) or morphine (Stolt et al., 2013). In functional activity at human opioid receptors in G protein bioluminescent resonance energy transfer (BRET) assay showed that maximum

efficacy of mitragynine at human MOR was 34% compared with DAMGO. Mitragynine could fully inhibit the effect of U-50,488, a KOR agonist, with $pA_2$ of 1.4 (Kruegel et al., 2016). The fact that mitragynine served as antagonist at KOR may have additional effects and become a candidate for use as treatment for opioid addiction (Carroll and Carlezon, 2013; Helal et al., 2017). Antidepressant effect of kratom had been reported in traditional use and in some studies (Cinosi et al., 2015; Halpenny, 2017). In mice model of depression, mitragynine reduced immobility time in forced swimming test and tail suspension test. Moreover, it was shown that mitragynine reduced corticosterone level in mice exposed to forced swimming- and tail suspension tests (Idayu et al., 2011). Anti-addictive property of KOR antagonists had been tested. KOR antagonists blocked stress-induced potentiation of drug reward, stress-induced reinstatement of drug seeking behavior, and escalation of drug consumption in long access models (Whitfield et al., 2015).

Physical dependence observed by jumping and Straub tail reactions show that, when given with morphine, 10 mg/kg mitragynine could reduce jumping behavior to the same level as chronic treatment of 10 mg/kg mitragynine alone (**Figure 5C** and **7A**) and 30 mg/kg mitragynine could reduce Straub tail reaction (**Figure 5D** and **7B**). Reduction of withdrawal symptoms might due to the fact that mitragynine blocked neuronal calcium channels that participated in opioid withdrawal (Matsumoto et al., 2005b). Kratom side effects and withdrawal in human were reviewed by Singh et al. (2016). In brief, individuals faced both physical and psychological symptoms. Physical symptoms included irritability, muscle pain, cramps and diarrhea where psychological symptoms included restlessness, tension, aggression, sadness, delusion, hallucination, and craving. People who consume high amount of kratom were more likely to develop severe dependence and withdrawal symptoms. However, those symptoms usually lasted only for 1-3 days (Singh et al., 2014). Mitragynine withdrawal was similar to opioid withdrawal symptoms and it could be reversed by administration of opioid agonist (Mackay and Abrahams, 2018).

Moreover, mitragynine reduced the effect of methamphetamine in drug discrimination test. Boyer et al. (2008) performed *in vitro* radioligand binding assay and found inhibition on radioligand binding effects by mitragynine at $\alpha_2$ adrenergic, dopamine $D_2$, serotonin $5HT_{2C}$ and serotonin $5HT_7$ receptors. One study showed that

methanolic extract of mitragynine reduced both positive and negative symptoms of psychosis in mice via inhibition of dopaminergic ($D_2$) and serotonergic (5-$HT_{2A}$/5-$HT_{2C}$) receptors. (Vijeepallam et al., 2016). Here, we speculate involvement of dopaminergic and serotonergic pathways in mitragynine effects on reduction of methamphetamine discrimination.

Furthermore, acquisition of methamphetamine induced conditioned place preference was attenuated in rats pretreated with 30 mg/kg mitragynine. D'Adamo et al. (2012) tested the effect of dopamine receptor antagonists on 3,4-methylenedioxy-metamphetamine (MDMA), an amphetamine analogue, conditioned place preference acquisition and expression. They reported that dopamine $D_2$ receptor antagonists, haloperidol and raclopride, at high doses attenuated acquisition of MDMA induced conditioned place preference. Hoffman and Donovan (1995) reported the effect of haloperidol and raclopride, along with other dopamine receptors antagonists, in attenuation of acquisition of amphetamine conditioned place preference. Together, we hypothesized that mitragynine effects on methamphetamine conditioned place preference was mediated at least by blockade of dopamine $D_2$ receptor antagonism.

From our results and other studies, it showed that mitragynine served as MOR partial agonist and KOR competitive antagonist. This is similar to buprenorphine. Buprenorphine is a partial agonist at MOR and competitive antagonist at KOR (Virk et al., 2009). Upon activation of MOR, G-protein pathway mediates analgesic effect of opioid where beta arrestin pathway mediates side effects, especially respiratory depression and constipation (Dahan, 2016). Like mitragynine, buprenorphine also showed bias antagonism at MOR for G-protein coupling over beta arrestin (Kruegel et al., 2016). It had low abuse liability and respiratory depression. Thus, buprenorphine has been approved for use in treatment of opioid dependence in replacement therapy or opioid maintenance treatment (Soyka, 2017). All these results support the use of kratom in traditional medicine, informal use or self-medication in the treatment for opioid addiction, withdrawal and for cessation of opioid analgesics (Vicknasingam et al., 2010; Prozialeck et al., 2012). Mitragynine could also reduce the effect of methamphetamine in drug discrimination and conditioned place preference models. However, further studies are still needed to provide more evidences supporting utilization of mitragynine as pharmacotherapy for methamphetamine addiction.

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# APPENDIX

## Tables of Experimental Results

**Table 1** Result for experiment 1: Drug discrimination of drugs on rats trained to discriminate 1 mg/kg methamphetamine.

| Treatment (mg/kg) | Mean percent depression on methamphetamine-paired lever ± SEM | Number of animal |
|---|---|---|
| Saline | 12.99 ± 4.759 | 6 |
| Methamphetamine 0.1 | 24.13 ± 12.08 | 6 |
| Methamphetamine 0.3 | 52.78 ± 12.43 | 6 |
| Methamphetamine 0.6 | 82.54 ± 10.43 | 6 |
| Methamphetamine 1.0 | 91.95 ± 4.971 | 6 |
| mitragynine 1 | 19.86 ± 9.858 | 6 |
| mitragynine 5 | 35.57 ± 12.83 | 6 |
| mitragynine 10 | 80.88 ± 10.37 | 6 |
| morphine 1 | 4.639 ± 1.814 | 6 |
| morphine 2 | 86.09 ± 6.592 | 4 |

**Table 2** Result for experiment 2: Conditioned place preference of mitragynine

| Treatment (mg/kg) | Mean time change in drug-paired compartment (sec) | Number of animal |
|---|---|---|
| Saline | 8.800 ± 16.8 | 10 |
| Morphine 5 | 207.4 ± 29.02 | 7 |
| Mitragynine 5 | 12.88 ± 26.52 | 8 |
| Mitragynine 10 | 72.29 ± 45.01 | 7 |
| Mitragynine 30 | 143.4 ± 28.29 | 8 |
| Mitragynine 90 | 143.0 ± 33.69 | 12 |
| Methamphetamine 1 | 161.0 ± 23.08 | 9 |
| Methamphetamine 2 | 165.8 ± 15.13 | 8 |

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**Table 3** Result for experiment 3: Acute mitragynine withdrawal

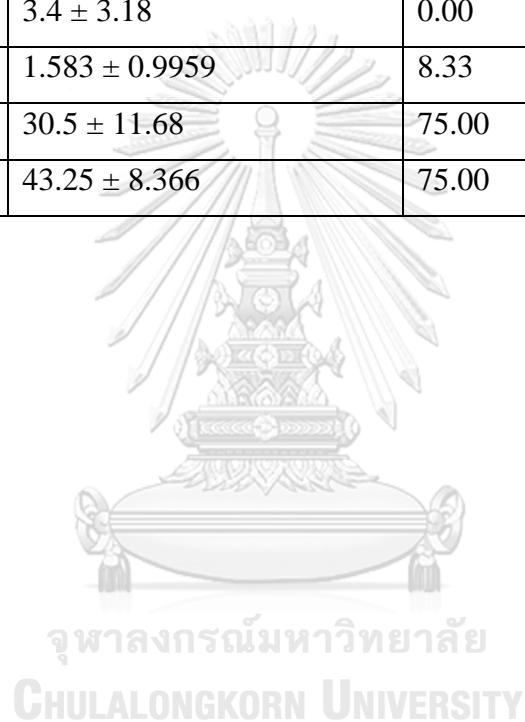| Treatments (mg/kg) | Number of jumping ± SEM | Percent of animal exhibited Straub tail reaction | Number of animal |
|---|---|---|---|
| Saline | 0.875 ± .6391 | 0.00 | 8 |
| Morphine 100 | 49.17 ± 11.08 | 83.33 | 12 |
| Mitragynine 5 | 3.4 ± 3.18 | 0.00 | 10 |
| Mitragynine 10 | 1.583 ± 0.9959 | 8.33 | 12 |
| Mitragynine 30 | 30.5 ± 11.68 | 75.00 | 12 |
| Mitragynine 60 | 43.25 ± 8.366 | 75.00 | 8 |

**Table 4** Result for experiment 4: Chronic mitragynine withdrawal

| Treatments (mg/kg) | Number of jumping ± SEM | Percent of animal exhibited Straub tail reaction | Number of animal |
|---|---|---|---|
| Saline | 0 ± 0 | 0 | 8 |
| Morphine | 38.25 ± 6.858 | 100 | 8 |
| Mitragynine 10 | 28.6 ± 11.08 | 80 | 5 |
| Mitragynine 30 | 42.5 ± 14.68 | 100 | 6 |

**Table 5** Result for experiment 5: Effect of mitragynine on acquisition of morphine conditioned place preference

| Treatment (mg/kg) | Time change in drug-paired compartment (sec) | Number of animal |
|---|---|---|
| Saline | -24.83 ± 13.22 | 6 |
| Mitragynine 0 | 219.6 ± 31.11 | 7 |
| Mitragynine 5 | 124 ± 28.21 | 9 |
| Mitragynine 10 | 30.38 ± 35.72 | 8 |
| Mitragynine 30 | 8.375 ± 27.3 | 8 |

**Table 6** Result for experiment 6: Effect of mitragynine on expression of mitragynine conditioned place preference

| Treatment (mg/kg) | Time change in drug-paired compartment (sec) | Number of animal |
|---|---|---|
| Mitragynine 0 | 164.3 ± 38.1 | 8 |
| Mitragynine 10 | 30.38 ± 35.72 | 8 |
| Mitragynine 30 | 8.375 ± 27.3 | 8 |

**Table 7** Result for experiment 7: Effect of mitragynine on chronic morphine withdrawal

| Treatments (mg/kg) | Number of jumping ± SEM (time) | Percent of animal exhibited Straub tail reaction | Number of animal |
|---|---|---|---|
| Saline | 0.8571 ± 0.8571 | 0 | 7 |
| Mitragynine 0 | 38.25 ± 6.858 | 83.33 | 8 |
| Mitragynine 10 | 19.83 ± 2.088 | 83.33 | 6 |
| Mitragynine 30 | 28.5 ± 7.702 | 50 | 6 |

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**Table 8** Result for experiment 8: Effect of mitragynine on methamphetamine discrimination

| Treatment | Percent depression on methamphetamine-paired lever ± SEM | Number of animals |
|-----------|----------------------------------------------------------|-------------------|
| MG 0 | 78.99675 ± 10.80323 | 6 |
| MG 1 | 72.72266 ± 10.49602 | 6 |
| MG 5 | 66.18671 ± 19.57458 | 6 |

**Table 9** Result for experiment 9: Effect of mitragynine on acquisition of methamphetamine conditioned place preference

| Treatment (mg/kg) | Time change in drug-paired compartment (sec) | Number of animal |
|---|---|---|
| Saline | -58.94 ± 23.29 | 10 |
| MG 0 | 261.4 ± 24.91 | 8 |
| MG 5 | 171.9 ± 24.03 | 10 |
| MG 10 | 198.1 ± 29.26 | 8 |
| MG 30 | -83.5 ± 24.05 | 6 |

**VITA**

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