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## **Milnacipran Enhances the Control of Impulsive Action by Activating D<sub>1</sub>-like Receptors in the Infralimbic Cortex**

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## **Abstract**

*Rationale:* Elevated impulsivity is often observed in patients with depression. We 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. *Objectives:* Our goal was to identify whether D<sub>1</sub>-like and/or D<sub>2</sub>-like receptors in the infralimbic cortex (IL), the ventral portion of the medial prefrontal cortex, mediates the milnacipran-enhanced impulse control in a three-choice serial reaction time task. *Methods:* The rats were bilaterally injected with SCH23390, a selective D<sub>1</sub>-like receptor antagonist, (0.3 or 3 ng/side) or eticlopride, a selective D<sub>2</sub>-like receptor antagonist, (0.3 or 1 µg/side) into the IL after acute intraperitoneal administration of milnacipran (10 mg/kg). *Results:* Intra-IL SCH23390 injections reversed the milnacipran-enhanced impulse control, whereas injections of eticlopride into the IL failed to block the effects of milnacipran on impulsive action. *Conclusions:* This is the first report that demonstrates a critical role for D<sub>1</sub>-like receptors of the IL in milnacipran-enhanced control of impulsive action.

## **Key words**

Response inhibition; inhibitory control; ventromedial prefrontal cortex; suicide; addiction; five-choice serial reaction time task

## Introduction

Impaired control of impulsivity is often observed in depressed patients (Maalouf et al. 2011; Perroud et al. 2011). Higher impulsivity can also be a risk factor for drug addiction and suicide (Corruble et al. 2003; Perry et al. 2005; Diergaarde et al. 2008; McGirr et al. 2008). Substance abuse and/or suicide attempts in patients with depressive disorders have emerged in recent years (Swendsen et al. 1998; Arsenault-Lapierre et al. 2004). Therefore, a significant issue concerns whether some antidepressants could enhance the control of impulsivity.

We recently reported that milnacipran, an antidepressant, suppressed impulsive action in rats (Tsutsui-Kimura et al. 2009). However, the neural mechanisms underlying the effects of milnacipran on impulsive action have not been identified. Milnacipran is a potent serotonin/noradrenaline reuptake inhibitor (SNRI,  $K_i = 151$  nM and 68 nM, respectively) (Stahl et al. 2005). 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) (Carboni et al. 1990; Pozzi et al. 1994; Yamamoto and Novotney 1998; Stahl et al. 2005). Indeed, acute administration of milnacipran increases extracellular concentrations of dopamine in the mPFC (Kitaichi et

al. 2005, 2008).

The rat mPFC is implicated in various aspects of impulsivity (Passeti et al. 2002; Chudasama et al. 2003; Gill et al. 2010; Loos et al. 2010). The infralimbic cortex (IL), the ventral portion of the mPFC, plays a pivotal role in the control of impulsive action (Chudasama et al. 2003; Murphy et al. 2005; Tsutsui-Kimura et al. 2010). A previous study reported that dopamine release in the mPFC plays a role in enhancing the control of impulsive behavior (Sokolowski and Salamone 1994; Loos et al. 2010). Dopamine receptors have been classified into five subtypes, D<sub>1</sub>-D<sub>5</sub>, based on the sequences of their encoding genes (Cooper et al. 2003). Pharmacological studies have demonstrated that D<sub>1</sub> and D<sub>5</sub> receptors, namely D<sub>1</sub>-like receptors, are linked to a stimulation of adenylyl cyclase, whereas D<sub>2</sub>-D<sub>4</sub> receptors, namely D<sub>2</sub>-like receptors, are linked to an inhibition of cAMP production (Cooper et al. 2003; Jose et al. 2007). Both types of dopamine receptors are distributed throughout the rat mPFC (Richfield et al. 1989; Gaspar et al. 1995).

The present aim was to investigate the role of D<sub>1</sub>-like and D<sub>2</sub>-like receptors of the IL in the milnacipran-enhanced control of impulsive action. Thus, we used systemic and intracranial drug injections to manipulate behavioral performance in the three-choice serial reaction time task (3-CSRTT, Tsutsui-Kimura et al. 2009), which is a

simplified (but reliable) version of the five-choice serial reaction time task (5-CSRTT, Robbins 2002) that measures impulsive action in rats.

## **Materials and Methods**

### *Subjects*

Twenty-two 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 nine weeks old (270–290 g), we 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-benzazepine (SCH23390) hydrochloride and S(-)-eticlopride hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). SCH23390 is more selective for D<sub>1</sub> and D<sub>5</sub> receptors (> 1000-fold) than for D<sub>2</sub>, D<sub>3</sub>, and D<sub>4</sub> receptors (Bourne 2001). Eticlopride is a selective D<sub>2</sub> and D<sub>3</sub> antagonist (K<sub>i</sub> = 0.5, and 0.16 nM, respectively) and also a

preferential D<sub>4</sub> antagonist (K<sub>i</sub> = 27 nM) (Tang et al. 1994). Eticlopride has little affinity for D<sub>1</sub> and D<sub>5</sub> receptors (IC<sub>50</sub> > 100,000 nM) (Hall et al. 1985). Milnacipran hydrochloride was generously donated by Asahi-Kasei Co. Ltd. (Tokyo, Japan) and administered at a volume of 3 ml/kg. All three 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. The curved rear wall of each chamber contained nine 2.5 cm<sup>2</sup> 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).

### *Three-choice serial reaction time task*

The training procedure and the task sequence that were employed in the 3-CSRTT are detailed in previous reports (Ohmura et al. 2009; Tsutsui-Kimura et al. 2009). Briefly, when the task started, the house light was illuminated. After a fixed inter

trial interval (ITI: 5 s), one of three 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, we did not set a time restriction for this task. Each session consisted of 100 trials. Training was conducted for one session per day and six 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.

We used six behavioral parameters described as follows:

- (a) Premature responses (no. per session)
- (b) Accuracy (percentage of correct responses):  $[\text{correct responses} / (\text{correct and incorrect responses})] \times 100$
- (c) Omissions (no. per session):  $[\text{omission errors} / \text{total trials}] \times 100$
- (d) Perseverative responses (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. We set the criteria for determining stable performance as follows: the change in premature

responses stayed within  $\pm 25\%$ , the accuracy stayed within  $\pm 5\%$ , and the number of omissions were less than 20 for at least three 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 (Paxinos and Watson 1996). 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 four-day recovery period prior to retraining.

### *Drug treatment schedule*

Prior to testing, the rats were retrained for at least one 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  $\mu$ l 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 IL according to a Latin Square design. 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. A different group of rats was used for each experiment (SCH23390 or eticlopride).

#### *Basal performance*

We used the data from the last three days of training to provide a preoperative baseline, and we 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  $\mu\text{m}$  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 *Three-choice serial reaction time task*). Each measure was analyzed separately using two-factor 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 we used in this study. We injected milnacipran and SCH23390 (or eticlopride) with five combinations (D1-D5) to 10 rats (A-J) using Latin Square design. We 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 (Myers and Well 2010, pp. 404-406). 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

The alpha level was set to 0.05 for ANOVA. Multiple comparisons testing using the Holm method (Holm 1979) was performed where a significant main effect of the dose was observed. All statistical procedures were conducted using SPSS (version 15.0 J).

## Results

### *Histological analysis*

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

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

Figure 2A shows the effects of intra-IL 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  $\times$  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-IL 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).

*The effects of intra-IL injections of eticlopride on milnacipran-enhanced the control of impulsive action*

Figure 2B shows the effects of intra-IL 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  $\times$  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-IL 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).

### *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.

## Discussion

Consistent with our previous study, systemic administration of milnacipran decreased the number of premature responses (Tsutsui-Kimura et al. 2009). This milnacipran-induced decrease in the number of premature responses was blocked by injections of SCH23390, a selective D<sub>1</sub>-like receptor antagonist, into the IL, whereas intra-IL injections of eticlopride, a selective D<sub>2</sub>-like receptor antagonist, failed to inhibit the effect of milnacipran on impulsive action (Figure 2). In addition, intra-IL 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 IL 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 (Harrison et al. 1997; Carli and Samanin 2000; Fletcher et al. 2007; Navarra et al. 2008; Sun et al. 2010; Pattij et al. 2012). Intra-IL 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 (Neumeier et al. 2003) and a serotonin 2C receptor agonist, albeit these affinities are relatively weak (Millan et al. 2001). However, these effects would induce a decrease of premature responding rather than an increase (Winstanley et al. 2003; Fletcher et al. 2007), 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-IL injections of eticlopride failed to reverse the effect of milnacipran on impulsive action (Figure 2B). Since we were skeptic whether 1  $\mu\text{g}/\text{side}$  of eticlopride had enough antagonistic action to the  $D_2$ -like receptors in the IL, we examined the effects of higher dose (3  $\mu\text{g}/\text{side}$ ) of eticlopride on milnacipran-suppressed premature response. However, 3  $\mu\text{g}/\text{side}$  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 (Supplemental Figure S1). Blockade of dopamine  $D_2$  receptors in the rat mPFC was reported to induce inhibition of locomotor activity in a dose-dependent manner (Radcliffe and Erwin 1996), suggesting that high dose of intra-IL injection of eticlopride impaired motor activity in our study. Thus, we could not determine whether 3  $\mu\text{g}/\text{side}$  of

eticlopride did not reverse the effect of milnacipran or the effect of 3  $\mu\text{g}/\text{side}$  of eticlopride on milnacipran-suppressed premature response was masked by increased the number of omission. Nevertheless, the facts that intra-IL injection of D<sub>1</sub>-like receptor antagonist almost completely blocked the effect of milnacipran on premature response (Figure 2A) and that D<sub>2</sub>-like receptors in the mPFC, especially in the IL, are sparsely distributed compared to D<sub>1</sub>-like receptors (Richfield et al. 1989; Gaspar et al. 1995; Le Moine and Gaspar 1998; Rivera et al. 2008; Oda et al. 2010) are damping the idea that D<sub>2</sub>-like receptors in the IL associate with milnacipran-enhanced impulse control.

Unfortunately, there is no direct evidence that 10 mg/kg of milnacipran increases DA release in the mPFC. However, Kitaichi et al. (2005 and 2008) demonstrated that extracellular dopamine level was significantly increased 40 min after intraperitoneal injection of higher dose of milnacipran (30 mg/kg). Although we used a lower dose (10 mg/kg) of milnacipran, it is likely that milnacipran increased dopamine levels in our present study because Kitaichi et al. (2008) showed 30 mg/kg of milnacipran dramatically increased dopamine levels (approximately up to 300%) and we restricted feeding and food-restricted animals show higher C<sub>max</sub> in milnacipran (Sakai et al. 1994).

Based on the present results and those of previous studies, we conclude that

D<sub>1</sub>-like receptors in the IL play an important role in milnacipran-enhanced impulse control though we could not completely rule out the possibility of the contribution of D<sub>2</sub>-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 (Robinson et al. 2008; Liu et al. 2009). It is possible that these drugs activate D<sub>1</sub>-like receptors in the IL 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 (Carr and Sesack 2000; Gabbott et al. 2005; Dalley et al. 2007). Nucleus accumbal dopamine is part of the neural circuit that is thought to mediate impulsive action (Cole and Robbins 1987; Pattij et al. 2006). 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. (2001) 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 (King et al. 1997). There is a bi-directional projection between the mPFC and the ventral tegmental area (VTA), which is predominantly-comprised of dopaminergic neurons (Van Eden et al. 1987; Vertes 2004; Gabbott et al. 2005). Some of D<sub>1</sub>-like receptors are localized on pyramidal cells in the IL that project to the VTA (Gaspar et al. 1995; Lu et al. 1997), suggesting that the IL 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 (Carr et al. 1999), suggesting that pyramidal cells in the IL could directly modulate accumbal dopaminergic activities. Thus, it is feasible that milnacipran stimulates D<sub>1</sub>-like receptors in the IL 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 (Sokolowski and Salamone 1994; Loos et al. 2010). Meanwhile, increased dopamine release in the NAc stimulates impulsive behavior (Cole and Robbins 1987; Pattij et al. 2006). 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 (Leland et al. 2006) and in animals (van Gaalen et al. 2006; Milstein et al. 2010). However, inhibition of the noradrenaline transporters by atomoxetine induces an increase of dopamine release in the mPFC without affecting dopamine release in the NAc (Bymaster et al. 2002) and consequently enhances impulse control (Robinson et al. 2008; Tsutsui-Kimura et al. 2009). 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 depressive patients with elevated impulsivity should be considered in the future.

In conclusion, our data suggest that milnacipran suppresses impulsive action by stimulating D<sub>1</sub>-like receptors in the IL though we could not completely rule out the possibility of the contribution of D<sub>2</sub>-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.

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## Table

Table 1 An example of a Latin Square design

	<b>O1</b>	<b>O2</b>	<b>O3</b>	<b>O4</b>	<b>O5</b>
<b>R1</b>	62.5 (D1)	14.0 (D2)	43.5 (D3)	46.5 (D4)	5.02 (D5)
<b>R2</b>	22.5 (D2)	11.5 (D3)	20.5 (D4)	25.0 (D5)	32.5 (D1)
<b>R3</b>	20.5 (D3)	47.5 (D4)	24.5 (D5)	41.5 (D1)	22.0 (D2)
<b>R4</b>	46.5 (D4)	40.5 (D5)	61.5 (D1)	23.0 (D2)	49.0 (D3)
<b>R5</b>	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).*

## Figure legends

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 IL region (closed circles) for (**C**) the SCH23390 experiment and (**D**) the eticlopride experiment, 2.7 mm and 3.2 mm anterior to the bregma.

Figure 2. The effects of intra-IL 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-IL SCH23390 (0, 0.3, or 3 ng per side;  $n = 10$ ) or eticlopride (0, 0.3, or 1  $\mu\text{g}$  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-IL injections of SCH23390 (SCH) with systemic milnacipran (Mil) on behavioral parameters of the 3-CSRTT. The rats received systemic

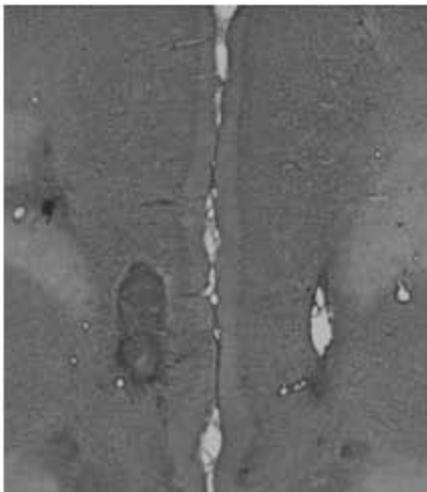
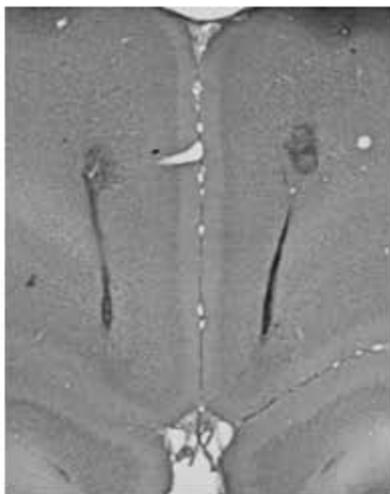
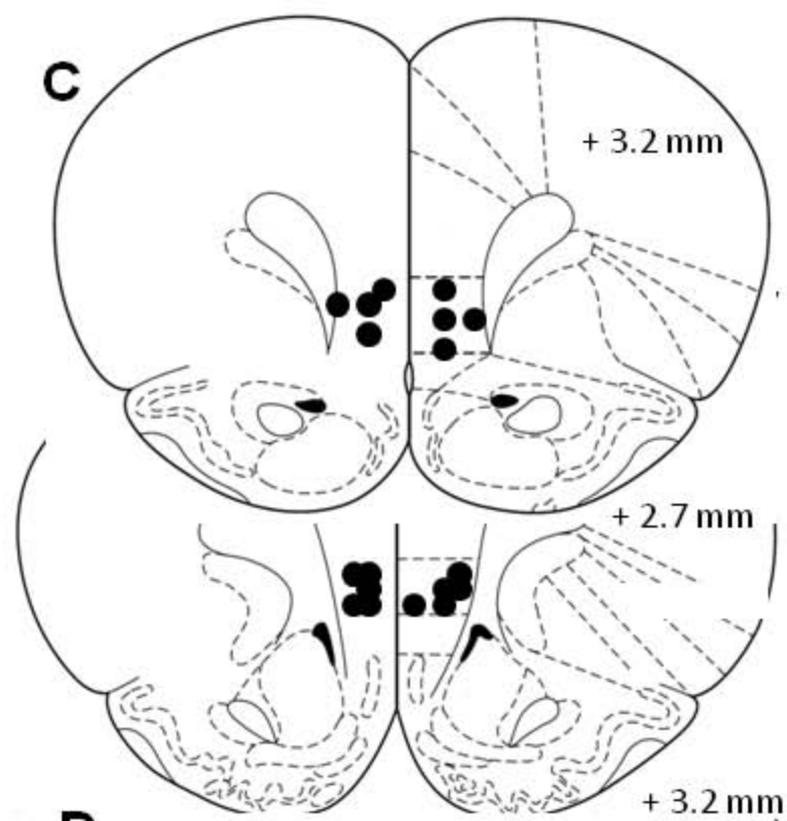
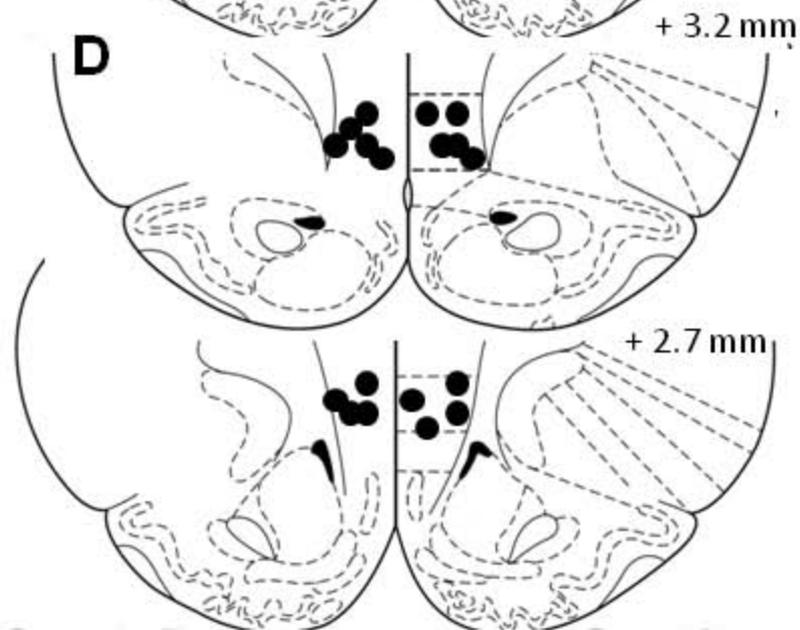
milnacipran (0 or 10 mg/kg) and intra-IL SCH23390 (0, 0.3, or 3 ng per side; n = 10).

The bars represent the mean, and the lines represent the SEM.

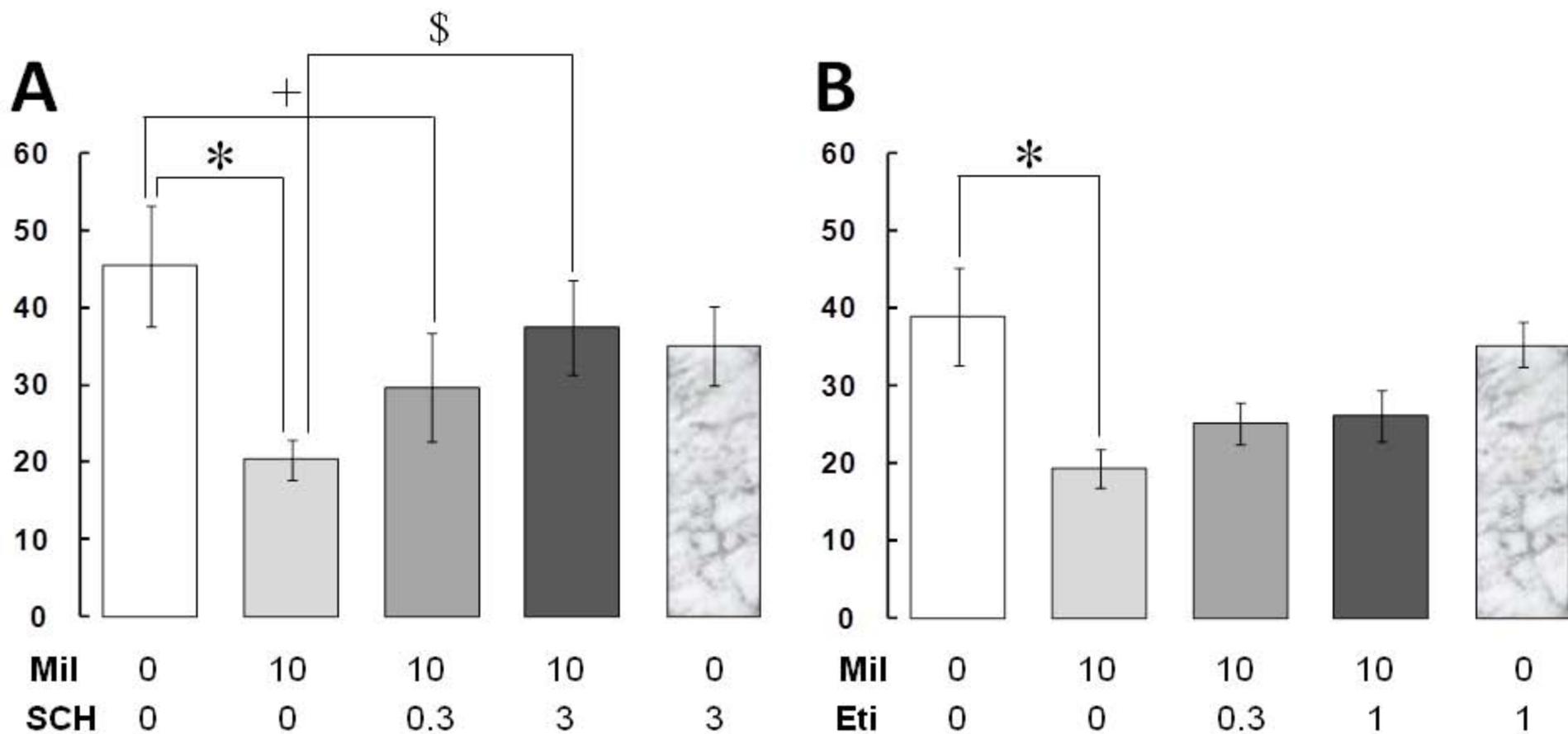
Figure 4. The effects of intra-IL injections of eticlopride (Eti) with systemic milnacipran (Mil) on behavioral parameters of the 3-CSRTT. Rats received systemic milnacipran (0 or 10 mg/kg) and intra-IL eticlopride (0, 0.3, or 1 µg per side; n = 10).

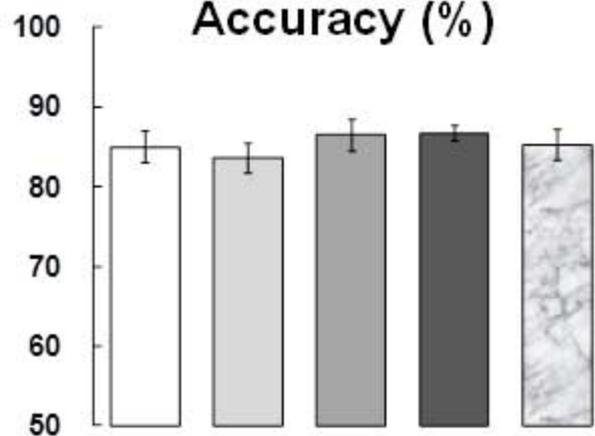
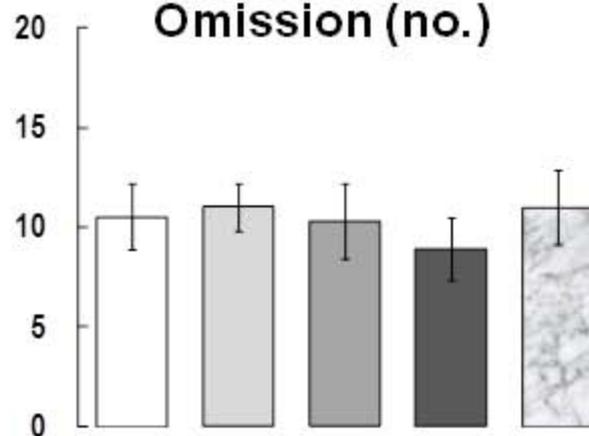
The bars represent the mean, and the lines represent the SEM.

Figure 5. Basal performance. Three preoperative (Pre-ope), three postoperative (Post-ope), and five 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.

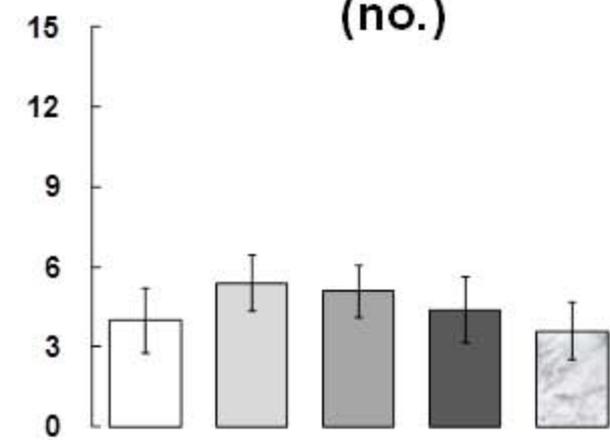
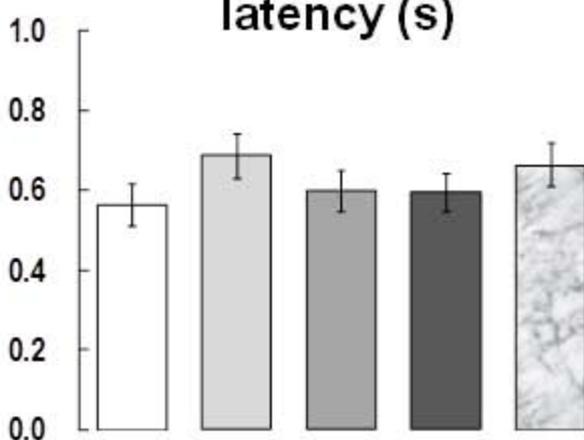
**A****B****C****D**

# Premature responses (no.)



**Accuracy (%)****Omission (no.)**

- Vehicle-Vehicle
- Mil-Vehicle
- Mil-SCH (0.3 ng)
- Mil-SCH (3 ng)
- Vehicle-SCH (3 ng)

**Perseverative response (no.)****Correct response latency (s)****Reward latency (s)**