# 学位論文

**Is milnacipran a promising agent to suppress impulsive behavior?**

**（ミルナシプランは有望な衝動性抑制薬であるか？）**

**２０１４年３月**

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# Contents

# Lists of Presented Papers and Conference Presentation

## A part of the present study was published in the papers below:

1. **Tsutsui-Kimura I**, Ohmura Y, Izumi T, Kumamoto H, Yamaguchi T, Yoshida T, Yoshioka M. Milnacipran enhances the control of impulsive action by activating $D_1$-like receptors in the infralimbic cortex. Psychopharmacology (Berl). 2013 Jan;225(2):495-504.

2. **Tsutsui-Kimura I**, Yoshida T, Ohmura Y, Izumi T, Yoshioka M. Repeated Milnacipran Administration Remediates Elevated Impulsive Action in Rats with Lesions of the Ventromedial Prefrontal Cortex. Journal of Neuroscience, submitted (JN-RM-4637-13).

## A part of the present study was presented at the conferences below:

1. **Tsutsui-Kimura I**, Ohmura Y, Izumi T, Kumamoto H, Yamaguchi T, Yoshida T, Yoshioka M. Milnacipran Enhances Inhibitory Control of Impulsive Action through Stimulating D1-like receptors in the Infralimbic Cortex. 第 85 回日本薬理学会年会, Mar. 14th－16th, 2012, Kyoto.

2. **Tsutsui-Kimura I**, Yoshida T, Ohmura Y, Izumi T, Yoshioka M. Chronic Milnacipran Remedies Rats with Elevated Impulsive Action via Reconstructing the Dendritic Spines and Excitatory Currents in the Ventromedial Prefrontal Cortex. 第 23 回 日本臨床精神薬理学会／第 43 回 日本神経精神薬理学会 合同年会, Oct. 24th－26th, 2013, Okinawa.

# CHAPTER 1

# General Introduction

## Introduction

Impulsive behavior is broadly defined as “actions that are poorly conceived, prematurely expressed, unduly risky, or inappropriate to the stimulation and that often result in undesirable outcomes”[1]. An action that is prematurely expressed is one of the simplest forms of impulsive behavior, and I focused on this type of impulsive behavior, often referred as “impulsive action”.

***Is impulsivity evil?*** Impulsive behavior can be viewed as everyday normal behavior (*e.g.,* start walking during red light at an intersection, impulsive shopping, *etc.*). Normal levels of impulsivity occasionally increase the probability of success. It is depending on the circumstancies whether normal impulsivity resuts in positive or negative outcomes[2]. However, as described later, many studies have suggested that abnormally high levels of impulsivity is defined as one of core symptoms in attention-deficit hyperactivity disorder, borderline personality disorder, bipolar disorder, mania, and substance abuse (Diagnostic and Statistical Manual of Mental Disorders-IV)[3] and also defined as one of peripheral symptoms in schizophrenia, major depression, and anxiety disorder[4]. Moreover, elevated impulsivity in mood disorder patients increases a risk of suicidal behavior[5-7]. Nevertheless, only a few treatments, amphetamine, methylphenidate, and atomoxetine have been approved as therapeutic agents for suppressing elevated impulsivity[8, 9]. Therefore, developing novel therapeutic agents for disorders characterized by excessive levels of impulsivity are strongly needed.

In this chapter, I first introduce two operant tasks assessing rodent impulsive action, the 5-choice serial reaction time task and the 3-choice serial reaction time task, which I previously developed[10]. Second, I will refer to the recent works examining the effects of psychoactive drugs on impulsive action. Finally, I will introduce the animal models currently developed for screening the therapeutic agents for treating elevated impulsivity.

## Abbreviations

| | |
|---|---|
| ADHD | attention-deficit hyperactivity disorder |
| 3-CSRTT | 3-choice serial reaction time task |
| 5-CSRTT | 5-choice serial reaction time task |
| CPP | 3-(R)-2-carboxypiperazine-4-propyl-l-phosphonic acid |
| DSM-IV | Diagnostic and Statistical Manual of Mental Disorders-IV |
| mPFC | medial prefrontal cortex |
| NAc | nucleus accumbens |
| NMDA | N-methyl-D-aspartate |
| PCP | phencyclidine |
| PFC | prefrontal cortex |
| SNRI | serotonin-noradrenaline reuptake inhibitor |
| SSRI | selective serotonin reuptake inhibitor |
| vmPFC | ventromedial prefrontal cortex |

## Impulsive action in rodents

Robbins and his colleagues had designed the 5-choice serial reaction time task (5-CSRTT)[11, 12] based on human 5-Choice Test of Serial Reaction[13]. The 5-CSRTT has been originally developed to measure attentional performance and neural mechanisms underlying the cognitive function in rats. This task requires rodents to make nose poke into 1 of 5 target holes that is illuminated briefly (less than 1 s) and pseudo randomly. Nose poke before the presentation of the light stimulus is recognized as impulsive action. The 5-CSRTT is superior in terms of capability of simultaneously measuring impulsive action, attentional function, motor activity, and appetite/motivation. The 5-CSRTT allows investigating the neural mechanisms of impulsive action and the roles of central neurotransmitter systems underlying the effects of drugs on impulsive action[12, 14], and screening agents that could enhance control of impulsive action.

Although the 5-CSRTT is useful, it takes a long time to train the animals involved[15, 16]. I had speculated that the number of target holes is a critical factor that determines the time required for the completion of training because the more the number of target holes, the more spatial attention is required. Therefore, I established a 3-choice serial reaction time task (3-CSRTT) by decreasing the number of target holes from 5 to 3[10]. I successfully saved the training time at least for 4 weeks using the 3-CSRTT. I also evaluated the pharmacological validity of the 3-CSRTT as an appropriate assessment method for impulsive action using nicotine, which is well characterized to provoke impulsive-like action in the 5-CSRTT[17, 18], and atomoxetine, which is well characterized to suppress impulsive action in the 5-CSRTT[19, 20]. The 3-CSRTT is a simpler preclinical model of impulsive action and contributes to developing a new therapeutic agent for psychiatric disorders associated with higher impulsivity.

## The neural basis of impulsive action

One of most widely accepted theories of neural mechanisms that underlie impulsive action is associated with the fronto-striatal system: impulsive action is mediated by cortico-accumbal interactions[21-23]. Nucleus accumbal dopamine is part of the neural circuit that is thought to stimulate impulsive action[24, 25]. On the

other hand, there is a growing body of evidence that psychiatric patients with higher impulsivity commonly exhibit impairments of the prefrontal cortex (PFC)[26-29]. The rat medial prefrontal cortex (mPFC) is comparable to the human PFC in terms of structural and functional characteristics[30]. More precisely, Chudasama *et al.*[31] found that lesions of the ventral part of the mPFC (ventromedial prefrontal cortex: vmPFC) selectively elevate impulsive action in rats. I also demonstrated that nicotine evokes impulsive action via stimulating $\alpha 4\beta 2$ nicotinic acetylcholine receptors in the vmPFC, but not in the dorsal part of the mPFC[32]. It has been reported that dopamine release in the mPFC has a role in suppressing impulsive behavior in rats[33, 34]. Indeed, most drugs suppressing impulsive behavior (*e.g.,* noradrenaline reuptake inhibitors and atypical antipsychotics; see **Therapeutic agents for elevated impulsivity**) stimulate dopamine release in the mPFC in rats[35, 36]. ***Nevertheless, there is no direct evidence that drugs suppress impulsive behavior via stimulating dopamine system in the mPFC. In addition, the subtypes of dopamine receptors ($D_1$-$D_5$) involved in the suppressing effect of drugs on impulsive behavior have not been revealed.***

## Therapeutic agents for elevated impulsivity

For decades, the stimulant medications methylphenidate, and mixed amphetamine salts (not approved in Japan) have been the most common drugs used in the treatment of attention-deficit hyperactivity disorder (ADHD), a disorder usually first diagnosed in childhood, and categorized into 3 types: Predominantly Inattentive Types, Predominantly Hyperactive-Impulsive Type, and Combined Type in Diagnostic and Statistical Manual of Mental Disorders-IV (DSM-IV)[3]. The stimulants ameliorate deficits in impulse control in ADHD patients[37, 38]. The non-stimulant atomoxetine was introduced in the United States in 2002. It is a selective noradrenaline reuptake inhibitor also ameliorates deficits in impulse control in ADHD patients[39, 40] and in laboratory rodents[10, 19]. However, elevated impulsivity is a problematic symptom observed not only in ADHD but also in borderline personality disorder, bipolar disorder, mania, substance abuse, schizophrenia, major depression, and anxiety disorder[4]. Therefore, a significant issue concerns whether other psychoactive drugs could enhance the control of impulsivity. Here I introduce the recent efforts determining the effects of mood

stabilizers, antipsychotics, antidepressants, anxiolytics, and non-clinical drugs on impulsive action below.

*Mood stabilizers*

It is difficult to meet criteria without higher impulsivity in bipolar disorder in DSM-IV[3]. Higher impulsivity in the bipolar patients is a significant matter because the level of impulsivity is proportional to the risk of suicide in bipolar patients[6, 7]. Therefore, it is an important issue whether the mood stabilizers, the medications for bipolar disorder, could suppress impulsive action. My collegues recently showed that lithium administration suppressed impulsive action independent of the anorexic effect in rats[41] whereas valproic acid and carbamazepine did not affect impulsive action[41, 42]. A previous animal study showed that lithium administration significantly decreased dopamine release in the nucleus accumbens while mildly increasing dopamine release in the mPFC. This effect, however, did not reach a statistical significance[43].

*Antipsychotics*

Although impulsivity is not diagnostic criterion, impulsive behavior is characteristic of schizophrenic patients[44, 45]. There are several preclinical studies comparing the effects of classical (first generation) and atypical (second generation) antipsychotics on impulsive action. The classical antipsychotic haloperidol, a dopamine $D_2$ receptor antagonist, did not suppress impulsive action in normal rodents, but it suppressed elevated impulsive action in animal models of schizophrenia[46] (detailed in **The animal models of elevated impulsive action**) though another researcher denied the effects of haloperidol on the elevated impulsive action[47]. The atypical antipsychotic clozapine, a $D_2$ and serotonin$_{2A}$ receptor antagonist, shows higher affinity for serotonin$_{2A}$ than $D_2$ receptor and suppress impulsive action in both normal rodents and animal models[46-48]. Moreover, disruptions of inhibitory control of impulsive action induced by repeated phencyclidine administration are prevented by chronic clozapine treatment[47]. Thus, compared to haloperidol, the anti-impulsive effects of cloxzapine were consistent in previous studies. A previous study demonstrated that acute clozapine produced greater increases in extracellular dopamine levels in the mPFC than in the NAc whereas acute haloperidol significantly increased extracellular dopamine levels in the NAc but not in the mPFC[49]. These dissociable neurochemical characteristics between haloperidol

and clozapine could be partly attributable to different anti-impulsive effects. Aripiprazole is a new antipsychotic drug with a partial agonistic action at $D_2$ and serotonin$_{1A}$ receptors and antagonistic action at serotonin$_{2A}$ receptor[50, 51]. The suppressive effect of aripiprazole on impulsive action was not as well as that of clozapine, while attentional function was improved by aripiprazole[48].

*Antidepressants*

Impaired control of impulsivity is often observed in major depressive disorder patients[52, 53]. Recent prospective studies demonstrated that higher impulsivity increase suicide risk in depressed patients[6, 7], underscoring the need for addressing the effect of antidepressants on impulsive action. A Tricyclic antidepressant desipramine, which inhibits the reuptake of noradrenaline and to a lesser extent serotonin, suppressed the rodent impulsive action but it was often accompanied by increased ignored response and prolonged latency to collect rewards[18, 54], suggesting that decresed impulsive-like response could be due to impaired motivation or motor activity. Selective serotonin reuptake inhibitors (SSRIs) are widely used as first-line therapy for major depression. The effect of SSRIs on impulsive action is not consistent in the previous studies[10, 55, 56, 57], suggesting that acute effect of SSRIs on impulsive action is not strong as other impulsivity-suppressing agents. Serotonin-noradrenaline reuptake inhibitors (SNRIs) are newly developed antidepressants. ***I previously found that acute administration of milnacipran suppressed impulsive action in rats without changes in attentional, motor, motivational functions[10]. The suppressing effects of milnacipran is worth noting because this is the only drug has both anti-impulsive and anti-depressive effects at this time.*** Acute administration of Tricyclic antidepressants and SNRIs stimulates dopamine release in the mPFC[58-60] while acute SSRIs increase dopamine release both in the mPFC and NAc[61, 62].

*Anxiolytics*

Subjects with anxiety disorders have been reported to be more impulsive than the controls[63, 64]. Unfortunately, diazepam, a typical anxiolytic drug and a benzodiazepine receptor agonist, impairs inhibitory control of impulsive action in human[65] and rodents[66]. However, I recently found that tandospirone, a relatively new anxiolytic and a partial agonist of the serotonin$_{1A}$ receptor, suppresses rodents' impulsive action in a dose-dependent manner[67]. Acute administration of

tandospirone is reported to stimulate dopamine release in the mPFC[68] though the effects of the drug on accumbal dopamine release have not been examined.

*Non-clinical drugs*

I previously found that intracerebroventricular injections of the preferential α4β2 nicotinic acetylcholine receptor antagonist dihydro-β-erythroidine suppresses impulsive action in rodents without affecting attentional, motivational, or motor function[69] presumably via blocking α4β2 nicotinic acetylcholine receptors in the NAc[32]. Y379268, a group II metabotropic glutamate receptor agonist, stimulates dopamine release in the mPFC[70]. Moreover, LY379268 suppressed phencyclidine-induced impulsive behavior in rats[71].

Although these candidate drugs discussed above were promising as new anti-impulsive drugs, the effects of these drugs on elevated impulsive action in animal models remain unknown (except for haloperidol, clozapine, and LY379268). ***Because the effects of drugs on impulsive action in normal animals and animal models are often dissociable[46, 47], it is required to examine the effect of the candidate drugs on impulsive action using animal models exhibiting higher impulsivity. To examine the effects of repeated administration of the candidate drugs on impulsive action is also required because therapeutic agents were generally administered chronically in clinical practice.***

## The animal models of elevated impulsive action

According to the growing awareness of higher impulsivity in psychiatric disorders, two animal models exhibiting higher impulsive action were currently developed. Both animal models show the prefrontal impairment which is commonly observed in psychiatric patients with higher impulsivity.

*Repeated administration of phencyclidine (PCP)*

PCP is a dissociative anesthetic that acts as a noncompetitive antagonist at N-methyl-D-aspartate (NMDA) glutamate receptors. PCP intoxication produces a psychosis-like state similar to that in schizophrenia[72-76]. PCP-induced cognitive dysfunction could be attributable to the loss of spines in the PFC[77, 78]. Repeated

administration of PCP disturbed almost all behavioral parameters including impulsive action in the 5-CSRTT[47, 79].

*Microinjection of NMDA receptor antagonist into the mPFC*
This animal model reflects dysfunctional glutamate neurotransmission in the prefrontal cortex implicated in aspects of cognitive deficits in schizophrenia[80]. In this model, a competitive NMDA receptor antagonist, 3-(R)-2-carboxypiperazine-4-propyl-l-phosphonic acid (CPP) is injected into the mPFC and it induces poor accuracy, increased impulsive action and decreased motivation and speed of responding in the 5-CSRTT[81, 82].

Both types of animal models were well reflecting aspects of cognitive deficits in schizophrenia, such as attentional impairments and deficits in executive functions and used for screening of the antipsychotics[46-48]. However, these animal models elicit various cognitive dysfunctions, making it difficult to exclude the possibility that the increased impulsive action is due to the deficit of attentional function or motivation to the task. For instance, disturbance of attentional function and/or motivation to the task could diminish goal-directed behaviors and increase random responses to the target holes, leading to a false-positive regarding impulsive action. Indeed, reward-unrelated responses (*i.e.,* responses during the time-out period) were increased in the repeated PCP animals[47]. In addition, the suppressing effect of drugs on elevated impulsive action could be a false-positive in these models. Therefore, ***establishing a new animal model selectively exhibiting an impaired control of impulsive action is required for more precise screening of anti-impulsive drugs.***

## Aims of this thesis

Elucidating the mechanisms of suppressing effects of dugs discussed above on impulsive action will contribute to accelerating the development of anti-impulsive drugs. Then, I determined the neural mechanisms of suppressing effect of acute administration of milnacipran on impulsive action in normal rats (CHAPTER 2).

Current animal models of elevated impulsive action could elicit false-positive regarding impulsive action and effects of drugs on impulsive action. Therefore, I established a new animal model by employing the findings of

Chudasama *et al.*[31] in which selective disturbance of inhibitory control of impulsive action was obseved in rats with lesions of the vmPFC (CHAPTER 3). Then, I examined the effects of repeated administration of milnacipran on elevated impulsive action observed in that model (CHAPTER 3).

# CHAPTER 2

## The Neural Mechanisms of Suppressing Effects of Acute Milnacipran on Impulsive Action in Normal Rats


## Abstract

Elevated impulsivity is often observed in patients with depression. I recently found that milnacipran, an antidepressant and a serotonin/noradrenaline reuptake inhibitor, could enhance impulse control in rats. However, the neural mechanisms underlying the effects of milnacipran on impulsive action remain unclear. Milnacipran increases not only extracellular serotonin and noradrenaline but also dopamine specifically in the medial prefrontal cortex, which is one of brain regions responsible for impulsive action.

Our goal was to identify whether dopamine $D_1$-like and/or $D_2$-like receptors in the ventromedial prefrontal cortex (vmPFC), mediates the milnacipran-enhanced impulse control in a 3-choice serial reaction time task.

The rats were bilaterally injected with SCH23390, a selective $D_1$-like receptor antagonist, (0.3 or 3 ng/side) or eticlopride, a selective $D_2$-like receptor antagonist, (0.3 or 1 μg/side) into the vmPFC after acute intraperitoneal administration of milnacipran (10 mg/kg).

Intra-vmPFC SCH23390 injections reversed the milnacipran-enhanced impulse control, whereas injections of eticlopride into the vmPFC failed to block the effects of milnacipran on impulsive action.

This is the first report that demonstrates a critical role for $D_1$-like receptors of the vmPFC in milnacipran-enhanced control of impulsive action.



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## Abbreviations

| | |
|---|---|
| ANOVA | analysis of variance |
| 3-CSRTT | 3-choice serial reaction time task |
| 5-CSRTT | 5-choice serial reaction time task |
| ITI | inter trial interval |
| mPFC | medial prefrontal cortex |
| NAc | nucleus accumbens |
| PFC | prefrontal cortex |
| SCH23390 | R(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl -2,3,4,5-tetrahydro-1H-3-benzazepine |
| SNRI | serotonin-noradrenaline reuptake inhibitor |
| VTA | ventral tegmental area |
| vmPFC | ventromedial prefrontal cortex |

## Introduction

Impaired control of impulsivity is often observed in depressed patients[52, 53]. Higher impulsivity can also be a risk factor for drug addiction and suicide[83-86]. Substance abuse and/or suicide attempts in patients with depressive disorders have emerged in recent years[87, 88]. Therefore, a significant issue concerns whether some antidepressants could enhance the control of impulsivity.

I recently reported that milnacipran, an antidepressant, suppressed impulsive action in rats[10]. However, the neural mechanisms underlying the effects of milnacipran on impulsive action have not been identified. Milnacipran is a serotonin/noradrenaline reuptake inhibitor (SNRI, $K_i$=151 nM and 68 nM, respectively)[89]. Although the affinity of milnacipran for dopamine transporters is extremely low ($K_i$>10,000 nM), noradrenaline transporters take up not only extracellular noradrenaline but also dopamine in some specific brain regions, such as the medial prefrontal cortex (mPFC)[89-92]. Indeed, acute administration of milnacipran increases extracellular concentrations of dopamine in the mPFC[59, 60].

The rat mPFC is implicated in various aspects of impulsivity[31, 34, 92, 94]. The ventromedial prefrontal cortex (vmPFC) plays a pivotal role in the control of impulsive action[31, 32, 82]. A previous study reported that dopamine release in the mPFC plays a role in enhancing the control of impulsive behavior[33, 34]. Dopamine receptors have been classified into five subtypes, dopamine $D_1$-$D_5$, based on the sequences of their encoding genes[95]. Pharmacological studies have demonstrated that $D_1$ and $D_5$ receptors, namely $D_1$-like receptors, are linked to a stimulation of adenylyl cyclase, whereas $D_2$-$D_4$ receptors, namely $D_2$-like receptors, are linked to an inhibition of cAMP production[95, 96]. Both types of dopamine receptors are distributed throughout the rat mPFC[97, 98].

The present aim was to investigate the role of $D_1$-like and $D_2$-like receptors of the vmPFC in the milnacipran-enhanced control of impulsive action. Thus, I used systemic and intracranial drug injections to manipulate behavioral performance in the 3-choice serial reaction time task (3-CSRTT)[10], which is a simplified (but reliable) version of the 5-choice serial reaction time task (5-CSRTT)[12] that measures impulsive action in rats.

## Materials and Methods

### Subjects

Male Wistar/ST rats supplied by Nippon SLC Co. Ltd. (Hamamatsu, Japan) were used. They were housed in groups of four under an alternating light-dark cycle (light from 7 p.m. to 7 a.m.) at approximately 21 °C and relative humidity 40–50%. When the rats were 9 weeks old (270–290 g), I started to restrict their food intake. Thereafter, their body weights were maintained at 85% of those under free-feeding conditions. The food provided to the rats in the home cages was purchased from CLEA JAPAN, Inc. (Tokyo, Japan), and the rats were fed after each daily session of the 3-CSRTT. Water was available ad libitum. The treatment of animals was in compliance with the Guidelines for the Care and Use of Laboratory Animals of the Animal Research Committee of Hokkaido University.

### Drugs

R(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepin (SCH23390) hydrochloride and S(-)-eticlopride hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). SCH23390 is more selective for $D_1$ and $D_5$ receptors (>1000-fold) than for $D_2$, $D_3$, and $D_4$ receptors[99]. Eticlopride is a selective $D_2$ and $D_3$ antagonist ($K_i$=0.5, and 0.16 nM, respectively) and also a preferential $D_4$ antagonist ($K_i$=27 nM)[100]. Eticlopride has little affinity for $D_1$ and $D_5$ receptors ($IC_{50}$>100,000 nM)[101]. Milnacipran hydrochloride was generously donated by Asahi-Kasei Co. Ltd. (Tokyo, Japan) and administered at a volume of 3 ml/kg. All 3 compounds were dissolved in 0.9% saline (pH=6.5–6.8).

### Apparatus

Aluminum operant chambers measuring 26×26×26 cm (Med Associates Inc., St. Albans, VT, USA) were used (**Apendix 1**). The curved rear wall of each chamber contained nine 2.5 $cm^2$ holes that were 2.2 cm deep. Each hole had an infrared photocell beam for detection of nose poke responses and a 2.8 W bulb at its rear. Every other hole was sealed such that only the three centrally positioned holes were accessible. A food magazine was located on the opposite wall of the chamber, and a house light was located at the top of this wall. The apparatus was controlled by a computer program written in the MED-PC language (Med Associates Inc., St. Albans, VT, USA).

**3-choice serial reaction time task**

The training procedure and the task sequence that were employed in the 3-CSRTT are detailed in previous reports[10, 102] (**Appendix 2**). Briefly, when the task started, the house light was illuminated. After a fixed inter trial interval (ITI: 5 s), one of 3 holes was briefly illuminated (stimulus duration) in a random order so that a rat could not predict which hole would be illuminated. Nose poking during the ITI was recorded as a premature response, which is an index of impulsive action. Nose poking into the lit hole while it was illuminated or within 5 s of limited hold was recorded as a correct response, and the rat was rewarded by the delivery of a palatable food pellet (45 mg each, dustless precision pellets, Bio-serv, Frenchtown, NJ, USA). Nose poking into another hole was recorded as an incorrect response. When a rat failed to nose poke within the limited hold, it was recorded as an omission. After a food pellet had been delivered to and collected by the rat, the house light was switched off for 2 s to allow the rat to eat the pellet before the next trial was automatically started. The start of the next ITI was signaled by turning on the house light. Additional nose poking into any of the three holes prior to food collection was recorded as a perseverative response. Premature responses, incorrect responses, omissions, and perseverative responses resulted in a 5 s time out period during which the house light was extinguished. Because the trial was initiated automatically, I did not set a time restriction for this task. Each session consisted of 100 trials. Training was conducted for one session per day and 6 sessions per week.

At the beginning of the training schedule, the stimulus duration lasted 30 s. Depending on individual performances, the stimulus duration progressively reduced to 1 s (15, 10, 5, 3, 2, 1.5, and 1 s). When a rat attained > 80% accuracy (the percentage of correct responses) and < 20 omissions in a session, the stimulus duration was reduced in the next session.

I used six behavioral parameters described as follows:

(a) *Premature response* (no. per session)

(b) *Accuracy* (percentage of correct responses): [correct responses / (correct and incorrect responses)] ×100

(c) *Omission* (no. per session): [omission errors / total trials] ×100

(d) *Perseverative response* (no. per session)

(e) *Correct response latency* (s): the mean time between stimulus onset and nose poke in the correct hole

(f) *Reward latency* (s): the mean time between reward delivery and nose poke in

the food magazine

The completion of the training was determined as reaching the target phase (stimulus duration 1 s) and exhibiting stable performance. After completion of the training, the stimulus duration was fixed at 1 s regardless of performance. I set the criteria for determining stable performance as follows: the change in premature responses stayed within ± 25%, the accuracy stayed within ± 5%, and the number of omissions were less than 20 for at least 3 consecutive sessions.

**Surgery**

After completing the training, the rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and fixed in a stereotaxic frame (Narishige, Tokyo, Japan). Stainless steel guide cannulas (24 gauge, 9 mm long) were bilaterally implanted with coordinates 3.2 mm posterior to the bregma, 0.7 mm lateral to the midline, and 2.0 mm ventral to the dura[103]. Dummy cannulas (30 gauge) were inserted that penetrated to the tip of the guide cannulas. After surgery, the rats were housed individually and allowed a 4-day recovery period prior to retraining.

**Drug treatment schedule**

Prior to testing, the rats were retrained for at least 1 week until their performance restabilized for three consecutive sessions. Each drug session was conducted with more than a two-day interval.

The rats were gently restrained, and the dummy cannulas were removed and replaced with 30-gauge stainless steel injection cannulas (11.3 mm long) attached to a polyethylene tube. The tips of the injectors extended beyond the guide cannulas by 2.3 mm. SCH23390 (0, 0.3, or 3 ng in 0.5 μl saline per side, n=10) or eticlopride (0, 0.3, or 1 μg in 0.5 μl saline per side, n=10) were infused at 0.5 μl/min into the vmPFC according to a Latin Square design. For additional testing, intra-vmPFC eticlopride (0 or 3 μg/side; n=12) injections were infused. The solution was infused over a period of 1 min at constant flow using a microinjection pump (Carnegie Medicine, Sweden), and the injector was left in place for 1 min after injection to allow for diffusion.

Fifty minutes before the microinjection of SCH23390 or eticlopride, the rats were given intraperitoneal administrations of saline or milnacipran (10 mg/kg). Behavioral testing was conducted 10 min after the injection of SCH23390 or eticlopride (**Appendix 3**). A different group of rats was used for

each experiment (SCH23390 or eticlopride).

**Basal performance**

I used the data from the last 3 days of training to provide a preoperative baseline, and I used data from the last three days of retraining to provide a postoperative baseline. The experimental baseline was assessed a day before the testing day.

**Histology**

Following the completion of the experiments, the rats were deeply anaesthetized with urethane (1 g/kg, i.p.) and were transcardially perfused with 0.9% saline followed by paraformaldehyde. The brains were then removed and postfixed with paraformaldehyde overnight. Next, the brains were transferred to 30% sucrose. Coronal sections were cut at 60 μm on a freezing microtome and stained with toluidine blue, and the placements of cannula tips were determined using a light microscope. Only data from rats with correct injections were included in the analysis.

**Data analysis**

Six behavioral measures were analyzed (see **3-choice serial reaction time task**). Each measure was analyzed separately using two-factor analysis of variance (ANOVA) for repeated measures with dose as within-subject factor and rank of the injection dose as a between-subject factor.

Table 1 shows an example of Latin Square design I used in this study. I injected milnacipran and SCH23390 (or eticlopride) with five combinations (D1-D5) to 10 rats (A-J) using Latin Square design. I designed the order of the injection dose as (R1) D1-D2-D3-D4-D5 for rat A and B, (R2) D2-D3-D4-D5-D1 for rat C and D, (R3) D3-D4-D5-D1-D2 for rat E and F, (R4) D4-D5-D1-D2-D3 for rat G and H, and (R5) D5-D1-D2-D3-D4 for rat I and J. In this case, the order of the dose injection was counterbalanced but there were still five ranks of the injection dose (R1)-(R5). Then, if one includes the rank of the injection dose into the ANOVA as between-subjects, it contributes to reducing the error term[104]. Order of the treatment (see Table 1) was not included in ANOVA because baseline performance was stable throughout our experiments as shown in Figure 5

For the additional experiment examing the effect of higher dose (3 μg/side) of eticlopride on milnacipran-suppressed premature response, each

behavioral measure was analyzed separately using two-factor repeated measures ANOVA. One factor is vehicle or milnacipran and another factor is vehicle or eticlopride.

The alpha level was set to 0.05 for ANOVA. Multiple comparisons testing using the Holm method[105] was performed where a significant main effect of the dose was observed. All statistical procedures were conducted using SPSS (version 15.0 J).

**Table 1.** An example of a Latin Square design

| | **O1** | **O2** | **O3** | **O4** | **O5** |
|---|---|---|---|---|---|
| **R1** | 62.5 (D1) | 14.0 (D2) | 43.5 (D3) | 46.5 (D4) | 5.02 (D5) |
| **R2** | 22.5 (D2) | 11.5 (D3) | 20.5 (D4) | 25.0 (D5) | 32.5 (D1) |
| **R3** | 20.5 (D3) | 47.5 (D4) | 24.5 (D5) | 41.5 (D1) | 22.0 (D2) |
| **R4** | 46.5 (D4) | 40.5 (D5) | 61.5 (D1) | 23.0 (D2) | 49.0 (D3) |
| **R5** | 33.5 (D5) | 29.0 (D1) | 20.0 (D2) | 24.0 (D3) | 26.5 (D4) |

*Note*: As an example, the mean of the number of premature responses in milnacipran-SCH23390 experiment was used. The same experimental design was used in milnacipran-eticlopride experiment.

R: Rank of injection dose. Two rats each were assigned to each row.

O: Order of the treatment.

D: Dose.

D1: Vehicle-vehicle.

D2: Milnacipran-vehicle.

D3: Milnacipran-SCH23390 (0.3 ng).

D4: Milnacipran-SCH23390 (3 ng).

D5: Vehicle-SCH23390 (3 ng).

## Results

### Histological analysis

Figure 1 shows representative photomicrographs and illustrations indicating the locations of the cannula tips in the vmPFC region of rats that were included in the study. Of 22 implanted rats, 2 rats were excluded because the cannulas were located outside the target region, resulting in n=20.

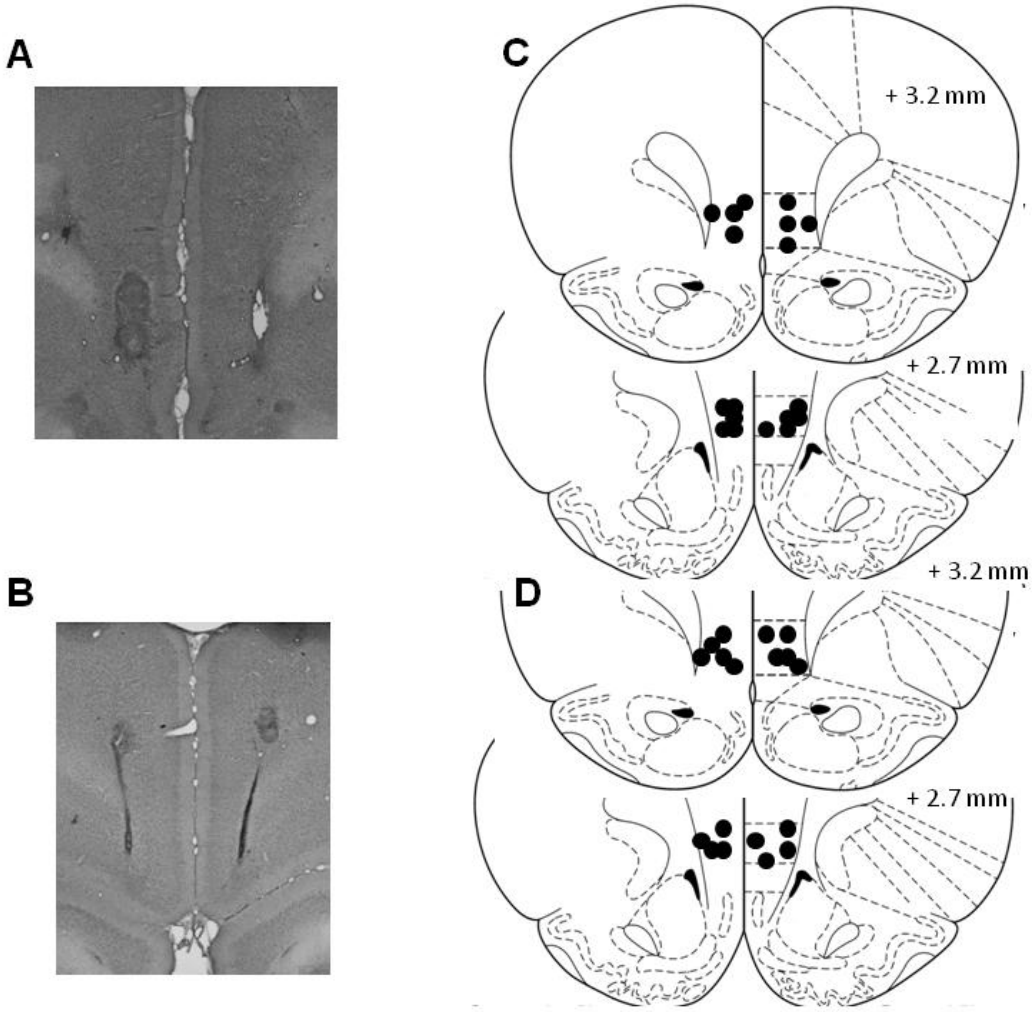



**Figure 1**. Representative photomicrographs of a coronal section (**A**) +2.7 mm from the bregma (SCH23390 experiment) and (**B**) +3.2 mm from the bregma (eticlopride experiment). The dark staining indicates the injection cannula path. Schematic diagrams showing the placements of cannula tips in the vmPFC region (closed circles) for (**C**) the SCH23390 experiment and (**D**) the eticlopride experiment, 2.7 mm and 3.2 mm anterior to the bregma.

**The effects of intra-vmPFC injections of SCH23390 on milnacipran-enhanced the control of impulsive action**

Figure 2A shows the effects of intra-vmPFC injections of SCH23390 on the milnacipran-enhanced control of impulsive action. Two-factor ANOVA revealed a significant main effect of the dose ($F_{4,\ 20}$=7.11, $P$<0.05). Dose×rank of the injection dose interaction was not significant ($F_{16,\ 20}$=1.33, *NS*). Multiple comparisons using the Holm method revealed that systemic milnacipran alone and in combination with 0.3 ng injections of SCH23390 per side significantly decreased the number of premature responses compared to vehicle treatment. This effect of milnacipran on premature responses was significantly blocked by intra-vmPFC injections of 3 ng SCH23390 per side. No other 3-CSRTT variable was significantly affected by the administration of milnacipran or the injection of SCH23390 (accuracy, $F_{4,\ 20}$=0.75, *NS*; omission, $F_{4,\ 20}$=0.24, *NS*; perseverative response, $F_{4,\ 20}$=1.04, *NS*; correct response latency, $F_{4,\ 20}$=1.49, *NS*; reward latency, $F_{4,\ 20}$=0.41, *NS*) (Figure 3).

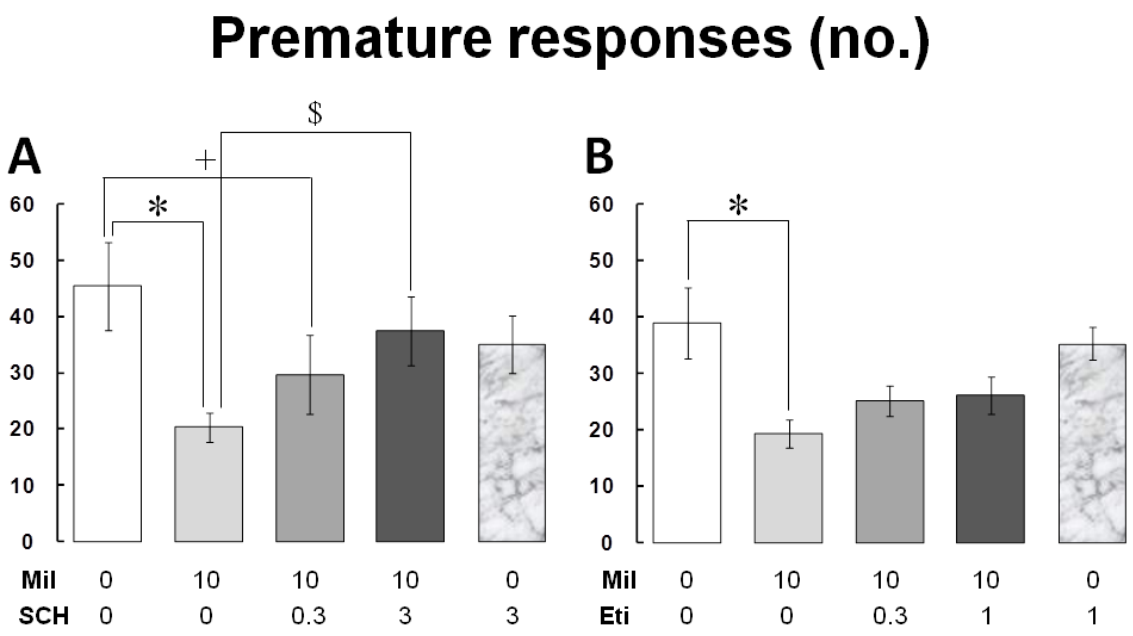



**Figure 2**. The effects of intra-vmPFC injections of SCH23390 (SCH) (**A**) and eticlopride (Eti) (**B**) on the enhancement of impulse control by systemic milnacipran (Mil). The rats received either systemic milnacipran (0 or 10 mg/kg) and or intra-vmPFC SCH23390 (0, 0.3, or 3 ng per side; n=10) or eticlopride (0, 0.3, or 1 μg per side; n=10). The bars represent the mean, and the lines represent the SEM. **P*< 0.007, vehicle treatment vs. milnacipran treatment; $^{+}$*P*<0.008, vehicle treatment vs. milnacipran with SCH23390 (0.3

ng/side); $^{\$}P$<0.01, milnacipran treatment vs. milnacipran with SCH23390 (3 ng/side) (with the Holm method).

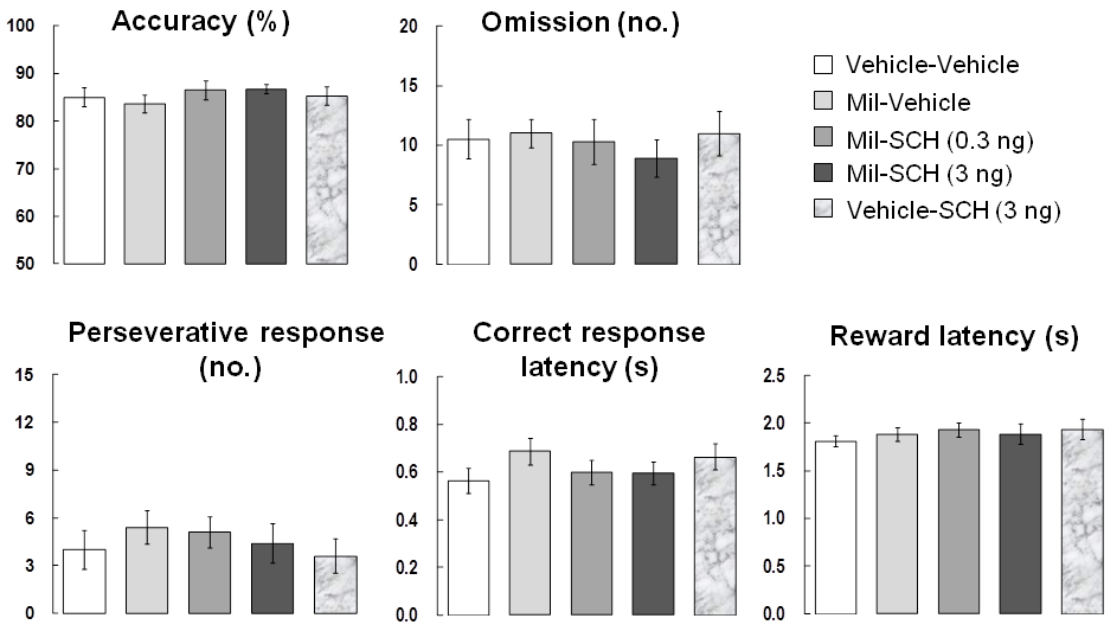



**Figure 3**. The effects of intra-vmPFC injections of SCH23390 (SCH) with systemic milnacipran (Mil) on behavioral parameters of the 3-CSRTT. The rats received systemic milnacipran (0 or 10 mg/kg) and intra-vmPFC SCH23390 (0, 0.3, or 3 ng per side; n=10). The bars represent the mean, and the lines represent the SEM.

## The effects of intra-vmPFC injections of eticlopride on milnacipran-enhanced the control of impulsive action

Figure 2B shows the effects of intra-vmPFC injections of eticlopride on milnacipran-suppressed impulsive action. Two-factor ANOVA revealed a significant main effect of the dose ($F_{4,\ 20}$=6.08, $P$<0.05). Dose × rank of the injection dose interaction was not significant ($F_{16,\ 20}$=1.04, *NS*). Multiple comparisons using the Holm method revealed that systemic milnacipran significantly decreased the number of premature responses compared to vehicle treatment. In contrast to SCH23390, this effect of milnacipran on premature responses was unchanged by intra-vmPFC injections of eticlopride for all doses, nor were there significant effects of milnacipran or eticlopride on other behavioral parameters in the 3-CSRTT (accuracy, $F_{4,\ 20}$=0.39, *NS*; omission, $F_{4,\ 20}$=2.80, *NS*; perseverative response, $F_{4,\ 20}$=2.76, *NS*; correct response latency, $F_{4,\ 20}$=2.69, *NS*; reward latency, $F_{4,\ 20}$=0.93, *NS*) (Figure 4).

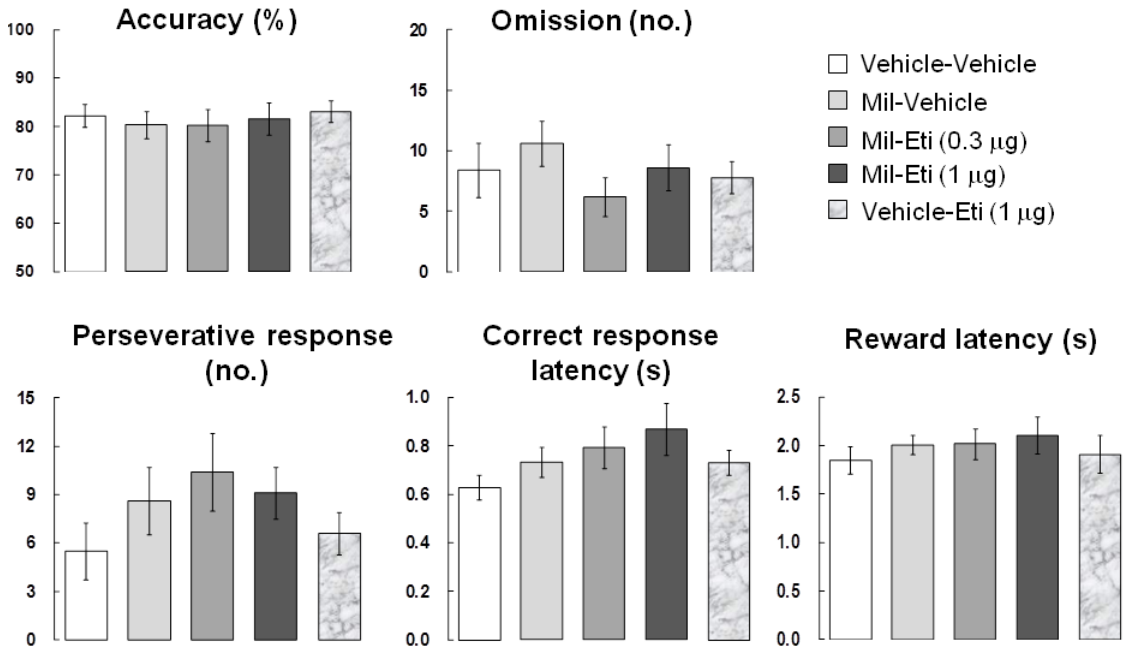



**Figure 4**. The effects of intra-vmPFC injections of eticlopride (Eti) with systemic milnacipran (Mil) on behavioral parameters of the 3-CSRTT. Rats received systemic milnacipran (0 or 10 mg/kg) and intra-vmPFC eticlopride (0, 0.3, or 1 μg per side; n=10). The bars represent the mean, and the lines represent the SEM.

## Basal performance

Figure 5 shows the preoperative, postoperative, and experimental basal performance levels for premature responses, accuracy, and omissions for all rats, which were assessed over eleven sessions. Repeated measures ANOVA revealed no significant effects of days on premature responses ($F_{10, 90}$=1.29, *NS*: $F_{10, 90}$=0.89, *NS*), accuracy ($F_{10, 90}$=1.57, *NS*: $F_{10, 90}$=0.66, *NS*), or omissions ($F_{10, 90}$=1.89, *NS*: $F_{10, 90}$=1.52, *NS*) in the SCH23390 and eticlopride experiments, respectively. This analysis indicated that basal performance remained stable throughout the experiments.

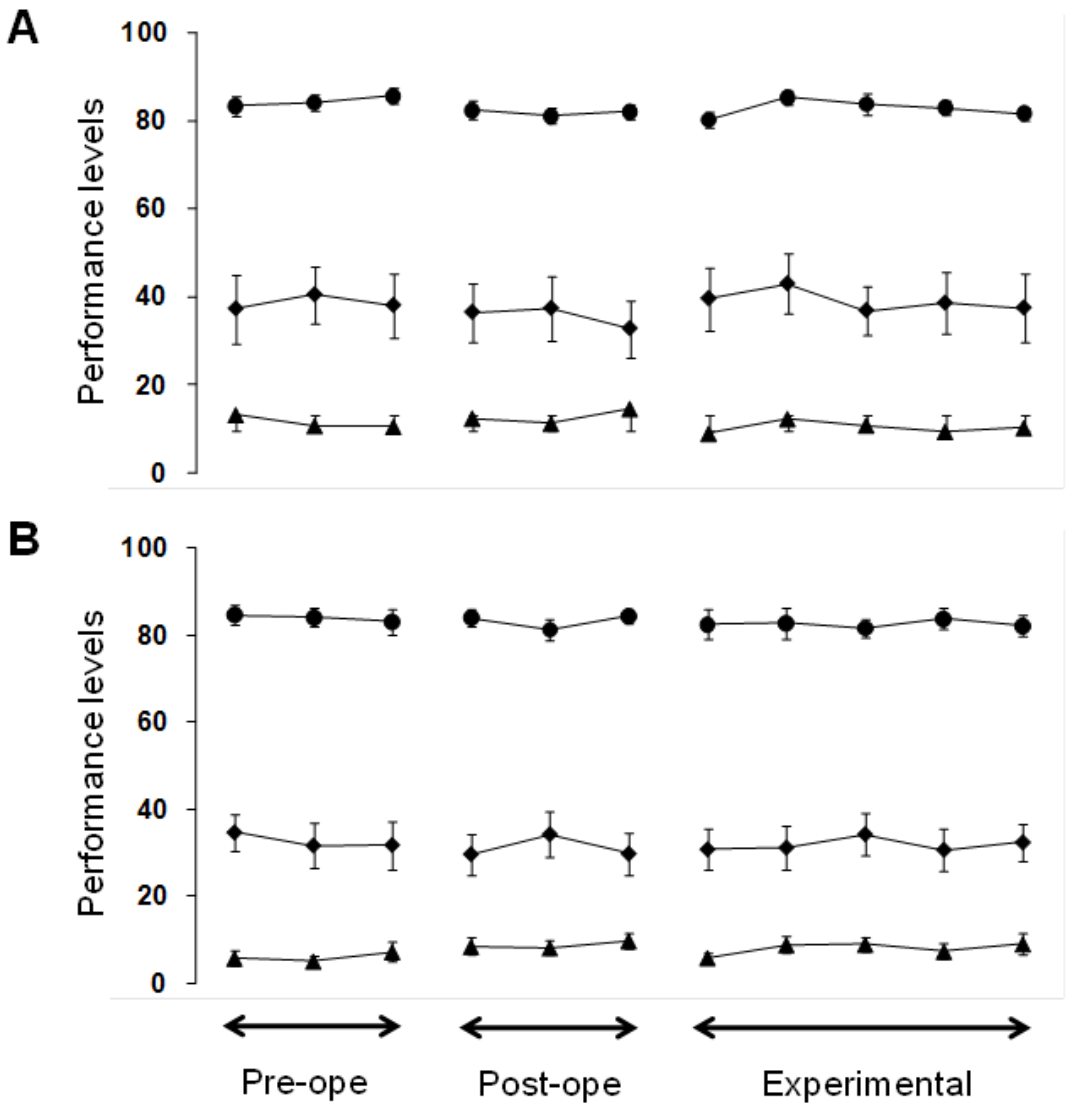



**Figure 5.** Basal performance. Three preoperative (Pre-ope), 3 postoperative (Post-ope), and 5 experimental basal performance levels of premature responses, accuracy, and omissions for the rats from (**A**) the SCH23390 experiment (n=10) and (**B**) the eticlopride experiment (n=10). Closed diamond: number of premature responses; closed circle: accuracy (percent); closed triangle: number of omissions. The vertical lines represent the SEM. No significant differences were detected using repeated measures ANOVA for each variable.

**The effects of intra-vmPFC injections of high dose of eticlopride on milnacipran-enhanced the control of impulsive action**

2×2 repeated measures ANOVA revealed significant effects of milnacipran ($F_1$,

$_{11}$=30.45, $P$<0.05) and eticlopride ($F_1$, $_{11}$=30.45, $P$<0.05) but not milnacipran×eticlopride interaction ($F_1$, $_{11}$=2.40, *NS*) on premature responses, suggesting that there were significant independent effects of milnacipran and eticlopride on premature responses but no synergistic effects between milnacipran and eticlopride (Figure 6). There were significant main effects of eticlopride but not milnacipran on omissions ($F_1$, $_{11}$=55.33, $P$<0.05), correct response latency ($F_1$, $_{11}$=21.64, $P$<0.05), and reward latency ($F_1$, $_{11}$= 44.25, $P$<0.05). However, there was a milnacipran×eticlopride interaction merely on correct response latency ($F_1$, $_{11}$=5.88, $P$<0.05), implying that although eticlopride alone prolonged correct response latency, there was also a synergistic effect of milnacipran and eticlopride on correct response latency. Neither milnacipran×eticlopride interaction, main effects of milnacipran, nor eticlopride were significant in accuracy or perseverative responses.

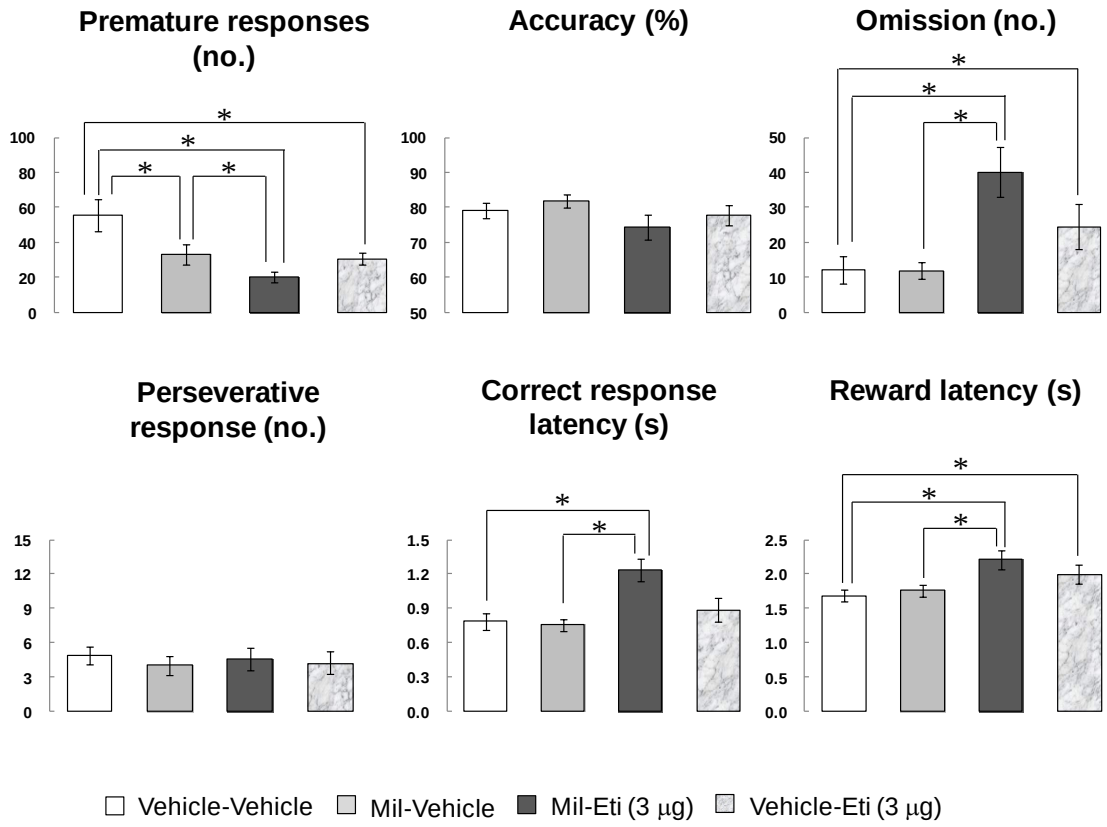



**Figure 6.** The effects of intra-vmPFC injections of eticlopride (Eti) with systemic milnacipran (Mil) on behavioral parameters of the 3-CSRTT. Rats received systemic milnacipran (0 or 10 mg/kg) and intra-vmPFC eticlopride (0 or 3 μg per side; n=12). The bars represent the mean, and the lines represent the SEM. $^{*}P$<0.05 (with Holm method).

## Discussion

Consistent with my previous study, systemic administration of milnacipran decreased the number of premature responses[10]. This milnacipran-induced decrease in the number of premature responses was blocked by injections of SCH23390, a selective $D_1$-like receptor antagonist, into the vmPFC, whereas intra-vmPFC injections of eticlopride, a selective $D_2$-like receptor antagonist, failed to inhibit the effect of milnacipran on impulsive action (Figure 2). In addition, intra-vmPFC SCH23390 injections without systemic administration of milnacipran caused no effect on impulsive action (Figure 2A). These results indicated that microinjections of 3 ng SCH23390 per side into the vmPFC elicited impulsive action by antagonizing the effects of milnacipran but not by antagonizing the effects of tonic endogenous dopamine.

Naturally, systemic milnacipran increases the extracellular levels of serotonin and noradrenaline as well as dopamine, and all of these neurotransmitters are involved in impulsive action[106-111]. Intra-vmPFC injection of SCH23390, however, almost completely reversed the milnacipran-improved control of impulsive action (Figure 2A), suggesting that the milnacipran-induced decrease in premature responses may not be affected by milnacipran-increased extracellular serotonin or noradrenaline levels.

SCH23390 is also a serotonin 2A receptor antagonist[112] and a serotonin 2C receptor agonist, albeit these affinities are relatively weak[113]. However, these effects would induce a decrease of premature responding rather than an increase[108, 114], indicating that the effects of SCH23390 on milnacipran-suppressed impulsive action are not due to its actions on serotonin 2A or 2C receptors.

In the present study, intra-vmPFC injections of eticlopride failed to reverse the effect of milnacipran on impulsive action (Figure 2B). Since I was skeptic whether 1 μg/side of eticlopride had enough antagonistic action to the $D_2$-like receptors in the vmPFC, I examined the effects of higher dose (3 μg/side) of eticlopride on milnacipran-suppressed premature response. However, 3 μg/side of eticlopride itself rather decreased the number of premature response and increased the number of omission and prolonged latency to correct response and collection of reward (Figure 6). Blockade of dopamine $D_2$ receptors in the rat mPFC was reported to induce inhibition of locomotor activity in a dose-dependent manner[115], suggesting that high dose of intra-vmPFC injection

of eticlopride impaired motor activity in our study. Thus, I could not determine whether 3 μg/side of eticlopride did not reverse the effect of milnacipran or the effect of 3 μg/side of eticlopride on milnacipran-suppressed premature response was masked by increased the number of omission. Nevertheless, the facts that intra-vmPFC injection of $D_1$-like receptor antagonist almost completely blocked the effect of milnacipran on premature response (Figure 2A) and that $D_2$-like receptors in the mPFC, especially in the vmPFC, are sparsely distributed compared to $D_1$-like receptors[97, 98, 116, 117, 118] are damping the idea that $D_2$-like receptors in the vmPFC associate with milnacipran-enhanced impulse control.

Yamauchi *et al.*[60] demonstrated that extracellular dopamine level was significantly increased 60 min after intraperitoneal injection of milnacipran (10 mg/kg), suggesting that systemic milnacipran increased dopamine levels in this study. Based on the present results and those of previous studies, I conclude that $D_1$-like receptors in the vmPFC play an important role in milnacipran-enhanced impulse control though I could not completely rule out the possibility of the contribution of $D_2$-like receptors. This finding is the first to elucidate the action site of milnacipran for its effects on impulsive action.

It should be noted that some other noradrenaline transporter inhibitors also suppress impulsive action[20, 119]. It is possible that these drugs activate $D_1$-like receptors in the vmPFC and enhance inhibitory control of impulsive action as well as milnacipran. Further studies are required to determine whether there is a common mechanism underlying suppressing effects of noradrenaline transporter inhibitors on impulsive action.

**Possible neural circuits**

One of most widely accepted theories of neural mechanisms that underlie impulsive action is associated with the fronto-striatal system: impulsive action is mediated by cortico-accumbal interactions[21-23]. Nucleus accumbal dopamine is part of the neural circuit that is thought to mediate impulsive action[24, 25]. There is anatomical and physiological evidence that the mPFC acts as an important regulator of dopamine transmission in the nucleus accumbens (NAc). Jackson *et al*.[120] demonstrated that electrical stimulation of the mPFC at physiologically relevant frequencies inhibited dopamine release in the NAc. Moreover, dopamine depletion in the mPFC led to an increase of basal dopamine levels in the NAc shell[121]. There is a bi-directional projection between the mPFC and the ventral tegmental area (VTA), which is predominantly-comprised of

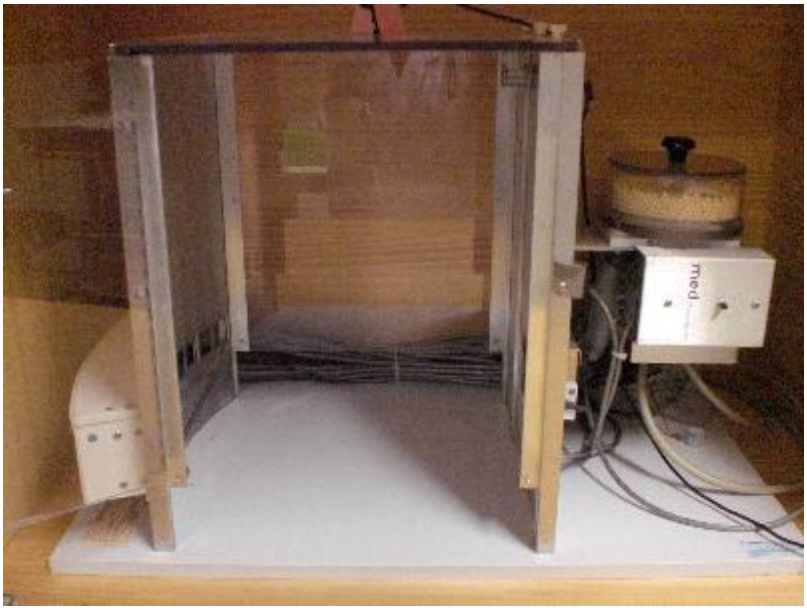
dopaminergic neurons[22, 122, 123]. Some of $D_1$-like receptors are localized on pyramidal cells in the vmPFC that project to the VTA[98, 124], suggesting that the vmPFC could indirectly modulate accumbal dopaminergic activities by modulating the VTA. It should also be noted that some of the dopamine terminals in the mPFC form synapses with pyramidal cells that directly project to the NAc[125], suggesting that pyramidal cells in the vmPFC could directly modulate accumbal dopaminergic activities. Thus, it is feasible that milnacipran stimulates $D_1$-like receptors in the vmPFC and thereby attenuates accumbal dopaminergic activities via a direct and/or indirect pathway, resulting in suppressed impulsive action.

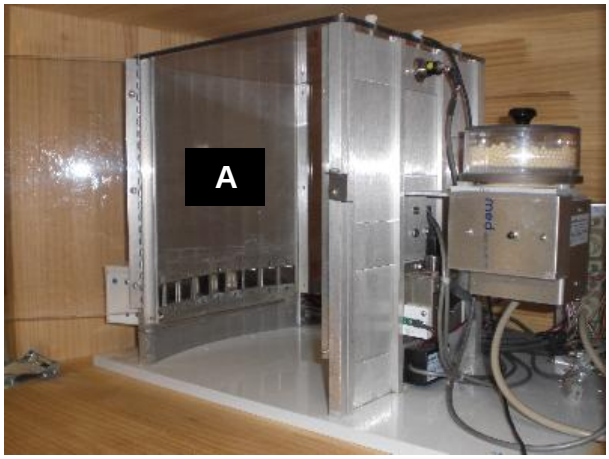
**Clinical implications**

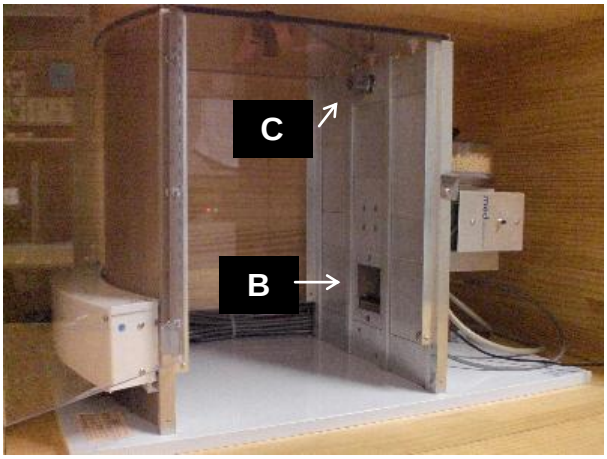
As previously mentioned, dopamine release in the mPFC plays a role in enhancing the control of impulsive behavior[33, 34]. Meanwhile, increased dopamine release in the NAc stimulates impulsive behavior[24, 25]. Administrations of drugs that activate the dopamine system not only in the mPFC but also in the NAc induce rather impaired impulse control in humans[126] and in animals[18, 127]. However, inhibition of the noradrenaline transporters by atomoxetine induces an increase of dopamine release in the mPFC without affecting dopamine release in the NAc[35] and consequently enhances impulse control[10, 20]. Similar to atomoxetine, milnacipran inhibits the noradrenaline transporter and suppresses impulsive action. Moreover, milnacipran is an antidepressant, whereas atomoxetine is not. Thus, the use of milnacipran for animal models with elevated impulsivity should be considered in the CHAPTER 3.

In conclusion, my data suggest that milnacipran suppresses impulsive action by stimulating $D_1$-like receptors in the vmPFC though I could not completely rule out the possibility of the contribution of $D_2$-like receptors. Elevated impulsive action is often observed in depressive disorders and could increase the risk of drug addiction and suicide. Revealing the neural mechanism of milnacipran-dependent effects on impulsive action will contribute to the development of novel strategies for treatment of depressive disorders that are associated with high impulsivity.

## **Appendix 1.** A photograph of the 9-hole apparatus

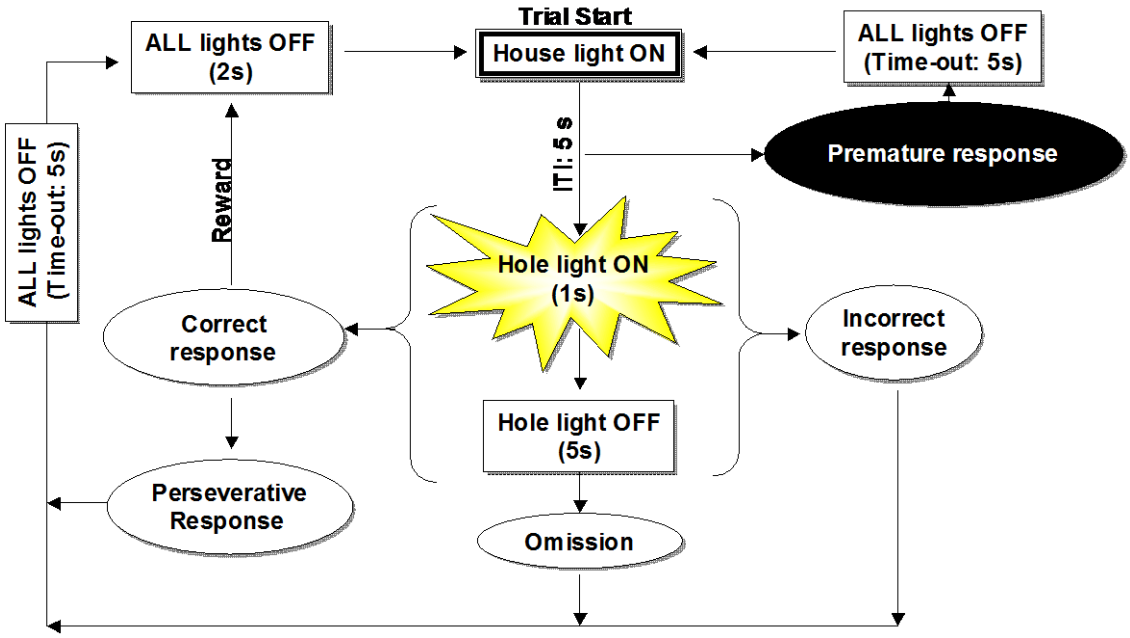




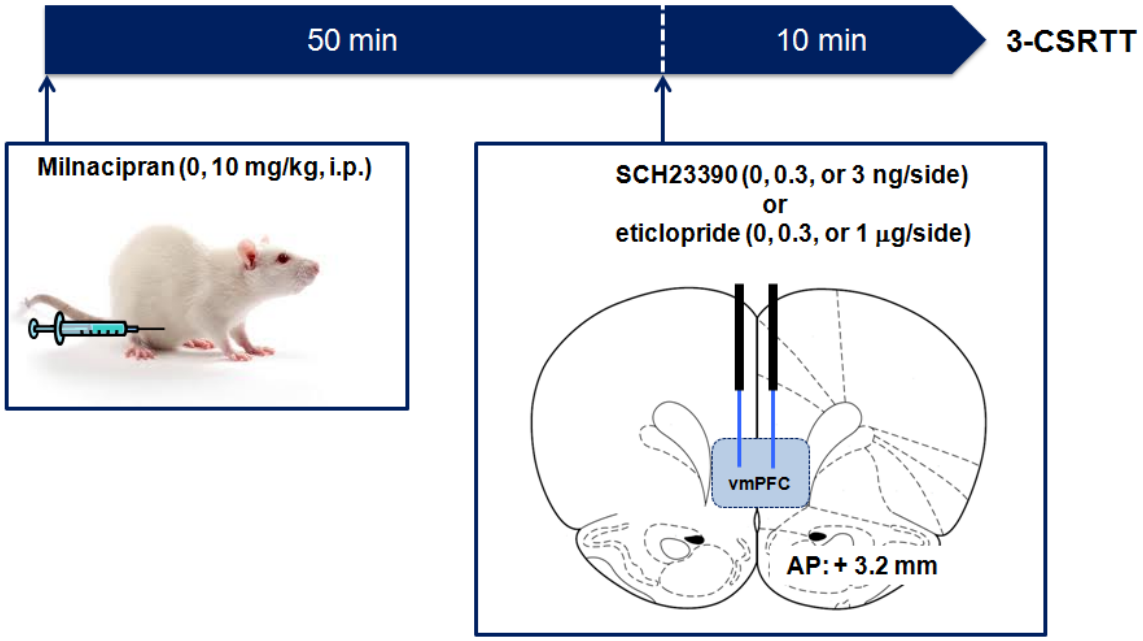

(**A**) The curved rear wall of each chamber contained nine 2.5 cm square holes. Each hole had an infra-red photocell beam for detection of nose poke responses and a 2.8 W bulb at its rear. Every other hole was sealed so that only the three centrally positioned holes were accessible. (**B**) A food magazine was located on the opposite wall of the chamber, and (**C**) a house light was located at the top of this wall.

## **Appendix 2.** A schematic diagram of the task procedure in the 3-CSRTT




When the task started, the house light was illuminated. After a fixed inter-trial interval (ITI: 5 s), one of three hole lights was illuminated randomly and briefly (stimulus duration: 1 s). Nose poking during the ITI was recorded as a premature response and resulted in turning off all lights (time-out: 5 s), and followed by restarting of the same trial. This parameter was regarded as an index of impulsive action. Nose poking into the lit hole while it was illuminated or within 5 s limited hold was recorded as a correct response and was rewarded by the delivery of a palatable food pellet. Additional nose poking into any of the three holes prior to food collection was recorded as a perseverative response and resulted in a 5 s time-out. This parameter was regarded as an index of compulsive behavior. Nose poking into another hole was recorded as an incorrect response and resulted in 5 s time-out. Correct response latency, an index of motor activity, and reward latency, an index of motivation and/or appetite, was also measured. Reward latency was the time between a correct response and nose poking into the food magazine. When a rat failed to nose poke within the limited hold, it was recorded as an omission and resulted in a 5 s time-out. This parameter was also regarded as an index of motivation and/or appetite. After a food pellet had been delivered to and collected by a rat, the house light was turned off for 2 s to allow the rat to eat the pellet before the next trial was automatically started. The start of the next ITI was signaled by the turning on the house light.

**Appendix 3.** A schematic diagram of the drug treatment schedule




The rats were gently restrained, and the dummy cannulas were removed and replaced with 30-gauge stainless steel injection cannulas (11.3 mm long) attached to a polyethylene tube. The tips of the injectors extended beyond the guide cannulas by 2.3 mm. SCH23390 (0, 0.3, or 3 ng in 0.5 μl saline per side, n=10) or eticlopride (0, 0.3, or 1 μg in 0.5 μl saline per side, n=10) were infused at 0.5 μl/min into the vmPFC according to a Latin Square design. For additional testing, intra-vmPFC eticlopride (0 or 3 μg/side; n=12) injections were infused. The solution was infused over a period of 1 min at constant flow using a microinjection pump, and the injector was left in place for 1 min after injection to allow for diffusion. Fifty minutes before the microinjection of SCH23390 or eticlopride, the rats were given intraperitoneal administrations of saline or milnacipran (10 mg/kg). Behavioral testing was conducted 10 min after the injection of SCH23390 or eticlopride.

# CHAPTER 3

## The Effects of Repeated Milnacipran Treatment on Elevated Impulsive Action in Rats with Lesions of the Ventromedial Prefrontal Cortex


## Abstract

Elevated impulsivity is often observed in several psychiatric disorders, such as attention-deficit/hyperactivity disorder and bipolar disorder, in which the impairment of the prefrontal cortex is commonly observed. I recently found that milnacipran, a serotonin/noradrenaline reuptake inhibitor, could suppress impulsive action in normal rats. However, whether milnacipran could suppress elevated impulsive action in rats with lesions of the ventromedial prefrontal cortex (vmPFC), which is functionally comparable to the human prefrontal cortex, remains unknown.

Selective lesions of the vmPFC were made using quinolinic acid in rats previously trained on a 3-choice serial reaction time task. Sham rats received phosphate buffered saline. Following a period of recovery, milnacipran (0 or 10 mg/kg/day × 14 days) was orally administered 60 min prior to testing on the 3-choice task. After 7 days of drug cessation, Western blotting, immunohistochemistry, electrophysiological analysis, and morphological analysis were conducted.

Lesions of the vmPFC increased impulsive action and repeated administration of milnacipran ameliorated the increased impulsivity, not only during the dosing period but also after the cessation of drug treatment. Repeated administration of milnacipran remediated the protein levels of mature brain-derived neutrophic factor and postsynaptic density-95, dendritic spine density, and excitatory currents in the few surviving neurons in the vmPFC of vmPFC-lesioned rats.

The findings of this study suggest that vmPFC-lesioned rats could facilitate screening for drugs that suppress elevated impulsivity, and the repeated administration of milnacipran could be a novel strategy for the treatment of psychiatric disorders that are associated with high impulsivity.



Revised version of a paper submitted in 2013, *The Journal of Neuroscience* (JN-RM-4637-13).

## Abbreviations

| | |
|---|---|
| ACSF | artificial cerebrospinal fluid |
| AMPA | alpha-amino-3-hydroxy-5-methyl-4- isoxazole-propionic acid |
| ANOVA | analysis of variance |
| AP | anteriorposterior |
| BDNF | brain-derived neurotrophic factor |
| DW | distilled water |
| dmPFC | dorsomedial prefrontal cortex |
| EPSC | excitatory postsynaptic current |
| GABA | γ-aminobutyric acid |
| GAPDH | glyceraldehyde 3-phosphate dehydrogenase |
| ITI | inter trial interval |
| MIL | milnacipran |
| mBDNF | mature BDNF |
| mPFC | medial prefrontal cortex |
| mRNA | messenger ribonucleic acid |
| NAc | nucleus accumbens |
| NBQX | 2,3-Dioxo-6-nitro-1,2,3,4- tetrahydrobenzo [f]quinoxaline-7-sulfonamide |
| NeuN | Neuronal Nuclei |
| NMDA | N-methyl-D-aspartate |
| PBS | phosphate buffered saline |
| PFC | prefrontal cortex |
| proBDNF | precursor BDNF |
| PSD-95 | postsynaptic density-95 |
| (R)-CPP | 3-((R)-2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid |
| SNRI | serotonin-noradrenaline reuptake inhibitor |
| SSRI | selective serotonin transporter inhibitor |
| VTA | ventral tegmental area |
| vmPFC | ventromedial prefrontal cortex |
| 3-CSRTT | 3-choice serial reaction time task |

## Introduction

Elevated impulsivity is defined as one of the core symptoms in attention-deficit/hyperactivity disorder, bipolar disorder, mania, borderline personality disorder, and substance abuse in Diagnostic and Statistical Manual of Mental Disorders, fourth edition[3]. Moreover, elevated impulsivity appears as a peripheral symptom in schizophrenia[128, 129] and major depression[52, 53]. Higher impulsivity can also be a risk factor for drug addiction and suicide[83, 85, 86]. However, only a few drugs (*e.g.,* atomoxetine and methylphenidate) are clinically available for treating elevated impulsivity though many experimental drugs have been found to suppress impulsive action in laboratory animals[130]. Therefore, it is a significant concern whether other clinically available drugs can suppress higher impulsivity.

It has been reported that psychiatric patients with higher impulsivity commonly exhibit impairments of the prefrontal cortex (PFC)[26-29]. The rat medial prefrontal cortex (mPFC) is comparable to the human PFC in terms of structural and functional characteristics[30]. Furthermore, Chudasama *et al*.[31] found that lesions of the ventral part of the mPFC (ventromedial prefrontal cortex: vmPFC) selectively elevate impulsive action in rats. Murphy *et al.*[82] demonstrated that the injection of an N-methyl-D-aspartate (NMDA) receptor antagonist into the rat vmPFC also elevates impulsive action. Therefore, impairments of the rat vmPFC could mimic the elevated impulsivity in psychiatric disorders.

I recently reported that acute milnacipran, an antidepressant and a serotonin/noradrenaline reuptake inhibitor (SNRI), suppressed impulsive action in normal rats[10] by stimulating dopamine $D_1$-like receptors in the vmPFC (CHAPTER 2). As previously mentioned, the fact that psychiatric patients with higher impulsivity commonly exhibit impairments of the PFC pose the question that acute milnacipran might not remedy elevated impulsivity in such psychiatric patients because their $D_1$-like receptors in the mPFC might be impaired. Interestingly, however, Mannari *et al*.[131] reported that the repeated administration of duloxetine, another SNRI, increases the protein levels of the brain-derived neurotrophic factor (BDNF) in the mPFC, suggesting that the repeated administration of SNRIs might induce plastic changes in the mPFC.

The present aim was to investigate whether the repeated administration of milnacipran could suppress elevated impulsive action in vmPFC-lesioned rats by inducing plastic changes in the few surviving neurons of the vmPFC. I

assessed the rats' impulsive action using a 3-choice serial reaction time task[10], which is a simplified (but reliable) version of the 5-choice serial reaction time task[12] that measures impulsive action. I also investigated the neural mechanisms that underlie the suppressive effect of repeated milnacipran on elevated impulsive action using histological and electrophysiological techniques.

## Materials and Methods

### Subjects

Male Wistar/ST rats supplied by Nippon SLC Co. Ltd. (Hamamatsu, Japan) were used in this study. They were housed in groups of 4 under an alternating light-dark cycle (light from 7 p.m. to 7 a.m.) at approximately 21°C and relative humidity 40–50%. When the rats were 9 weeks old (270–290 g), I started to restrict their food intake. Thereafter, their body weights were maintained at 85% of those under free-feeding conditions. The treatment of animals was in compliance with the Guidelines for the Care and Use of Laboratory Animals of the Animal Research Committee of Hokkaido University.

### Apparatus

I used the same apparatus detailed in CHAPTER 2 controlled by a computer program written in the MED-PC language (Med Associates Inc., St. Albans, VT, USA).

### 3-choice serial reaction time task

The training procedure, the task sequence, and the behavioral parameter employed in the 3-CSRTT were detailed in the CHAPTER 2.

### Excitotoxic lesion of the vmPFC

After completing the training, the rats were tested over 7 consecutive daily sessions on the standard task (ITI = 5 s, stimulus duration = 1 s) to establish a stable pre-operative baseline. Subsequently, the rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Rats received infusions of 0.09 M quinolinic acid (Tocris, Bristol, UK) or 0.01 M phosphate buffered saline (PBS) according to the following stereotaxic coordinates (mm from bregma or from dura): anteriorposterior (AP) + 2.5; lateral ± 0.7, dorsoventral −4.5 (0.4μl) and AP + 3.0; L ± 0.7, DV −4.5 (0.4 μl)[31]. Injections were made using a microsyringe mounted in a Harvard infusion pump and connected to a 30-gauge stainless steel cannula. The quinolinic acid solution was prepared freshly each day. The injection volume was infused over a period of 4 min (0.1 μl/min) and cannula was left in place for a further 2 min. After surgery, the rats were housed individually and allowed a 5-day recovery period prior to retraining.

**Post-surgical behavioral testing**

After recovery, the rats were tested over 10 consecutive daily sessions on the standard task. Only the data of the last 7 days were used as the post-operative baseline. Subsequently, rats were gently held and milnacipran (10 mg/kg) or distilled water (DW; 3 ml/kg) was administered via esophagus with a gastric sonde needle 60 min before testing on the 3-CSRTT for 14 days. Following that, the rats were tested without drug administration over 7 consecutive daily sessions on the standard task to establish the post-experimental baseline. I divided rats into 4 groups (nonlesioned-DW, nonlesioned-MIL, lesioned-DW, and lesioned-MIL) based on the number of premature response of pre-operative baseline to avoid generating the difference in basal impulsivity among groups of rats.

Milnacipran hydrochloride was generously donated by Asahi-Kasei Co. Ltd. (Tokyo, Japan) and dissolved in DW (pH = 6.5–6.8) at a volume of 3 ml/kg. The dose was chosen on the basis of our previous study[10]. The drug administration design was determined based on reports that demonstrated the pharmacokinetics of milnacipran[132, 133]. The half-life of the drug was gradually prolonged as once-daily oral administration was repeated and the residual drugs were capable to be detected in the cerebrum at 24 hr after 7th dosing. After the 14th dosing, the blood concentration of the drug reached a maximum concentration at 1 hr and then declined with half-life of 11 hr. However, the blood concentration of the residual milnacipran at 24 hr after cessation of the drug treatment declined as the same level as maximum concentration of 3 mg/kg of milnacipran which could not suppress impulsive action in our previous study[10] That is, the blood concentration of milnacipran would be below the effective blood concentration 1 day after the cessation of the drug treatment. Then, the residual milnacipran was almost completely eliminated from the rat body within approximately 3 days after the cessation of drug (**Appendix 1**).

**Microinjection of AMPA and NMDA receptor antagonists into the vmPFC**

Eighteen rats received the training of the 3-CSRTT for this study. After completing the training, the rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and fixed in a stereotaxic frame (Narishige, Tokyo, Japan). Stainless steel guide cannulas (24 gauge, 9 mm long) were bilaterally implanted according to the following stereotaxic coordinates (mm from bregma or from dura): AP + 3.2; lateral ± 0.7, dorsoventral −2.0[103]. Dummy cannulas (30 gauge) were

inserted to penetrate the tip of the guide cannulas. After surgery, the rats were housed individually and allowed a 4-day recovery period prior to retraining. Prior to testing, the rats were retrained for at least 1 week until their performance re-stabilized. On the testing day, the rats were gently restrained, and the dummy cannulas were removed and replaced with 30-gauge stainless steel injection cannulas (11.3 mm long) attached to a polyethylene tube. The tips of the injectors extended beyond the guide cannulas by 2.3 mm. Nine rats received intra-vmPFC injection of 2,3-Dioxo-6-nitro-1,2,3,4- tetrahydrobenzo [*f*]quinoxaline-7-sulfonamide (NBQX; 0, 0.1, and 1 μg/side) disodium salt (Tocris, USA), a selective and competitive AMPA receptor antagonist, 10 min before the 3-CSRTT. Another 9 rats received intra-vmPFC infusions of 3-((R)-2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid ((R)-CPP; 0, 1, and 10 ng/side) (Tocris, USA) 10 min prior to the 3-CSRTT. The injection volume (0.5 μl) was infused over a period of 1 min (0.5 μl/min) and cannula was left in place for a further 1 min. Each drug session was conducted with more than a 2-day interval. The order of the drug injection was counterbalanced by using a Latin Square design. The placements of cannula tips were determined using a Nissle staining after completing the behavioral experiment.

**Western blotting**

Following the completion of the behavioral experiments, 24 rats were deeply anaesthetized with urethane (1 g/kg, i.p.). Then, the brains were removed and 1-mm coronal sections through the dorsomedial prefrontal cortex (dmPFC) and the vmPFC were collected. Subsequent procedures are detailed in Song *et al.*[134]. The primary antibodies used in this study were as follows: rabbit anti-BDNF antibody (1:1000; SC-546; Santa Cruz Biotechnology, TX, USA), rabbit anti-Synapsin I antibody (1:100,000; ab64581; Abcam, Cambridge, UK), rabbit anti-postsynaptic density-95 (PSD-95) antibody (1:10,000, ab18258; Abcam), and mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies (1:10,000,000; MAB374; Millipore, MA, USA). The rabbit anti-BDNF antibody could detect both precursor and mature BDNF (proBDNF and mBDNF, respectively). The selectivity of the primary antibodies was confirmed with absorption tests using the BDNF blocking peptide (SC-546-P; Santa Cruz Biotechnology), the synapsin I peptide (ab64580; Abcam), and the PSD-95 peptide (ab18661; Abcam).

**Immunohistochemistry and cell counting**

Following the completion of the behavioral experiments, 20 rats were deeply anaesthetized with urethane (1 g/kg, i.p.) and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. The brains were then removed and postfixed with 4% paraformaldehyde overnight at 4°C. The procedures used in immunostaining for Neuronal Nuclei (NeuN) and cell counting were detailed by Shikanai *et al.*[135]. Briefly, immunoperoxidase for NeuN, a marker for neural cells, was performed using the avidin–biotin immunoperoxidase technique with the primary antibody: mouse anti-NeuN antibody (1:1000; MAB377; Millipore). Subsequently, I counted the number of NeuN-positive cells in 3 rostrocaudal sections every 20 μm in the dmPFC and vmPFC (between 2.5 and 3.7 mm anterior to the bregma) and assessed them by the automated selection of cells within the unit areas (200×200 μm) using a densitometric video image analysis system (MCID Elite; InterFocus Imaging Ltd, Cambridge, UK). Then, the number of NeuN-positive cells was averaged in the respective regions of the dmPFC and vmPFC.

**Electrophysiological recording**

Following the completion of the behavioral experiments, 16 rats were deeply anesthetized with $CO_2$ and decapitated, and coronal brain slices through the mPFC (300 μm thick) were cut with a Leica VT1000S (Germany) slicer in ice-cold low-$Na^+$ solution with a specific composition (in mM): 120 Choline-Cl, 3 KCl, 8 $MgCl_2$, 28 $NaHCO_3$, 1.25 $NaH_2PO_4$, and 22 glucose, bubbling with 95% $O_2$ and 5% $CO_2$. For recovery, slices were incubated for 60 min in normal artificial cerebrospinal fluid (ACSF) containing the following (in mM): 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 1.25 $NaH_2PO_4$, 26 $NaHCO_3$, and 20 glucose, pH 7.4 at 25°C. Whole-cell patch-clamp recordings were made from vmPFC neurons that satisfied the following criteria: found at (1) 400-800 μm inside from the pia, (2) the infralimbic area defined in Van Eden and Uylings[136], in coronal acute slices using an upright microscope (BX51WI; Olympus, Tokyo, Japan) equipped with an infrared-CCD camera system (modified DP72; Olympus) in normal bathing solution at 32°C. The resistance of the patch pipette was 3–5 MΩ when filled with intracellular solution containing (in mM): 6 KCl, 130 $K_D$-gluconate, 10 NaCl, 10 HEPES, 0.5 EGTA, 0.1 $CaCl_2$, 2 $MgCl_2$, 4 Na-ATP, and 0.4 Na-GTP, with 0.05% lucifer yellow (Sigma, MO, USA) at pH 7.3 adjusted with KOH. Whole-cell recordings were obtained from layer V pyramidal neurons in the

vmPFC. Neurons were voltage clamped at -70 mV or +40 mV for alpha-amino-3-hydroxy-5-methyl-4- isoxazole-propionic acid (AMPA) or NMDA receptor-mediated excitatory postsynaptic currents (EPSCs), respectively. EPSCs were evoked by stimulating (1– 10 μA; 0.05 Hz) apical dendrites and basal dendrites with glass pipettes with a tip diameter of ~15 μm (filled with ACSF) using a stimulus isolater (ISO-flex, A.M.P.I. Jerusalem, Israel). Data were acquired with an Axopatch 200B amplifier and pCLAMP 9 software (Molecular Devices, CA, USA). Picrotoxin (100 μM; Tocris), a γ-aminobutyric $acid_A$ ($GABA_A$) receptor antagonist, was present in the ASCF to isolate EPSCs.

**Dendrite/spine analysis**

A single apical dendrite emerged from the apex of the pyramidal soma. On average, apical dendrites were located 279 ± 33.4 μm from the soma. Basal dendrites emerged from the base of the pyramidal soma. On average, basal dendrites were located 84 ± 15.4 μm from the soma. Images were taken with a confocal laser-scanning microscope FV1000 (Olympus). Z-Stacks were acquired with 0.5 μm steps. For apical dendrites, an average of 10.5 dendrites from 3-5 neurons were imaged for each animal (n=4 animals per group). For basal dendrites, an average of 14 dendrites from 3-5 neurons were imaged for each animal (n=4 animals per group). Images were deconvolved using Image J software, and a dendrite/spine analysis was performed using the 3D automated software Spiso[137], which analyzes dendritic length, spine density, and spine head diameter. After Spiso processing, a human operator blinded to the conditions verified that all spines had been appropriately identified and manually corrected any errors in spine identification. Because spine size is correlated with synaptic strength, I further investigated the effect of milnacipran on the head diameter of the 2 most prominent spine types: immature (<0.3 μm) and mature (>0.3 μm)[138].

**Data analysis**

Behavioral parameters (see *Behavioral training*) were standardized with each value of the pre-operative baseline. The spine head diameter of each bin (0.1 μm) was standardized with the entire number of spines. The peak amplitude was measured on the basis of the averaged waveform of evoked EPSCs (5 consecutive trials). Most parameters were analyzed separately using a two-factor analysis of variance (ANOVA) with the lesion and the drug as

between-subject factors. In cases in which there was a significant lesion×drug interaction, it was followed by a one-factor ANOVA. For behavioral parameters and electrophysiological recording, a three-factor ANOVA was conducted by adding the phase (post-operative, experimental, or post-experimental) or stimulation intensity (1-10 μA, 1 μA steps) as within-subject factors, respectively.

The alpha level was set at 0.05. All statistical procedures were conducted using SPSS (version 15.0 J).

## Results

### Repeated administration of milnacipran remediated elevated impulsive action in vmPFC-lesioned rats

Table 1 shows the non-standardized data of pre-operative baseline measured in the 3-CSRTT.

**Table. 1** Non-standardized data of the behavioral parameters during the pre-operative baseline.

| Behavioral parameters | Group | Pre-operative baseline |
|---|---|---|
| Premature response (no.) | Nonlesioned-DW | 37.51±2.63 |
| | Nonlesioned-MIL | 40.12±2.67 |
| | Lesioned-DW | 29.11±3.64 |
| | Lesioned-MIL | 32.54±3.65 |
| Perseverative response (no.) | Nonlesioned-DW | 4.57±0.48* |
| | Nonlesioned-MIL | 5.87±0.55 |
| | Lesioned-DW | 4.67±0.78* |
| | Lesioned-MIL | 7.69±0.74 |
| Accuracy (%) | Nonlesioned-DW | 73.80±2.65 |
| | Nonlesioned-MIL | 79.55±2.02 |
| | Lesioned-DW | 75.87±3.27 |
| | Lesioned-MIL | 78.86±1.87 |
| Omission (no.) | Nonlesioned-DW | 14.59±2.06 |
| | Nonlesioned-MIL | 14.15±1.54 |
| | Lesioned-DW | 12.67±2.50 |
| | Lesioned-MIL | 12.65±1.00 |
| Correct response latency (s) | Nonlesioned-DW | 0.84±0.09 |
| | Nonlesioned-MIL | 0.70±0.06 |
| | Lesioned-DW | 0.74±0.09 |
| | Lesioned-MIL | 0.76±0.06 |
| Reward latency (s) | Nonlesioned-DW | 1.59±0.06 |
| | Nonlesioned-MIL | 1.72±0.07 |
| | Lesioned-DW | 1.57±0.06 |
| | Lesioned-MIL | 1.73±0.09 |

*Note*: *$P$ < 0.05, vs. Lesioned-MIL with Bonferroni's correction.

One-factor ANOVA revealed that there was no difference between groups in premature response ($F_{3, 59}$=2.50, *NS*), accuracy ($F_{3, 59}$=1.14, *NS*),

omission ($F_{3, 59}$=0.26, *NS*), correct response latency ($F_{3, 59}$=0.89, *NS*), and reward latency ($F_{3, 59}$=2.66, *NS*) while there was a significant main effect of perseverative response ($F_{3, 59}$=4.89, $P$<0.05). Multiple comparison of Bonfferoni's method detected that Lesioned-MIL group showed more perseverative response ($P$<0.05) compared to Nonlesioned-DW and Lesioned-DW groups. These results suggest that basal levels of impulsive action, attentional function, food appetite, motivation to the task, and motor activity were not different between groups before receiving experimental treatments though there was pre-existing differences in compulsive behavior between groups.

Premature responses showed clear changes due to excitotoxic lesions of the vmPFC and repeated administration of milnacipran, accompanied by a significant phase×lesion×drug interaction ($F_{2, 112}$=5.98, $P$<0.05, Figure 1A). A two-factor ANOVA revealed that there were significant main effects of the lesion in premature responses during the post-operative baseline ($F_{1, 56}$=31.43, $P$<0.05) and experimental period ($F_{1, 56}$=19.35, $P$<0.05). In the experimental period, there was a main effect of the drug ($F_{1, 56}$=18.28, $P$<0.05). However, there was a significant lesion×drug interaction in the post-experimental baseline ($F_{1, 56}$=22.02, $P$<0.05), and the one-factor ANOVA indicated that there was a significant difference in the premature response between the Lesioned-DW and Lesioned-MIL groups ($F_{1, 27}$=4.31, $P$<0.05) (Figure 1B). The results suggest that lesions of the vmPFC elevated impulsive action and repeated administration of milnacipran suppressed it. Moreover, surprisingly, the effect of milnacipran on the premature response persisted even after the cessation of the drug treatment.

Preservative response, an index of compulsive behavior, was affected by repeated treatment with milnacipran, accompanied by a significant phase×lesion×drug interaction ($F_{2, 112}$=5.71, $P$<0.05, Figure 1C). A two-factor ANOVA revealed that there was no significant main effect of the lesion in perseverative responses during any time phase. There were significant main effects of the drug in the perseverative response during the experimental phase ($F_{1, 56}$=8.25, $P$<0.05) and post-experimental phase ($F_{1, 56}$=6.23, $P$<0.05). There was no significant lesion×drug interaction in the perseverative response in any time phase. This result indicates that repeated milnacipran administration suppressed compulsive-like perseverative responses during dosing period and after the cessation of drug treatment. There was no significant main effect or interaction in any behavioral parameters (Figure 1D-G), except for premature response and perseverative response.

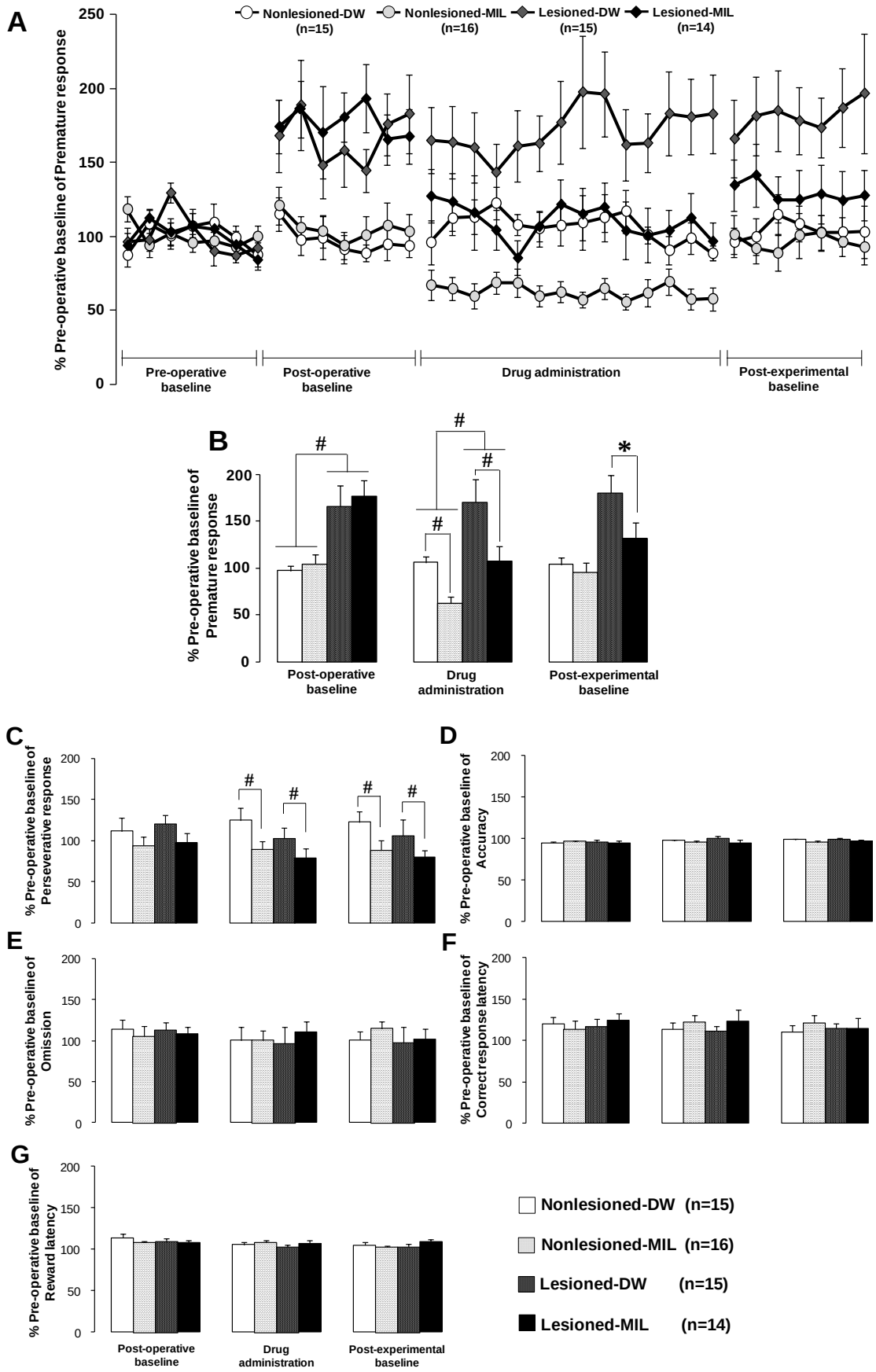
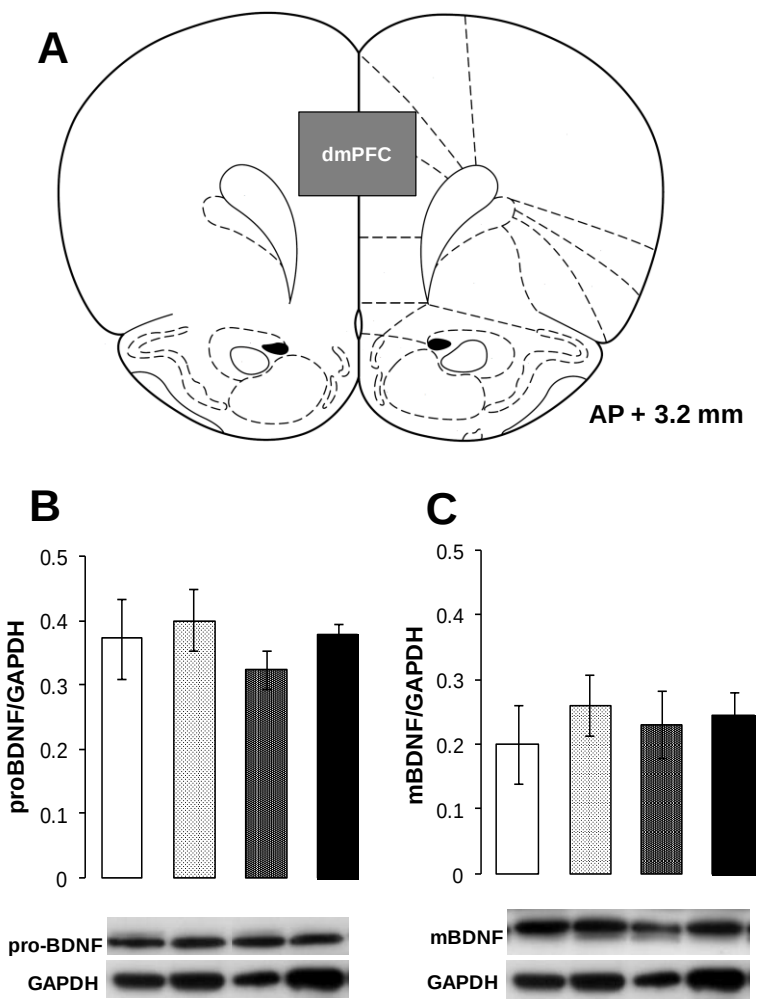

A
Nonlesioned-DW (n=15)
Nonlesioned-MIL (n=16)
Lesioned-DW (n=15)
Lesioned-MIL (n=14)
% Pre-operative baseline of Premature response
Pre-operative baseline
Post-operative baseline
Drug administration
Post-experimental baseline
B
% Pre-operative baseline of Premature response
Post-operative baseline
Drug administration
Post-experimental baseline
C
% Pre-operative baseline of Perseverative response
D
% Pre-operative baseline of Accuracy
E
% Pre-operative baseline of Omission
F
% Pre-operative baseline of Correct response latency
G
% Pre-operative baseline of Reward latency
Post-operative baseline
Drug administration
Post-experimental baseline
Nonlesioned-DW (n=15)
Nonlesioned-MIL (n=16)
Lesioned-DW (n=15)
Lesioned-MIL (n=14)

**Figure 1.** Summary of the main effects of the vmPFC lesions and repeated milnacipran administration on behavioral parameters in the 3-CSRTT.

(**A**) The temporal changes of the percent pre-operative baseline of premature response of Nonlesioned-DW rats (open circles, n=15), Nonlesioned-MIL rats (closed circles, n=16), Lesioned-DW rats (open diamonds, n=15), and Lesioned-MIL rats (closed diamonds, n=14). Lines represent SEM. Mean percent pre-operative baseline of (**B**) premature response (**C**) perseverative response, (**D**) percent accuracy, (**E**) omission, **(F**) correct response latency, and (**G**) reward latency of the 4 groups of rats averaged over the final 7 days of post-operative baseline and 14 days of experimental baseline. The bars represent the mean, and the lines represent the SEM. #$P < 0.05$, a main effect of the lesion or drug with a two-factor ANOVA. *$P < 0.05$, Lesioned-DW vs. Lesioned-MIL with a one-factor ANOVA. DW: distilled water, MIL: milnacipran.

## Repeated administration of milnacipran reversed the mBDNF deficit in the vmPFC of vmPFC-lesioned rats

Mannari *et al.*[131] demonstrated that repeated administration of duloxetine, another SNRI, increases the protein levels of mBDNF in the rat mPFC. mBDNF could induce neurogenesis, dendritic elongation, and spinogenesis in adult rat brain[139-142]. Then, I examined whether repeated administration of milnacipran increased mBDNF levels in the vmPFC of vmPFC-lesioned rats (Figure 2D-F). I also examined the protein levels of mBDNF in the dmPFC as a control (Figure 2A-C). As for the dmPFC, there was no main effect of the lesion or drug in the protein levels of proBDNF, a precursor protein of mBDNF, ($F_{1, 20}$=0.57, *NS*; $F_{1, 20}$=0.12, *NS*, respectively) or mBDNF ($F_{1, 20}$=0.42, *NS*; $F_{1, 20}$=0.23, *NS*, respectively) (Figure 2B and C). In contrast, lesions of the vmPFC significantly reduced the protein levels of proBDNF in the vmPFC ($F_{1, 20}$=5.78, $P$<0.05), but the repeated administration of milnacipran had no effect (Figure 2E). There was a significant lesion×drug interaction in mBDNF levels of the vmPFC ($F_{1, 20}$=5.90, $P$<0.05), and a one-factor ANOVA revealed that there was a significant difference in mBDNF levels between the Lesioned-DW and Lesioned-MIL groups ($F_{1, 10}$=5.45, $P$<0.05) (Figure 2F). The results suggest that mBDNF levels in the vmPFC were impaired by lesions of the vmPFC and the impairment was rescued by repeated administration of milnacipran.


A
dmPFC
AP + 3.2 mm
B
proBDNF/GAPDH
0.5
0.4
0.3
0.2
0.1
0
pro-BDNF
GAPDH
C
mBDNF/GAPDH
0.5
0.4
0.3
0.2
0.1
0
mBDNF
GAPDH

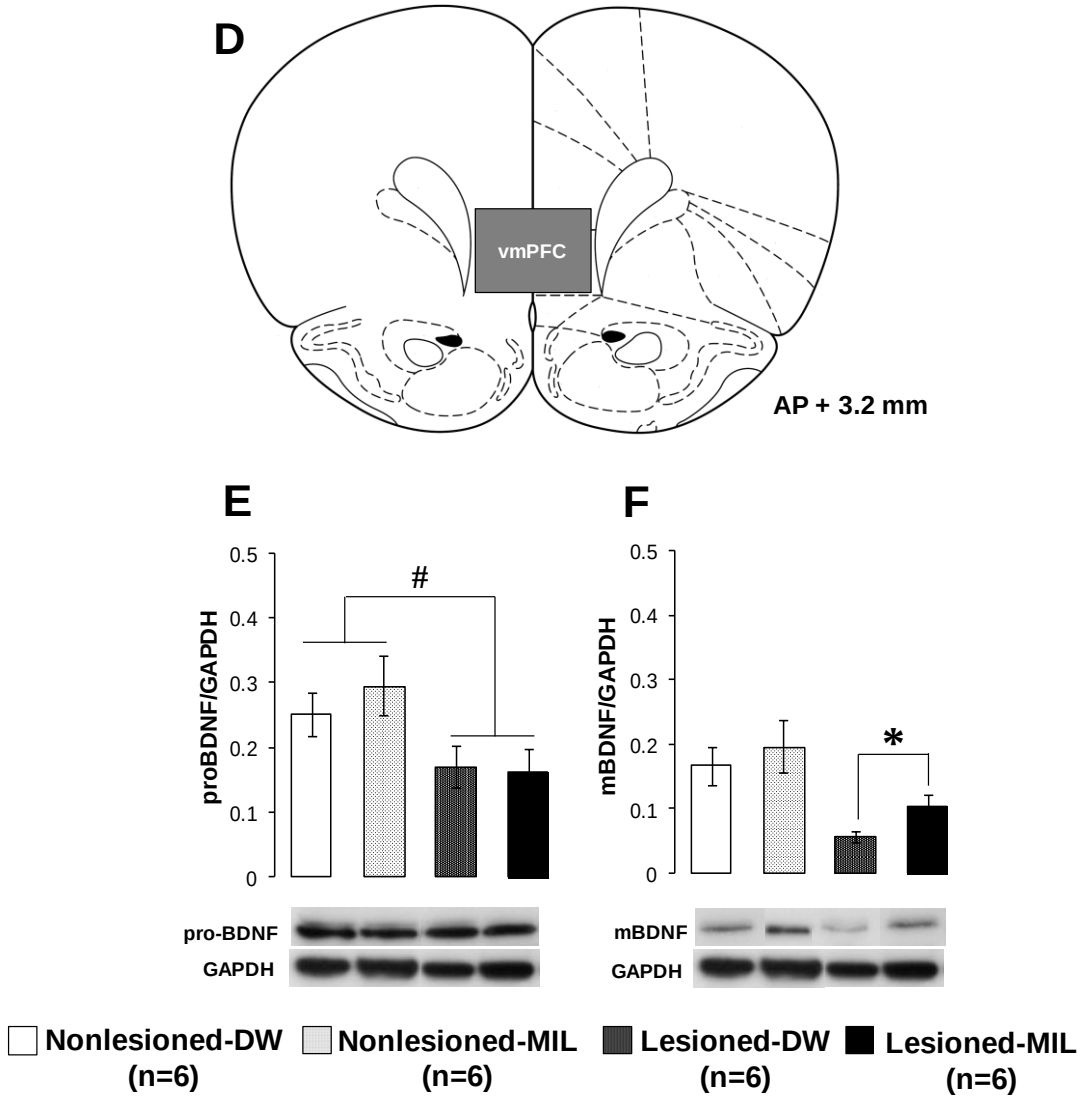



**Figure 2.** The effects of lesions of the vmPFC and repeated milnacipran administration on protein levels of pro- and mBDNF in the mPFC.

A schematic representation of sections that were used for Western blotting; (**A**) dmPFC and (**D**) vmPFC. The bars represent the means of the levels of (**B**) pro- and (**C**) mBDNF in the dmPFC and (**E**) pro- and (**F**) mBDNF in the vmPFC. The protein bands were detected around 32 kDa (proBDNF), 14 kDa (mBDNF), and 38 kDa (GAPDH). The lines represent the SEM. $^{\#}P < 0.05$, a main effect of the lesion with a two-factor ANOVA. $*P < 0.05$, lesioned-DW vs. lesioned-MIL with a one-factor ANOVA. DW: distilled water, MIL: milnacipran.

**Repeated administration of milnacipran did not elicit neurogenesis in the vmPFC of vmPFC-lesioned rats**

As shown in Figure 2F, repeated administration of milnacipran reversed mBDNF depletion in rats with vmPFC lesions. mBDNF is thought to elicit neurogenesis and reconstruct the plasticity in adult rat brain[139, 141]. I speculated that repeated milnacipran administration might elicit functionally-matured neural cells in the vmPFC and reconstruct the neural network in that area in rats with vmPFC lesions. Then, I examined whether the number of neural cells in the vmPFC were increased by repeated administration of milnacipran in vmPFC-lesioned rats (Figure 3A-D). I also analyzed the number of neural cells in the dmPFC as a control. As expected, excitotoxic lesions of the vmPFC significantly decreased the number of neural cells in the vmPFC ($F_{1, 16}$=20.31, $P$<0.05, Figure 3F) but not in the dmPFC ($F_{1, 16}$=0.41, *NS*, Figure 3E). However, there was no lesion×drug interaction in the number of neural cells in the vmPFC ($F_{1, 16}$=0.45, *NS,* Figure 3F). Thus, repeated administration of milnacipran did not reverse the decreased number of neural cells in the vmPFC of vmPFC-lesioned rats.

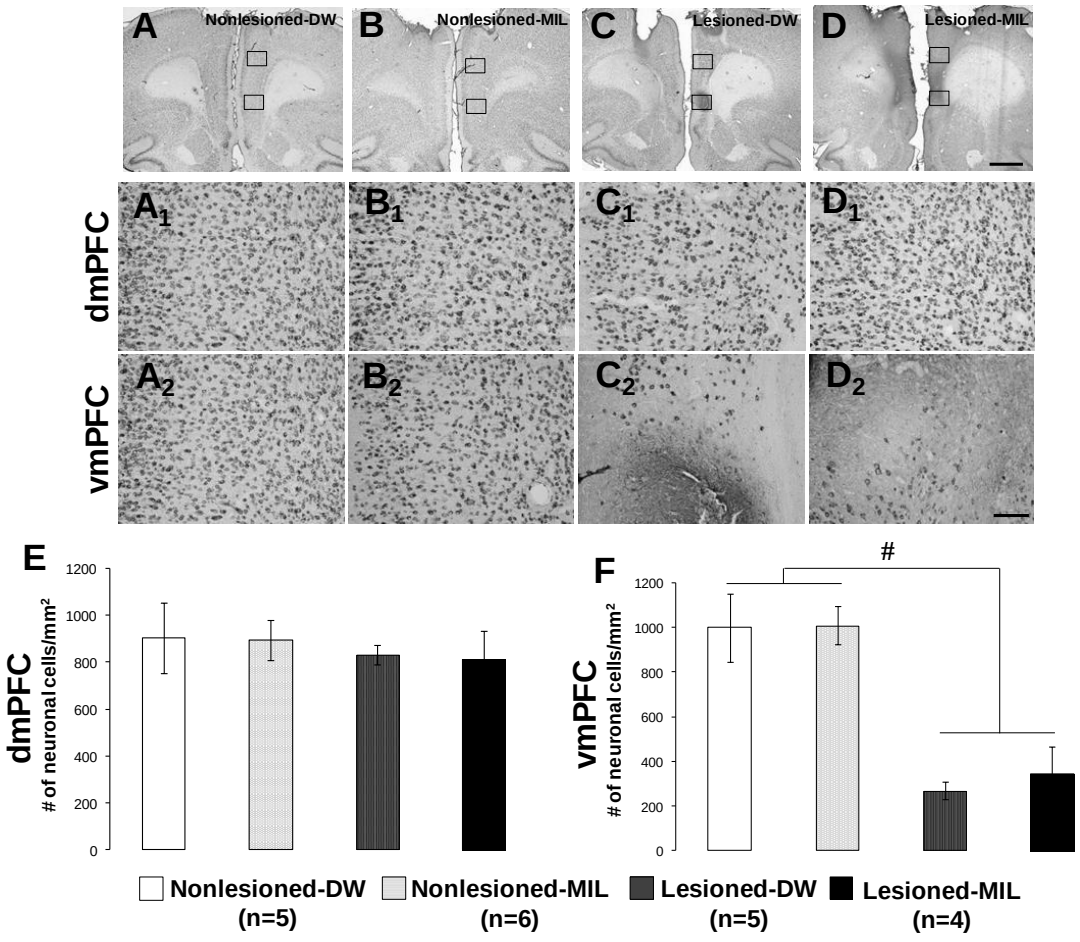


**Figure 3.** The effects of lesions of the vmPFC and repeated milnacipran administration on the number of neuronal cells in the mPFC.

A low-power photograph of NeuN immunolabeling in the mPFC of (**A**) Nonlesioned-DW, (**B**) Nonlesioned-MIL, (**C**) Lesioned-DW, and (**D**) Lesioned-MIL rats. High-power photographs showing NeuN expression in the (**$A_1$-$D_1$**) dmPFC and (**$A_2$-$D_2$**) vmPFC. The bars represent the mean number of neural cells/$mm^2$ in the (**E**) dmPFC and (**F**) vmPFC. The lines represent the SEM. $^{\#}P < 0.05$, a main effect of the lesion with a two-factor ANOVA. Scale bars: (**D**), 1 mm; (**$D_2$**), 100 μm. DW: distilled water, MIL: milnacipran.

## Repeated administration of milnacipran induced spine remodeling in the vmPFC of vmPFC- lesioned rats

Contrary to our expectation, repeated milnacipran treatment did not induce neurogenesis in the vmPFC (Figure 3F). However, there was another possible neural change underlying the mechanisms by which repeated milnacipran induced persistent remediation of elevated impulsive action. Repeated administration of milnacipran increased the protein levels of mBDNF in the vmPFC (Figure 2F) which enables producing dendric elongation and/or spinogenesis in the corresponding area[140, 142]. Then, I examined whether the dendritic elongation, spinogenesis, and spine head enlargement in the vmPFC of vmPFC-lesioned rats were stimulated by repeated treatment of milnacipran.

The number of apical and basal dendritic branch points in the vmPFC (Figure 4A-D) was decreased by lesions of the vmPFC ($F_{1, 38}$=7.39, $P$<0.05 and $F_{1, 38}$=5.40, $P$<0.05, respectively), but this dendritic impairment was not reversed by repeated administration of milnacipran in either apical ($F_{1, 38}$=1.02, *NS*, Figure 4E left) or basal ($F_{1, 38}$=1.49, *NS*, Figure 4E right) dendritic branches.

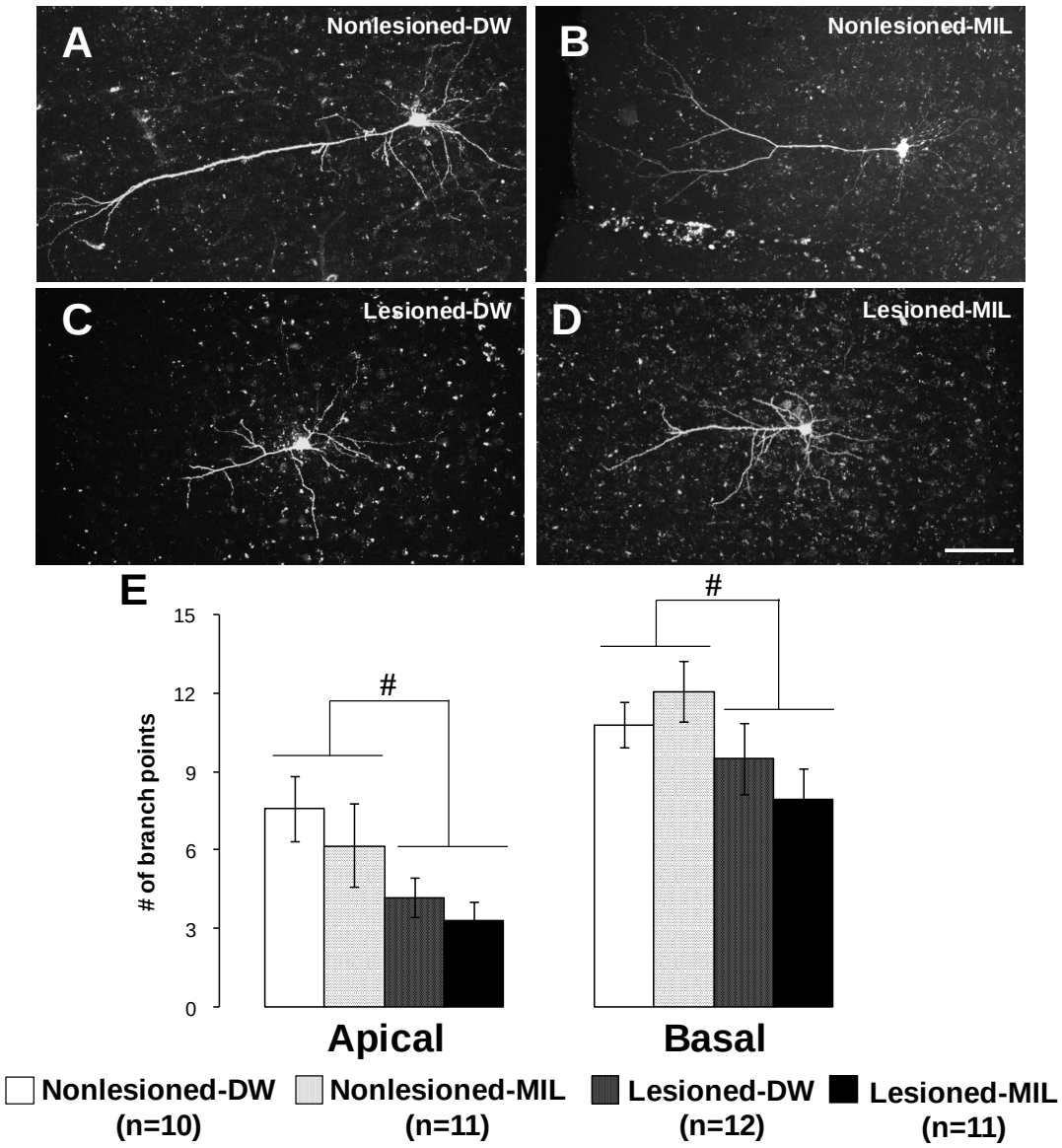



**Figure 4.** The effects of lesions of the vmPFC and repeated milnacipran administration on the number of dendritic branch points in the layer V pyramidal neurons of the vmPFC.

Representative images of Z-stack projections showing apical tuft dendrites from layer V pyramidal neurons in the vmPFC of (**A**) Nonlesioned-DW, (**B**) Nonlesioned-MIL, (**C**) Lesioned-DW, and (**D**) Lesioned-MIL rats. (**E**) Bar graph showing the mean number of branch points in the apical (left) and basal (right) dendrites in the 4 groups of rats. The lines represent the SEM. Scale bar: (**D**) 100 μm. $^{\#}P < 0.05$, a main effect of the lesion with a two-factor ANOVA. DW: distilled water, MIL: milnacipran.

Similarly, the spine density of the apical dendrites (Figure 5A-D) was decreased by lesions of the vmPFC ($F_{1, 164}$=21.59, $P$<0.05), but this spine atrophy was not ameliorated by the repeated administration of milnacipran ($F_{1, 164}$=2.60, *NS,* Figure 5I). In the basal dendrites (Figure 5E-H), however, the impairment of spine density by lesions of the vmPFC ($F_{1, 222}$=35.83, $P$<0.05) was reversed by the repeated administration of milnacipran. A two-factor ANOVA revealed that there was a significant lesion×drug interaction ($F_{1, 222}$=8.09, $P$<0.05) and a one-factor ANOVA revealed that there was a significant difference in spine density between the Lesioned-DW and Lesioned-MIL groups ($F_{1, 124}$=38.01, $P$<0.05) (Figure 5J).

A three-factor ANOVA revealed that there was a significant bin×lesion×drug interaction in the sizes of both apical ($F_{6, 984}$=5.20, $P$<0.05, Figure 5K) and basal ($F_{6, 1332}$=4.29, $P$<0.05, Figure 5L) dendritic spines. A two-factor ANOVA indicated that lesions of the vmPFC increased the ratio of immature spines in apical ($F_{1, 164}$=24.01, $P$<0.05 for bin 0.2-0.3) and basal ($F_{1, 222}$=17.39, $P$<0.05 for bin 0.2-0.3) dendrites and decreased the ratio of mature spines in apical ($F_{1, 164}$=35.22, $P$<0.05 for bin 0.4-0.5; $F_{1, 164}$=20.67, $P$<0.05 for bin 0.5-0.6; $F_{1, 164}$=9.68, $P$<0.05 for bin 0.6-0.7, Figure 5K) and basal ($F_{1, 222}$=8.60, $P$<0.05 for bin 0.5-0.6, Figure 5L) dendrites. There was a significant lesion×drug interaction regarding mature spines in apical ($F_{1, 164}$=5.49, $P$<0.05 for bin 0.3-0.4, Figure 5K) and basal ($F_{1, 222}$=7.69, $P$<0.05 for bin 0.3-0.4, Figure 5L) dendrites, and a one-factor ANOVA revealed that there were significant differences in the ratio of spines whose head diameters were 0.3-0.4 μm between the Lesioned-DW and Lesioned-MIL groups ($F_{1, 74}$=9.70, $P$<0.05 for apical dendrites; $F_{1, 124}$=6.64, $P$<0.05 for basal dendrites). These results suggest that repeated administration of milnacipan did not remediate dendritic atrophy but induced spinogenesis and spine head enlargement in the the vmPFC of vmPFC-lesioned rats.

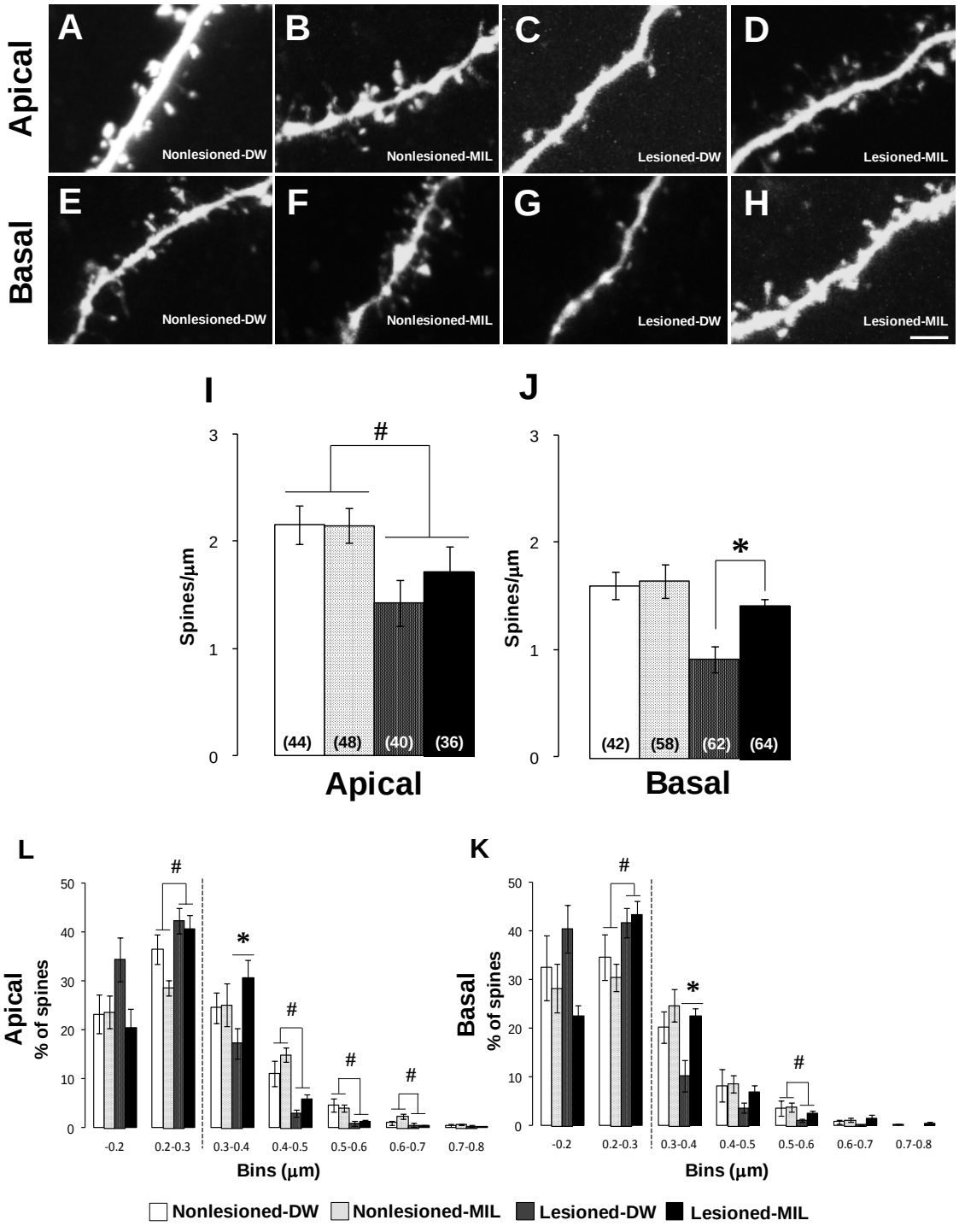



**Figure 5.** The effects of lesions of the vmPFC and repeated milnacipran administration on the dendritic spine density/morphology in layer V pyramidal neurons of the vmPFC.

Representative images showing spine density and morphology in (**A-D**) apical and (**E-H**) basal dendrites of 4 groups of rats. Bar graphs comparing spine density in (**I**) apical and (**J**)

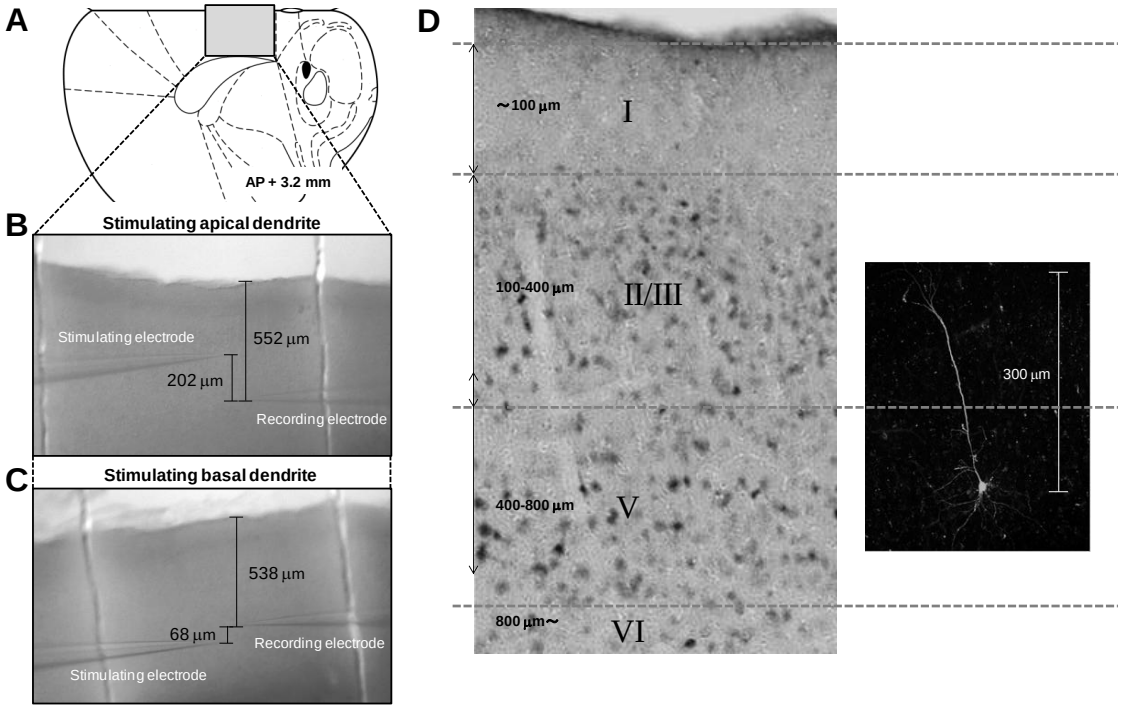
basal dendrites of the 4 groups of rats. The lines represent the SEM. The spine head diameters of (**K**) apical and (**L**) basal dendritic spines are plotted next to each other in which the spines were binned by size per group using 0.1 μm bins (numbers below the bar graph represent the lower bound of the bin). Dashed lines divide spine types into immature (<0.3 μm) and mature (>0.3 μm) types. The lines represent the SEM. Scale bar: (**H**) 5 μm. $^{\#}P$ < 0.05, a main effect of the lesion with a two-factor ANOVA. *$P$ < 0.05, Lesioned-DW vs. Lesioned-MIL with a one-factor ANOVA. DW: distilled water, MIL: milnacipran.

**Repeated administration of milnacipran ameliorated impaired excitatory currents in the basal dendritic neural circuits in the vmPFC of vmPFC-lesioned rats**

As shown in Figure 5, repeated treatment of milnacipran reversed decreased spine density in the basal dendrites and increased the ratio of mature spines in both apical and basal dendrites in the vmPFC of vmPFC-lesioned rats. Considering the majority of excitatory synapses in the brain occur on dendritic spines, I hypothesized that repeated milnacipran treatment ameliorates impaired excitatory currents in the vmPFC of vmPFC-lesioned rats. Then, I analyzed AMPA and NMDA EPSCs in the layer V pyramidal neurons of the vmPFC when stimulating apical and basal dendrites (Figure 6A-D). A three-factor ANOVA revealed that there were significant stimulation intensity×lesion×drug interactions in the AMPA EPSCs when stimulating apical ($F_{10, 610}$=3.56, $P$<0.05, Figure 6E and G) and basal ($F_{10, 360}$=9.44, $P$<0.05, Figure 6F and H) dendrites. However, a subsequent two-factor ANOVA did not find any significant main effect of the lesion ($F_{1, 40}$=0.09, *NS*) or drug ($F_{1, 40}$=0.35, *NS*) or a lesion×drug interaction ($F_{1, 40}$=0.63, *NS*) in AMPA EPSCs when stimulating apical dendrites (Figure 6G). In contrast, there was a significant lesion×drug interaction ($F_{1, 40}$=4.16, $P$<0.05, Figure 6F and H) in AMPA EPSCs when stimulating basal dendrites, and a one-factor ANOVA revealed that there was a significant difference in AMPA EPSCs between the lesioned-DW and lesioned-MIL groups ($F_{1, 20=}$6.25, $P$<0.05, Figure 6H).

A three-factor ANOVA found that there were significant stimulation intensity×lesion×drug interactions in the NMDA EPSCs when stimulating apical ($F_{10, 240}$=2.51, $P$<0.05, Figure 6I and K) and basal ($F_{10, 240}$=2.53, $P$<0.05, Figure 6J and L) dendrites. Subsequently, a two-factor ANOVA revealed a significant lesion×drug interaction in NMDA EPSCs when stimulating basal ($F_{1, 40}$=4.15, $P$<0.05) but not apical ($F_{1, 40}$=0.80, *NS,* Figure 6K) dendrites, and a one-factor

ANOVA revealed that there was a significant difference in NMDA EPSCs between the Lesioned-DW and Lesioned-MIL groups ($F_{1,\ 20}$=8.26, $P$<0.05, Figure 6L). There was a main effect of the lesion ($F_{1,\ 40}$=5.59, $P$<0.05) in NMDA EPSCs when stimulating apical dendrites (Figure 6K). The results suggest that repeated milnacipran ameliorated impaired AMPA and NMDA currents especially in the basal dendritic neural circuits in the vmPFC of vmPFC-lesioned rats.



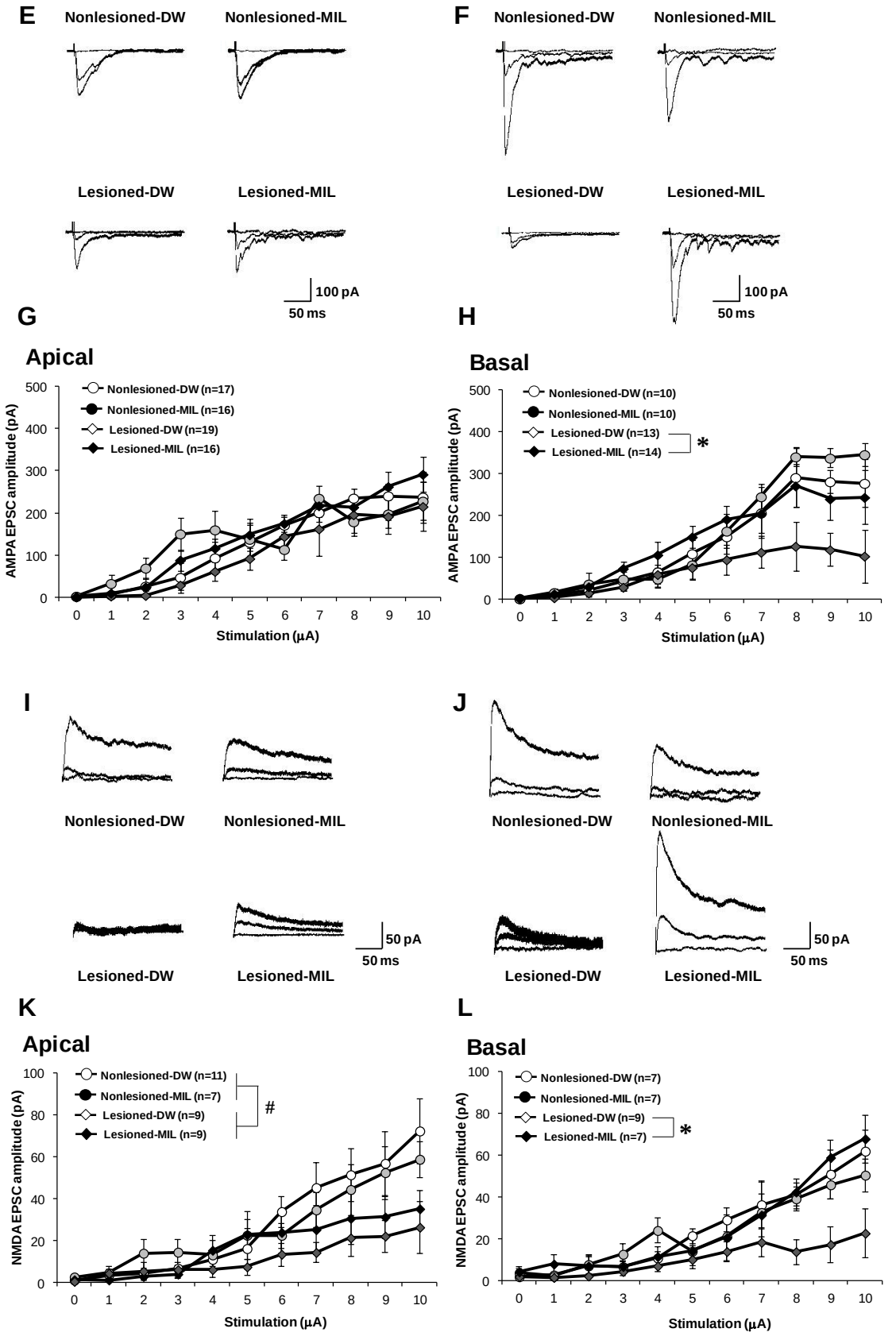

E
Nonlesioned-DW
Nonlesioned-MIL
Lesioned-DW
Lesioned-MIL
100 pA
50 ms
F
Nonlesioned-DW
Nonlesioned-MIL
Lesioned-DW
Lesioned-MIL
100 pA
50 ms
G
Apical
AMPA EPSC amplitude (pA)
Nonlesioned-DW (n=17)
Nonlesioned-MIL (n=16)
Lesioned-DW (n=19)
Lesioned-MIL (n=16)
Stimulation (μA)
H
Basal
AMPA EPSC amplitude (pA)
Nonlesioned-DW (n=10)
Nonlesioned-MIL (n=10)
Lesioned-DW (n=13)
Lesioned-MIL (n=14)
*
Stimulation (μA)
I
Nonlesioned-DW
Nonlesioned-MIL
Lesioned-DW
Lesioned-MIL
50 pA
50 ms
J
Nonlesioned-DW
Nonlesioned-MIL
Lesioned-DW
Lesioned-MIL
50 pA
50 ms
K
Apical
NMDA EPSC amplitude (pA)
Nonlesioned-DW (n=11)
Nonlesioned-MIL (n=7)
Lesioned-DW (n=9)
Lesioned-MIL (n=9)
#
Stimulation (μA)
L
Basal
NMDA EPSC amplitude (pA)
Nonlesioned-DW (n=7)
Nonlesioned-MIL (n=7)
Lesioned-DW (n=9)
Lesioned-MIL (n=7)
*
Stimulation (μA)

**Figure 6.** The effects of lesions of the vmPFC and repeated milnacipran administration on excitatory currents in the layer V pyramidal neurons in the vmPFC.
Electrophysiological characterization of pyramidal neurons in the vmPFC using whole-cell patch-clamp recording. (**A**) vmPFC position and representative photographs of a coronal slice submerged in our recording chamber depicting the position of an electrode for the recording of EPSCs when stimulating (**B**) apical (239 ± 33.4 μm from soma) and (**C**) basal (77 ± 9.2 μm from soma) dendrites. (**D**) Only vmPFC neurons that satisfied the criteria (see Materials and Methods) were used for this study. Examples of AMPA EPSCs evoked by 1-, 5-, and 10–μA stimulation of (**E**) apical and (**F**) basal dendrites of layer V pyramidal neurons of the 4 groups of rats. Average AMPA EPSC amplitude in (**G**) apical and (**H**) basal dendrites of layer V pyramidal neurons of the 4 groups of rats. Examples of NMDA EPSCs evoked by 1-, 5-, and 10-μA stimulation of (**I**) apical and (**J**) basal dendrites of layer V pyramidal neurons of the 4 groups of rats. Average NMDA EPSC amplitude in (**K**) apical and (**L**) basal dendrites of layer V pyramidal neurons of the 4 groups of rats. $^{\#}P < 0.05$, a main effect of the lesion with a two-factor ANOVA. $^{*}P < 0.05$, Lesioned-DW vs. Lesioned-MIL with a one-factor ANOVA. DW: distilled water, MIL: milnacipran.

## Repeated administration of milnacipran reversed impaired post- but not presynaptic strength in the vmPFC of vmPFC-lesioned rats

There were at least 3 possible mechanisms by which repeated milnacipran reversed decreased excitatory currents in the vmPFC of vmPFC lesioned rats; repeated milnacipran treatment improved the function of (1) presynaptic glutamatergic release, (2) postsynaptic excitatory receptors, or (3) both (1) and (2) in the vmPFC of vmPFC-lesioned rats. The Synapsin I protein is a member of the synapsin family that are neuronal phosphoproteins. Because the Synapsin I protein is associated with the cytoplasmic surface of synaptic vesicles, the Synapsin I is widely used as a marker for presynaptic function. The PSD-95 scaffolding protein has been identified as a marker for synaptic strength. Therefore, I examined the protein levels of Synapsin I and PSD-95 in the vmPFC of 4 groups of rats using Western blotting methodology. A two-factor ANOVA revealed that there was no main effect of the lesion ($F_{1, 20}$=3.26, *NS*) or drug ($F_{1, 20}$=0.01, *NS*) or a lesion×drug interaction ($F_{1, 20}$=1.81, *NS*) in the protein levels of Synapsin I, a marker for presynaptic function, indicating that Synapsin I levels in the vmPFC were not affected by lesions of the vmPFC or drug treatment (Figure 7A). Conversely, lesions of the vmPFC significantly reduced the protein levels of PSD-95, a marker for postsynaptic function, in the vmPFC ($F_{1, 20}$=6.39, $P$<0.05).

There was a significant lesion×drug interaction in PSD-95 levels in the vmPFC ($F_{1,\ 20}$=4.78, $P$<0.05), and a one-factor ANOVA revealed that there was a significant difference in protein levels of PSD-95 between the Lesioned-DW and Lesioned-MIL groups ($F_{1,\ 10}$=4.21, $P$<0.05, Figure 7B). The results suggest that protein levels of PSD-95 in the vmPFC were impaired by lesion of the vmPFC and it was rescued by repeated milnacipran, while protein levels of Synapsin I were intact.

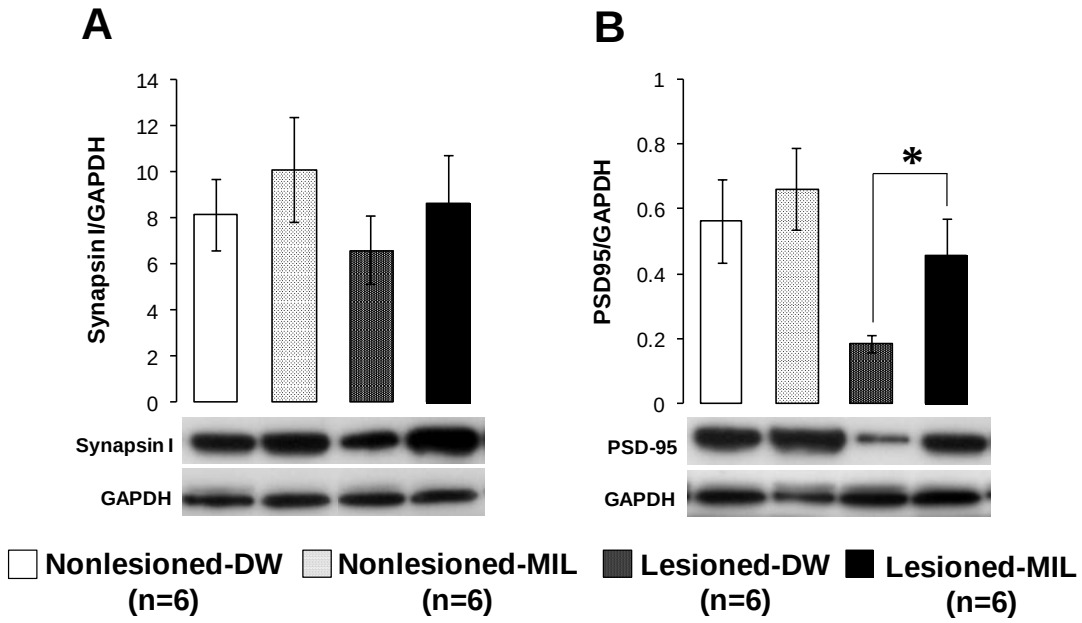


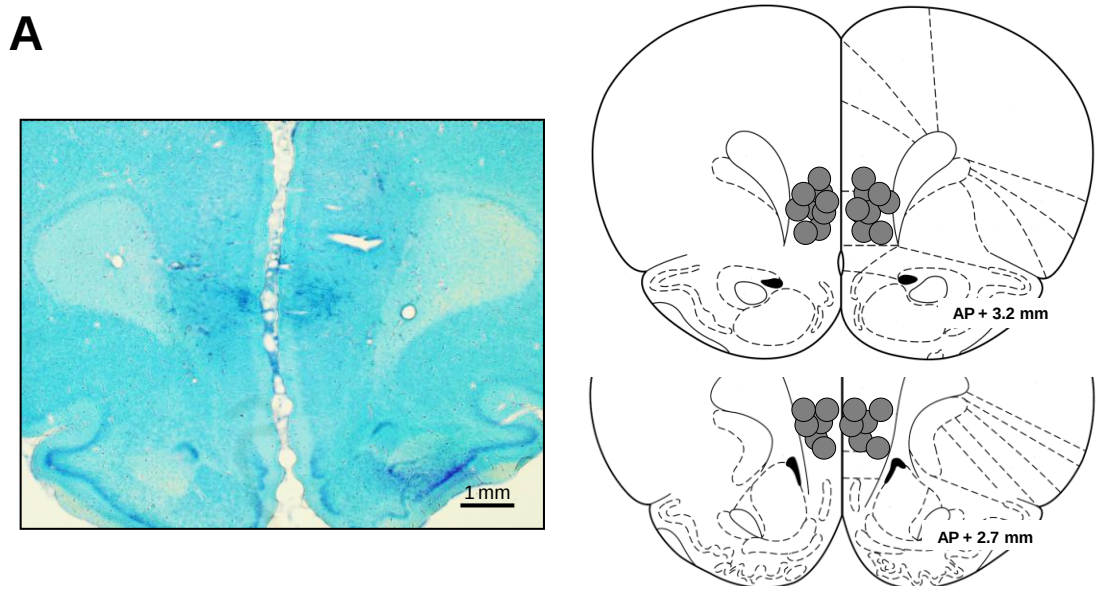

**Figure 7.** The effects of lesions of the vmPFC and repeated milnacipran administration on the protein levels of Synapsin I and PSD-95 in the vmPFC

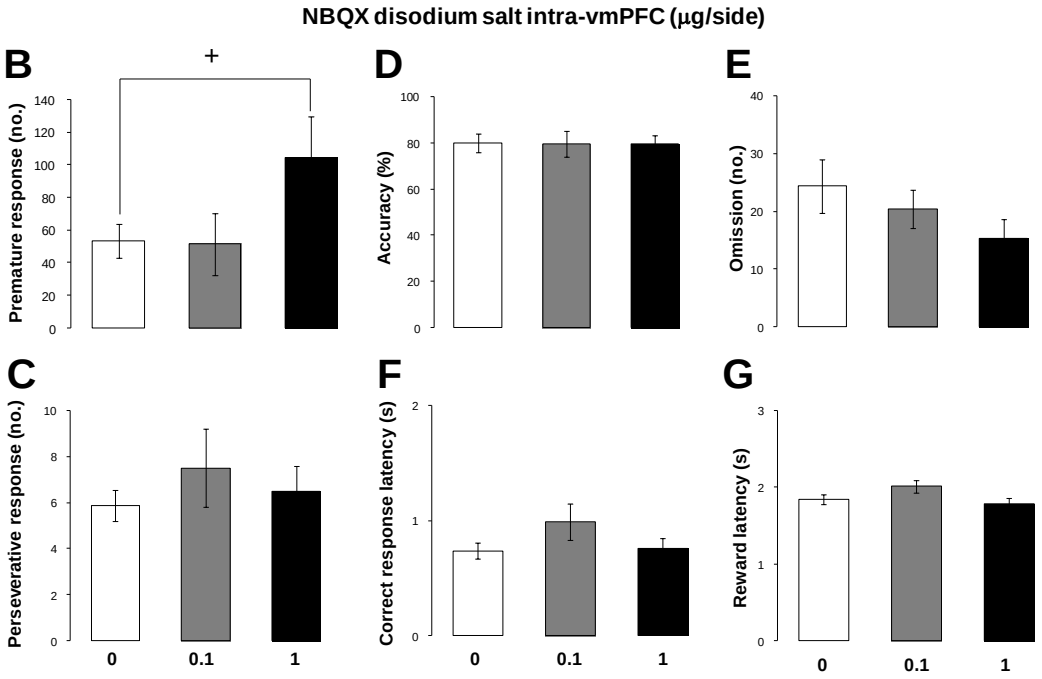
The bars represent the means of the levels of (**A**) Synapsin I and (**B**) PSD-95 in the vmPFC of the 4 groups of rats. The protein bands were detected around 70 kDa (Synapsine I), 80 kDa (PSD-95), and 38 kDa (GAPDH). The lines represent the SEM. *$P$ < 0.05, Lesioned-DW vs. Lesioned-MIL with a one-factor ANOVA. DW: distilled water, MIL: milnacipran.

**AMPA and NMDA receptors in the vmPFC have a crucial role in the regulation of impulsive action in normal rats**

Finally, I examined the effects of intra-vmPFC injections of NBQX and (R)-CPP on behavioral parameters in the 3-CSRTT in normal rats (Figure 8A). A one-factor ANOVA revealed that there was a main effect of NBQX on premature responses ($F_{2,\ 24}$=8.00, $P$<0.05, Figure 8B), but no effect on any other parameters (Figure 8C-G). Subsequent post hoc comparisons with Bonferroni's

correction revealed that 1 μg/side of NBQX significantly increased the number of premature response ($P$<0.05, Figure 8B), indicating that AMPA receptors in the vmPFC play a critical role in the inhibitory control of impulsive action. One-factor ANOVA revealed that there was a main effect of (R)-CPP on premature responses ($F_{2, 24}$=9.13, $P$<0.05, Figure 8H) and omission ($F_{2, 24}$=5.86, $P$<0.05, Figure 8K), but no effect on any other parameters (Figure 8I, J, L, and M). Bonferroni's correction revealed that 10 ng/side of (R)-CPP significantly increased the number of premature responses (Figure 8H) and omission ($P$<0.05, Figure 8K), indicating that NMDA receptors in the vmPFC play a critical role in the inhibitory control of impulsive action and motivational/appetitive function.


A
1 mm
AP + 3.2 mm
AP + 2.7 mm


NBQX disodium salt intra-vmPFC (µg/side)
B
Premature response (no.)
+
D
Accuracy (%)
E
Omission (no.)
C
Perseverative response (no.)
F
Correct response latency (s)
G
Reward latency (s)
0
0.1
1

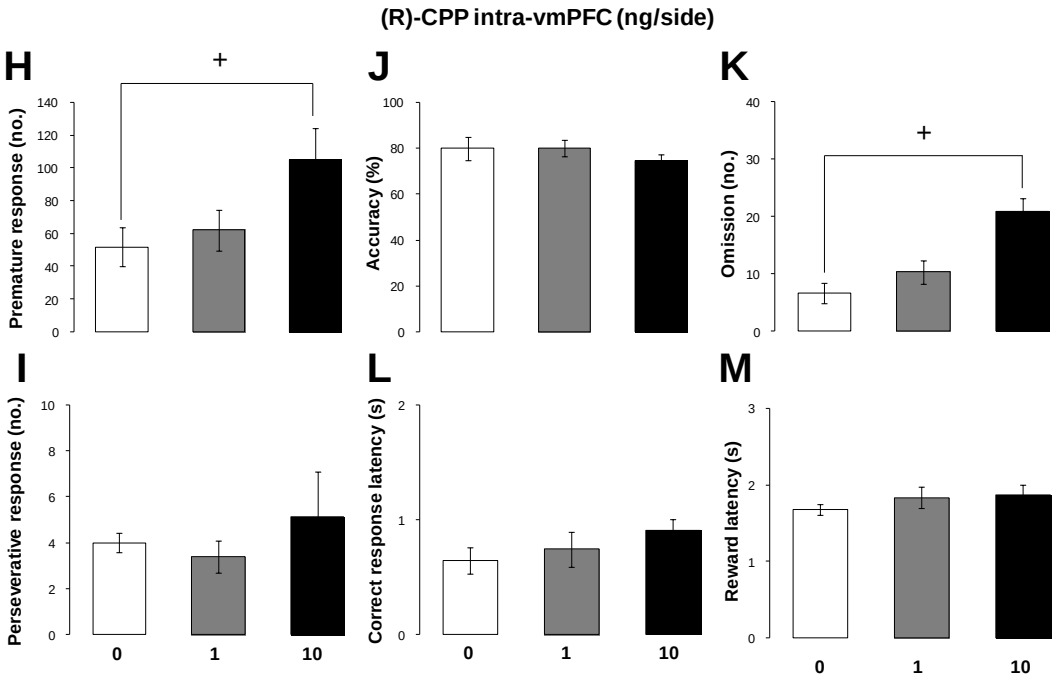



**Figure 8.** The roles of the excitatory signaling in the vmPFC in normal rats

(**A**) A representative photomicrograph and schematic diagrams of coronal sections + 2.7 and +3.2 mm from the bregma. The gray circles indicate the placements of cannula tips in the vmPFC region of rats used in this experiment. The effect of intra-vmPFC infusion of NBQX on (**B**) premature response, (**C**) perseverative response, (**D**) percent accuracy, (**E**) omission, (**F**) correct response latency, and (**G**) reward latency in the 3-CSRTT. The effect of intra-vmPFC infusion of (R)-CPP on (**H**) premature response, (**I**) perseverative response, (**J**) percent accuracy, (**K**) omission, (**L**) correct response latency, and (**M**) reward latency in the 3-CSRTT. The lines represent the SEM. $^{+}P<0.05$ with Bonferroni's correction.

## Discussion

Consistent with the results of a previous study[31], lesions of the vmPFC provoked impulsive action (Figure 1A and B). This lesion-induced increase in the number of premature responses was reversed by the repeated administration of milnacipran. Interestingly, the suppressive effect of repeated milnacipran on premature responses persisted for at least a week, even after the cessation of the treatment (Figure 1A and B).

It is likely that there was somewhat acute suppressive effect of milnacipran on elevated impulsive action in vmPFC-lesioned rats during the drug administration phase because the suppressive effects of milnacipran on impulsive action during the drug administration phase was stronger than those during the post-experimental phase though the difference was not statistically significant (Figure 1A). However, it should be noted that the suppressive effects of milnacipran on impulsive action was still significant even after the cessation of drug treatment (*i.e*., post-experimetnal phase). Within 24 hr after the cessation of repeated administration of milnacipran, the drug concentration declined less than the maximum concentration of 3 mg/kg of acute milnacipran administration[132] which did not suppress impulsive action in our previous study[10]. Moreover the impulsive action was stably-suppressed during the post-experimental period (Figure 1A). Therefore, the persistent suppressive effect of repeated administration of milnacipran on impulsive action was not due to an influence of the residual drugs in the rat body but due to the plastic changes via stimulating BDNF signaling (discussed later).

There was no significant main effect or interaction in any behavioral parameters, except for premature response and perseverative response (Figure 1D-G), indicating that the changes of impulsive action induced by the manipulation of the lesion and repeated treatment with milnacipran were not due to the changes in attentional, motivational/appetitive, or motor function in this study.

Repeated milnacipran treatment also suppressed compulsive-like perseverative response in both lesioned and nonlesioned groups of animals (Figure 1C). Several case reports suggested the potential efficacy of SNRIs treatments in obsessive-compulsive disorder patients[143]. However, basal level of compulsivity in the MIL groups was higher than that in the DW groups of animals (Table 1). Then, I cannot exclude the effect of basal differences of compulsivity

between groups on the current results. Further studies focusing on perseverative response would be required to determine whether milnacipran could suppress compulsivity. It should be noted that the difference of basal compulsivity between MIL and DW groups was unlikely to affect the effect of the lesion manipulation and the drug treatment on impulsive action in this study. This is because (1) the basal performance levels of premature response were not different between groups (Table 1), (2) compulsivity shows little correlation with impulsivity[144], and (3) the neural basis of these phenotypes could be separable; the orbitofrontal cortex rather than the vmPFC is responsible for regulating compulsive behavior[31, 145].

I can speculate that repeated milnacipran treatment increased mBDNF levels by promoting the cAMP-CREB (cAMP-related Element Binding Protein) pathway, which is one of the widely accepted theories regarding the effects of antidepressants on mBDNF[146]. It should be also noted that repeated milnacipran treatment did not affect the protein levels of proBDNF in vmPFC-lesioned rats (Figure 2E). To my knowledge, it has not been reported that proBDNF, a precursor protein of mBDNF, has a neurotrophic action. Repetated milnacipran administration might stimulate both the generation of proBDNF and the processing of mBDNF from proBDNF because the repeated administration of milnacipran increased the protein levels of mBDNF with maintaining its precursor protein at the same level as in the Lesioned-DW group (Figure 2E). The effect of milnacipran treatment on protein levels of mBDNF was limited to vmPFC in lesioned rats (Figure 2) though the drug was administered systemically. Although I cannot explain this phenomenon adequately at this time, the similar result was observed in previous reports. They showed that drug treatment increased messenger ribonucleic acid (mRNA) of BDNF in the PFC or hippocampus of lesioned/stressed animals but not of control animals[147, 148].

In the present study, milnacipran treatment did not restore the decreased number of neural cells in the vmPFC (Figure 3F) while improving the functional properties of surviving neurons. Although the repeated administration of selective serotonin transporter inhibitors (SSRIs) or SNRIs might induce neurogenesis in the adult rat hippocampus[149-151], several studies have denied the birth of new neurons in the adult mammalian PFC[152-156]. Thus, it is unlikely that repeated milnacipran-reversed elevated impulsive action in the vmPFC-lesioned rats was due to neurogenesis in the vmPFC in this study.

Repeated milnacipran administration remedied basal rather than apical

dendritic spine density (Figure 5I and J) and excitatory currents (Figure 6E-L) in the vmPFC of vmPFC-lesioned rats. The apical dendritic spines of layer V pyramidal neurons in the mPFC receive projections from other brain regions[157, 158] while the basal dendritic spines receive local projections from other pyramidal and/or GABAergic neurons in the mPFC[159]. From this perspective, milnacipran treatment might reconstruct the basal dendritic spines and strengthen the local network of the vmPFC. The lesion manipulation and repeated administration of milnacipran did not affect the protein levels of Synapsine I in the vmPFC (Figure 7A). In contrast, repeated milnacipran treatment restored the protein levels of PSD-95 in the vmPFC of vmPFC-lesioned rats (Figure 7B), suggesting that the remodeling of excitatory currents in the vmPC by the repeated administration of milnacipran was likely due to the retrieval of the function of postsynaptic AMPA and NMDA receptors. Indeed, mBDNF induces the translocation of AMPA and NMDA receptors to the postsynaptic membrane[160-162]. Both AMPA and NMDA receptors in the vmPFC have a critical role in the suppression of impulsive action (Figure 8B and H). Although Murphy *et al*.[163] reported no significant effect of intra-vmPFC NBQX, an AMPA receptor antagonist, on premature response, it is probably because they used lower doses of the drug.

The milnacipran treatment might have induced plastic changes not only in the vmPFC but also in the other brain regions. However, to our knowledge, there is so far no evidence that a SNRI induces plastic changes in the brain regions responsible for impulsive behavior except for the vmPFC. For example, Larsen *et al*.[164] demonstrated that repeated administration of venlafaxine, another SNRI, increased mBDNF mRNA levels in the dorsal but not in the ventral hippocampus. However, it is the ventral but not dorsal part of the hippocampus that associated with inhibition of impulsive action[165]. Therefore, it is unlikely that the milnacipran treatment rescued elevated impulsivity via improving hippocmpal function in the current study though I cannot exclude the possibility of pathways leading to plastic changes other than BDNF in the ventral hippocampus. Thus, it is plausible that repeated treatment of milnacipran suppressed impulsivity through inducing plastic changes in the vmPFC.

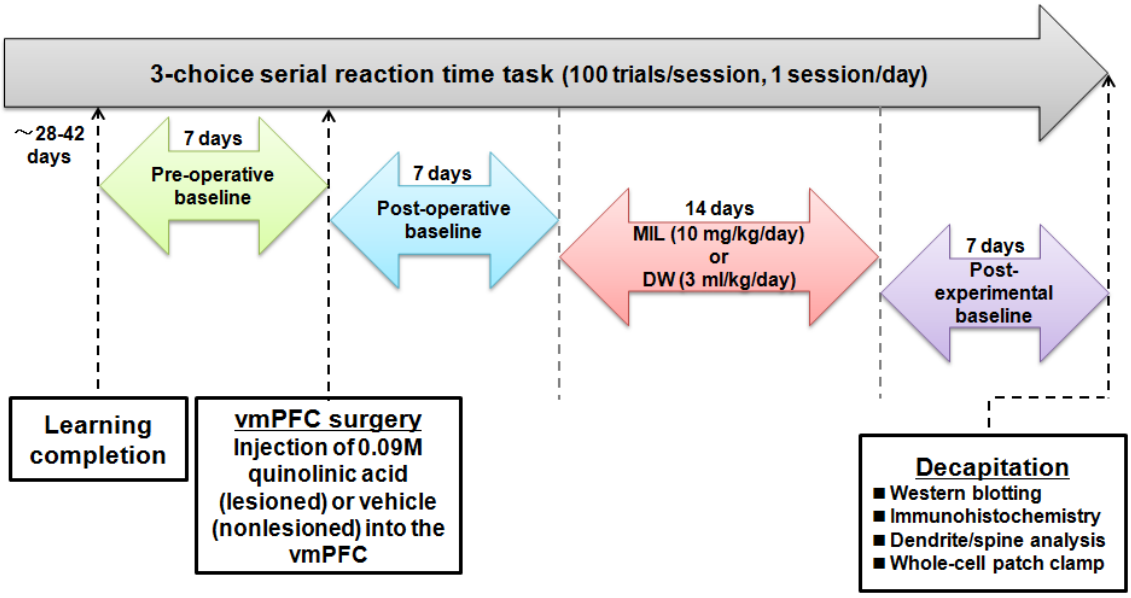
The acute administration of milnacipran suppresses impulsive action by enhancing the dopamine system in the vmPFC (CHAPTER 2), while its repeated administration activates the mBDNF system in the vmPFC, offering a strong strategy for the treatment of psychiatric patients with elevated impulsivity. It is also worth noting that levomilnacipran SR, an active enantiomer of milnacipran,

is currently undergoing phase III clinical trials as a candidate antidepressant in the USA and Canada[166], even though milnacipran is a classic antidepressant in Japan and in European countries. Thus, milnacipran and levomilnacipran SR would be promising candidates to treat depression comorbid with heightened impulsivity.

Considering the results of the current study and previous findings demonstrating that the neurotrophic effects of SSRIs[167] and the acute administration of noradrenaline reuptake inhibitors (NRIs) suppress impulsive action[19, 20, 168, 169], SSRIs, NRIs and milnacipran could be promising therapies for psychiatric disorders with high impulsivity. However, the acute administration of SSRIs lacks anti-impulsive effects[10, 55] and the effects of NRIs on depression have not yet established. It should be noted that milnacipran is an established antidepressant and both the acute and repeated administration of milnacipran suppress impulsivity.

In conclusion, my data suggest that repeated milnacipran treatment essentially ameliorates the dysregulation of impulsive action in vmPFC-lesioned rats presumably through enhancing the protein levels of mBDNF and reconstructing dendritic spines and excitatory currents in the vmPFC. Rats with ventromedial prefrontal lesions could mimic elevated impulsivity observed in several psychiatric disorders, such as attention-deficit/hyperactivity disorder, borderline personality disorder, schizophrenia, and depression. My findings will contribute to the development of novel strategies for the treatment of psychiatric disorders that are associated with high impulsivity.

## **Appendix 1.** A schematic diagram of the experimental design




Male Wistar/ST rats were trained on the 3-CSRTT. After completing the training, the rats were tested over 7 consecutive daily sessions on the standard task to establish a stable pre-operative baseline. Subsequently, the rats received infusions of 0.09 M quinolinic acid or 0.01 M PBS according to the following stereotaxic coordinates (mm from bregma or from dura): anteriorposterior (AP) + 2.5; lateral ± 0.7, dorsoventral −4.5 (0.4μl) and AP + 3.0; L ± 0.7, DV −4.5 (0.4 μl). After surgery, the rats were housed individually and allowed a 5-day recovery period prior to retraining. Following that, the rats were tested over 10 consecutive daily sessions on the standard task. Only the data of the last 7 days were used as the post-operative baseline. Subsequently, rats were gently held and milnacipran (10 mg/kg) or DW (3 ml/kg) was administered via esophagus with a gastric sonde needle 60 min before testing on the 3-CSRTT for 14 days. Following that, the rats were tested without drug administration over 7 consecutive daily sessions on the standard task to establish the post-experimental baseline. I divided rats into 4 groups (nonlesioned-DW, nonlesioned-MIL, lesioned-DW, and lesioned-MIL) based on the number of premature response of pre-operative baseline to avoid generating the difference in basal impulsivity among groups of rats. After 7 days of drug cessation, Western blotting, immunohistochemistry, dendrite/spine analysis, and whole-cell patch clamp were conducted. MIL: Milnacipran, DW: distilled water.

# CHAPTER 4

# General Discussion

## Summary

In CHAPTER 2, I examined the neural mechanisms of the effects of acute administration of milnacipran on impulsive action in normal rats. Acute systemic administration of milnacipran-induced decrease in the number of premature responses was blocked by injections of SCH23390, a selective dopamine $D_1$-like receptor antagonist, into the vmPFC, whereas intra-vmPFC injections of eticlopride, a selective $D_2$-like receptor antagonist, failed to inhibit the effect of milnacipran on impulsive action. The results indicated milnacipran suppresses impulsive action by stimulating $D_1$-like receptors in the vmPFC.

In CHAPER 3, I examined the effects of repeated administration of milnacipran on elevated impulsive action in animal models of high impulsivity. Excitotoxic lesions in the vmPFC selectively disrupted the control of impulsive action. This lesion-induced increase in the number of premature responses was reversed by the repeated administration of milnacipran. Surprisingly, the therapeutic effect of milnacipran on impulsivity persisted even after the cessation of the drug treatment, indicating that milnacipran has an etiotropic effect and caused plastic changes. The electrophysiological and histochemical data support this interpretation; repeated milnacipran reconstructed dendritic spines and excitatory currents in the few surviving neurons in the vmPFC.

## The roles of the fronto-striatal dopaminergic system in suppressive effects of acute milnacipran administration on impulsive behavior in normal rats

I summarized the putative mechanisms of the effects of acute systemic administration of milnacipran on impulsive action schematically in Figure 1 to help readers understand the following discussion.

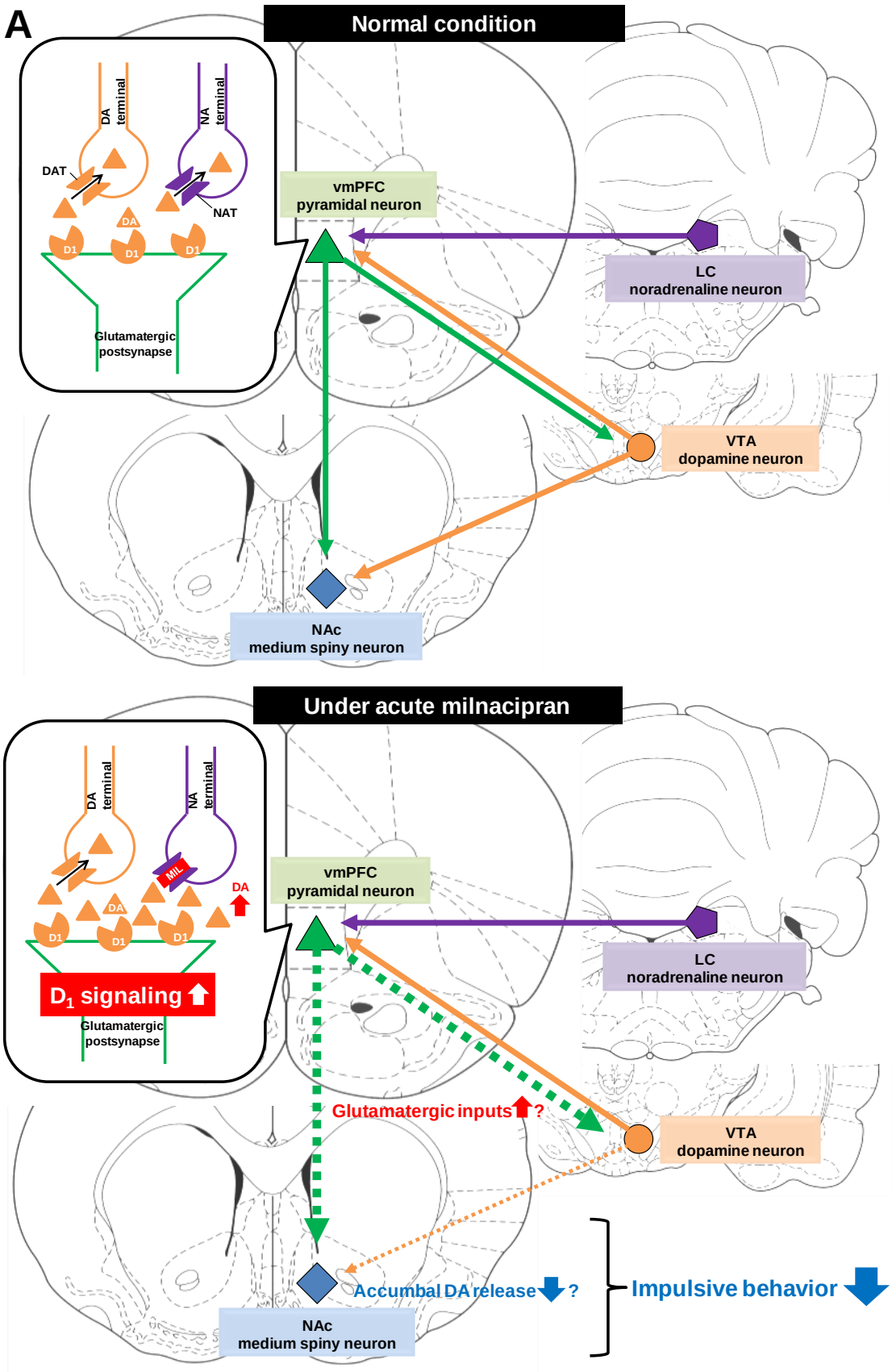

A
Normal condition
DA terminal
NA terminal
DAT
NAT
DA
D1
D1
D1
Glutamatergic postsynapse
vmPFC pyramidal neuron
LC noradrenaline neuron
VTA dopamine neuron
NAc medium spiny neuron
Under acute milnacipran
MIL
DA
D1 signaling
Glutamatergic inputs ?
Accumbal DA release ?
Impulsive behavior

**Figure 1.** Hypothetical model of the effects of acute administration of milnacipran on impulsive action in normal animals

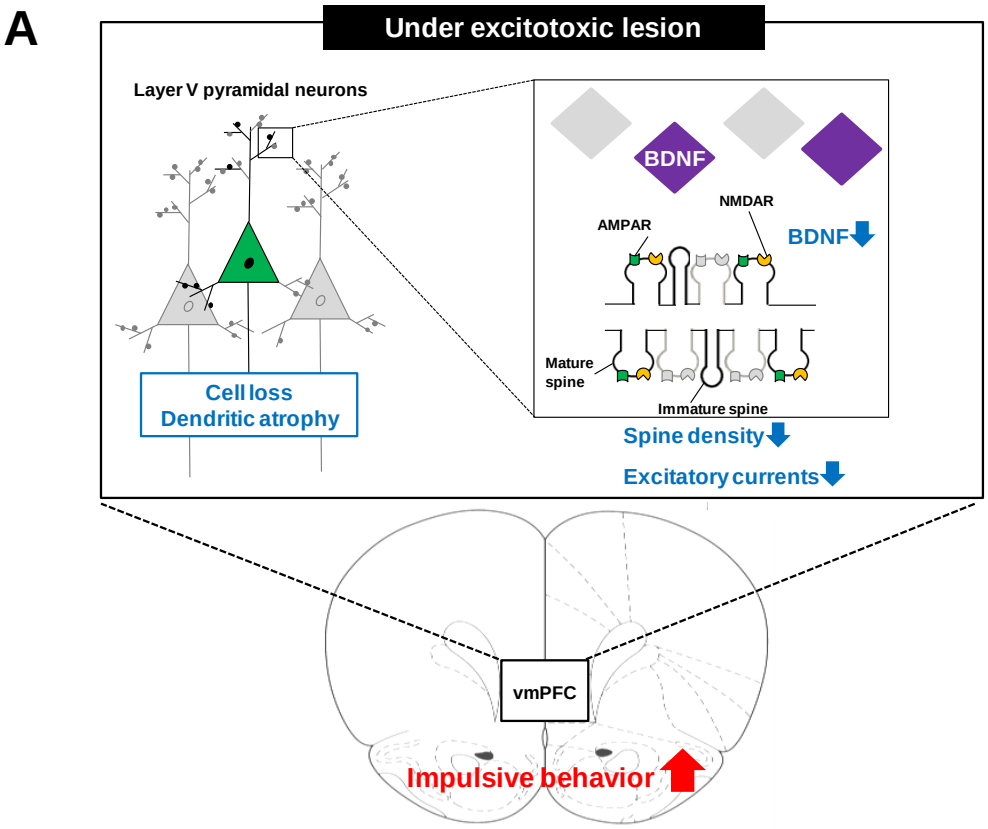
Schematic diagrams showing putative neural circuits modulating impulsive action under (**A**) normal condition and (**B**) acute administration of milnacipran. Green arrows indicate glutamatergic innovation from the vmPFC to the ventral tegmental area (VTA) and the nucleus accumbens (NAc). A purple arrow indicates noradrenergic innovation from the locus coeruleus (LC) to the vmPFC. Orange arrows indicate dopaminergic innervations to the vmPFC and the NAc. (**A**) Under normal conditions, noradrenaline transporters in the vmPFC reuptake not only extraxellular noradrenaline but also dopamine. DA release in the vmPFC is involved in the regulation of DA release in the NAc by directly and/or indirectly via modulating activities of VTA dopaminergic neurons. Previous studies demonstrated that dopamine release in the vmPFC suppress impulsive action while dopamine release in the NAc has an opposite role in modulating impulsive action. (**B**) Milnacipran inhibits noradrenaline transporters and increases extracellular dopamine levels in the vmPFC, resulting in facilitating $D_1$-like signaling. Presumablry, milnacipran-enhanced $D_1$-like signaling might reduce dopamine release in the NAc through dilectly and/or indirectly via modulating activities of VTA dopaminergic neurons. As a net effect, systemic acute milnacipran injection suppresses impulsiveaction in normal rodents. Dashed lines indicate hypothetical pathways. DA: dopamine, NA: noradrenaline, DAT: dopamine transporter, NAT: noradrenaline transporter, MIL: Milnacipran, $D_1$: $D_1$-like receptor, vmPFC: ventromedial prefrontal cortex, NAc: neucleus accumbens, VTA: ventral tegmental area, LC: locus coeruleus.

Milnacipran is a serotonin/noradrenaline reuptake inhibitor (SNRI, $K_i$=151 nM and 68 nM, respectively)[89]. Although the affinity of milnacipran for dopamine transporters is extremely low ($K_i$>10,000 nM), noradrenaline transporters take up not only extracellular noradrenaline but also dopamine in some specific brain regions, such as the mPFC[89-92]. Indeed, acute administration of milnacipran increases extracellular concentrations of dopamine in the mPFC[59, 60]. Dopamine release in the mPFC has been implicated in suppressing impulsive behavior in rats[33, 34]. This is the first to directly elucidate that acute milnacipran suppresses impulsive action via stimulating $D_1$-like receptor in the vmPFC (CHAPTER 2). Moreover, here is anatomical and physiological evidence that the mPFC acts as an important regulator of dopamine transmission in the nucleus accumbens (NAc), which stimulates impulsive action[120, 121]. Thus, milnacipran stimulates $D_1$-like receptor in the

vmPFC and it might reduce dopamine release in the NAc resulting in suppressing impulsive action. Most drugs suppressing impulsive behavior (*e.g.*, noradrenalin reuptake inhibitors and atypical antipsychotics; see **General Introduction**) stimulate dopamine release in the mPFC in rodents[35, 36]. It is worth noting whether this $D_1$ theory could be applied in other anti-impulsive drugs in the future.

## The roles of the prefrontal BDNF signaling in suppressive effects of repeated milnacipran administration on elevated impulsive action in rats with vmPFC lesions

The putative mechanisms of suppressing effect of repeated milnacipran on elevated impulsive action are different from those of acute administration of the drug. I summarized the putative mechanisms of the effects of repeated systemic administration of milnacipran on elevated impulsive action observed in the animal model of higher impulsivity schematically in Figure 2 to help readers understand the following discussion.



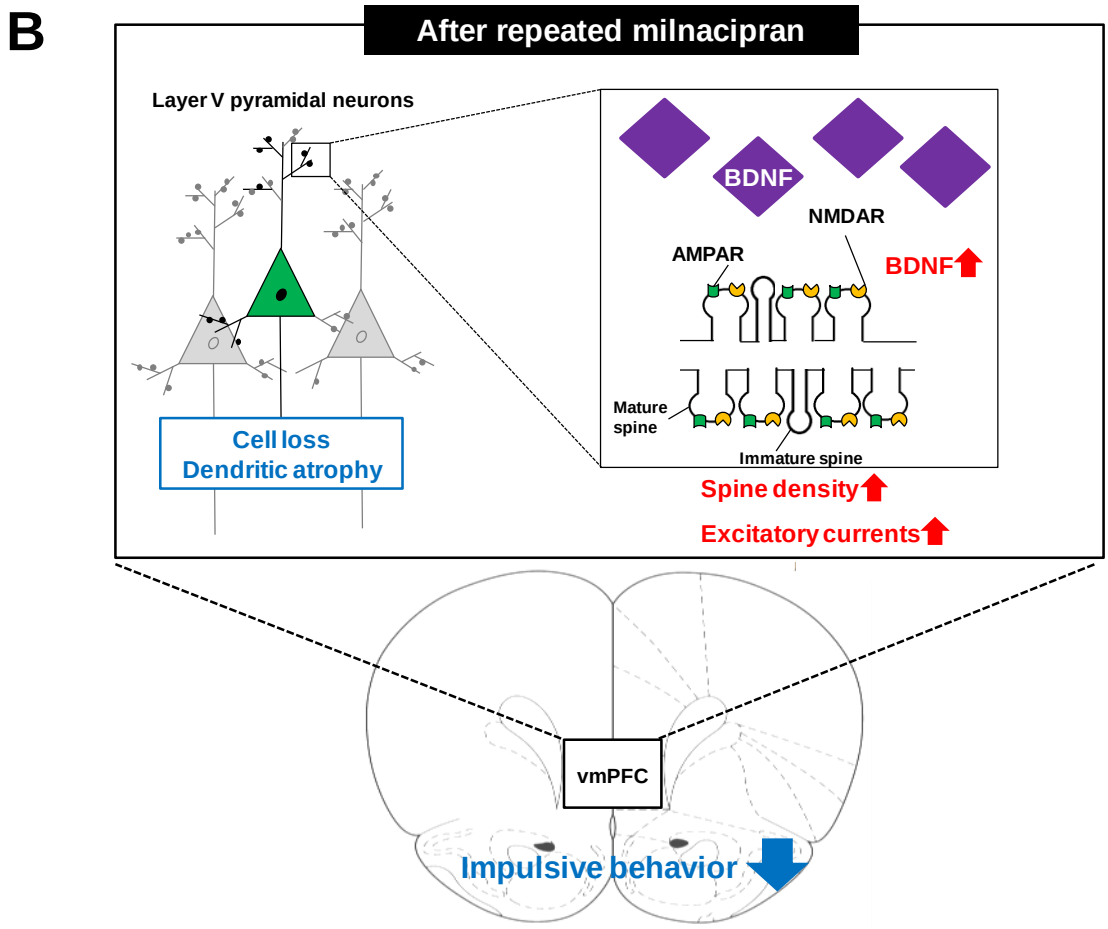



**Figure 2.** Hypothetical model of the effects of repeated administration of milnacipran on elevated impulsive action in animal models of high impulsivity

Schematic diagrams showing putative neural mechanisms modulating impulsive action (**A**) under excitotoxic lesions of the vmPFC and (**B**) after repeated administration of milnacipran. (**A**) Exitotoxic lesions with 0.09 M quinolinic acid induced excessive levels of impulsive action, reduced BDNF signaling, neural cell loss, dendritic atrophy, spine loss, decreased ratio of mature spine, impaired excitatory currents. (**B**) After cessation of 14 days of repeated milnacipran treatment, impulsive action was persistently suppressed. In parallel with behavioral changes, repairement of BDNF signaling, remodeling of spines and reconstructing the excitatory currents in the few surving pyramidal neurons in the layer V of the vmPFC were observed though repeated milnacipran might not induce neurogenesis or dendritic elongation. vmPFC: ventromedial prefrontal cortex, BDNF: brain-derived neurotrophic factor, AMPAR: AMPA receptor, NMDAR: NMDA receptor.

The animal model exhibiting higher impulsivity was made by excitotoxic lesions in the vmPFC. This animal model showed neural cell loss, dendritic atrophy, spine loss/atrophy, and impaired excitatory currents in the vmPFC (CHAPTER 3). This lesion-induced increase in the number of premature responses was reversed by the repeated administration of milnacipran and it persisted for at least a week, even after the cessation of the treatment. Repeated administration of milnacipran restored the protein levels of mBDNF in the vmPFC of vmPFC-lesioned rats. Repeated milnacipran administration did not elicit neurogenesis or dendritic elongation but did recover the decreased spine density and increased the proportion of mature spines in the vmPFC of vmPFC-lesioned rats. Furthermore, repeated milnacipran treatment ameliorated impaired AMPA and NMDA EPSCs in the layer V pyramidal neurons in the vmPFC of vmPFC-lesioned rats. Therefore, repeated milnacipran treatment radically improved the dysregulation of impulsive action in vmPFC-lesioned rats, which could be attributable to the mBDNF-induced remediation of the spine density and the excitatory currents in the surviving layer V pyramidal neurons in the vmPFC.

## Limitations and Future Directions

I elucidated that $D_1$-like receptor in the vmPFC is involved in the regulation of impulsive action in CHAPTER 2. This result could contribute to further understanding of the fronto-striatal dopaminergic system as putative neural basis of impulsive action. However, it still remains unknown how the enhanced $D_1$-like signaling suppress dopamine release in the NAc. There is a bi-directional projection between the mPFC and the ventral tegmental area (VTA), which is predominantly-comprised of dopaminergic neurons[22, 122, 123]. Some of $D_1$-like receptors are localized on pyramidal cells in the vmPFC that project to the VTA dopamine neurons[98, 124], suggesting that the vmPFC could indirectly modulate accumbal dopaminergic activities by modulating the VTA. It should also be noted that some of the dopamine terminals in the PFC form synapses with pyramidal cells that directly project to the NAc[125], suggesting that pyramidal cells in the vmPFC could directly modulate accumbal dopaminergic activities. Thus, it is required to determine whether $D_1$-like signaling in the vmPFC attenuates accumbal dopaminergic activities via a direct and/or indirect pathway, resulting

in suppressed impulsive action.

I demonstrated the neural mechanisms underlying the persisted suppressive effects of repeated milnacipran on elevated impulsive action in animal models of high impulsivity in CHAPTER 3. My findings that repeated milnacipran treatment repaired the functions of few surviving neurons in the vmPFC of lesioned rats are attributable to the suppressive effects of repeated milnacipran on elevated impulsive action. However, I cannot exclude a possibility that repeated milnacipran treatment induced plastic changes in brain regions other than the vmPFC because it was correlational study I showed in CHAPTER 3. Thus, additional experiments showing the causal relationship between the retrieval of functions in the vmPFC and persisted suppressive effects of repeated milnacipran treatment are required in the future.

In this thesis, I examined the effects of acute/repeated administration of milnacipran on impulsive action using the 3-CSRTT. Nevertheless, impulsive action is only an aspect of impulsive behavior, which consists of several other subordinate concepts[1]. Among them, a growing body of data from preclinical studies has shown that a behavior, which reflects impulsive decision making, often referred to as “impulsive choice”, is also well characterized[14]. The level of impulsive choice in rodents could be assessed by using the delay discounting task[170, 171]. While there are shared brain areas and receptors responsible for these two behaviors, there are also dissociable brain areas and receptors[130]. Indeed, the effects of drugs on impulsive behavior often depend on the type of task employed, possibly because each task assesses different aspects of impulsivity[172]. From this point of view, in future studies, it will be desirable to examine whether drugs used in this thesis affect other aspects of impulsive behavior such as impulsive choice.

## Concluding remarks

My findings suggest that acute milnacipran could suppress impulsive behavior by enhancing $D_1$-like signaling in the PFC. In addition, repeated milnacipran could suppress impulsive behavior on psychiatric patients even after the cessation of the treatment by upregulating the excitatory signaling in the impaired PFC via stimulating BDNF pathway. “Is milnacipran a promising agent to suppress impulsive behavior?” The answer is “YES” at the stage of animal

study. I hope the proposed mechanisms underlying the suppressing effects of acute/repeated milnacipran on impulsive behavior will contribute to accelerating the development of anti-impulsive drugs.

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