ANTI-ADDICTIVE ACTIVITY OF QUINUCLIDINE DERIVATIVES IN NICOTINE-INDUCED CONDITIONED PLACE PREFERENCE TEST IN MICE

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A Thesis Submitted in Partial Fulfillment of the Requirements
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ฤทธิ์ต้านการติดยาของอนุพันธ์ควินิวคลิดีนในการทดสอบพฤติกรรมการชอบสถานที่แบบมีเงื่อนไขที่เหนี่ยวนำด้วยนิโคตินในหนูเมาส์

น.ส.สรัญดา เนียนพานิช

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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สรัญดา เนียนพานิช : ฤทธิ์ต้านการติดยาของอนุพันธ์ควินิวคลิดีนในการทดสอบพฤติกรรมการชอบสถานที่แบบมีเงื่อนไขที่เหนี่ยวนำด้วยนิโคตินในหนูเมาส์. (ANTI-ADDICTIVE ACTIVITY OF QUINUCLIDINE DERIVATIVES IN NICOTINE-INDUCED CONDITIONED PLACE PREFERENCE TEST IN MICE) อ.ที่ปรึกษาหลัก : ผศ. ภญ. ดร.รัชนี รอดศิริ, อ.ที่ปรึกษาร่วม : อ. ภก. ดร.พัฒนชัย ลิมปิกิรติ

อนุพันธ์ควินิวคลิดีน *(s)*-T1 *(s)*-T2 และ *(s)*-T6 เป็นลิแกนด์ของตัวรับนิโคตินิกชนิดแอลฟ่า3เบต้า4 ตัวรับนิโคตินิกชนิดแอลฟ่า3เบต้า4 พบการแสดงออกมากในมีเดียลฮาบินูลา มีเดียลฮาบินูลาเชื่อมต่อกับวิถีการให้รางวัลในสมองและมีบทบาทในผลการให้รางวัลที่เหนี่ยวนำโดยนิโคติน สารที่เป็นตัวต้านต่อตัวรับนิโคตินิกชนิดแอลฟ่า3เบต้า4 หลายชนิดได้ถูกนำมาศึกษาฤทธิ์ต้านการเสพติดในแบบจำลองสัตว์ทดลองของการเสพติดนิโคติน การศึกษานี้มีวัตถุประสงค์เพื่อประเมินฤทธิ์และกลไกการต้านการเสพติดของอนุพันธ์ควินิวคลิดีน *(s)*-T1 *(s)*-T2 และ *(s)*-T6 ในการทดสอบพฤติกรรมการชอบสถานที่แบบมีเงื่อนไขที่เหนี่ยวนำด้วยนิโคตินในหนูเมาส์ ในการทดลองที่ 1 หนูเมาส์ได้รับนิโคติน (0.5 มก./กก.), *(s)*-T1 (1, 3 หรือ 10 มก./กก.), *(s)*-T2 (1, 3 หรือ 10 มก./กก.) หรือ *(s)*-T6 (1, 3 หรือ 10 มก./กก.) ทางใต้ผิวหนังและทดสอบการเคลื่อนที่และพฤติกรรมการชอบสถานที่แบบมีเงื่อนไข นิโคตินและ *(s)*-T6 10 มก./กก. ลดเวลาการเคลื่อนที่ ($P < 0.05$) ในการทดสอบพฤติกรรมการชอบสถานที่แบบมีเงื่อนไข นิโคตินเพิ่มคะแนน CPP อย่างมีนัยสำคัญเมื่อเปรียบเทียบกับกลุ่มควบคุม ($P < 0.05$) ในขณะที่ *(s)*-T1 *(s)*-T2 และ *(s)*-T6 ลดคะแนน CPP อย่างมีนัยสำคัญเมื่อเปรียบเทียบกับกลุ่มนิโคติน ($P < 0.05$) ในการทดลองที่ 2 หนูได้รับวาเรนนิคลีน (1 มก./กก.), *(s)*-T1 (1, 3 หรือ 10 มก./กก.), *(s)*-T2 (1, 3 หรือ 10 มก./กก.) หรือ *(s)*-T6 (1 หรือ 3 มก./กก.) ทางใต้ผิวหนัง 30 นาทีก่อนได้รับนิโคติน วาเรนนิคลีน, *(s)*-T1 (1, 3, และ 10 มก./กก.), *(s)*-T2 (1 และ 3 มก./กก.) และ *(s)*-T6 (1 และ 3 มก./กก.) ลดคะแนน CPP อย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มที่ได้รับนิโคตินอย่างเดียว ($P < 0.05$, $P < 0.001$, $P < 0.05$, $P < 0.05$, $P < 0.01$, $P < 0.05$, $P < 0.05$, $P < 0.05$ ตามลำดับ) *(s)*-T1 *(s)*-T2 และ *(s)*-T6 ทุกขนาดเมื่อให้ร่วมกับนิโคตินไม่มีผลต่อเวลาการเคลื่อนที่ ในการทดลองที่ 3 หนูได้รับยาเหมือนการทดลองที่ 2 ที่ 40 นาทีหลังจากได้รับนิโคตินเก็บสมองส่วนนิวเคลียสแอคคัมเบน สเตรียตรัม ฮิปโปแคมปัสและพรีฟรอนทอลคอร์เท็ก วัดระดับโดปามีนและ DOPAC ด้วยเครื่อง LC/MS/MS *(s)*-T1 (1, 3, และ 10 มก./กก.), *(s)*-T2 (3 และ 10 มก./กก.) และ *(s)*-T6 (1 และ 3 มก./กก.) ยับยั้งการเพิ่มขึ้นของโดปามีนที่เหนี่ยวนำด้วยนิโคตินในสเตรียตรัม นอกจากนี้ *(s)*-T1 (3 มก./กก.) และ *(s)*-T6 (1 และ 3 มก./กก.) ยับยั้งการเพิ่มขึ้นของโดปามีนที่เหนี่ยวนำด้วยนิโคตินในพรีฟรอนทอลคอร์เท็ก โดยสรุป *(s)*-T1 *(s)*-T2 และ *(s)*-T6 สามารถป้องกันการชอบสถานที่แบบมีเงื่อนไขที่เหนี่ยวนำด้วยนิโคติน โดยกลไกเกี่ยวข้องกับการป้องกันการเพิ่มขึ้นของนิโคตินในสมองที่เกี่ยวข้องกับการเรียนรู้ความสัมพันธ์ของสิ่งกระตุ้นกับผลการให้รางวัลของนิโคติน

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Saranda Nianpanich : ANTI-ADDICTIVE ACTIVITY OF QUINUCLIDINE DERIVATIVES IN NICOTINE-INDUCED CONDITIONED PLACE PREFERENCE TEST IN MICE. Advisor: Asst. Prof. RATCHANEE RODSIRI, Ph.D. Co-advisor: PATANACHAI LIMPIKIRATI, Ph.D.

Quinuclidine derivatives *(s)*-T1, *(s)*-T2, and *(s)*-T6 are the ligands of $\alpha_3\beta_4$ nicotinic acetylcholine receptor (nAChR). $\alpha_3\beta_4$ nAChRs are highly expressed in medial habenula (MHb). The MHb connects to the brain rewarding pathway and mediates nicotine induce rewarding effect. Various $\alpha_3\beta_4$ nicotinic acetylcholine antagonists have been investigated for their anti-addictive effect in animal models of nicotine addiction. The present study was aimed to evaluate the anti-addictive effects and mechanisms of quinuclidine derivatives, *(s)*-T1, *(s)*-T2, and *(s)*-T6 in nicotine-induced conditioned place preference (CPP) in mice. In the first experiment, mice received either nicotine (0.5 mg/kg, s.c.), *(s)*-T1 (1, 3, or 10 mg/kg, s.c.), *(s)*-T2 (1, 3, or 10 mg/kg, s.c.), or *(s)*-T6 (1, 3 or 10 mg/kg, s.c.) and performed locomotor activity and CPP. Nicotine and *(s)*-T6 (10 mg/kg) reduced locomotion time ($P < 0.05$). In CPP model, nicotine significantly increased CPP score compared to control ($P < 0.05$), while *(s)*-T1, *(s)*-T2, and *(s)*-T6 significantly decreased CPP scores compared nicotine-treated mice ($P < 0.05$). In the second experiment, mice received either varenicline (1 mg/kg, s.c.), *(s)*-T1 (1, 3, or 10 mg/kg, s.c.), *(s)*-T2 (1, 3, or 10 mg/kg, s.c.), or *(s)*-T6 (1 or 3 mg/kg, s.c.) 30 min prior to nicotine administration. CPP and locomotor activity were performed. Varenicline, *(s)*-T1 (1, 3, and 10 mg/kg), *(s)*-T2 (1 and 3 mg/kg) and *(s)*-T6 (1 and 3 mg/kg) significantly decreased CPP scores compared to nicotine treatment alone ($P < 0.05$, $P < 0.001$, $P < 0.05$, $P < 0.05$, $P < 0.01$, $P < 0.05$, $P < 0.05$, $P < 0.05$, respectively). All doses of *(s)*-T1, *(s)*-T2, and *(s)*-T6 plus nicotine treatment had no effect on locomotion time. In the third experiment, mice received the same treatment as in the second experiment, nucleus accumbens (NAc), striatum, hippocampus and prefrontal cortex (PFC) were collected 40 min after nicotine administration. Dopamine and DOPAC levels were determined using LC/MS/MS. *(s)*-T1 (1, 3 and 10 mg/kg), *(s)*-T2 (3 and 10 mg/kg) and *(s)*-T6 (1 and 3 mg/kg) block nicotine-induced dopamine elevation in the striatum. In addition, *(s)*-T1 (3 mg/kg) and *(s)*-T6 (1 and 3 mg/kg inhibited nicotine-induced dopamine elevation in PFC. In conclusion, *(s)*-T1, *(s)*-T2, and *(s)*-T6 can prevented nicotine-induced CPP. The mechanism of action involved the prevention of nicotine induced dopamine elevation in the brain areas involving cue-associate learning of rewarding effect of nicotine.

Field of Study:	Pharmacology and Toxicology	Student's Signature .....
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		Co-advisor's Signature .....

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Lastly, I would give the credit of this success to my parents, colleagues and supportive friends who are beside me when I was dishearten. They provided the positive energy and unconditionally love that make me pass though.

Saranda Nianpanich

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ABBREVIATION

μL = microliter(s)

/ = per

% = percentage

± = plus-minus sign

α = alpha

β = beta

ANS = autonomic nervous system

ANOVA = analysis of variance

BBB = blood brain barrier

B.W = body weight

CBT = cognitive-behavioral therapy

CMC = carboxy methyl cellulose

CNS = central nervous system

COPD = chronic obstructive pulmonary disease

CPP = Conditioned place preference

CYP450 = cytochrome P450

CV = coefficient variance

DA = dopamine

DOPAC = 3,4-dihydroxyphenylacetic acid

Fig = figure

g = gram(s)

h = hour

hERG = human Ether-a-go-go Related Gene

HIP = hippocampus

HVA = homovanillic acid

IC_{50} = half maximum inhibitory concentration

IS = internal standard

kg = kilogram(s)

mg = milligram(s)

min = minute(s)

mL = mililiter(s)

n = number

NAc = nucleus accumbens

nAChR = nicotinic acetylcholine receptor

NRT = nicotine replacement therapy

ng = nanogram(s)

NSS = normal saline solution

PFC = prefrontal cortex

RE = recovery error

LC-MS = liquid chromatography and mass spectrometry

SC = subcutaneous

S.E.M = standard error of mean

STR = striatum

CHAPTER 1

INTRODUCTION

1.1 Background and Rationale

Smoking is a major health problem worldwide (1). Smoking leads to cancers, chronic respiratory infection, and cardiovascular diseases. Nicotine is the main component in tobacco which is the cause of addiction. Several drugs have been approved for the treatment of nicotine addiction including nicotine replacement therapy, bupropion SR, and varenicline (2).

In the brain reward pathway, nicotine activates nicotinic acetylcholine receptors (nAChR) located on cell bodies of dopaminergic neurons in the ventral tegmental area (VTA) causing dopamine release to the nucleus accumbens (NAc) and leading to rewarding effect of nicotine (3). nAChR is a pentameric ligand-gate ion channel, consisting of five subunits (4). Several subtypes of nAChR subunits are identified including alpha, beta, gamma and delta subunits (4). The composition of nAChR varies in each brain area and is responsible for various types of behaviors (4). $\alpha 4\beta 2^*$ nAChRs, which is the target of varenicline, are abundant in the VTA (4). $\alpha 3\beta 4^*$ nAChR, one of the drug targets for the treatment of nicotine addiction, are highly expressed in the medial habenula (MHb) and interpeduncular nucleus (IPN) (4) Recently, the anti-addictive effect of several $\alpha 3\beta 4$ nAChR antagonists have been widely investigated. AT1001, a non-competitive $\alpha 3\beta 4$ nAChR antagonist, inhibited nicotine self-administered in rats (5). Conotoxin TxID, the $\alpha 3\beta 4$ nAChR antagonist, inhibited nicotine-induced CPP in mice (6). The present study aimed to investigate

the anti-addictive effects of quinuclidine derivatives, which are $\alpha 3\beta 4$*nAChR ligands, against nicotine addiction using conditioned place preference mouse model.

Quinuclidine derivatives used in this study are quinuclidine anti-1,2,3-triazoles. These compounds are designed and developed based on the potent ligands of $\alpha 7$ nAChR QDN8 (7). Previous study showed that (R)-enantiomer of quinuclidine derivatives bound to $\alpha 7$ nAChRs, whereas (S)-enantiomer of quinuclidine derivatives selectively bound to $\alpha 3\beta 4$ nAChRs (7). In this study, *(s)*-T1, *(s)*-T2 and *(s)*-T6 are selected because they have high affinity and selectively to $\alpha 3\beta 4$ nAChRs (7). In vitro functional study revealed that *(s)*-T1 and *(r)*-T2 served as $\alpha 3\beta 4$ nAChR agonists, whereas *(r)*-T1 and *(s)*-T2 were $\alpha 3\beta 4$ nAChR antagonist (7). Previous study showed that *(s)*-[18F]T1 was uptaken into the mouse brains (8). In vitro cardiotoxicity also reported IC50 of *(s)*-T6 to human ether-a-go-go-related gene (hERG) potassium channels was 120 nM (9).

Conditioned place preference (CPP) has been widely used to investigate rewarding and aversive effects of a drug in animals. Generally, CPP apparatus composes of two compartments with different wall and floor color or pattern to generate different environmental cues. Principally, animals are trained to pair between a given drug and an environmental cue in one compartment of CPP apparatus. After training, animals are allowed to freely select between two compartments in the CPP apparatus. If a given drug has rewarding property, animal would prefer the paired environmental cue. On the other hand, if a given drug has

adverse effect to the animals, animals are likely to avoid the paired compartment (10).

Dopamine plays a key role in the brain reward pathway (11). In this study, dopamine levels in the nucleus accumbens, striatum, hippocampus, and prefrontal cortex were measured to elucidate the anti-addictive mechanism of quinuclidine derivatives against nicotine. Liquid chromatography and mass spectrometry (LC/MS) will be used to detect dopamine and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in mouse brain tissue.

1.2 Objectives

1. To evaluate anti-addictive activity of quinuclidine derivatives; *(s)*-T1, *(s)*-T2 or *(s)*-T6, in nicotine-induced conditioned place preference in mice

2. To determine the effect of quinuclidine derivatives; *(s)*-T1, *(s)*-T2 or *(s)*-T6 on dopamine and its metabolite levels in mouse brains

1.3 Hypothesis

Quinuclidine derivatives; *(s)*-T1, *(s)*-T2 or *(s)*-T6 protect against nicotine-induced conditioned place preference by preventing the increased dopamine levels caused by nicotine.

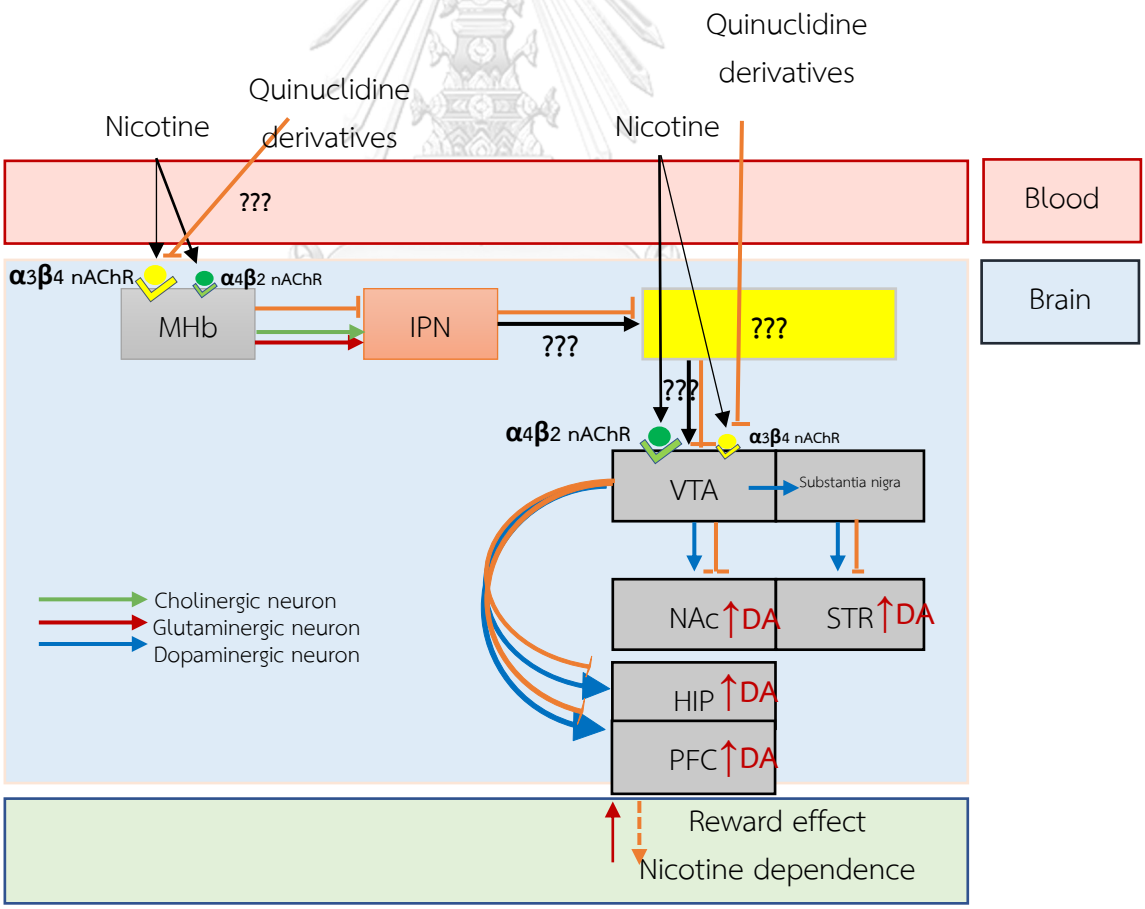
1.4 Research design

Experimental design

1.5 Benefits of the study

The results from this study will support the development of quinuclidine derivatives as a potential treatment in nicotine dependence.

1.6 Conceptual framework



CHAPTER 2

LITERATURE REVIEW

2.1 Smoking and smoking cessation

According to WHO report 2018, smoking was a major health problem and a risk factor of death in smokers and secondhand smokers (1). In 2018, approximately 10.7 million smokers were reported in Thailand (1). Although, the number of smokers tends to decline from 2008 to 2018, 81,521 death were related to smoking (1). Smoking causes atherosclerosis and thrombosis leading to various cardiovascular diseases. Other diseases relating to deaths from tobacco used include cancers and chronic respiratory infection (1, 12-14).

Cigarette contains over 4,000 chemicals such as nicotine, tar, ammonia, and aromatic hydrocarbon (15). Nicotine is the main compound in cigarettes which is a cause of smoking addiction. Nicotine affects the autonomic nervous system (ANS) causing tachycardia, high blood pressure and muscle relaxation (16). Nicotine also modulates the central nervous system (CNS) leading to relaxation, pleasure, anorexia, anxiety and memory enhancement (17, 18). Since nicotine activates the brain reward pathway leading to pleasure sensations, smokers are craving when they stop smoking. Nicotine addicts repeatedly smoke to relieve craving and prevent withdrawal symptoms. These are the signs of substance use disorder according to DSM-V criteria (19).

About 70 percent of smokers received the advice for smoking cessation from healthcare providers (12). The successful smoking cessation strategy is the combination of therapeutic education, behavioral support, and medication therapy.

Patients are educated about diseases caused by smoking, benefits of quitting smoke, and the smoking cessation treatment. The 5As steps; ask, advise, assess, assist, and arrange, are applied to all cases. Then, the severity of addiction, environment causes of addiction, and the motivation of tobacco quitting are identified followed by 5Rs strategy; relevance, risks, rewards, roadblocks, and repetition (2). Behavior therapy is recommended for smokers with psychological factors from personal, social, and family causes because these factors are the important barriers for quitting smoking (20). The cognitive-behavioral therapy (CBT) is also recommended to provide the skill training, stress management, and intervening to increase social support (21). Medication therapy is used in patients who have history of severe tobacco addiction and have motivation to stop smoking (2).

The United State Food and Drug Administration (U.S. FDA) has approved seven medications for smoking cessation including various formulations of nicotine replacement therapy (NRT); gum, inhaler, lozenges, patch, and nasal spray, bupropion sustained release (SR), and varenicline (21). Off-label medications such as nortriptyline, clonidine, and cytisine have also been used either in combination with the first-line drug or in patients who do not response to the first-line therapy (21, 22).

Nicotine replacement therapy (NRT) is indicated for tobacco consumers with motivated and not motivated to stop. Nicotine in these formulations will displace nicotine from tobacco, thus smokers can reduce the quantity of smoking. Nicotine in NRTs can also stimulate nicotinic receptor in the brain reward pathway and then reduce craving and withdrawal symptoms (21). The combination of NRTs is recommend in smokers who have high consumption of cigarettes per day (21). The

initial dose of NRTs depends on the number of cigarettes using per day and time to first cigarette of smoke (Table 1).

Table 1 The recommend dose of nicotine replacement therapy (21).

Time to First cigarette in the morning	Number of cigarettes per day		
	10-19	20-30	> 30
< 5 mins	Patch (0.9 mg/h) +/- oral NRT	Patch (0.9 mg/h) +/- oral NRT	2 Patch (0.9 mg/h) +/- oral NRT
< 30 mins	Patch (0.9 mg/h)	Patch (0.9 mg/h) +/- oral NRT	Patch (0.9 mg/h) +/- oral NRT
< 60 after waking	Oral NRT	Patch (0.9 mg/h)	Patch (0.9 mg/h) +/- oral NRT
> 60 mins after waking		Oral NRT	Oral NRT

Bupropion SR is recommended to use in smokers who have motivation to quit smoking. Bupropion is an alternative choice for smokers who fail to NRTs and smokers who prefer non-nicotine medication. Bupropion is an anti-depressant drug acting by blocking the dopamine and noradrenaline reuptake transporters resulting in the reduction of craving and withdrawal symptoms (2). Bupropion SR is available in 150 mg sustained release tablets. Patients should take 150 mg once daily for 3 days, then twice daily until the end of treatment course which is 7 to 9 or 12 weeks. Then, bupropion SR is recommended for 6 months after quitting for long-tern treatment. Patients should take bupropion SR for 1-2 weeks before stop smoking (21).

Varenicline is the newest medication for smoking cessation therapy. Varenicline is an $\alpha_4\beta_2$ and $\alpha_3\beta_4$ nicotinic receptor partial agonist. As a partial agonist, it can displace nicotine from nicotinic receptor but produce lesser effect on the brain rewarding pathway than does nicotine. Thus, varenicline effectively reduces craving and withdrawal symptoms and can also delay smoking relapse (2, 20). Varenicline is available in 0.5 and 1 mg tablets. The initial dose of varenicline is 0.5 mg/day for days 1-3, then 0.5 mg twice a day for days 4-7 and 1 mg twice a day in days 8-14. Patients should start varenicline 1 week before stop smoking. The continuous dose is 1 mg twice daily for 3 weeks to 6 months (21).

European Network for Smoking and Tobacco Prevention aisbl (ENSP) guideline 2016 (21) and the Centers for Disease Control and Prevention (CDC) guideline 2020 (22) have recommended seven medications approved by U.S. FDA for the first-line therapy. The choice of smoking cessation pharmacotherapy is based on patient preference, disease conditions, and contraindications (20, 21). Monotherapy is preferred for most cases; however, combination therapy can be used to reduce side effects and used in patients with monotherapy failure (2). According to Handbook for the treatment of tobacco addiction in patients with chronic diseases in Thailand, four medications including nicotine gum, patch, bupropion, and varenicline have been recommended (23).

A meta-analysis of 83 randomized trials assessing the rate abstinence at six months after medications treatment showed that varenicline produced the greatest abstinence rate of 33.2%, whereas the abstinence rate of NRTs and bupropion was about 19 to 26.5% (21). The combination therapy of long-term patch and nicotine

gum or spray showed the highest abstinence rate of 36.5% with higher side effects presented (Table 2) (21).

The side effects of NRTs are headache, dizziness, nausea and irritate at the sites of absorption. Moreover, the compliance of NRT is reduced during long-term therapy, so their efficacy decreases. The side effects of bupropion include skin reaction, insomnia, headache, and dizziness. Bupropion is contraindicated in patients with epilepsy, history of alcoholism, and in combination with CYP2B6 inducers and inhibitors. The most common side effects of varenicline are nausea, insomnia, and headache. Varenicline is recommend in patients with comorbidity diseases including renal failure, cardiovascular diseases, human immunodeficiency virus (HIV) infection, psychiatric disorders, chronic obstructive pulmonary disease (COPD), and heart disease (21, 22).

Table 2 Efficacy of smoking cessation therapy (21).

Pharmacotherapy	Estimated OR of abstinence (95% CI)	Estimated abstinent rate (95% CI)
Placebo	1.0	13.8
Monotherapy		
Nicotine patch	1.9 (1.7-2.2)	23.4 (21.3-25.8)
High dose nicotine patch	2.3 (1.7-3.0)	26.5 (21.4-32.5)
Nicotine inhaler	2.1 (1.5-2.9)	24.8 (19.1-31.6)
Nicotine gum	1.5 (1.2-1.7)	19.0 (16.5-21.9)
Bupropion	2.0 (1.8-2.2)	24.2 (22.2-26.4)
Varenicline	3.1 (2.5-3.8)	33.2 (28.9-25.1)

Table 2 (Continued)

Pharmacotherapy	Estimated OR of abstinence (95% CI)	Estimated abstinent rate (95% CI)
Combination therapy		
Patch + Inhaler	2.2 (1.3-2.6)	25.8 (17.3-36.5)
Patch + gum	2.6 (2.5-5.2)	26.5 (28.6-45.3)
Patch + Bupropion	2.5 (1.9-3.4)	28.9 (23.5-25.1)
Patch >14 weeks + NRT (gum/spray)	3.6 (2.5-5.2)	36.5 (28.6-45.3)

As varenicline showed the highest efficacy for reducing craving and withdrawal symptoms from nicotine addiction, nicotinic acetylcholine receptor (nAChR) becomes a main target for nicotine dependence treatment. Recently, new compounds interacting with nicotinic receptor, such as AT1001 and conotoxins, have been investigated for their effects in the treatment of nicotine addiction in animal models (5, 24, 25). The present study also aims to determine the effects of the nAChR ligands, quinuclidine derivatives, in animal models of nicotine dependence.

2.2 Nicotine and nicotinic acetylcholine receptor

Nicotine is a natural alkaloid compound found in *Nicotiana tabacum* (Solanaceae). The chemical structure of nicotine consists of pyridine and pyrrolidine rings (Figure 1) with the chemical formula $C_{10}H_{14}N_2$ and molecular weight 162.23 g/mol. Nicotine is a weak base with pKa of 8.0. and dissolved in water. After smoking, nicotine is absorbed through alveoli and can reach the brain within 10 seconds.

Nicotine is widely distributed to a liver, kidney, and spleen. It is metabolized and converted to six primary metabolites. Nicotine is converted by cytochrome P450 (CYP) 2A6 in the liver to cotinine which is the major metabolite. Cotinine has a long half-life of 18 hours (26). Therefore, it is a biomarker for nicotine intake. Nicotine and its metabolites are mainly excreted in urine (17, 26, 27). The renal excretion depends on renal blood flow and urine pH.

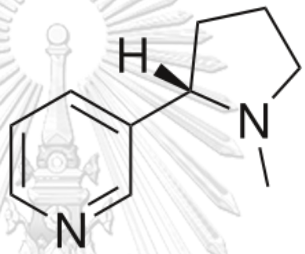
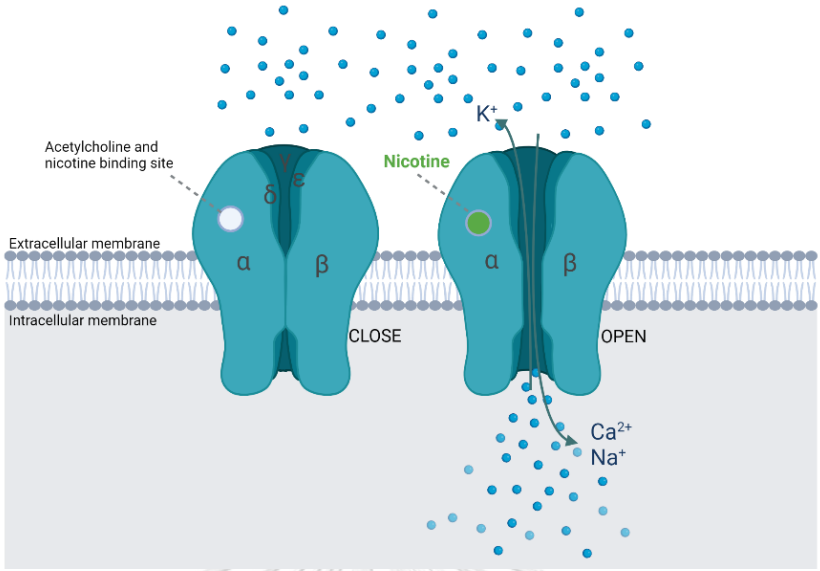


Figure 1 Chemical structure of nicotine

Nicotine acts as an agonist to nicotinic acetylcholine receptors (nAChR). Nicotinic acetylcholine receptor (nAChR) is a pentameric ligand-gated ion channel (28). It is composed of 5 subunits linked by a "cys-loop" disulfide bridge. Seventeen types of nAChR subunits have been reported in mammalians including 10 types of alpha (α1- α10), 4 types of beta (β1-β4), 1 type of gamma (γ1), delta (δ1), and epsilon (ε1) subunits. Alpha subunit is the binding site of acetylcholine and nicotine (Figure 2A) (4). Nicotinic acetylcholine receptor is classified into neuronal nicotinic receptors (Nn) and muscular nicotinic receptors (Nm) based on their expression areas. The neuronal nicotinic receptors are found in the autonomic ganglia and brain. These

receptors comprise of α2- α10 and β2- β4 subunits; for example, homomeric α7 and heteromeric α3β4*, α4β2*, α6β3* (Figure 2B) (4, 28). The muscular nicotinic receptors are found at the muscular junction. These receptors comprise of α1, β1, γ, δ, and ε subunits such as (α1)2β1$\delta\gamma$(4, 28). Activation of nAChR causes ion channel opening, Na^+ influx and K^+ efflux, and consequently neuronal activation and neurotransmitter release (Figure 2A).

A



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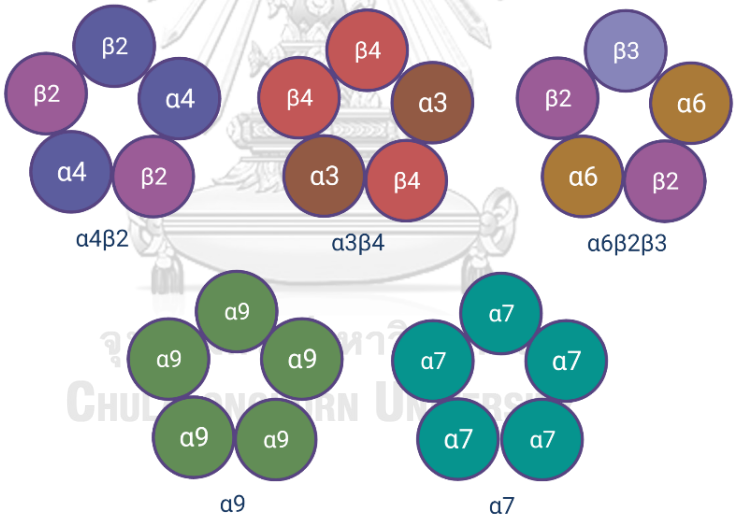


Figure 2 A) Structure and binding sites of nicotinic acetylcholine receptor B) Subtypes of nicotinic acetylcholine receptors

The composition of nAChR varies in each brain areas. Moreover, each type of nAChRs is responsible for different behaviors (Table 3) (29). $\alpha 4/\beta 2/\alpha 6/\beta 3$ are highly expressed in VTA and involved in reinforcement of nicotine (29) while $\alpha 3\beta 4$ nAChR is

abundantly expressed in the medial habenula (MHb) (30, Antolin-Fontes, 2015 #37, 31). In MHb, the activation of nAChRs on cholinergic neurons caused co-released of acetylcholine and glutamate into IPN (30). Previous study showed that the $\alpha_3\beta_4$ nAChR antagonists 18-methoxycoronaridine (18-MC) (35) and AuIB (32) given directly to the MHb can decrease nicotine-induced dopamine release in the NAc. In addition, systemic administration of 18-MC prevented nicotine-induced behavioral sensitization (33). 18-MC given directly to the MHb also attenuated nicotine self-administration in rats (34). Moreover, AT1001, a non-competitive $\alpha_3\beta_4$ nAChR antagonist, effectively reduced nicotine self-administration in rats (5, 35). Therefore, $\alpha_3\beta_4$ nAChR is the potential target for the treatment of nicotine addiction.

Table 3 The behavioral effects of nicotine activating nicotinic receptor subtypes (29)

Potential nAChR subtypes	Site of nicotine action	behavior
$\alpha_4/\beta_2/\alpha_6/\beta_3$	Mesolimbic DA system (VTA-NAc)	Reinforcement Hyperlocomotion
α_3/β_4; α_2/β_4	MHb and IPN	Hypolocomotion
α_7; $\alpha_4/\beta_2/\alpha_6/\beta_3$	VTA	Sensitization
α_4/β_2	Raphe and thalamus Amgydala, brainstem, thalamo-cortical pathway	Antinocicception Fear associated learning
α_7	Septo-hippocampal system	Anxiety
α_4/β_2, α_7	Septo-hippocampal system	Depression

2.3 Dopaminergic pathway associated with nicotine addiction

There are four main dopaminergic pathways in the CNS; tuberoinfundibular, nigrostriatal, mesocortical, and mesolimbic pathways (Figure 3) (36).

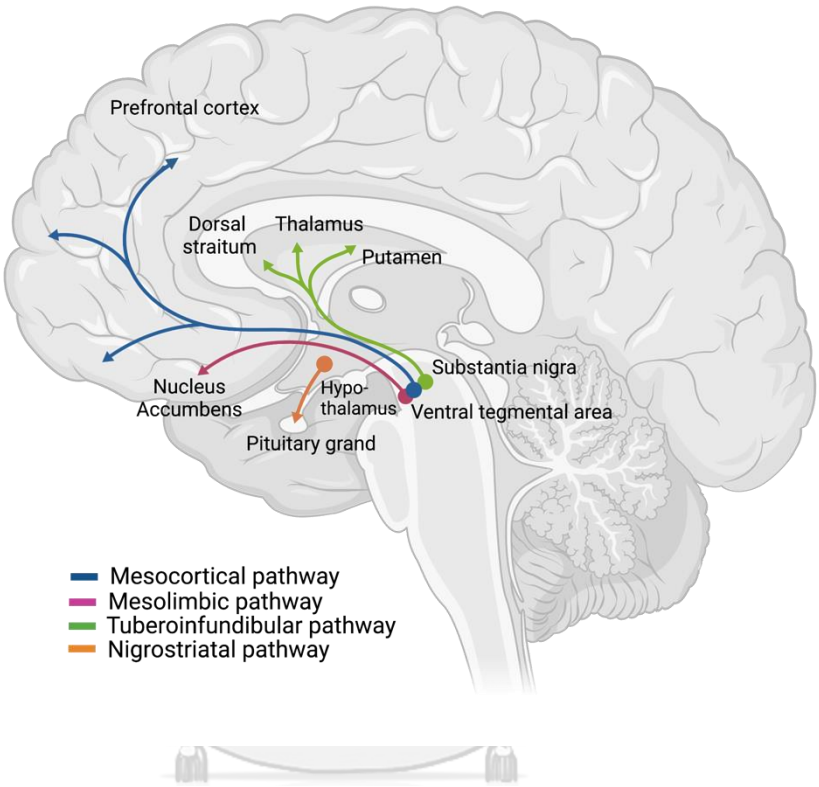


Figure 3 Dopaminergic pathways in the CNS (Adapted from 29)

Mesocorticolimbic system, dopaminergic neuron is originated from VTA to many areas in brain including NAc, striatum (medial caudate-putamen) (37), medial and orbital prefrontal cortex (38), and hippocampus leading to dopamine mediating learning, memory, emotions, and reward (3, 39, 40). Dopaminergic neurons in the VTA are regulated by GABA interneurons and glutaminergic neurons from pedunculo-pontine (PPT), laterodorsal tegmental nuclei (LDT) and amygdala. The main dopaminergic pathway responsible for the rewarding effect is the brain reward pathway. In the brain reward pathway, dopaminergic neurons from VTA project their

axons to NAc(37). Nicotine stimulates dopaminergic neurons in the VTA causing dopamine release in NAc and produces the pleasure feeling and reward effect (Figure 4). In addition, the activation of dopaminergic neurons in VTA by nicotine can modulate hippocampus function which is involved in long-term memory of rewarding effect of nicotine (41). The effects of nicotine on the dopaminergic neurons projecting to the medial prefrontal cortex (mPFC) involves conditioning and learning factors (contextual cue) related nicotine addiction(42, 43) and craving(44).

In the nigrostriatal pathway, dopamine cell bodies and dendrites locate in the substantia nigra and project their axons to caudate and putamen in dorsal striatum. This dopaminergic pathway regulates motor function. Although dopamine levels in the striatum is mainly regulated by the nigrostriatal dopaminergic pathway, the activation of dopaminergic neurons in VTA can also induce dopamine release in the striatum [3]. The striatum mediates habit learning(45). Thus, the striatum involves in the association between drug of abuse and environment cue leading to craving(45)

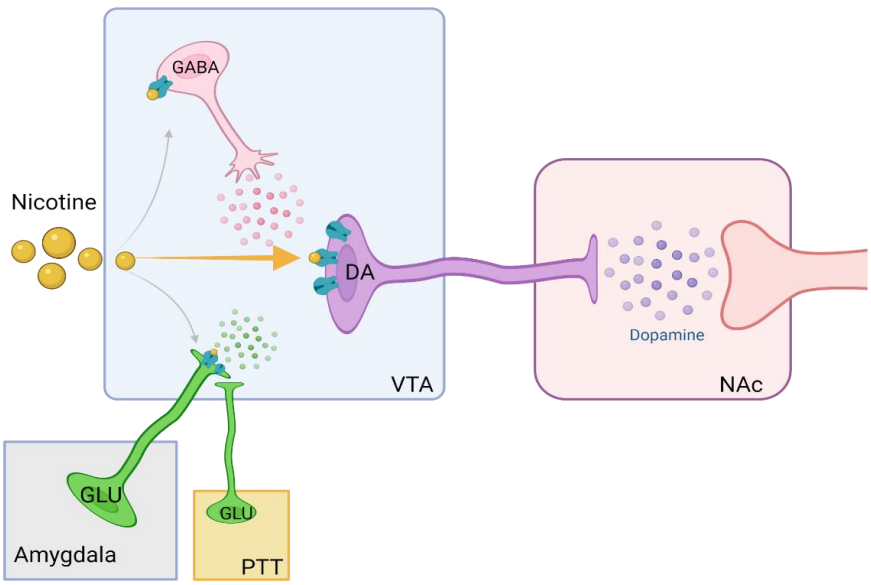


Figure 4 Nicotine acting in the brain rewarding pathway

2.4. Quinuclidine derivatives

Quinuclidine derivatives are developed from a potent ligand of α7 nAChR, QDN8 (Figure 5) (7). Quinuclidine derivatives are designed for improving the affinity and selectivity to α7, α3β4, and α4β2 nAChR. The chemical structure of quinuclidine derivatives composes of one benzene ring and two important pharmacophores; quinuclidine and triazole. The modification of structure was focused on the molecule or functional group at the benzene ring. This position plays a key role in the binding affinity and selectivity to nAChR subtypes. The hydrophobic side chain was added at either para or meta-phenyl to improve lipophilicity of the compounds. After structural modification, quinuclidine anti-1,2,3-triazoles are synthesized and studied for their nAChR binding affinity and selectivity (Table 4). Quinuclidine derivatives with an F-substituted at phenyl ring including T1 and T2 strongly bind to α3β4 nAChRs.

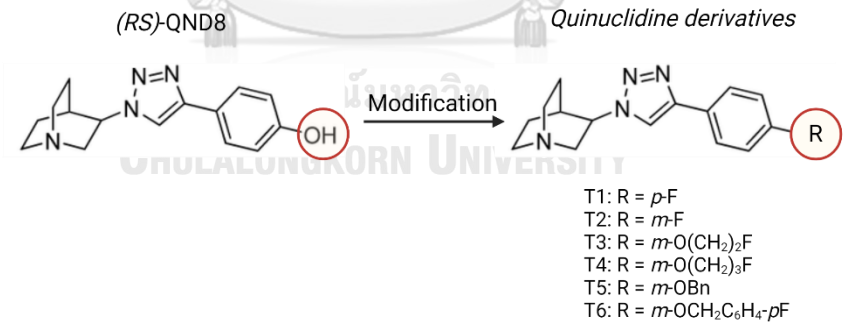
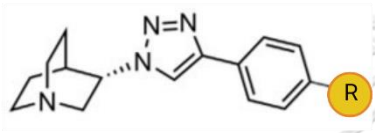


Figure 5 Quinuclidine derivatives modified from QND8 (7)

In vitro binding affinity study reported that (*R*)-enantiomer of T1-T6 selectively bound to α7 nAChRs, whereas (*S*)-enantiomer selectively bound to α3β4 nAChRs (7). Both of (*S*)- and (*R*)-enantiomers of quinuclidine derivatives had low binding

affinity and selectivity to $\alpha_4\beta_2$ nAChRs (Table 4). In addition, *in vitro* functional assay using HEK 293 cell expressing human $\alpha_3\beta_4$ nAChR revealed that *(s)*-T1 and *(r)*-T2 acted as $\alpha_3\beta_4$ nAChR agonists, whereas *(r)*-T1 and *(s)*-T2 were $\alpha_3\beta_4$ nAChR antagonist (7).

Table 4 In vitro binding affinities of quinuclidine derivatives to various subtypes of nicotinic acetylcholine receptor (7)



Com-pound	R	Inhibition constant Ki in mM Mean ± SD			Selectivity (inverse of respective Ki ratio)		
		α_7	$\alpha_3\beta_4$	$\alpha_4\beta_2$	α_7 vs $\alpha_3\beta_4$	α_7 vs $\alpha_4\beta_2$	$\alpha_3\beta_4$ vs $\alpha_4\beta_2$
T1	p-F	72.8±13	8.50±0.50	449±127	1/8.6	6.2	53
(r)-T1		73.0±15	1010±162	10436±1943	14	143	10
(s)-T1		174.5±66	3.09±0.10	515±64	1/56	3.0	167
T2	m-F	133±40	5.24±0.35	748±114	1/25	5.6	143
(r)-T2		117±4	362±27	5201±412	3/1	44	14
(s)-T2		660.5±139	2.25±0.42	519±20	1/294	1/1.3	231
T3	m-$O(CH_2)_2F$	98.7±39	20.9±0.7	1962±228	1/4.7	20	94
(r)-T3		38.8±8	558±34	7050±200	14	182	13
(s)-T3		74.9±20	11.8±0.3	1262±187	1/6.3	17	107
T4	m-$O(CH_2)_3F$	74.6±14	44.4±8.0	3894±252	1/1.7	52	88
(r)-T4		62.3±10	1628±11	9010±5034	26	145	5.5
(s)-T4		96±17	19.5±0.4	1980±117	1/5.0	20	102

Table 4 (continued)

Com-pound	R	Inhibition constant Ki in mM Mean ± SD			Selectivity (inverse of respective Ki ratio)		
		$\alpha7$	$\alpha3\beta4$	$\alpha4\beta2$	$\alpha7$ vs $\alpha3\beta4$	$\alpha7$ vs $\alpha4\beta2$	$\alpha3\beta4$ vs $\alpha4\beta2$
T5	m-OBn	91.3±9	7.57±2.9	668±7	1/12	7.3	88
(r)-T5		22.5±9	631±206	5059±374	28	225	8.0
(s)-T5		279±31	6.67±0.7	414±59	1/42	1.5	62
T6	m-$OCH_2C_6H_4F$	127±5	13.9±2.8	1013±107	1/9.1	8.0	73
(r)-T6		33.2±7	1090±163	6392±230	33	193	5.9
(s)-T6		149±42	7.17±1.2	537±11	1/21	3.6	75
QND8	p-OH	9.61±1.47	3.44±0.04	627±52	1/2.8	65	182
(r)-QND8		10.9±1.42	138±0	7389±42	12.7	678	54
(s)-QND8		29.3±0.18	2.48±0.04	461±89	1/11.8	16	186

Previous study showed that *(s)*-[^{18}F]T1 was uptaken into the mouse brains (8). *In vitro* permeability study reported that Pe value and IC_{50} for cardiotoxicity of (*s*)-T6 were 8.99 (±1.56) x 10^{-6} cm/s and 120 nM, respectively (9). These data suggested the promising pharmacokinetic and safety profiles of *(s)*-T1, *(s)*-T2 and *(s)*-T6 for further drug development.

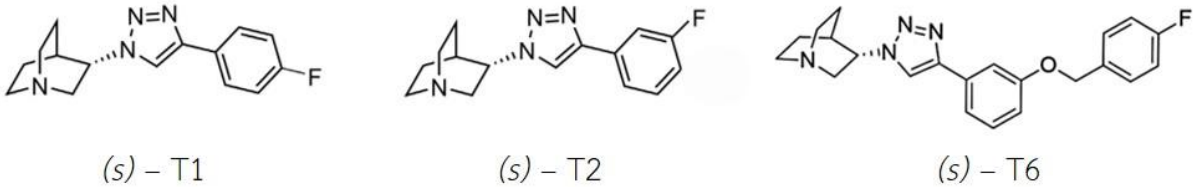


Figure 6 The structure of quinuclidine derivatives; *(s)*-T1, *(s)*-T2, and *(s)*-T6

2.5. Conditioned place preference

Conditioned place preference (CPP) has been widely used to investigate rewarding and aversive effects of a drug in animals (46). Principally, a drug is given repeatedly together with an environment cue in the CPP apparatus to generate the associative learning in animals (47). In general, CPP apparatus composes of a square box with two or three compartments (Figure 7). Each compartment has different wall and floor colors and/or patterns (48).

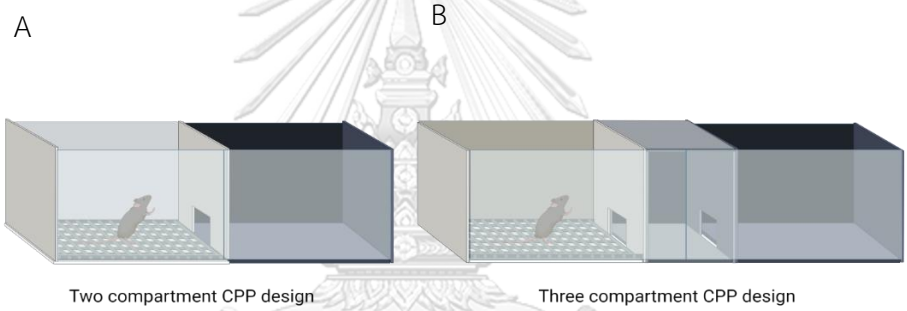


Figure 7 A) Two compartments CPP apparatus B) Three compartments CPP apparatus (48)

CPP procedure comprises of 3 phases, pre-conditioning, conditioning, and post-conditioning phases (46). In the pre-conditioning phase, a mouse is placed in the central of CPP apparatus. The mouse is allowed to freely explore the apparatus. The time that mouse spent in each compartment is recorded to evaluate the compartment bias. In the conditioning phase, the mouse is limited to stay in one compartment. Firstly, the mouse receives the drug(s) and is placed in a drug-paired compartment for defined amount of time. Then, the mouse receives saline and is placed in a saline-paired compartment for the same duration as in the drug-paired compartment. The mouse repeatedly undergoes in the condition phase for two to

twelve days. In the post-conditioning phase, the mouse is allowed to freely explore the apparatus. The time that the mouse spent in each compartment is recorded. CPP score is a parameter for assessment of either aversion or preference effects of the drug(s) given during the conditioning phase. CPP score is calculated by time that mice spent in drug-paired chamber during the post-conditioning phase minus time that mice spent in drug-paired chamber during the preconditioning phase. The significant positive CPP score is interpreted as a preference to the drug, while the significant negative CPP score value is interpreted as aversion to the drug (25, 46)

2.6. Dopamine level determination

Dopamine (DA) is a main neurotransmitter in the brain reward pathway. The precursor of dopamine is tyrosine. Tyrosine is converted to L-3,4-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase. L-DOPA is converted to dopamine by DOPA decarboxylase. Dopamine is metabolized by catechol-O-methyltransferase (COMT) and monoamine oxidase (MAO) to 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) (Figure 8).

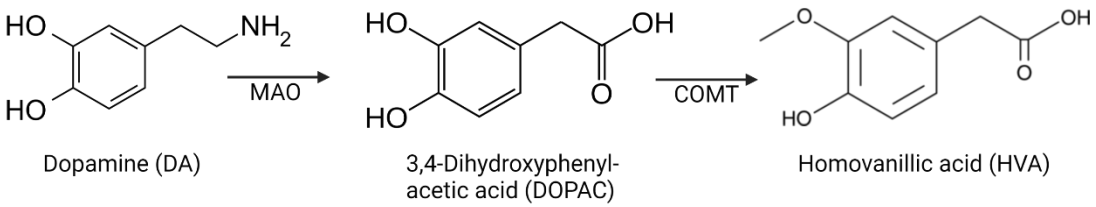


Figure 8 Dopamine and its main metabolites

This study used reversed-phase liquid chromatography and tandem mass spectrometry (LC/MS/MS) to determine level of dopamine and its metabolites in the brain tissue. In liquid chromatography, compounds are separated based on their different polarity and distribution between mobile phase and stationary phase. Subsequently, each compound is detected by mass spectrometry (MS). MS compose of ion source, mass analyzer, and detector. In mass spectrometry used in this study, a molecule is ionized in an electrospray ionization (ESI) source and its ion are analyzed by quadrupole mass analyzers. ESI can be set to either positive mode or negative mode based on an ionizable functional group of the compound. The positive mode is suitable for the functional groups which readily accept proton such as amide, amino, and other basic group, while the negative mode is usually used when the functional groups readily donate proton such as carboxylic, hydroxyl, and another acidic group. In quadrupole mass analyzer, ions with different m/z are filtered by a quadripolar electric field generated from direct and alternate currents (DC and AC). LC/MS/MS is a very useful tool to qualitatively and quantitatively analyze a compound in a sample because each compound has m/z value which indicate molecular masses and different pattern of fragmentation, which provides good specificity to compound identification. Targeted quantification by mass spectrometry using selective reaction monitoring (SRM) or multiple reaction monitoring (MRM) which are applicable in triple quadrupole provides tremendous selectivity and sensitivity to quantitative analysis. In our study, MRM on triple quadrupole mass analyzer was used in analyses of dopamine and its metabolites in the biological samples, while preliminary scans were performed in MS1 and product ion scan MS2 mode to choose optimal pairs of precursor and product ions for MRM

of each analyte. Tandem mass spectrometry composed of quadrupole 1 (Q1), multipole as a collision cell (q2), and quadrupole 3 (Q3) are filter for a selected m/z. In q2 collision cell, the selected ion was fragmented by collision with nitrogen gas. The energy of collision is controlled by acceleration voltage of q2. After fragmentation, resulting product ion will pass through the Q3 (MS2) to a detector. Q3 is set a scanning mode where ions with different m/z are detected at varied voltages of DC and AC and a mass spectrum of the product ions are acquired (49).

Chapter 3

Materials and Methods

3.1 Chemicals and reagents

Carboxymethylcellulose (Sigma–Aldrich St. Louis, MO, USA)

3,4-Dihydroxyphenylacetic acid (DOPAC) (Sigma–Aldrich St. Louis, MO, USA)

Dopamine hydrochloride (Sigma–Aldrich St. Louis, MO, USA)

Homovanillic acid; HVA (Sigma–Aldrich St. Louis, MO, USA)

(-)-Nicotine hydrogen tartrate (Sigma–Aldrich St. Louis, MO, USA)

Sodium dodecyl sulfate (Millipore, Billerica, MA, USA)

(s)-T1, *(s)*-T2, *(s)*-T6 (kindly provided by Dr. Jiradanai Sarasamkan, Faculty of Medicine, Khon Kaen University and Professor Dr. Opa Vajragupta, Faculty of Pharmaceutical Sciences, Chulalongkorn university)

Varenicline tartrate (Sigma–Aldrich St. Louis, MO, USA)

3.2 Equipment and instruments

Analytical balance (Mettler Toledo, Switzerland)

Conditioned place preference apparatus

LC/MS/MS (Agilent 1290 HPLC system and QTRAP6500, AB Sciex)

Locomotor activity box

VideoMOT2 system (TSE Systems, Germany)

Vortex mixer

3.3 Chemical preparation

Nicotine tartrate was dissolved in saline in the concentrations of 0.01 and 0.05 mg/ml (calculated as nicotine base) with pH adjusted to 7.3±0.1. Varenicline tartrate was dissolved in saline with 0.1 mg/ml concentration (calculated as varenicline base). *(s)*-T1, *(s)*-T2, and *(s)*-T6 were dissolved in 2% DMSO in 0.5% CMC in the concentrations of 0.1, 0.3, and 1 mg/ml.

3.4 Animals

Male C57BL/6N mice, 9-12 weeks old (20-30 g) (Nomura Siam International company, Bangkok, Thailand) were maintained under standard conditions (24 ± 2°C, 40-60% humidity, 12-h light cycle) with freely access to food and water. Mice were allowed to acclimatize for 1 week before the experiments. The experimental procedures were approved by the Institutional Animal Care and Use Committee, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand (approval number – 1933005 and 2133004).

3.5 Experimental design

There were three experiments in this study. The first experiment aimed to evaluate the addictive property of *(s)*-T1, *(s)*-T2, and *(s)*-T6. The second experiment was performed for determining the anti-addictive property of *(s)*-T1, *(s)*-T2, and *(s)*-T6 against nicotine. The third experiment was conducted to determine the effect of *(s)*-

T1, *(s)*-T2, and *(s)*-T6 and nicotine on dopamine, DOPAC and HVA levels in the nucleus accumbens, striatum, hippocampus, and prefrontal cortex.

3.5.1 Effect of *(s)*-T1, *(s)*-T2, and *(s)*-T6 on locomotor activity and conditioned place preference

Locomotor activity aimed to evaluate the sedative or stimulative property of *(s)*-T1, *(s)*-T2, and *(s)*-T6. Mice were divided into 11 groups (Table 5). Each mouse received one treatment as shown in Table 5. After injection, mice were placed in the open field for locomotor activity test. The video tracking was used to record locomotion time for 30 minutes. It was shown that *(s)*-T6 (10 mg/kg) treatment caused seizure in mice. Therefore, high dose of *(s)*-T6 (10 mg/kg) was not used in further experiments.

CPP aimed to evaluate the addictive property of *(s)*-T1, *(s)*-T2, and *(s)*-T6 compared to nicotine. Mice were divided into 10 groups (Group no. 1 – 10 in Table 5). Mice received one treatment in the morning as shown in Table 5 and saline in the afternoon during 4-day conditioning phase of CPP (Figure 11).

Table 5 Design groups, treatment and number of mice in experiment 1

Group No.	Group name	Treatment
1	Control	NSS (10 mL/kg s.c.)
2	Nicotine	Nicotine (0.5 mg/kg s.c.)
3	T1 (1)	*(s)*-T1 (1 mg/kg s.c.)
4	T1 (3)	*(s)*-T1 (3 mg/kg s.c.)
5	T1 (10)	*(s)*-T1 (10 mg/kg s.c.)
6	T2 (1)	*(s)*-T2 (1 mg/kg s.c.)
7	T2 (3)	*(s)*-T2 (3 mg/kg s.c.)
8	T2 (10)	*(s)*-T2 (10 mg/kg s.c.)
9	T6 (1)	*(s)*-T6 (1 mg/kg s.c.)
10	T6 (3)	*(s)*-T6 (3 mg/kg s.c.)
11	T6 (10)	*(s)*-T6 (10 mg/kg s.c.)

3.5.2 Effects of *(s)*-T1, *(s)*-T2 and *(s)*-T6 plus nicotine on conditioned place preference and locomotor activity

The second experiment aimed to evaluate the anti-addictive property of *(s)*-T1, *(s)*-T2 and *(s)*-T6 against nicotine. Mice were divided into 11 groups (Table 6). Varenicline was used as a positive control. According to Table 6, each mouse received “Treatment 1” followed by “Treatment 2”, 30 minutes apart. After injection of “Treatment 2”, mice were subject to the CPP experiment in a conditioning phase for 4 consecutive days (Figure 11). After 14-day wash-out period, locomotor activity was performed. Each mouse received two treatments as shown in Table 6, 30 minutes apart. After injection of treatment 2, mice immediately performed

locomotor activity. The video tracking was used to record locomotion time for 30 minutes.

Table 6 Design groups, treatments and number of mice in experiment 2

Group No.	Group name	Treatment 1	Treatment 2
1	Control	NSS (10 mL/kg s.c.)	NSS (10 mL/kg s.c.)
2	Nicotine	NSS (10 mL/kg s.c.)	Nicotine (0.5 mg/kg s.c.)
3	Varenicline	Varenicline (1 mg/kg s.c.)	Nicotine (0.5 mg/kg s.c.)
4	T1 (1)	*(s)*-T1 (1 mg/kg s.c.)	Nicotine (0.5 mg/kg s.c.)
5	T1 (3)	*(s)*-T1 (3 mg/kg s.c.)	Nicotine (0.5 mg/kg s.c.)
6	T1 (10)	*(s)*-T1 (10 mg/kg s.c.)	Nicotine (0.5 mg/kg s.c.)
7	T2 (1)	*(s)*-T2 (1 mg/kg s.c.)	Nicotine (0.5 mg/kg s.c.)
8	T2 (3)	*(s)*-T2 (3 mg/kg s.c.)	Nicotine (0.5 mg/kg s.c.)
9	T2 (10)	*(s)*-T2 (10 mg/kg s.c.)	Nicotine (0.5 mg/kg s.c.)
10	T6 (1)	*(s)*-T6 (1 mg/kg s.c.)	Nicotine (0.5 mg/kg s.c.)
11	T6 (3)	*(s)*-T6 (3 mg/kg s.c.)	Nicotine (0.5 mg/kg s.c.)

3.5.3 Effect of *(s)*-T1, *(s)*-T2 and *(s)*-T6 plus nicotine on dopamine and its metabolites levels in the nucleus accumbens, striatum, hippocampus, and prefrontal cortex

The third experiment aimed to evaluate the effects of *(s)*-T1, *(s)*-T2 and *(s)*-T6 and nicotine on dopamine, DOPAC and HVA levels in the nucleus accumbens,

striatum, hippocampus, and prefrontal cortex. Mice were divided into 11 groups as shown in Table 6. Each mouse received “Treatment 1” followed by “Treatment 2” (according to Table 6), with 30 minutes apart. Mice were sacrificed and their brains tissue were collected at 20, 40, and 60 minutes after injection of “Treatment 2” (Figure 9).

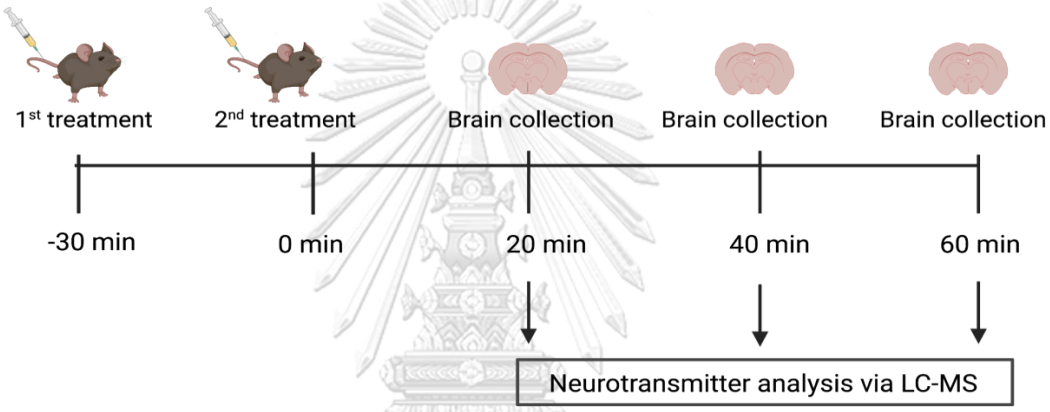


Figure 9 Timeline of brain collection

3.6 Conditioned place preference (CPP)

CPP apparatus consisted of two compartments. One compartment has straight line, black and white walls, and a black smooth floor (Figure 10). Another compartment has texture line, black and white walls, and a white mesh floor. The sliding partition is used for dividing the compartments. CPP test consists of 3 phases; 1-day pre-conditioning, 4-day conditioning and 1-day post-conditioning (Figure 11).

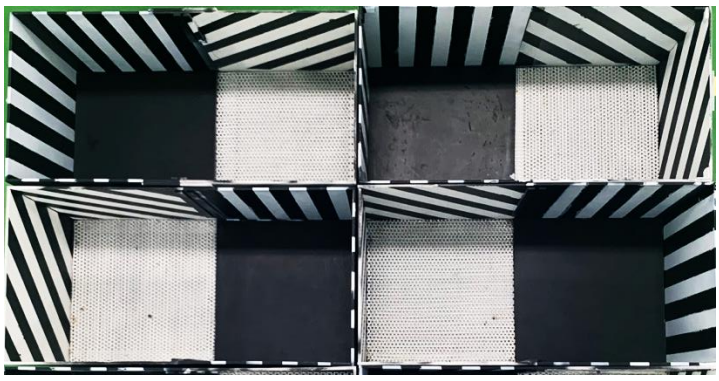


Figure 10 Conditioned place preference apparatus used in this study

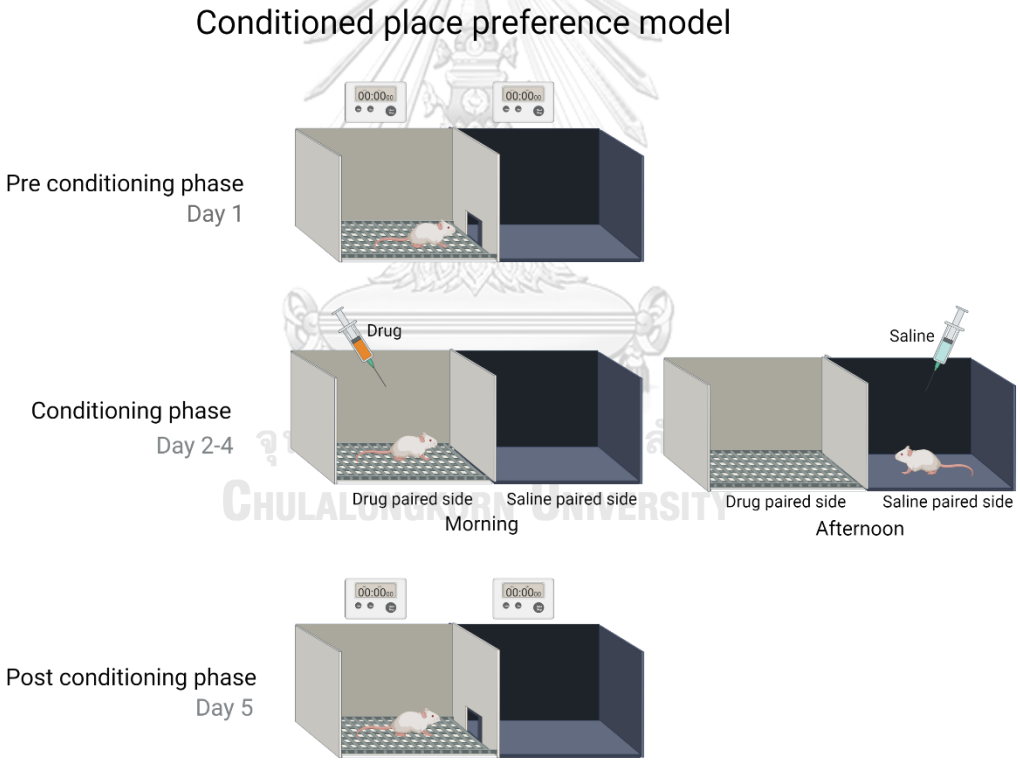


Figure 11 Conditioned place preference model procedure used in this study

In the pre-conditioning phase (day 1), mice can freely explore the two compartments without a removable wall between two sides for 15 minutes. Time that mice spent in each compartment was recorded by VideoMOT2.

In the conditioning phase (day 2-5), in the experiment 1, mice receive either NSS, nicotine, *(s)* -T1, *(s)* -T2 or *(s)* -T6 and were allowed to explore one compartment for 30 minutes in the morning. In the afternoon, mice received saline and were allowed to explore another compartment for 30 minutes. In the experiment 2, in the morning, mice received either NSS, *(s)*-T1, *(s)*-T2, *(s)*-T6 or varenicline (so called "Treatment 1", according to Table 6) and then received NSS or nicotine (so called "Treatment 2", according to Table 6), 30 minutes after "Treatment 1". Then mice were allowed to explore one compartment for 30 minutes. In the afternoon, mice received saline and were allowed to explore another compartment for 30 minutes.

In the post-conditioning phase (day 6), mice can freely explore the two compartments without a removable wall for 15 minutes. Time that each mouse spent in each compartment was recorded by VideoMOT2. CPP score was calculated as time that mice spent in drug-paired side during post-conditioning phase minus time that mice spent in drug-paired side during pre-conditioning phase.

3.7 Open field test

The open field apparatus was a white box (50 x 50 x 40 cm). The video tracking set was placed over the box and connected to VideoMOT2 software for real-time analysis of locomotor activity. The locomotion time was recorded for 30 minutes.

3.8 Brain sample preparation

Mice were sacrificed by cervical dislocation at 20, 40, and 60 minutes after injection of "Treatment 2" according to Table 6. Brains were quickly removed, then the nucleus accumbens, striatum, hippocampus and prefrontal cortex were dissected and snap frozen in liquid nitrogen. Brain samples were then kept in -80°C freezer until further analysis.

Brain tissue was homogenized with 10% acetonitrile mixed with 0.2% formic acid for 200-300 uL (1 mg/10 µL). Internal standards (10 ng/mL; dopamine-D4; 1000 ng/mL; 4-acetamidophenol) were added. Acetic acid approximately 40 µL (to the final concentration of 20%) was then added for protein precipitation. Brain homogenates were centrifuged at 28,000g for 15 min at 4°C. Supernatants were transferred to insert tube in HPLC vials and then injected onto the LC/MS/MS system by an autosampler. For method validation, brain tissues from untreated mice were used as a blank matrix.

3.9 LC/MS/MS method for determination of dopamine and its metabolite

3.9.1 LC/MS/MS conditions

A mass spectrometer QTRAP6500, AB Sciex (Triple quadrupole/ion trap with option for tendem MS) equipped with an electrospray ionization (ESI) ion source and a Micro LC M3 HPLC system was used to measure dopamine (DA), DOPAC, HVA levels in the brain samples, with the internal standards, dopamine-D4 and 4-acetamidophenol. The analytes were separated on an ACE-3 C18 column (50 × 1.0 mm, 3 μm, Bellefonte, PA, USA) at a column temperature 30°C. The gradient elution of mobile phases is shown in Table 7.

Table 7 HPLC gradient elution of mobile phases – A (water:formic acid 100:0.1) and B (acetonitrile:formic acid 100:0.1)

Gradient elution for Positive mode – ESI detection			Gradient elution for Negative mode – ESI detection		
Time (min)	Solvent A (%)	Time (min)	Solvent A (%)	Time (min)	Solvent A (%)
0	100	0	0	100	0
2	0	100	0.8	100	0
4	0	100	2.6	10	90
4.2	100	0	5	10	90
5	100	0	5.2	100	0

The flow rate was set at 50 µL/min and the injection volume was 5 µL. Dopamine and dopamine-D4 were detected in a positive mode of ESI while DOPAC, HVA, and 4-acetamidophenol were detected in a negative mode of ESI. Conditions were set as following: gas temperature 350°C, gas flow 10 L/min, ESI needle 4500 V, nebulizer pressure 35 psi. MS acquisition of dopamine, HVA, and DOPAC was performed in an MS1 scan, product ion scan (MS2), and a multiple reaction monitoring (MRM)-MS2 mode. MS1 scan rate of Q1 was 10 m/z per sec. An isolation window was 10 m/z and a scan rate of Q3 (for the product ion scan) was 10 m/z per sec. Collision energy used in Q2 was set at 10 to 30 volts.

3.9.2 Determination of neurotransmitters and internal standards from MS/MS

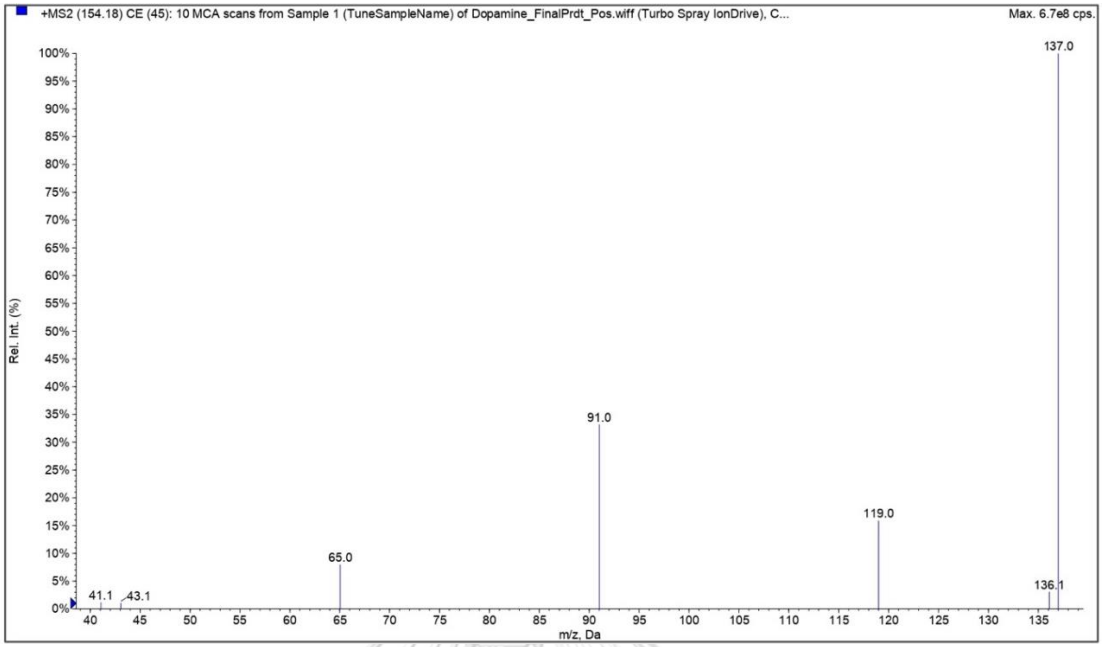
Full-scan MS1 mass spectra of dopamine and the internal standard (IS), dopamine-D4, showed that m/z of protonated ions $[M + H]^+$ were 153.8 and 157.8, respectively. For MS2, the mass-to-charge ratios of fragments of dopamine's major product ions after fragmentation were 137.0 and 91.0, and those of DA-D4's major product ions were 141.0 and 95.0. The most abundant ions in the product ion spectrum were 137.0 for dopamine (Figure 12A) and 141.0 for dopamine-D4 (Figure 12B).

Full-scan MS1 mass spectra of DOPAC, HVA and the IS, 4-acetamidophenol, showed that m/z of deprotonated ions $[M + H]^-$ were 166.8, 187.0 and 149.9, respectively. After fragmentation (MS2), the most abundant ions in the product ion

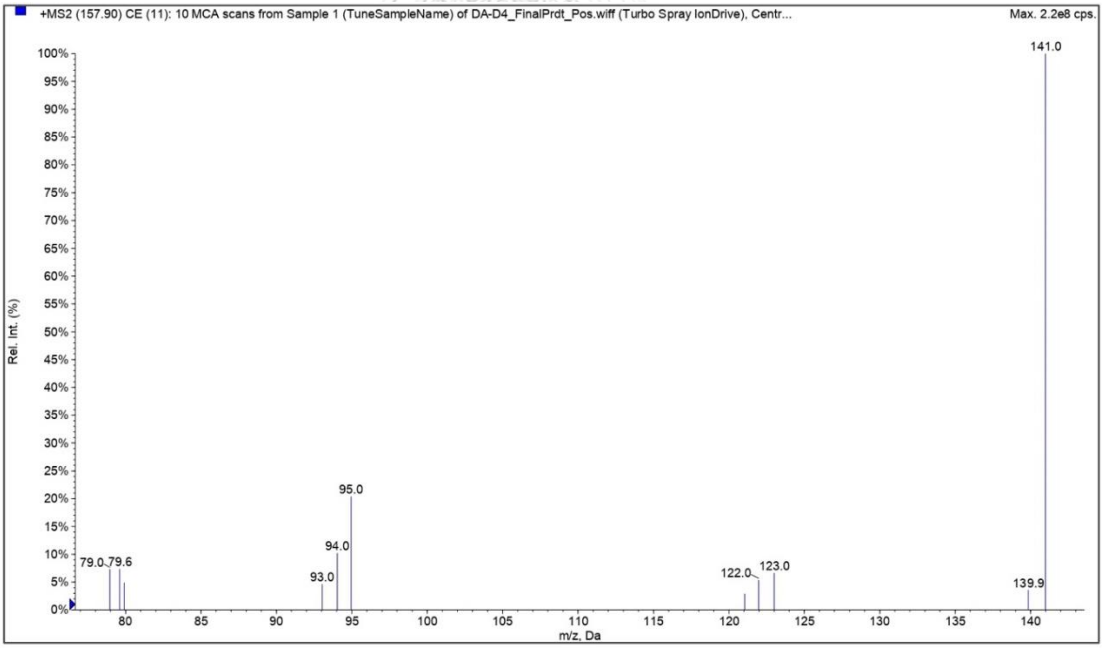
spectra were 122.9 for DOPAC (Figure 12C), 136.9 for HVA (Figure 12D) and 106.9 for 4-acetamidophenol (Figure 12E).

The retention times of dopamine, dopamine-D4, DOPAC, HVA, and 4-acetamidophenol as shown in extracted ion chromatograms of MRM, were 1.20, 1.20, 2.92, 3.05, and 2.88 minutes, respectively (Figure 13, 14).

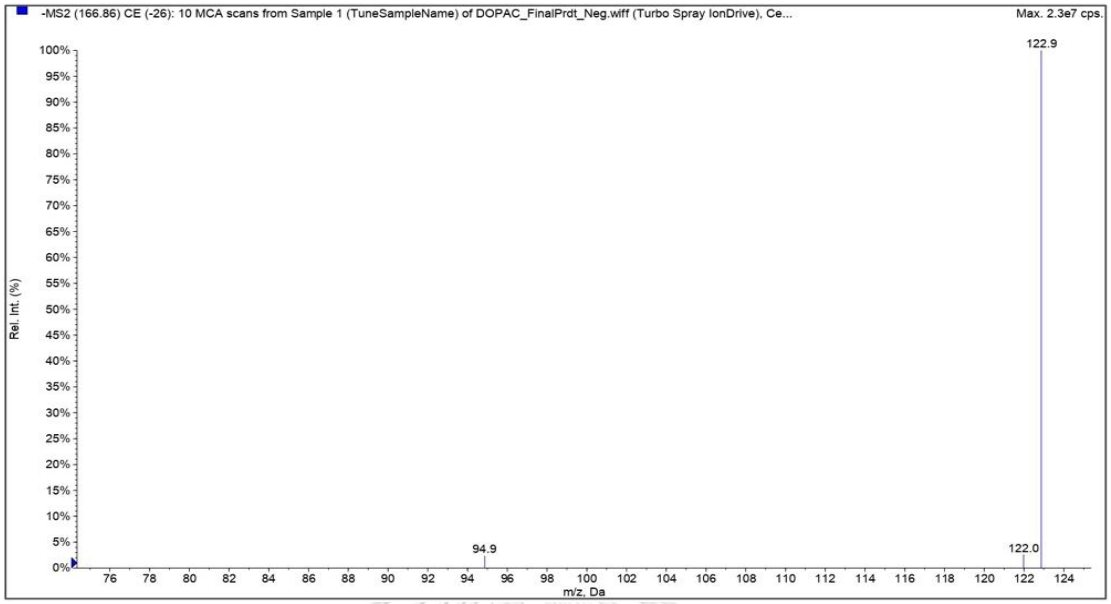
A Dopamine ([M+H]+ 153.8 -> product ions)



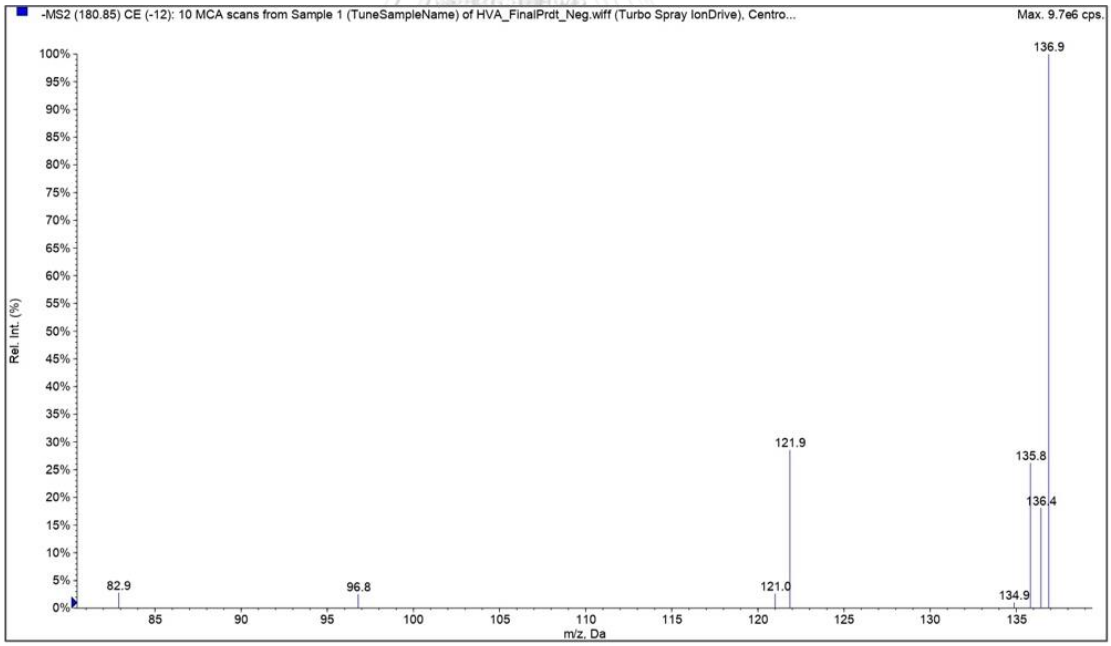
B Dopamine-D4 ([M+H]+ 157.8 -> product ions)



C DOPAC ([M-H]- 166.8 -> product ions)



D HVA ([M-H]- 187.0 -> product ions)



E 4-Acetamidophenol ([M-H]- 149.9 -> product ions)

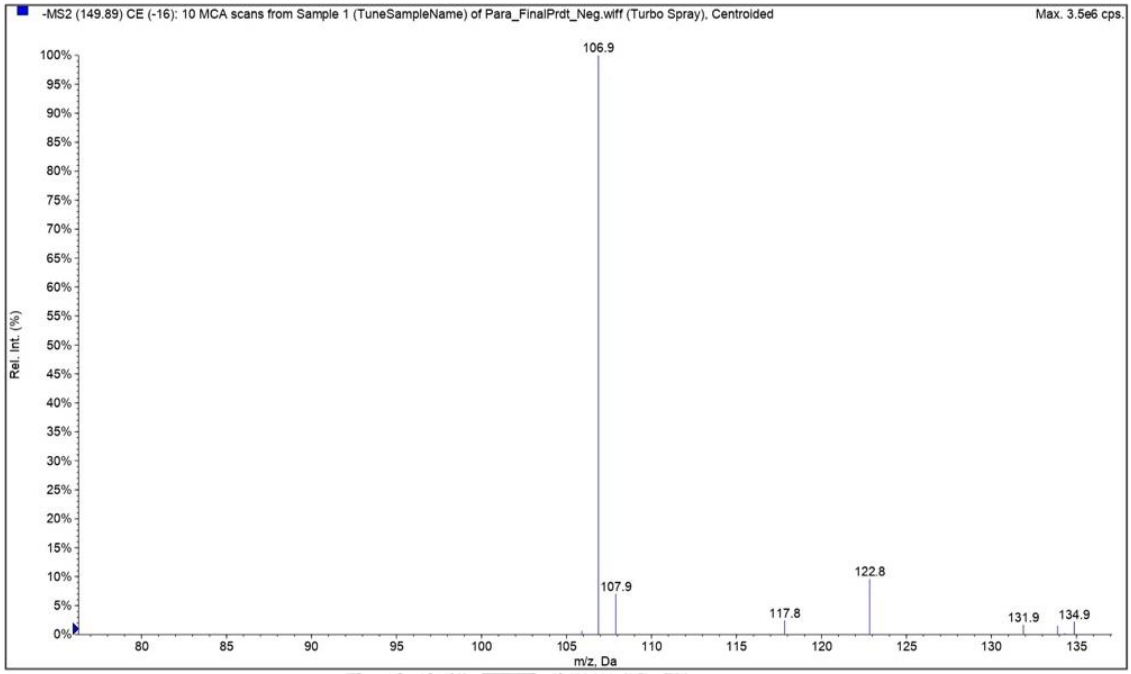


Figure 12 Product ion spectra for dopamine (A), dopamine-D4 (B), DOPAC (C), HVA (D), and 4-acetamidophenol (E)

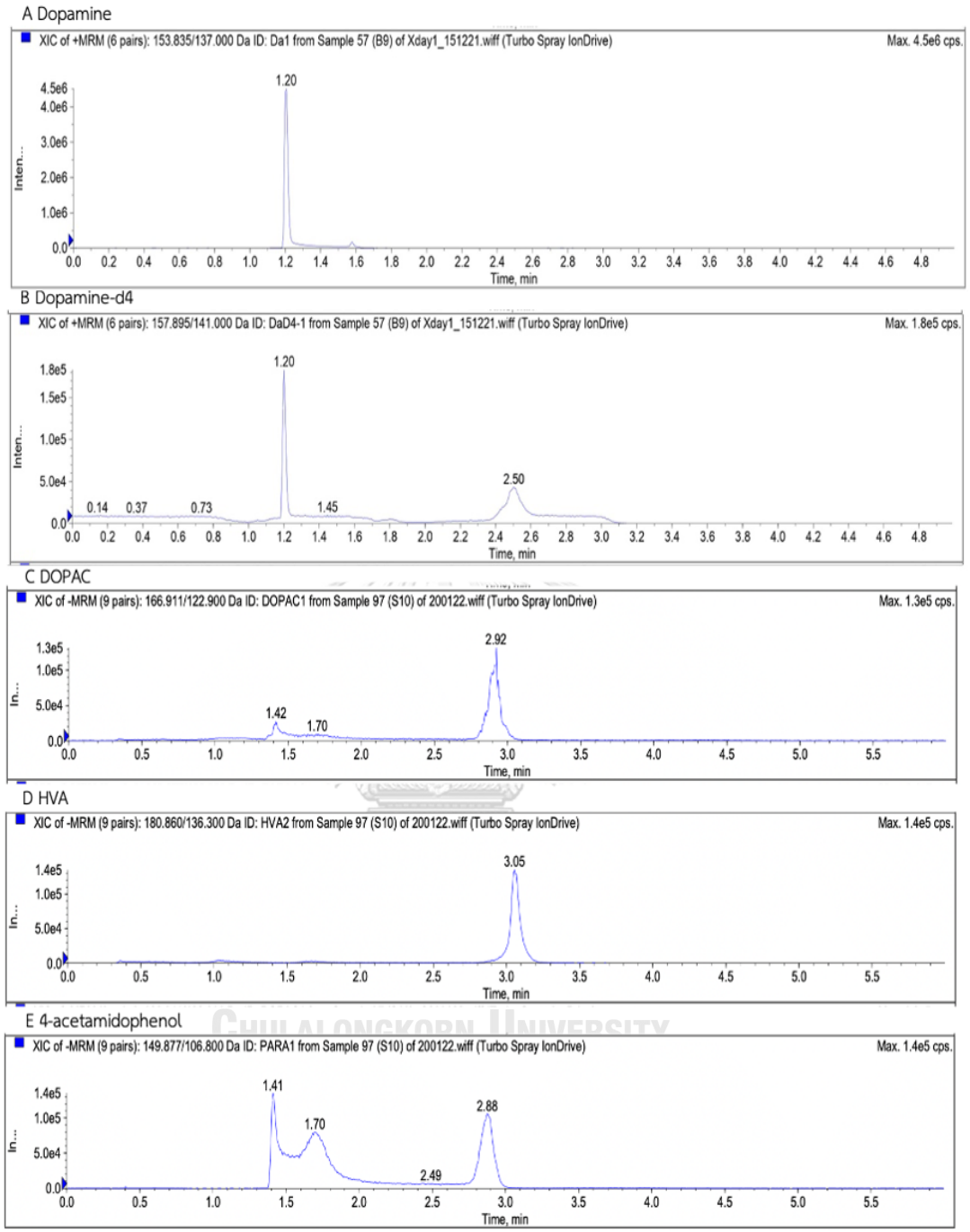


Figure 13 The LC/MS/MS chromatograms of dopamine (A), dopamine-D4 (B), DOPAC (C), HVA (D) and 4-acetamidophenol (E) in solvent

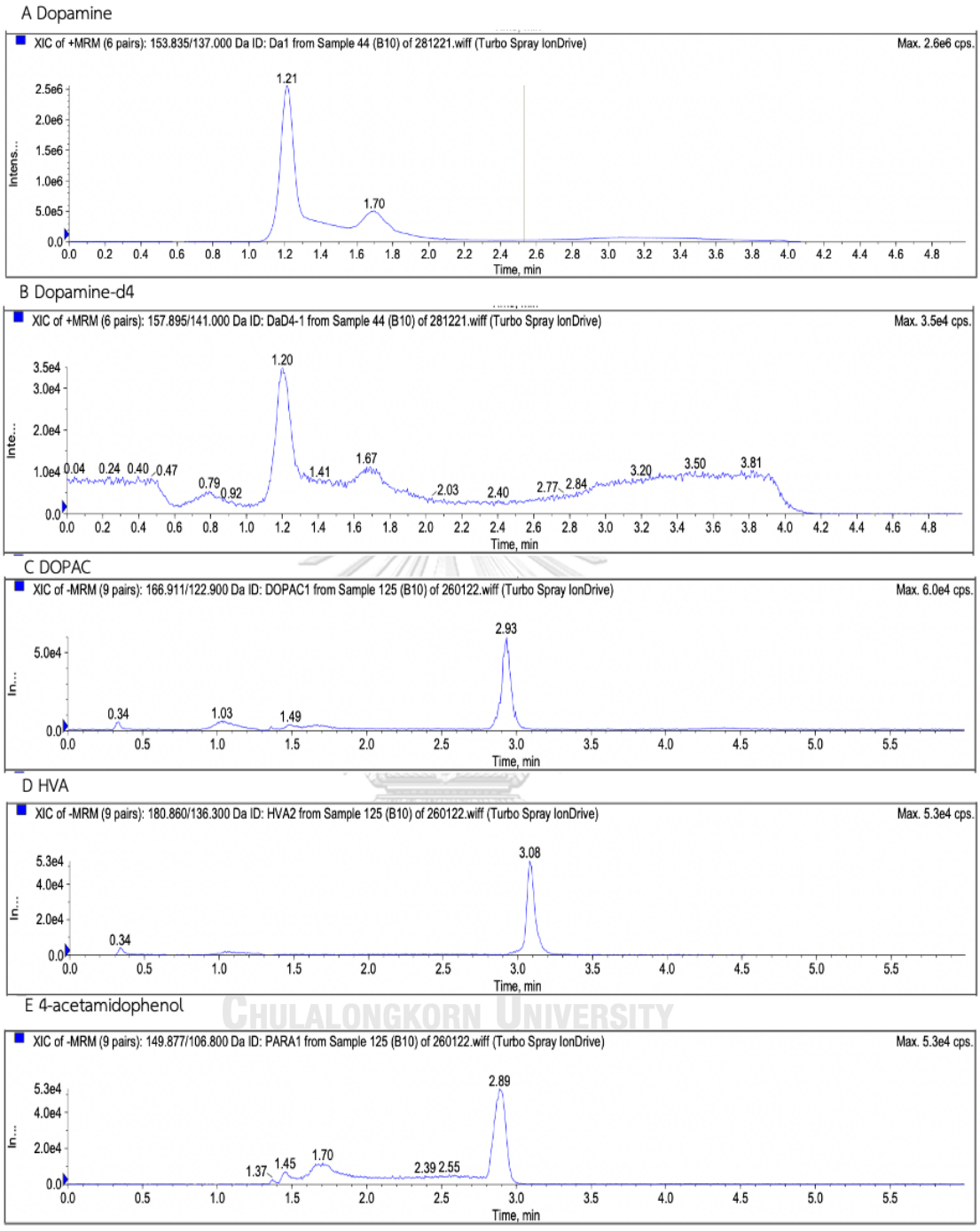


Figure 14 The LC/MS/MS chromatograms of dopamine (A), dopamine-D4 (B), DOPAC (C), HVA (D) and 4-acetamidophenol (E) in brain matrix

3.9.3 Method development

Upon method development, voltages of ESI and other ion optics and gas flow rates were optimized. These parameters were stated in the section 3.9.1 For MRM, pairs of precursors, product ion, and collision energy were choosing for dopamine and dopamine-D4 (IS), DOPAC, HVA, and 4-acetamidophenol (IS) MRM parameters were shown in Table 8.

Table 8 The MRM parameters for the determination of dopamine, DOPAC, HVA and the internal standards, dopamine-D4 and 4-acetamidophenol using LC/MS/MS

Chemical	Precursor ion (m/z)	Collision energy (V)	Product ion (m/z)	Retention time (m/z)
Dopamine	153.8	13.0	137.0	1.20
Dopamine-D4	157.9	13.0	141.0	1.20
DOPAC	166.9	-10.0	122.9	2.92
HVA	180.9	-10.0	136.3	3.05
4-acetamidophenol	149.9	-22.0	106.8	2.88

3.9.4 Method Validation

The validation methods followed the guideline of "FDA (US) [Bioanalytical Method Validation Guidance for Industry], May 2018." (50)

3.9.4.1 Calibration curves

Stock solutions of dopamine, DOPAC and HVA standard were prepared to the concentration of 1 mg/mL in water and then diluted with acetonitrile (10%) plus 0.2% formic acid to a selected concentration. A calibration curve was made by

spiking the standard solutions of dopamine, DOPAC and HVA to brain homogenates. The final concentration range for the standard curve of dopamine, DOPAC and HVA were 0.01, 0.1, 0.5, 1, 5, 10, 50, 100, 500, 1000 ng/ml, 1, 2, 5, 10, 50, 100, 200, 100, 500, 1000 ng/ml, and 10, 20, 50, 100, 150, 200, 400, 500, 800, 1000 ng/ml, respectively. The linear ranges and correlation coefficient of the calibration curve were calculated and shown in Table 9 and Figure 15. Y-axis of the calibration curve was the ratio of peak area of dopamine, DOPAC, HAV to that of IS. The correlation coefficient (R^2) was determined using the least-squares linear regression analysis. The linear range was reported where the correlation coefficient (R^2) was more than 0.99. All the solutions were freshly prepared for each experiment.

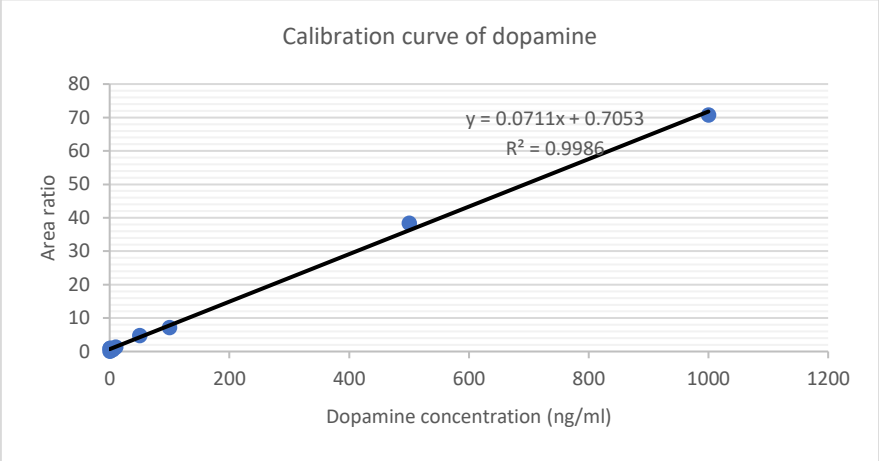
3.9.4.2 Limit of detection (LOD) and Limit of quantification (LOQ)

The quality control samples were prepared by spiking the standard solutions of dopamine (1, 10, and 1000 ng/ml), DOPAC (10, 100, and 1000 ng/ml) and HVA (50, 500, 1000 ng/ml) and internal standards to the brain homogenates. The signal-to-noise ratio was the comparison between the dopamine, DOPAC and HVA signals of the spiked brain homogenates to the signals of those in the non-spike brain homogenates. LOD was the concentration where the signal-to-noise was more than 3:1 whereas LOQ was the concentration where signal-to-noise was more than 10:1. LOD and LOQ of dopamine, DOPAC and HVA were summarized in Table 9.

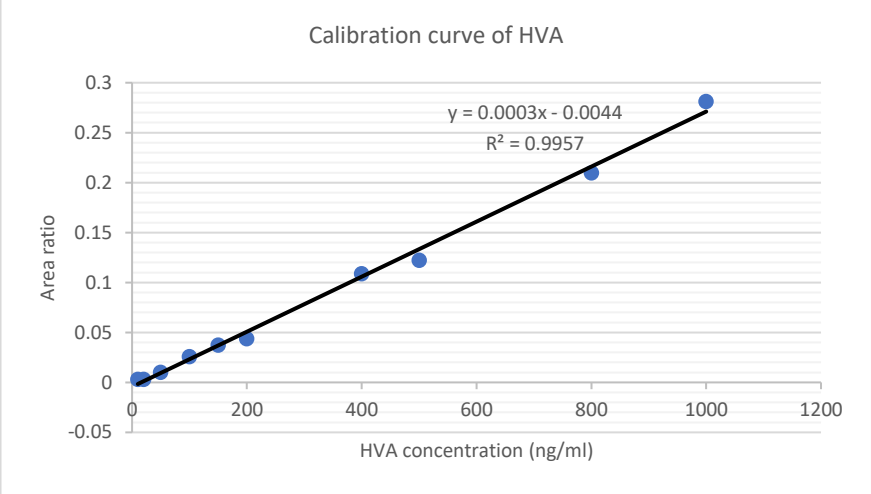
Table 9 The calibration of neurotransmitter by LC-MS/MS.

Chemical	Internal standard	Equations	Linear range (ng/ml)	Correlation Coefficient (R^2)	LOD (ng/ml)	LOQ (ng/ml)
Dopamine	Dopamine-D4	y = 0.0711x + 0.7053	0.1 - 1000	0.9986	0.10	0.50
DOPAC	4-acetamidophenol	y = 0.0003x - 0.0044	2 – 1000	0.9967	2.0	5.0
HVA	4-acetamidophenol	y = 0.0028x - 0.0189	10 - 1000	0.9966	10.0	50.0

A



B



C

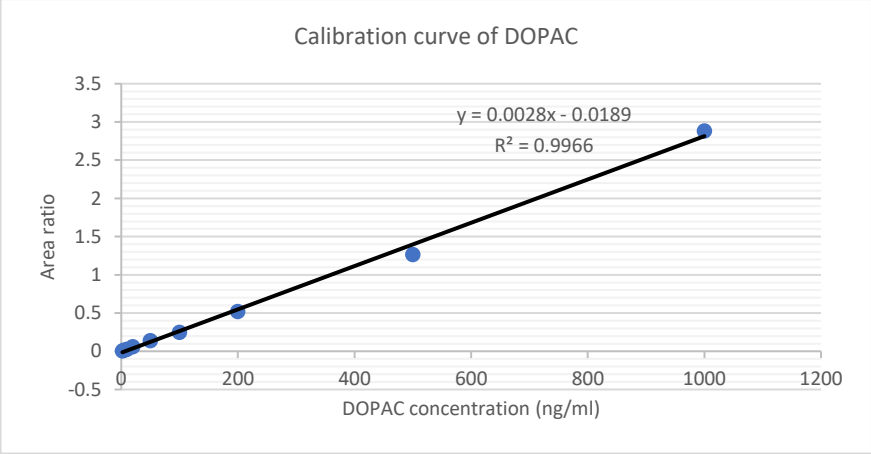


Figure 15 Calibration curve of dopamine (A), DOPAC (B) and HVA (C)

3.9.4.3 Precision and accuracy

Precision and accuracy were evaluated using three different concentrations of the quality control samples (low, medium, high) including (1, 10, and 1000 ng/ml of dopamine, 10, 100, and 1000 ng/ml of DOPAC, 50, 500, 1000 ng/ml of HVA). Intra-day precision was evaluated by 3 replicates per concentration within one day. Inter-day precision was evaluated for 3 replicates per concentration for 3 consecutive days. The precision limit was calculated as percentage of coefficient of variation (%CV).

$$\%CV = \frac{Standard\ deviation}{mean} \times 100$$

An acceptable of %CV was not more than 15% (50). Relative error (RE) is the ratio of the absolute error of the measurement to the actual value. Accuracy limit was calculated as 100% minus %RE and the acceptable of %accuracy was more than 85%(50).

$$\%RE = \frac{Measured\ value - actual\ value}{actual\ value} \times 100$$

The precision and accuracy of intra-day and inter-day testing were summarized in Table 10.

Table 10 Precision and accuracy of dopamine, DOPAC and HVA determined by LC/MS/MS

Chemical	Intra-day precision (%CV)			Inter-day precision (%CV)			Accuracy (%)			RE (%)		
	Low	Mid	High	Low	Mid	High	Low	Mid	High	Low	Mid	High
Dopamine	9.63	5.13	4.61	14.03	12.49	14.16	94.94	85.62	97.49	5.06	14.38	2.51
DOPAC	12.43	5.86	4.91	6.63	7.79	2.43	97.37	95.33	94.50	2.63	4.67	5.49
HVA	12.93	8.04	5.32	30.69	35.36	19.35	87.13	79.24	78.57	12.87	20.76	21.43

Intra-day and inter-day precision < 15, Accuracy > 85% (50)

3.9.4.5 Recovery

Recovery was evaluated to demonstrate the effect of sample preparation process. The standard solutions (1000 ug/ml of dopamine, DOPAC, and HVA) were spike to the brain homogenate either before or after homogenizer process. Percent recovery was evaluated from 3 replicates per concentration (Table 11).

$$\%\text{recovery} = \frac{(\text{Area ratio of STD/IS concentration before spike} - \text{Area ratio of STD/IS concentration after spike})}{\text{Area ratio of STD/IS concentration after spike}} x100$$

Table 11 Determination of neurotransmitters by LC-MS/MS: validation results on recovery

Analyte	Add before homogenization	Add after homogenization	% Recovery
Dopamine	990.37	1102.63	89.81
DOPAC	936.06	1058.14	88.46
HVA	957.39	1057.82	90.50

3.10 Data analysis

Statistical analysis was carried out using Statistical Package for the Social Science (SPSS) software. Results were presented as mean ± standard error of the mean (S.E.M). One-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test was used to analyze the differences between groups. Two-way ANOVA with time and treatment as factors followed by Tukey's post-hoc test was used to analyze the effects of nicotine and varenicline on dopamine and DOPAC levels at different timepoints. Differences was statistically significant at p value < 0.05.

CHAPTER 4

RESULTS

4.1 Effect of *(s)*-T1, *(s)*-T2, and *(s)*-T6 on locomotor activity

Mice were place in the locomotor box immediately after saline, *(s)*-T1, *(s)*-T2, or *(s)*-T6 treatment. Locomotor time was recorded for 30 min. One-way ANOVA revealed the effect of treatment on locomotion time ($F_{10,55}$ = 2.141, P < 0.05) (Figure 16A-C). Locomotion time decreased in mice receiving nicotine and *(s)*-T6 (10 mg/kg) compared to control (p < 0.05) (Figure 16C). In addition, seizure occurred in mice treated with *(s)*-T6 at 10 mg/kg. Thus, *(s)*-T6 at 10 mg/kg was excluded for the next experiments.

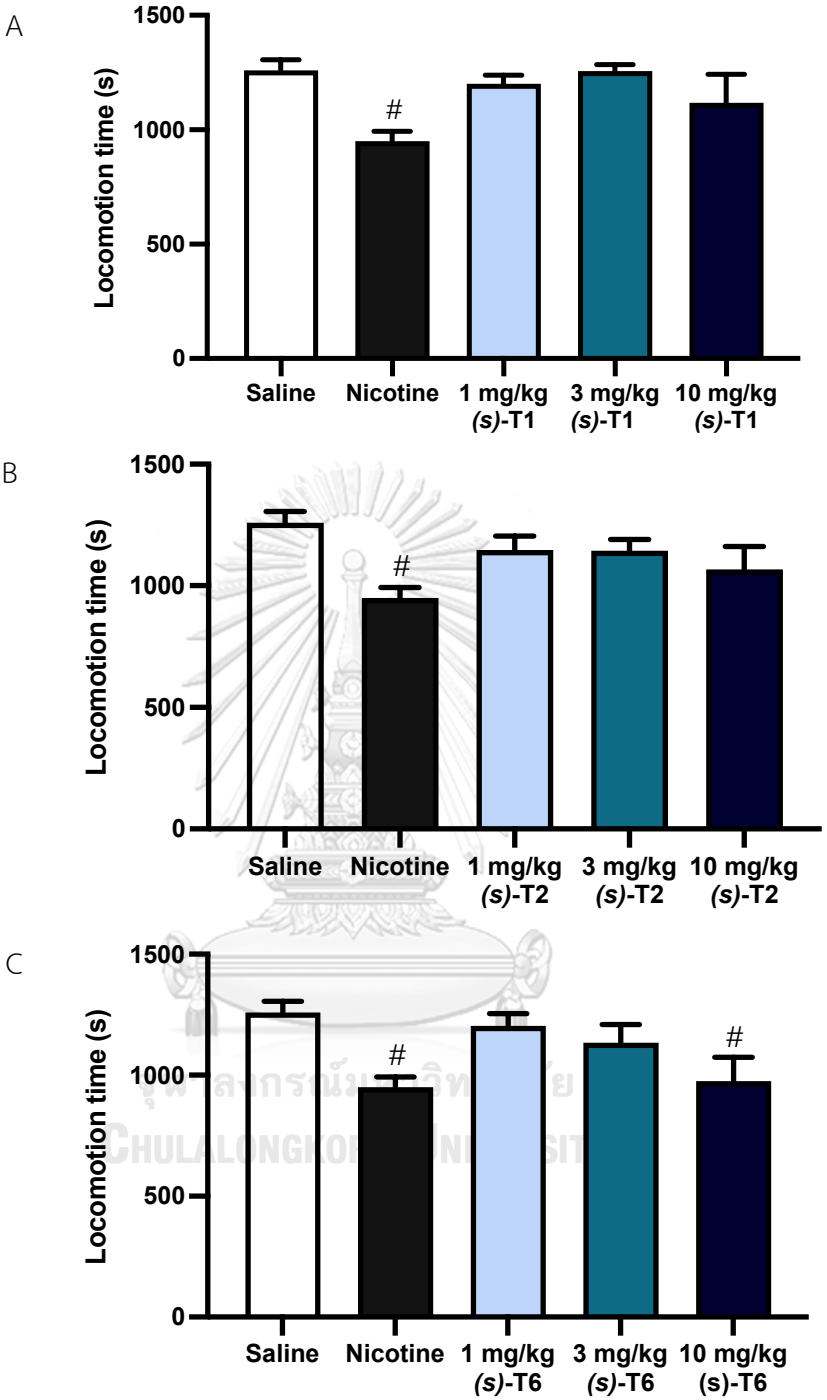


Figure 16 Effect of *(s)*-T1, *(s)*-T2, and *(s)*-T6 on locomotor activity. Mice received either nicotine, *(s)*-T1 (A), *(s)*-T2 (B), or *(s)*-T6 (C) and immediately performed locomotor activity. Locomotion time was recorded for 30 min. Data are presented as mean (+S.E.M.) (N = 6/group). $^{\#}P < 0.05$ compared to control group

4.2 The addictive effect of *(s)*-T1, *(s)*-T2, and *(s)*-T6 in conditioned place preference

To evaluate the addictive effect of *(s)*-T1, *(s)*-T2, and *(s)*-T6, each mouse received a single injection of either saline, nicotine (0.5 mg/kg, s.c.), *(s)*-T1 (1, 3, or 10 mg/kg), *(s)*-T2 (1, 3, or 10 mg/kg), or *(s)*-T6 (1 or 3 mg/kg) 30 min before placing in the drug-paired chamber during 4-day conditioning phase of CPP. One-way ANOVA showed no effect of treatment on CPP score ($F_{9,63}$ = 1.956, P > 0.05, Figure 17). However, Dunnett's post-hoc test showed that nicotine-treated mice spent more time in drug-paired chamber during post-conditioning phase than that during pre-conditioning phase with positive CPP score (+159.8 s, P < 0.05 vs control). In contrast, mice treated with *(s)*-T1 at 1, 3, and 10 mg/kg had the negative CPP scores (-21.00, -133.30, -94.29 s, respectively). The CPP scores of mice in *(s)*-T1 1, 3, and 10 mg/kg groups were not different from control group (Figure 17A).

Mice treated with *(s)*-T2 at 1, 3, and 10 mg/kg had the negative CPP scores (-76.29, -48.43, -52.86 s, respectively). The CPP scores of mice in *(s)*-T2 1, 3, and 10 mg/kg groups were not different from control group (Figure 17B).

Mice treated with *(s)*-T6 at 1 and 3 mg/kg had the negative CPP scores (-46.00 and -44.43 s, respectively). The CPP scores of mice in *(s)*-T6 1 and 3 mg/kg groups were not different from control group (Figure 17C).

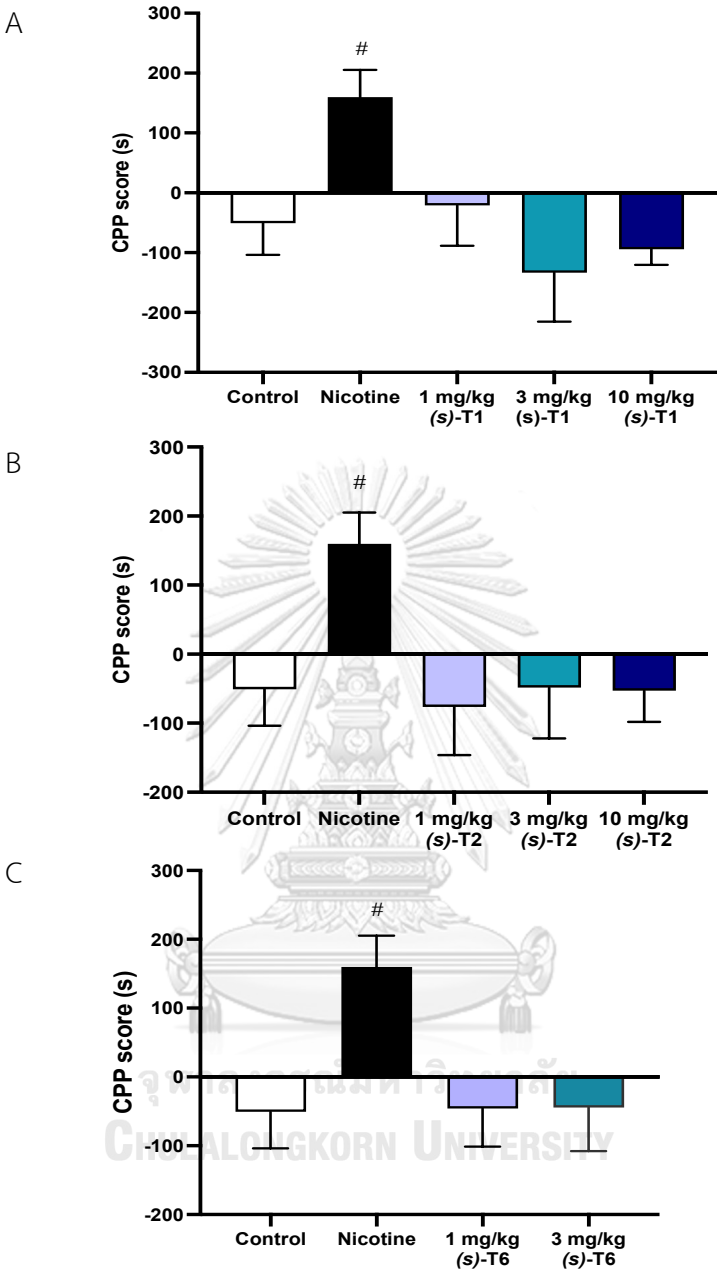


Figure 17 Effect of *(s)*-T1, *(s)*-T1, and *(s)*-T6 treatment in conditioned place preference model. *(s)*-T1 (1, 3 or 10 mg/kg, s.c.) or *(s)*-T2 (1, 3 or 10 mg/kg, s.c.) or *(s)*-T6 (1 or 3 mg/kg, s.c.) or nicotine (0.5 mg/kg, s.c.) or saline was given 30 min before CPP experiment during conditioning phase. Data are presented as mean (+ S.E.M.) (N = 6-9/group). CPP score was calculated as time spent in drug-paired chamber during post-conditioning phase minus time spent in drug-paired chamber during pre-conditioning phase. $^{\#}P < 0.05$ compared to control group.

4.3 The anti-addictive effect of *(s)*-T1, *(s)*-T2, and *(s)*-T6 induced by nicotine in conditioned place preference

To evaluate the anti-addictive effect of *(s)*-T1, *(s)*-T2, and *(s)*-T6 induced by nicotine, mice received the first treatment which were either saline, varenicline (1 mg/kg, s.c.), *(s)*-T1 (1, 3, or 10 mg/kg), *(s)*-T2 (1, 3, or 10 mg/kg), or *(s)*-T6 (1 or 3 mg/kg) 30 min before receiving the second treatment of either saline or nicotine (0.5 mg/kg, s.c.). Then mice were placed in the drug-paired chamber during 4-day conditioning phase of CPP. One-way ANOVA showed the effect of treatment on CPP score ($F_{10, 74}$ = 2.408, $P < 0.05$, Figure 18). Nicotine-treated mice spent more time in drug-paired chamber during post-conditioning phase than that during pre-conditioning phase with positive CPP score (+122.8 s, $P < 0.05$ vs control). Varenicline treatment reversed the nicotine effect showing by the negative CPP scores (-17.25 s, $P < 0.05$ vs nicotine group). In the same way, *(s)*-T1 at 1, 3, and 10 mg/kg also produced the negative CPP scores (-64.50, -31.50, -34.13 s, respectively). The CPP scores of mice in *(s)*-T1 1, 3, and 10 mg/kg plus nicotine groups were significantly lower than that of mice in nicotine group ($P < 0.001$, $P < 0.05$, $P < 0.05$, respectively) (Figure 18A).

(s)-T2 1 and 3 mg/kg-treated mice had negative CPP scores (-50.63 and -5.00 s, respectively), which were significantly lower than that of mice in nicotine group ($P < 0.01$ and $P < 0.05$, respectively). In contrast, mice treated with *(s)*-T2 10 mg/kg presented the positive CPP score (+27.88 s, $P > 0.05$ vs nicotine group) (Figure 18B),

suggesting that high dose *(s)*-T2 (10 mg/kg) failed to inhibit nicotine addiction in CPP model.

(s)-T6 1 and 3 mg/kg-treated mice had negative CPP scores (-4.75 and -14.67 s, respectively), which were significantly lower than that of mice in nicotine group ($P < 0.05$ and $P < 0.05$, respectively) (Figure 18C).

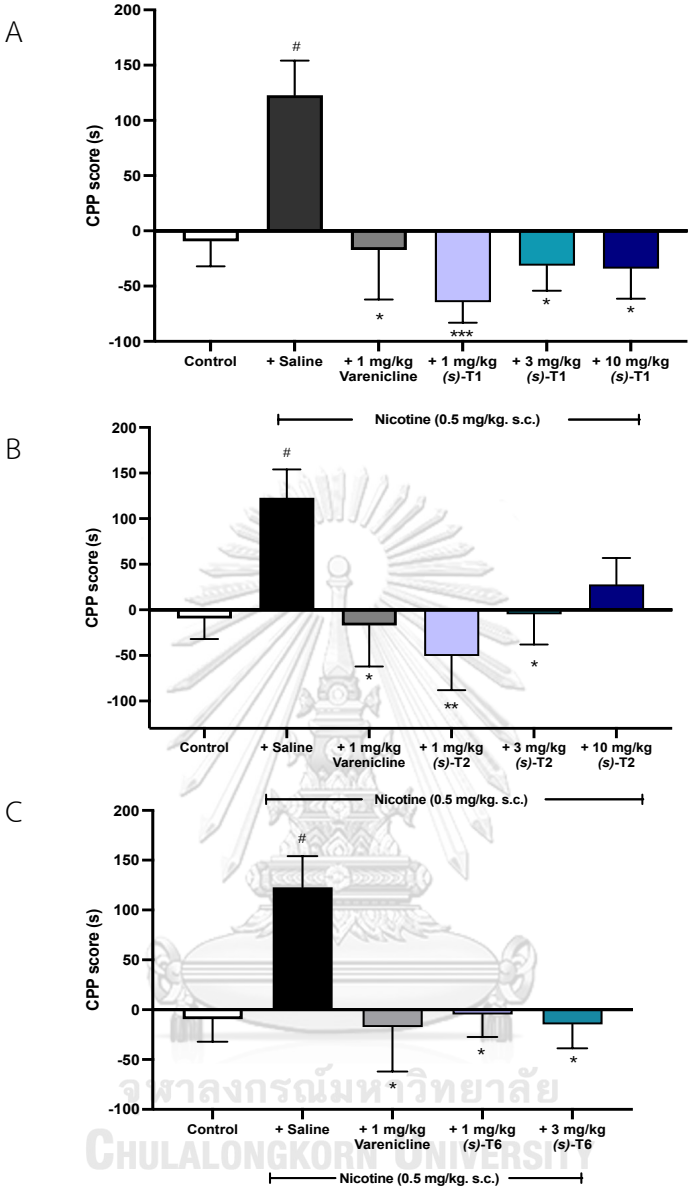


Figure 18 Effect of *(s)*-T1, *(s)*-T2, and *(s)*-T6 treatment in nicotine-induced conditioned place preference model. *(s)*-T1 (1, 3, or 10 mg/kg, s.c.), *(s)*-T2 (1, 3, or 10 mg/kg, s.c.), *(s)*-T6 (1 or 3 mg/kg, s.c.), varenicline (1 mg/kg, s.c.) or saline were given 30 min before nicotine (0.5 mg/kg, s.c.) during conditioning phase. Data are presented mean (+ S.E.M.) (N = 6-9/group). CPP score was calculated as time spent in drug-paired chamber in post-conditioning phase minus time spent in drug paired chamber in pre-conditioning phase. $^{\#}P < 0.05$ compared to control group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to nicotine group

4.4 Effect of *(s)*-T1, *(s)*-T2, and *(s)*-T6 plus nicotine on locomotor activity

Mice received either saline, *(s)*-T1, *(s)*-T2, or *(s)*-T6 and saline or nicotine 30 min apart. Then, mice were placed in the locomotor box immediately after the second treatment. Locomotion time were recorded for 30 min. One-way ANOVA revealed the effect of treatment on locomotion time ($F_{9,80}$ = 2.362 $P < 0.05$). However, Dunnett's post-hoc analysis showed no different between other treatment groups compared to nicotine group ($P > 0.05$) (Figure 19).

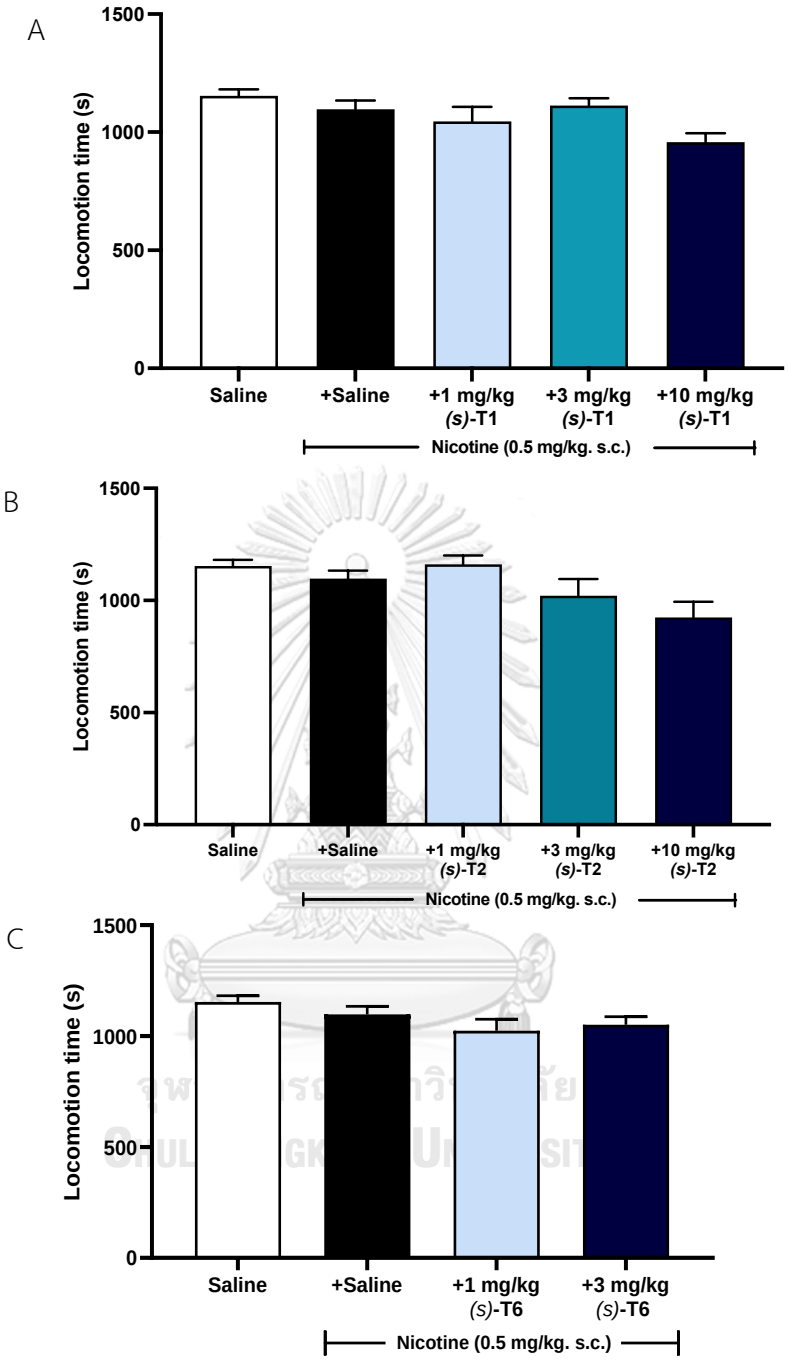


Figure 19 Effect of *(s)*-T1, *(s)*-T2, and *(s)*-T6 plus nicotine on locomotor activity. *(s)*-T1 (A), *(s)*-T2 (B), *(s)*-T6 (C) or saline were given followed by nicotine (0.5 mg/kg, s.c.) or saline 30 min apart. Locomotor activity was performed immediately after the second treatment. Locomotion time was recorded for 30 min. Data are presented as mean (+S.E.M.) (N = 6-9/group)

4.5 Effects of nicotine and nicotine plus varenicline on dopamine and DOPAC levels in the striatum and prefrontal cortex at different time points

To determine the effect of nicotine and nicotine plus varenicline on dopamine and DOPAC levels at different time points, mice received either saline or varenicline (1 mg/kg s.c.) followed by nicotine (0.5 mg/kg s.c.) 30 min later. Then, mice were cervical dislocated at 20, 40, and 60 min after nicotine treatment. The striatum and prefrontal cortex (PFC) were collected for analysis of dopamine and DOPAC levels using LC/MS/MS.

Two-way ANOVA showed no effect of time, treatment and time x treatment interaction on dopamine levels in the striatum after nicotine treatment ($F_{2, 33}$ = 0.252, $P > 0.05$, $F_{2, 33}$ = 1.397, $P > 0.05$, and $F_{4, 33}$ = 0.9644, $P > 0.05$, respectively). Tukey's post-hoc test showed that at 40 min, nicotine significantly increased dopamine levels compared to control ($P < 0.05$). In contrast, varenicline significantly reduced dopamine levels compared to nicotine-treated alone ($P < 0.05$) (Figure 20A).

Two-way ANOVA showed no effect of time, treatment and time x treatment interaction on DOPAC levels in the striatum after nicotine treatment ($F_{2, 27}$ = 1.112, $P > 0.05$, $F_{2, 27}$ = 8.242, $P > 0.05$, and $F_{4, 27}$ = 1.824, $P > 0.05$, respectively). Tukey's post-hoc test showed that at 60 min, nicotine significantly increased DOPAC levels compared to control ($P < 0.05$) (Figure 20B).

Two-way ANOVA revealed the effect of treatment and time on dopamine levels in PFC after nicotine treatment ($F_{2, 32}$ = 5.138, $P < 0.05$ and $F_{2, 32}$ = 3.783, $P <$

0.05, respectively) (Figure 21A). Turkey's post-hoc test showed that at 40 min, nicotine significantly increased dopamine levels compared to control ($P < 0.05$). In contrast, varenicline significantly reduced dopamine levels compared to nicotine-treated alone ($P < 0.05$) (Figure 21A).

Two-way ANOVA revealed the effect of treatment on DOPAC levels in PFC after nicotine treatment ($F_{2, 32} = 7.117$, $P < 0.05$) (Figure 21B). Tukey's post-hoc test showed that at 20 and 40 min, nicotine significantly increased DOPAC levels compared to control ($P < 0.05$). In contrast, varenicline significantly reduced DOPAC levels compared to nicotine-treated alone at 20 and 40 min ($P < 0.05$) (Figure 21B).

As nicotine significantly increased dopamine level in the striatum and PFC after 40 min administration, this time point was used to further investigation of *(s)*-T1, *(s)*-T2 and *(s)*-T6 plus nicotine effects on dopamine and DOPAC levels in the nucleus accumbens, striatum, hippocampus and prefrontal cortex.

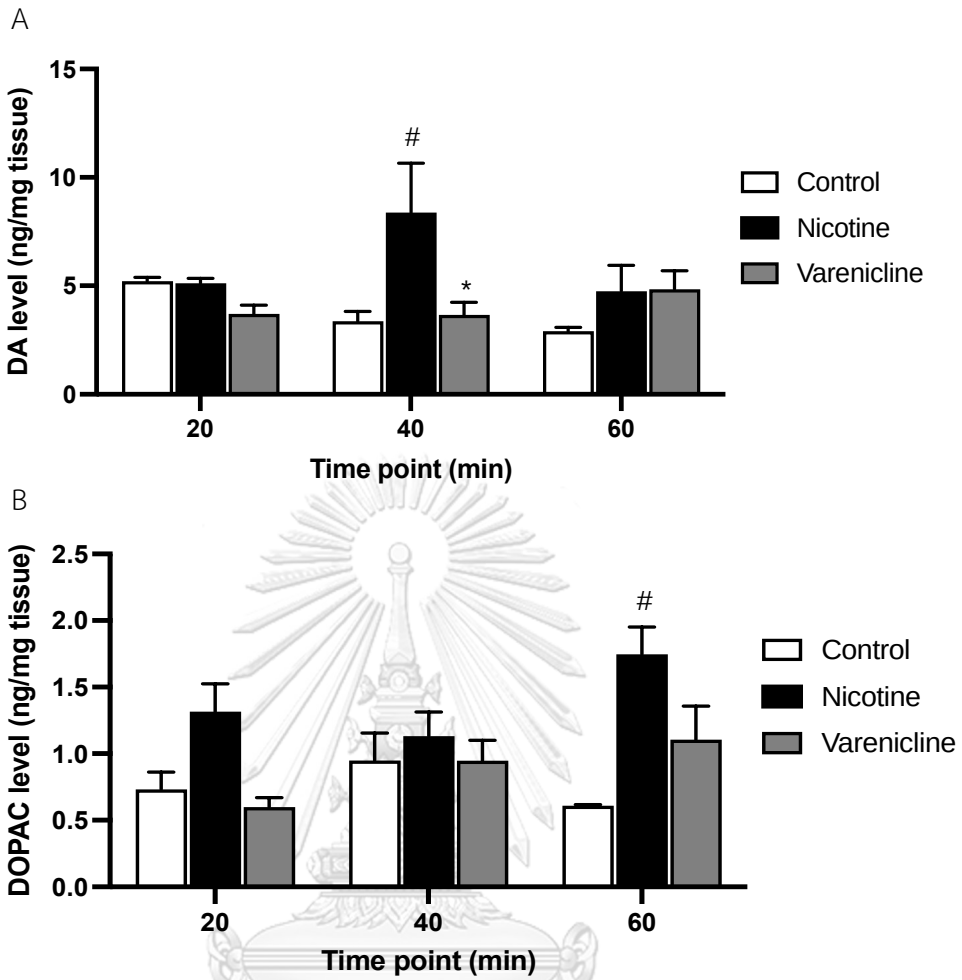


Figure 20 Effect of nicotine and nicotine plus varenicline on dopamine and DOPAC levels in the striatum. Varenicline (1 mg/kg s.c.) or saline were given followed by nicotine (0.5 mg/kg, s.c.) or saline 30 min apart. The striatum was dissected immediately after the second treatment at 20, 40, and 60 min. Dopamine (A) and DOPAC (B) levels were determined using LC/MS/MS. Data are presented as mean (+S.E.M.) (N = 3-9/group). $^{\#}P < 0.05$ compared to control group, $^{*}P < 0.05$ compared to nicotine group.

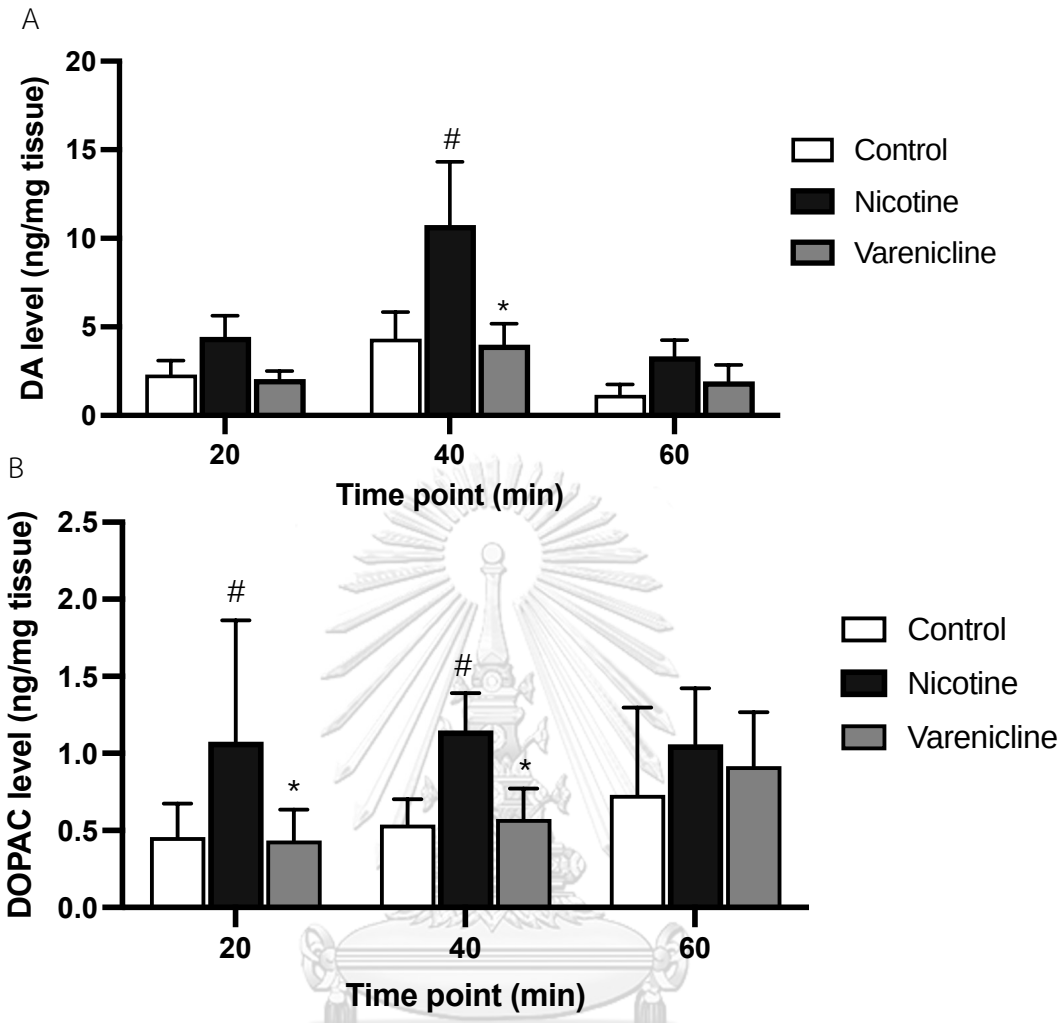


Figure 21 Effect of nicotine and nicotine plus varenicline on dopamine and DOPAC levels in the prefrontal cortex. Varenicline (1 mg/kg s.c.) or saline were given followed by nicotine (0.5 mg/kg, s.c.) or saline 30 min apart. The prefrontal cortex was dissected immediately after the second treatment at 20, 40, and 60 min. Dopamine (A) and DOPAC (B) levels were determined using LC/MS/MS. Data are presented as mean (+S.E.M.) (N = 3-7/group). $^{\#}P < 0.05$ compared to control group, $^{*}P < 0.05$ compared to nicotine group.

4.6 Effect of *(s)*-T1, *(s)*-T2, and *(s)*-T6 plus nicotine on dopamine and DOPAC levels in the nucleus accumbens

One-way ANOVA showed no effect of treatment on dopamine levels ($F_{10,41}$ = 1.978, P > 0.05) (Figure 22) and DOPAC levels in NAc ($F_{10,44}$ = 0.4645, P > 0.05) (Figure 23).

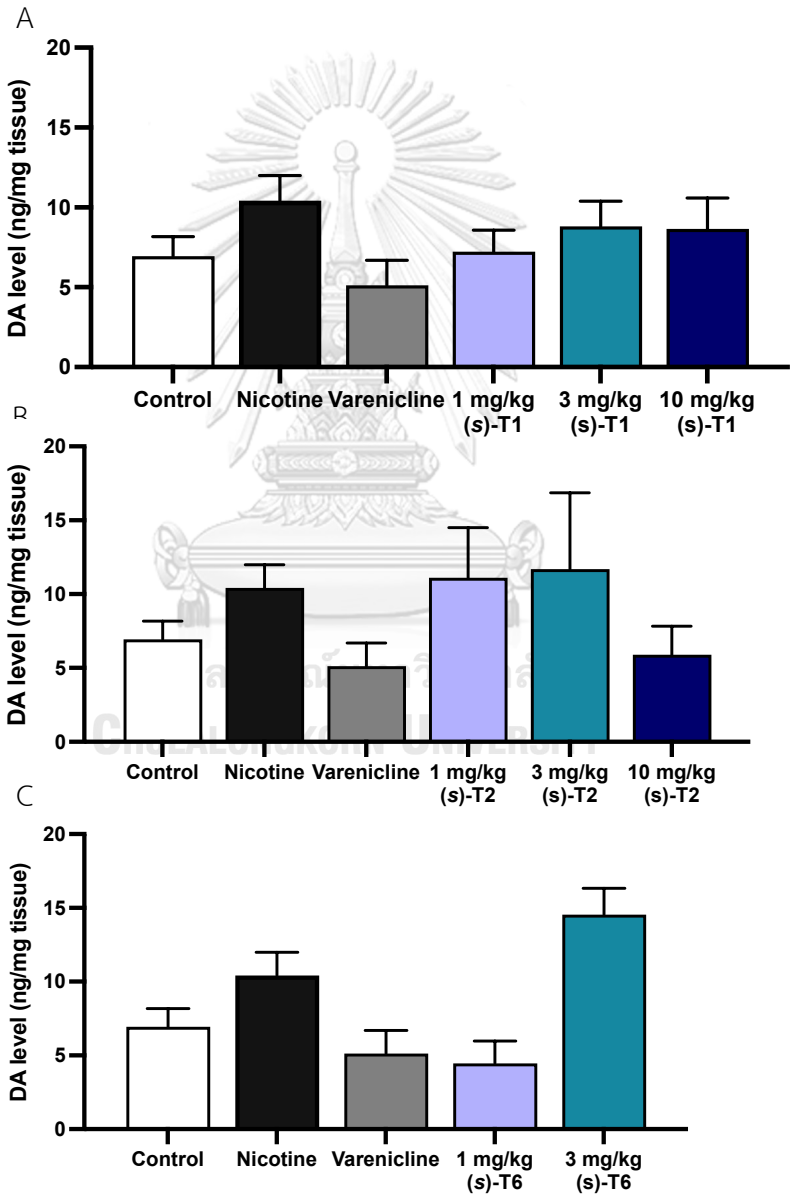


Figure 22 Effect of *(s)*-T1 (A), *(s)*-T2 (B), and *(s)*-T6 (C) plus nicotine on dopamine levels in NAc. *(s)*-T1, *(s)*-T2, *(s)*-T6 or saline were given followed by nicotine (0.5 mg/kg, s.c.) or saline 30 min apart. Mouse brain was dissected 40 min after the second treatment. Data are presented as mean (+S.E.M.) (N = 3-5/group).

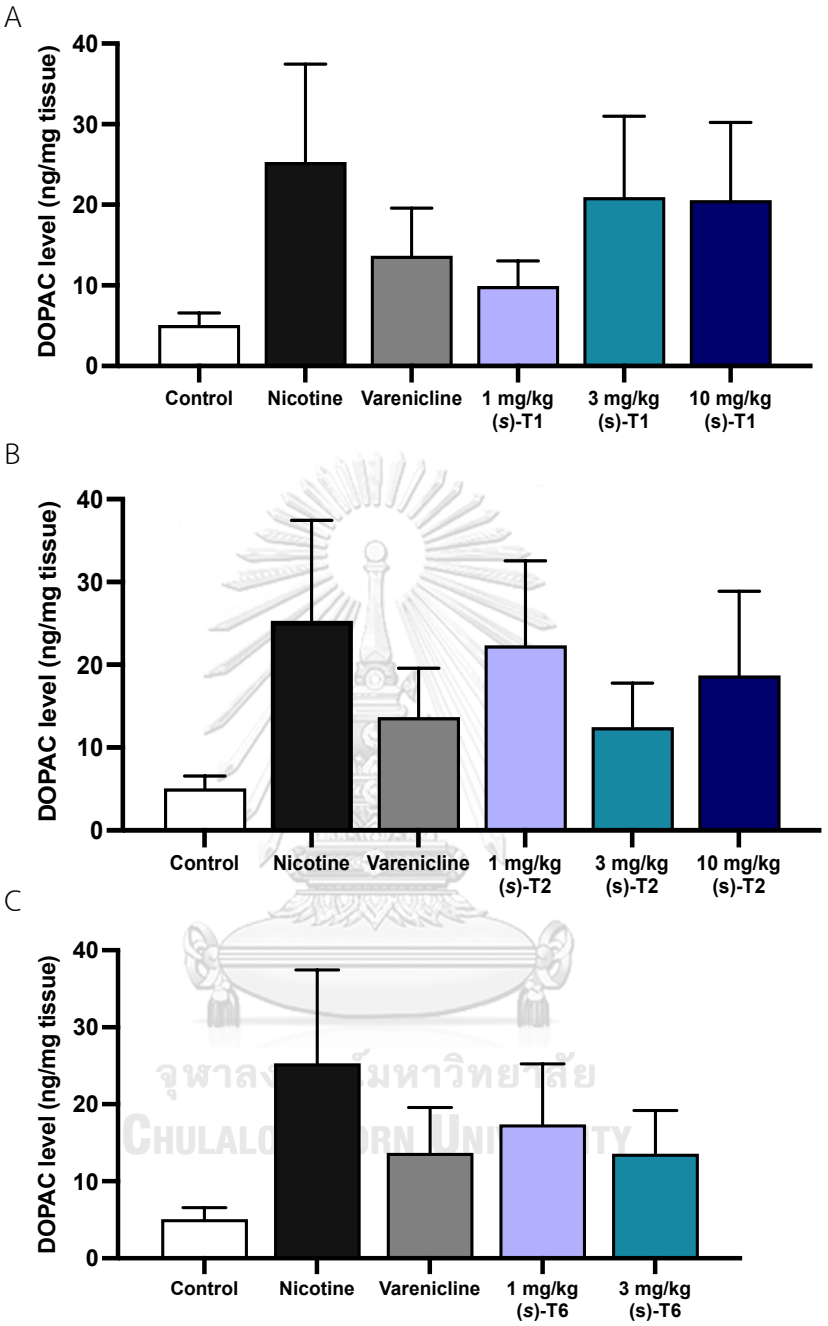


Figure 23. Effect of *(s)*-T1 (A), *(s)*-T2 (B), and *(s)*-T6 (C) plus nicotine on DOPAC levels in NAc. *(s)*-T1, *(s)*-T2, *(s)*-T6 or saline were given followed by nicotine (0.5 mg/kg, s.c.) or saline 30 min apart. Mouse brain was dissected 40 min after the second treatment. Data are presented as mean (+S.E.M.) (n = 5/group).

4.7 Effect of *(s)*-T1, *(s)*-T2, and *(s)*-T6 plus nicotine on dopamine and DOPAC levels in the striatum

One-way ANOVA showed effect of treatment on dopamine levels in the striatum ($F_{10,\ 71}$ = 2.015, P < 0.05) (Figure 24). Nicotine treatment significantly increased dopamine levels compared to control (P < 0.05). In contrast, varenicline, *(s)*-T1 (1, 3 and 10 mg/kg), *(s)*-T2 (3 and 10 mg/kg), and *(s)*-T6 (1 and 3 mg/kg) significantly reduced dopamine levels compared to nicotine treatment alone (P < 0.05) (Figure 24A-C).

One-way ANOVA showed no effect of *(s)*-T1, *(s)*-T2 and *(s)*-T6 on DOPAC levels in the striatum ($F_{10, 54}$ = 1.471, P > 0.05) (Figure 25).

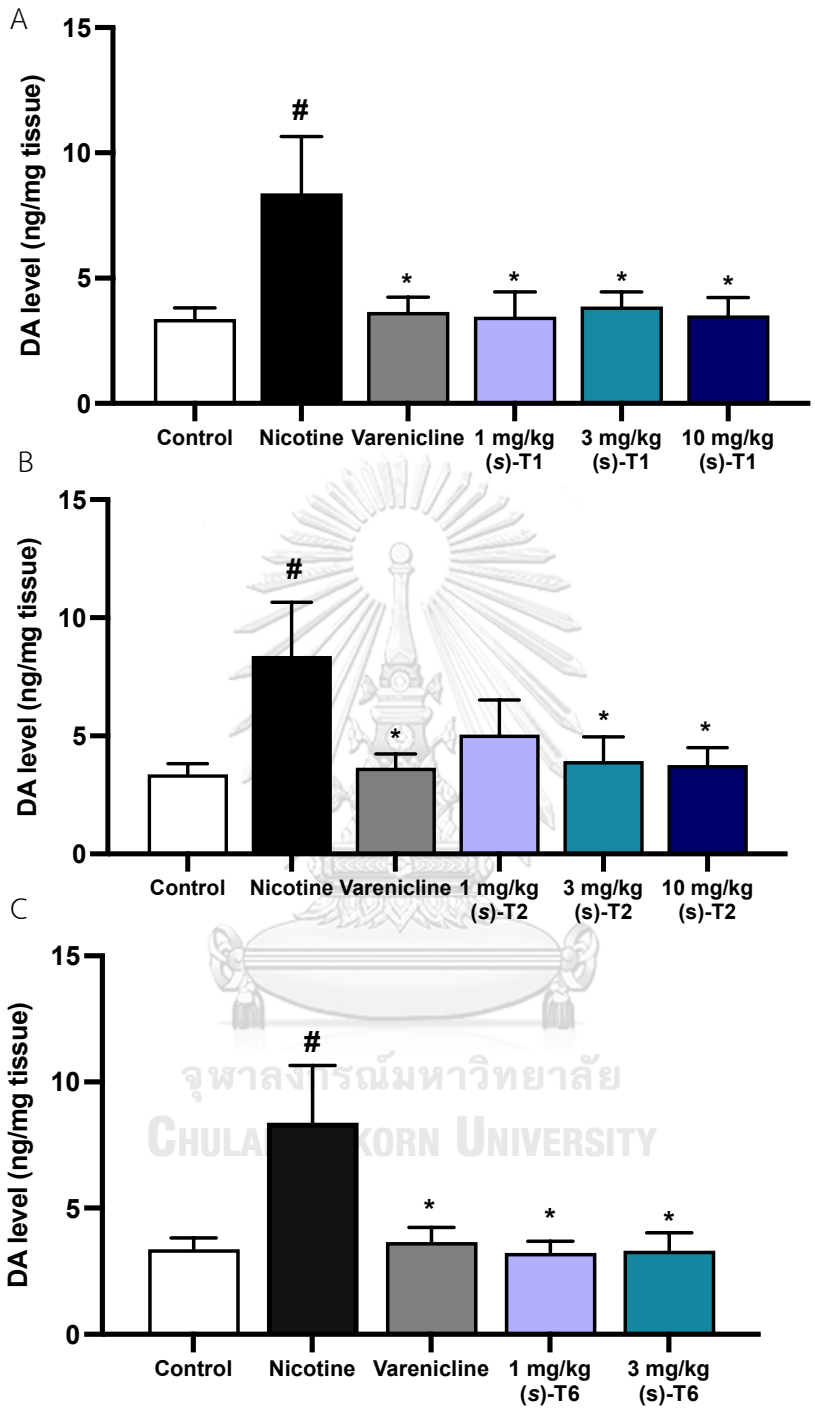


Figure 24 Effect of *(s)*-T1 (A), *(s)*-T2 (B), and *(s)*-T6 (C) plus nicotine on dopamine levels in the striatum. *(s)*-T1, *(s)*-T2, *(s)*-T6 or saline were given followed by nicotine (0.5 mg/kg, s.c.) or saline 30 min apart. Mouse brain was dissected 40 min after the second treatment. Data are presented as mean (+S.E.M.) (N = 6-9/group). $^{\#}P < 0.05$ compared to control group, $^{*}P < 0.05$ compared to nicotine group.

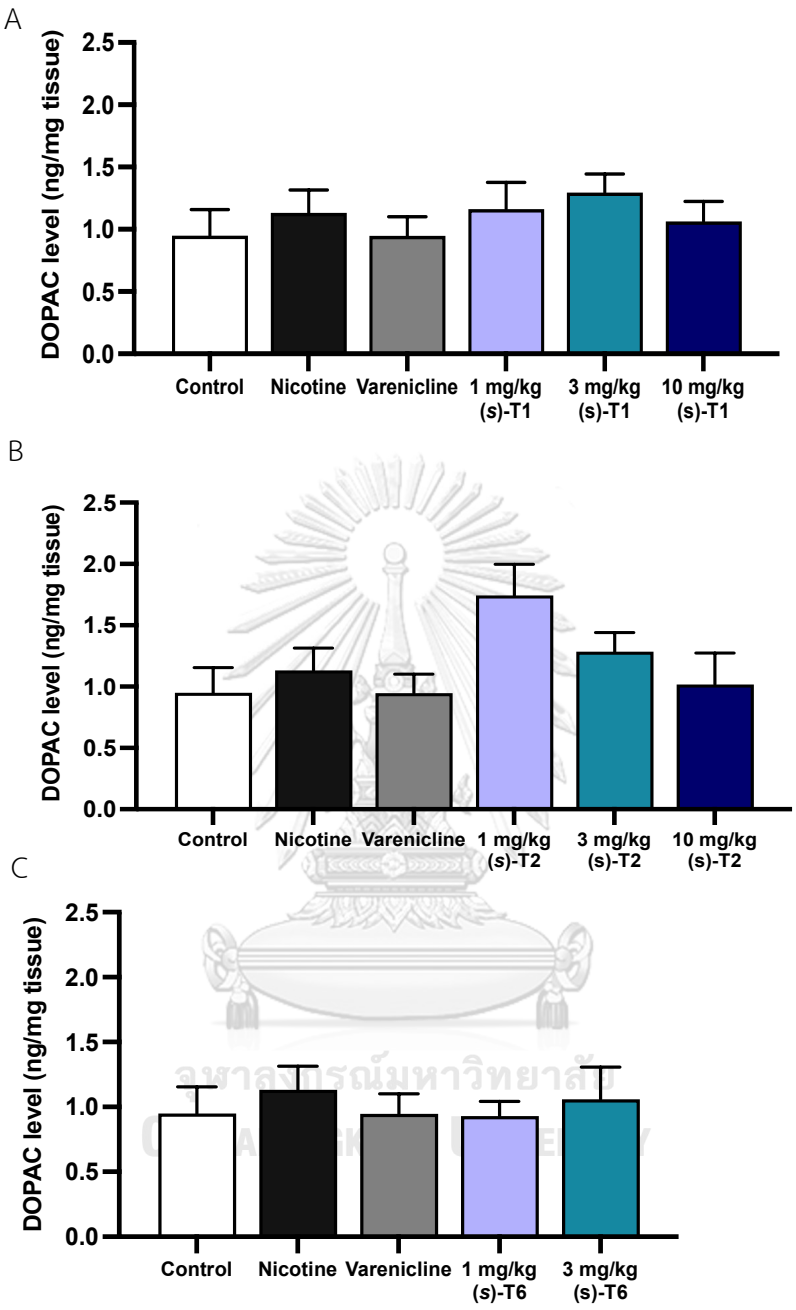


Figure 25 Effect of *(s)*-T1 (A), *(s)*-T2 (B), and *(s)*-T6 (C) plus nicotine on DOPAC levels in the striatum. *(s)*-T1, *(s)*-T2, *(s)*-T6 or saline were given followed by nicotine (0.5 mg/kg, s.c.) or saline 30 min apart. Mouse brain was dissected 40 min after the second treatment. Data are presented as mean (+S.E.M.) (N = 5-6/group).

4.8 Effect of *(s)*-T1, *(s)*-T2, and *(s)*-T6 plus nicotine on dopamine and DOPAC levels in the hippocampus

One-way ANOVA showed no effect of treatment on dopamine ($F_{10, 33}$ = 1.334, P > 0.05) (Figure 26) and DOPAC levels ($F_{10, 33}$ = 0.1761, P > 0.05) (Figure 27) in the hippocampus.

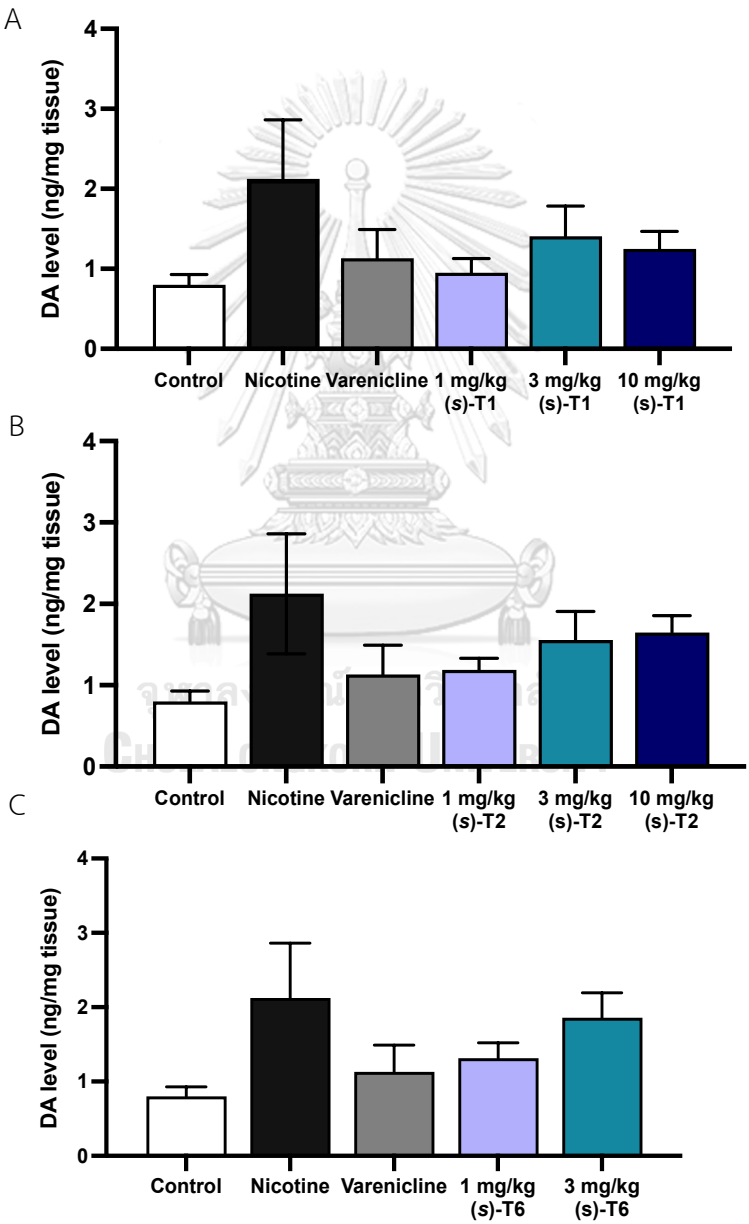


Figure 26 Effect of *(s)*-T1 (A), *(s)*-T2 (B), and *(s)*-T6 (C) plus nicotine on dopamine levels in the hippocampus. *(s)*-T1, *(s)*-T2, *(s)*-T6 or saline were given followed by nicotine (0.5 mg/kg, s.c.) or saline 30 min apart. Mouse brain was dissected 40 min after the second treatment. Data are presented as mean (+S.E.M.) (N = 4/group)

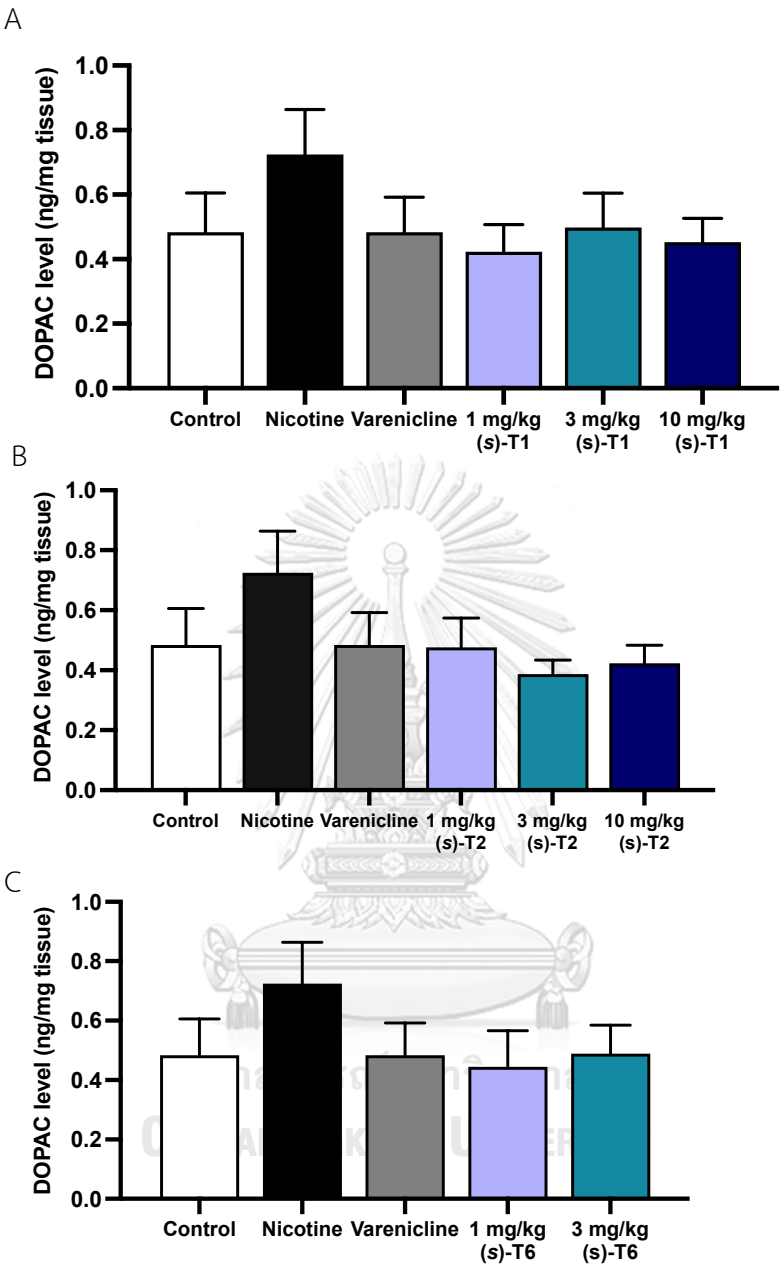


Figure 27 Effect of *(s)*-T1 (A), *(s)*-T2 (B), and *(s)*-T6 (C) plus nicotine on DOPAC levels in the hippocampus. *(s)*-T1, *(s)*-T2, *(s)*-T6 or saline were given followed by nicotine (0.5 mg/kg, s.c.) or saline 30 min apart. Mouse brain was dissected 40 min after the second treatment. Data are presented as mean (+S.E.M.) (N = 4/group).

4.9 Effect of *(s)*-T1, *(s)*-T2, and *(s)*-T6 plus nicotine on dopamine and DOPAC levels in the prefrontal cortex

One-way ANOVA showed the effect of treatment on dopamine levels in PFC ($F_{10, 56}$ = 1.553, P < 0.05) (Figure 28). Nicotine tended to increase dopamine levels in PFC, but this effect was not significantly different from control. However, Dunnett post-hoc analysis showed that *(s)*-T1 (3 mg/kg) and *(s)*-T6 (1 and 3 mg/kg) significantly reduced dopamine levels compared to nicotine treatment alone (P < 0.05) (Figure 28A, C).

One-way ANOVA showed no effect of treatment on DOPAC levels in PFC ($F_{10, 49}$ = 1.578, P > 0.05) (Figure 29).

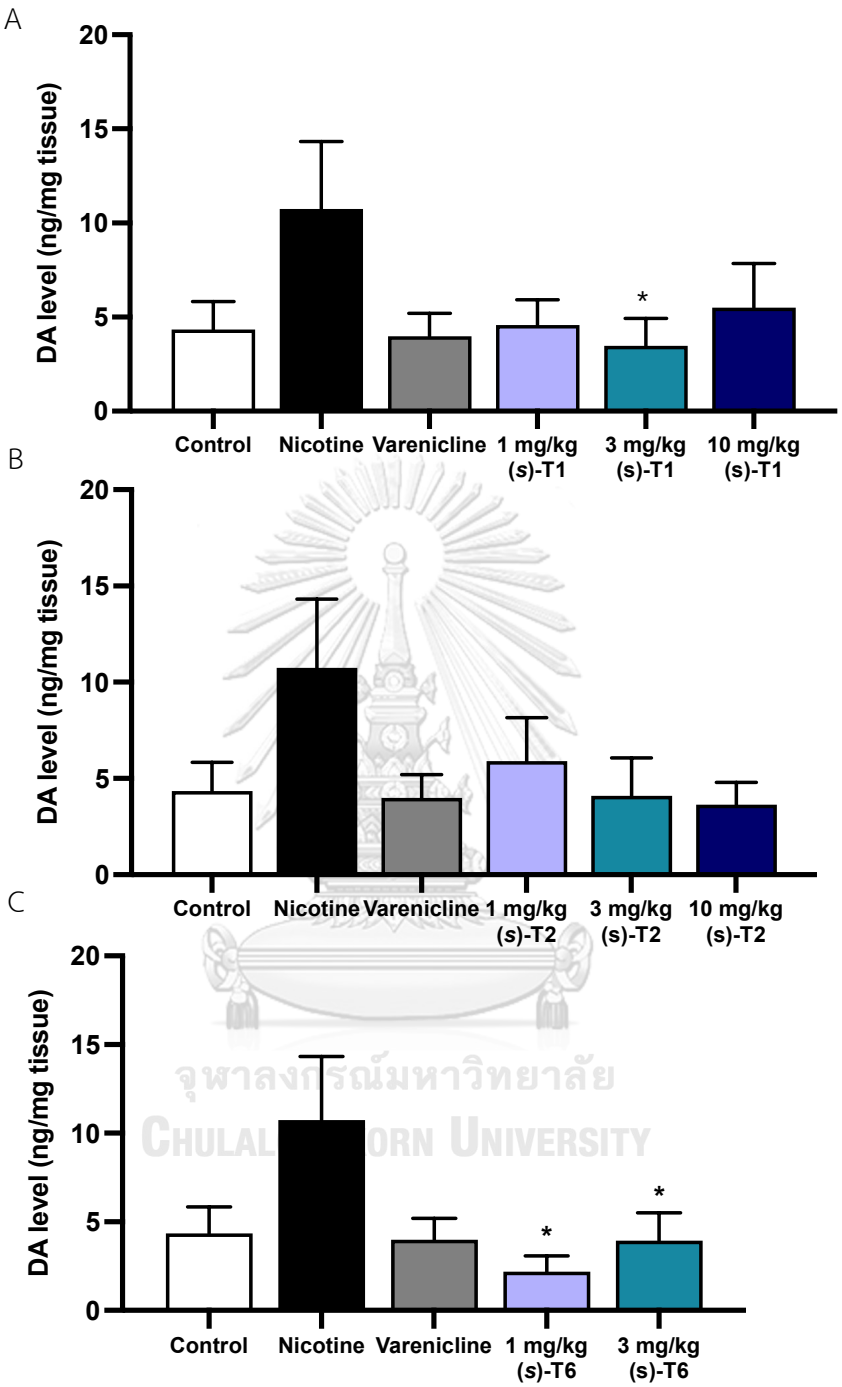


Figure 28 Effect of *(s)*-T1 (A), *(s)*-T2 (B), and *(s)*-T6 (C) plus nicotine on dopamine levels in the prefrontal cortex. *(s)*-T1, *(s)*-T2, *(s)*-T6 or saline were given followed by nicotine (0.5 mg/kg, s.c.) or saline 30 min apart. Mouse brain was dissected 40 min after the second treatment. Data are presented as mean (+S.E.M.) (N = 5-7/group). $^{*}P < 0.05$ compared to nicotine group.

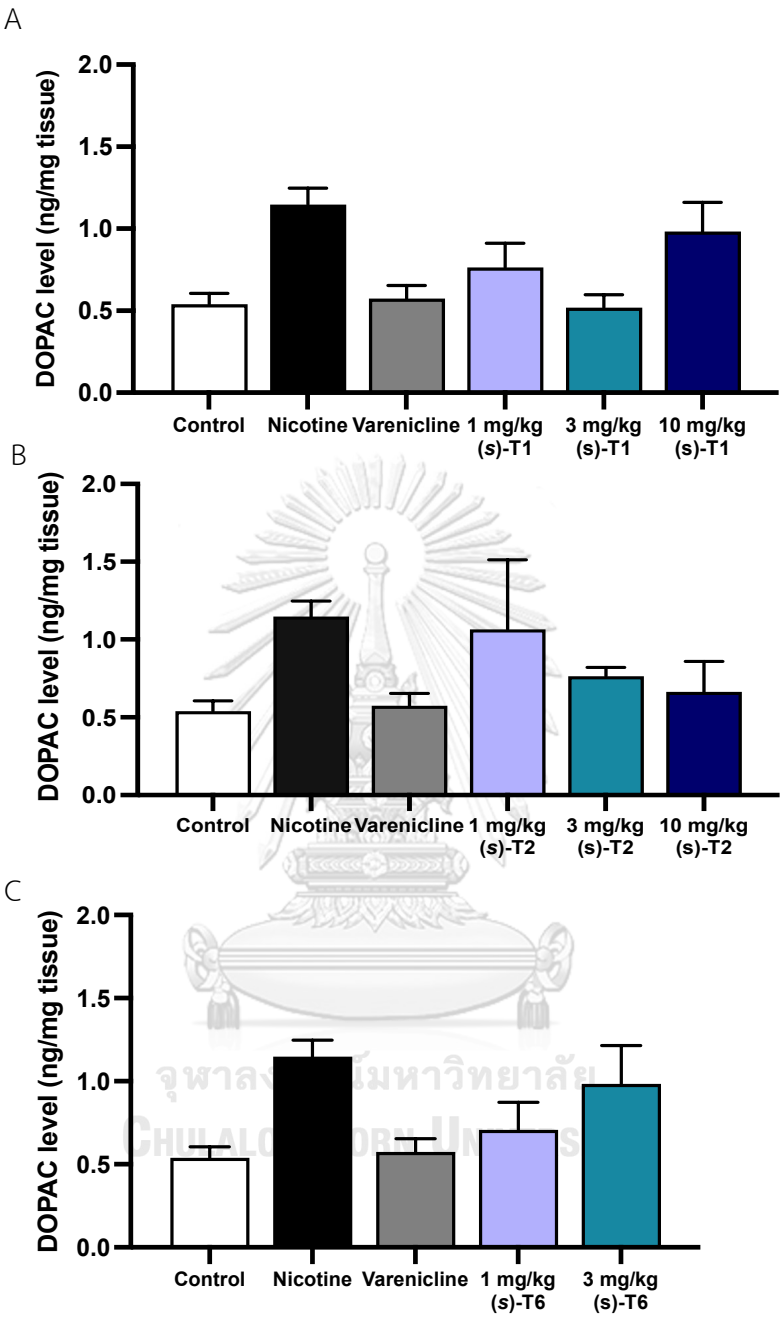


Figure 29 Effect of *(s)*-T1 (A), *(s)*-T2 (B), and *(s)*-T6 (C) plus nicotine on DOPAC levels in the prefrontal cortex. *(s)*-T1, *(s)*-T2, *(s)*-T6 or saline were given followed by nicotine (0.5 mg/kg, s.c.) or saline 30 min apart. Mouse brain was dissected 40 min after the second treatment. Data are presented as mean (+S.E.M.) (N = 5-6/group).

CHAPTER 5
DISCUSSION AND CONCLUSION

The present study aimed to determine the effects of quinuclidine derivatives *(s)*-T1, *(s)*-T2, and *(s)*-T6 on nicotine addiction using the conditioned place preference (CPP) model. The effects of these compounds and nicotine on dopamine and DOPAC levels were also determined in the specific brain areas. Varenicline, an $\alpha 4\beta 2$ nAChR partial agonist, was used as a positive control in this study. The results showed that varenicline as well as *(s)*-T1 (1, 3, and 10 mg/kg), *(s)*-T2 (1 and 3 mg/kg) and *(s)*-T6 (1 and 3 mg/kg) can prevent nicotine-induced CPP. All doses of *(s)*-T1 also inhibit nicotine-induced dopamine elevation in the striatum. In addition, *(s)*-T1 (3 mg/kg) inhibit nicotine-induced dopamine elevation in the prefrontal cortex. *(s)*-T2 (3 and 10 mg/kg) inhibited nicotine-induced dopamine elevation in the striatum. All doses of *(s)*-T6 also inhibited nicotine-induced dopamine elevation in striatum and prefrontal cortex.

Conditioned place preference (CPP) and conditioned place avoidance (CPA) has been used to investigate rewarding and aversive effects of nicotine and quinuclidine derivatives in this study. A significant positive response in time spent in the drug-paired chamber was interpreted as a CPP and reward effect. In contrast, a significant negative response in time spent in the drug-paired chamber was interpreted as a CPA or aversive effect (25, 46). In this study, nicotine (0.5 mg/kg s.c.) induced CPP in mice, indicating the reward effect of nicotine in the dose given which

is in agreement with previous study (51). All doses of *(s)*-T1, *(s)*-T2, and *(s)*-T6 had negative CPP score, indicating that these compounds have no rewarding effect. Previous study showed that high varenicline (2.5 mg/kg) can induce conditioned place aversion (CPA) (52). In this study, the negative CPP scores of *(s)*-T1, *(s)*-T2, and *(s)*-T6-treated mice were not significantly different from control mice, indicating that *(s)*-T1, *(s)*-T2, and *(s)*-T6 did not produce aversive effect.

Varenicline, the $\alpha4\beta2$ nicotinic receptor partial agonist, has been widely used clinically as the first-line therapy for smoking cessation (21). Recently, many compounds, which modulate $\alpha3\beta4$ nicotinic receptors, have been increasingly investigated for their effect on nicotine addiction. AT1001, the $\alpha3\beta4$ nAChR antagonist, dose-dependently blocked nicotine self-administration in rats (5). In addition, Conotoxin TxID, the $\alpha3\beta4$ nAChR antagonist, inhibited nicotine-induced CPP in mice (6). Moreover, 18- methoxycoronaridine (18-MC), the $\alpha3\beta4$ nAChR antagonist, given directly to the MHb can attenuate nicotine self-administration in rats (53). 18-MC also prevented nicotine-induced behavioral sensitization and decreased dopamine level in NAc in nicotine-sensitized mice (54). In the same way, this study showed that the $\alpha3\beta4$ nAChR ligands, *(s)*-T1, *(s)*-T2 and *(s)*-T6 also prevented nicotine-induced CPP in mice.

The agonistic and antagonistic effects of *(s)*-T1, *(s)*-T2 and *(s)*-T6 remains inconclusive. Previous *in vitro* studies using HEK293 cells expressing human $\alpha3\beta4$

nAChR showed that *(s)*-T1 (10 μM) was the $\alpha 3\beta 4$ nAChR agonist, while *(s)*-T2 (10 μM) was the $\alpha 3\beta 4$ nAChR antagonist (7). *(s)*-T6 is the potent ligand of $\alpha 3\beta 4$ nAChR (7) but its agonist or antagonist effect has not been examined. Because all doses of *(s)*-T1 and *(s)*-T6 can prevent nicotine-induced CPP, it is hypothesized that *(s)*-T1 and *(s)*-T6 antagonized $\alpha 3\beta 4$ nAChR in the MhB-IPN pathway resulting in the anti-addictive effects. In contrast, *(s)*-T2 dose-independently inhibit nicotine addiction in CPP model. This result suggested the $\alpha 3\beta 4$ nAChR partial agonist properties of *(s)*-T2 because high dose *(s)*-T2 can activate nAChR and produce positive CPP score (Figure 18). Since the previous *in vitro* study used one concentration of *(s)*-T1 and *(s)*-T2 to conclude their agonistic and antagonistic properties (7), the complete functional assay is needed to confirm agonistic and antagonistic properties of *(s)*-T1, *(s)*-T2, and *(s)*-T6.

Decreases in locomotion time can be interpreted as the results of motor impairment as well as central nervous system depression (55). Nicotine treatment decreased locomotor activity after administration (Figure 16). This is in agreement with previous study showing that nicotine (0.65 mg/kg) induced hypolocomotion (56). *(s)*-T1 and *(s)*-T2 given alone did not affect total locomotor activity (Figure 16) while *(s)*-T6 (10 mg/kg) decreased total locomotor after administration. This dose of (s)-T6 was not used in the other experiments because it induced seizure in some mice. *(s)*-T1, *(s)*-T2, and *(s)*-T6 given with nicotine showed no effect on locomotor activity

(Figure 19). It is noted that the reduced locomotor might affect the exploring activity of the animals during the conditioning phase of CPP.

In the mesocorticolimbic system, dopaminergic neurons are originated from VTA and project their axons to many brain areas including NAc, striatum, medial and orbital prefrontal cortex, and hippocampus (38). The mesocorticolimbic system plays a key role in learning, memory, emotions, and reward (3, 39, 40). Nicotine activates nicotinic receptor in the VTA causing dopamine release in NAc. Previous *in vivo* microdialysis study showed that nicotine (0.4 mg/kg s.c.) significantly increased extracellular dopamine in NAc 20, 40, and 60 min after injection in rats (57). Similarly, this study showed that nicotine (0.5 mg/kg s.c.) increased dopamine levels in the striatum and prefrontal cortex 40 min after administration and varenicline can inhibit dopamine elevation at this timepoints. Therefore, this time point was used for further investigation of the effects of *(s)*-T1, *(s)*-T2 and *(s)*-T6 on dopamine and DOPAC levels in brain areas in the mesocorticolimbic system.

$\alpha_4\beta_2$ nicotinic acetylcholine receptors are abundant in VTA, while $\alpha_3\beta_4$ nicotinic acetylcholine receptors are mainly found in the MHb (58). The activation of $\alpha_4\beta_2$ nAChR in the VTA produced dopamine release in NAc, resulting in rewarding effect (59). Varenicline, the $\alpha_4\beta_2$ nAChR partial agonist, blocked nicotine effect in the VTA, resulting in the reduction of dopamine levels in the NAc. In MHb, the activation of $\alpha_3\beta_4$ nAChR located on the cholinergic neurons caused glutamate and

acetylcholine co-release in the IPN. However, MHb and VTA connection pathway still unclear. Previous study showed that the $\alpha 3\beta 4$ nAChR antagonist 18-MC given directly to the MHb can decrease nicotine-induced dopamine release in the NAc (32). However, this study showed that *(s)*-T1, *(s)*-T2, and *(s)*-T6 showed no effect on nicotine-induced dopamine levels elevation in NAc.

Dopaminergic neurons in the VTA can regulate dopaminergic neurons in the nigrostriatal pathway (60). Thus, the activation of dopaminergic neurons in VTA can also induce dopamine release in the striatum (60). Striatum involves in the association between drug of abuse and environment cue which mediates drug craving (45). In this study, nicotine significantly increased dopamine in the striatum, while varenicline reverse the effect of nicotine. *(s)*-T1, *(s)*-T2, and *(s)*-T6 also blocked nicotine-induced dopamine elevation in the striatum. The results indicated that the anti-addictive effects of *(s)*-T1, *(s)*-T2, and *(s)*-T6 might be mediated by this mechanism.

Hippocampus involves in long-term memory of nicotine rewarding effect (61). The medial prefrontal cortex (mPFC) involves conditioning and learning factors (contextual cue) related nicotine addiction and craving (42-44). *(s)*-T1 and *(s)*-T6 inhibited nicotine-incuded increased in dopamine in PFC but *(s)*-T1, *(s)*-T2 and *(s)*-T6 had no effect on dopamine levels in the hippocampus. The effect of *(s)*-T1 and *(s)*-T6 in the PFC suggested the involvement of conditioned learning related to nicotine

addiction and craving. The effects of *(s)*-T1 and *(s)*-T6 on nicotine abstinence, withdrawal and reinstatement should be further investigated.

The results suggested the antagonistic effect of *(s)*-T1 and *(s)*-T6. Previous study showed that direct injection of mecamylamine, the non-selective nAChRs, into MHb resulted in nicotine withdrawal in mice (62) Thus, $\alpha 3\beta 4$ nAChRs antagonist may lead to withdrawal symptoms and cause unsuccessful smoking cessation. In addition, *(s)*-T6 at 10 mg/kg induced seizure in mice. These effects are the disadvantages of *(s)*-T1 and *(s)*-T6 for further drug development. The potential adverse effects of *(s)*-T1 and *(s)*-T6 should be further investigated. On the other hand, *(s)*-T2 might be a partial agonist to $\alpha 3\beta 4$ nAChRs according to the CPP result. Varenicline, the a4b2 partial agonist, can decrease nicotine craving and withdrawal (52) which promote nicotine abstinence. Thus, *(s)*-T2 has a potential to develop for a new therapy in nicotine addiction.

In conclusion, quinuclidines derivatives, *(s)*-T1, *(s)*-T2, and *(s)*-T6 protect against nicotine-induced conditioned place preference. The mechanisms of action involved the prevention of the increased dopamine levels caused by nicotine in various brain areas associated with cue-association learning of rewarding effect of nicotine.

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Appendix

Animal use certificate

Chulalongkorn University Animal Care and Use Committee

Certificate of Project Approval	☑ Original ☐ Renew
Animal Use Protocol No. 19-33-005	**Approval No. 19-33-005**

Protocol Title
Anti-addictive effect of quinuclidine derivatives in nicotine-induced conditioned place preference

Principal Investigator

Ratchanee RODSIRI, PhD

Certification of Institutional Animal Care and Use Committee (IACUC)

This project has been reviewed and approved by the IACUC in accordance with university regulations and policies governing the care and use of laboratory animals. The review has followed guidelines documented in Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes edited by the National Research Council of Thailand.

Date of Approval	**Date of Expiration**
April 1, 2019	March 31, 2021

Applicant Faculty/Institution
Faculty of Pharmaceutical Sciences, Chulalongkorn University, Phyathai Road., Pathumwan
BKK-THAILAND. 10330

Signature of Chairperson	**Signature of Authorized Official**
Name and Title **THONGCHAI SOOKSAWATE, Ph.D.** **Chairman**	Name and Title **PORNCHAI ROJSITTHISAK, Ph.D.** **Associate Dean (Research and Academic Service)**

The official signing above certifies that the information provided on this form is correct. The institution assumes that investigators will take responsibility, and follow university regulations and policies for the care and use of animals.

This approval is subjected to assurance given in the animal use protocol and may be required for future investigations and reviews.

Chulalongkorn University Animal Care and Use Committee

Certificate of Project Approval	☑ Original ☐ Renew
Animal Use Protocol No. 21-33-004	**Approval No. 21-33-004**

Protocol Title
Effect of quinuclidine derivatives on dopamine levels in the prefrontal cortex, hippocampus and striatum

Principal Investigator

Ratchanee RODSIRI, Ph.D.

Certification of Institutional Animal Care and Use Committee (IACUC)

This project has been reviewed and approved by the IACUC in accordance with university regulations and policies governing the care and use of laboratory animals. The review has followed guidelines documented in Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes edited by the National Research Council of Thailand.

Date of Approval	**Date of Expiration**
June 2, 2021	June 1, 2023

Applicant Faculty/Institution
Faculty of Pharmaceutical Sciences, Chulalongkorn University, Phyathai Road., Pathumwan
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Signature of Chairperson	**Signature of Authorized Official**
Name and Title **THONGCHAI SOOKSAWATE, Ph.D.** **Chairman**	SIGN HER Name and Title **PORNCHAI ROJSITTHISAK, Ph.D.** **Associate Dean (Research Affairs)**

The official signing above certifies that the information provided on this form is correct. The institution assumes that investigators will take responsibility, and follow university regulations and policies for the care and use of animals.

This approval is subjected to assurance given in the animal use protocol and may be required for future investigations and reviews.

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