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Noradrenaline reuptake inhibition increases control of impulsive action by activating D₁-like receptors in the infralimbic cortex

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Abstract

Higher impulsivity is a risk factor for criminal involvement, substance abuse, and suicide. However, only a few drugs are clinically available for the treatment of deficient impulse control. We recently proposed a strategy for identifying potential drugs to treat such disorders by investigating clinically available drugs that increase extracellular dopamine levels in the medial prefrontal cortex and stimulate dopamine D₁-like receptors without increasing extracellular dopamine levels in the ventral striatum. To determine whether this strategy is promising, we examined the effects of duloxetine, a serotonin-noradrenaline reuptake inhibitor that might meet these criteria, on impulsive action in adult male Wistar/ST rats using a 3-choice serial reaction time task. The effects of duloxetine on extracellular dopamine levels in the medial prefrontal cortex and nucleus accumbens, a part of the ventral striatum were evaluated using *in vivo* microdialysis, as the noradrenaline transporter transports dopamine in some brain regions. Our results showed that the administration of duloxetine reduced impulsive actions and increased extracellular dopamine levels in the mPFC but not in the nucleus accumbens. Microinjection of a selective D₁-like receptor antagonist into the infralimbic cortex blocked the suppression of impulsive action by duloxetine. In

addition, we demonstrated that the microinjection also blocked the suppression of impulsive action by atomoxetine, a noradrenaline reuptake inhibitor and an established anti-impulsive drug. These results support our proposed strategy for identifying and developing anti-impulsivity drugs.

Key words

Impulsivity, impulsive behavior, behavioral inhibition, SNRI, microdialysis, dopamine

1. Introduction

Higher impulsivity is a risk factor for criminal involvement, substance abuse, and suicide (Babinski et al., 1999; Corruble et al., 2003; McGirr et al., 2008; Ohmura et al., 2005), and is observed in psychiatric disorders such as attention-deficit/hyperactivity disorder (ADHD) (Losier et al., 1996), schizophrenia (Barr et al., 2008), substance abuse (Swann et al., 2004), bipolar disorder (Strakowski et al., 2009), and borderline personality disorder (Dom et al., 2006). While many experimental drugs have potential anti-impulsivity effects (Pattij and Vanderschuren, 2008), only a few are clinically available for treating this condition.

To further the development of anti-impulsivity drugs, we previously proposed the use of clinically-available drugs that increase extracellular dopamine levels in the medial prefrontal cortex and stimulate dopamine D₁-like receptor in the infralimbic cortex, a ventral part of the medial prefrontal cortex, without increasing extracellular dopamine levels in the ventral striatum (Ohmura et al., 2012). This proposal is based on previous studies showing that impulsive behavior is inhibited by dopaminergic functions in the medial prefrontal cortex (Sokolowski and Salamone, 1994) and promoted by those in the ventral striatum (Cole and Robbins, 1989; Pattij et al., 2007)

and on a study demonstrating that dopamine D₁-like receptors in the infralimbic cortex play a pivotal role in the control of impulsive action (Tsutsui-Kimura et al., 2013). As previously suggested (Ohmura et al., 2012), noradrenaline reuptake inhibitors might meet these criteria because the noradrenaline transporter sometimes transports dopamine in brain regions in which the dopamine transporter is sparsely expressed, such as the medial prefrontal cortex (Carboni et al., 1990; Pozzi et al., 1994; Stahl et al., 2005; Yamamoto and Novotney, 1998). In dopamine-transporter-rich brain regions, such as the ventral striatum, the noradrenaline transporter transports noradrenaline but not dopamine. Indeed, milnacipran, a potent serotonin/noradrenaline reuptake inhibitor (SNRI), suppresses impulsive action in rats (Tsutsui-Kimura et al., 2009).

Previous studies have indicated that drugs sometimes exert different effects despite having similar pharmacological properties. For example, paroxetine suppresses impulsive action (Humpston et al., 2013) whereas fluvoxamine does not (Tsutsui-Kimura et al., 2009), even though both drugs are selective serotonin reuptake inhibitors. Thus, to determine whether our strategy is promising, we examined the effects of duloxetine, another SNRI, on impulsive action by using a 3-choice serial reaction time task (Tsutsui-Kimura et al., 2009), which is a simplified (but reliable) version of the 5-

choice serial reaction time task (Robbins, 2002).

To determine whether the neural mechanisms underlying the anti-impulsive effects of duloxetine support our hypothesis, we used *in vivo* microdialysis to investigate whether duloxetine at a dose that exerts anti-impulsive effects also increases extracellular dopamine levels in the medial prefrontal cortex without affecting dopamine levels in the nucleus accumbens, a part of the ventral striatum. Furthermore, we examined whether the anti-impulsive effects of duloxetine could be blocked by microinjections of a D₁-like receptor antagonist into the infralimbic cortex. In addition, we repeated the same experiment, but using atomoxetine, a noradrenaline reuptake inhibitor and an established anti-impulsive drug, to confirm whether the same mechanisms underlie the anti-impulsive effects of these drugs.

2. Materials and methods

2.1. Subjects

Sixty-three male Wistar/ST rats were supplied by Nippon SLC Co. Ltd. (Hamamatsu, Japan). They were housed in groups of four under an alternating light-

dark cycle (light from 7 pm to 7 am) at approximately 21°C and a relative humidity of 40–50%. All testing was carried out during the dark phase of the cycle. Rats received 3-choice serial reaction time task training and were allocated to 1 of 4 experimental conditions as follows: 16 rats were used for acute duloxetine administration (Experiment 1), 14 rats were used for the measurement of dopamine and serotonin levels in the mPFC and NAc after acute administration of duloxetine (Experiment 2), 21 rats were used for acute duloxetine administration with microinjection of dopamine D₁-like receptor antagonist into the infralimbic cortex (Experiment 3), and 12 rats were used for acute atomoxetine administration with microinjection of dopamine D₁-like receptor antagonist into the infralimbic cortex (Experiment 4). When the rats were 9 weeks old (270–290 g), food intake was restricted to maintain their body weight at 85% of those under free-feeding conditions. The daily feed (CE-2, CLEA JAPAN, Inc., Tokyo, Japan) in the home cages was given after the daily sessions. The food intake in the home cage was 8–16 g during the training period and 8–12 g (plus 2–3 g of reward pellets consumed in the operant box; see also **2.3. 3-choice serial reaction time task** in **2. Methods**) during the experimental period. Water was available *ad libitum*. The treatment of animals complied with the guidelines of the Animal Research Committee of the Hokkaido University Graduate School of Medicine for the care and use of

laboratory animals.

2.2. Drugs

Duloxetine hydrochloride (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) and atomoxetine hydrochloride (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) were dissolved in saline and administered intraperitoneally at a volume of 5 ml/kg. R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine (SCH23390) hydrochloride (Sigma Aldrich, St. Louis, MO, USA) is more selective for dopamine D₁ and D₅ receptors (>1,000-fold) than for dopamine D₂, D₃, and D₄ receptors (Bourne, 2001). SCH23390 was dissolved in 0.01 M phosphate buffer saline (PBS) (pH 7.4) and microinjected into the infralimbic cortex bilaterally at a volume of 0.5 µl/side. Doses reported here are based on the molecular weight of the salt.

2.3. 3-choice serial reaction time task

The apparatus, training procedure, and task sequence employed in the 3-choice serial reaction time task have been described in detail in our 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 the three holes was illuminated briefly (stimulus duration, 1 s) in a pseudorandom order. Nose poking

during the ITI was recorded as a premature response, an index of impulsive action. Nose poking into the lit hole while it was illuminated or within a 5 s limited hold was recorded as a correct response and was rewarded by the immediate delivery of a reward pellet (45 mg each) (dustless precision pellets, Bio-serv, Frenchtown, NJ, USA) to the food magazine. The correct response latency was measured and regarded as an index of attentional function, motivation, and motor function. Reward latency was also measured and regarded as an index of motivation and/or appetite. The reward latency was the time between a correct response and nose poking into the food magazine. Nose poking into another hole was recorded as an incorrect response. Animals are required to pay attention to the holes to make a correct nose-poke response to the flashed hole. Therefore, accuracy in this task was regarded as an index of attentional function. When a rat failed to nose poke within the time limit, it was recorded as an omission. This parameter was regarded as an index of motivation and/or appetite. After the delivery and collection of the food pellet by the rat, the house light was switched off for 2 s to allow the rat to eat the pellet before the next trial automatically began. The start of the next ITI was signaled by illumination of the house light. Additional nose poking into any of the three holes before food collection was recorded as a perseverative response. This parameter was regarded as an index of compulsive behavior. Premature responses,

incorrect responses, omissions, and perseverative responses resulted in a 5-s time-out period during which the all lights were extinguished. The responses during time-out were also counted. Because the trial was initiated automatically, we did not set a time restriction. Each session consisted of 100 trials. All rats in the present study finished 100 trials within 45 min. Training was conducted for one session per day and five sessions per week.

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

We evaluated the rats using the following seven behavioral parameters: (a) Premature responses (count per session); (b) Accuracy (percentage of correct responses): $[\text{correct responses} / (\text{correct and incorrect responses})] \times 100$; (c) Omissions (count per session); (d) Percentage of perseverative responses: $[\text{perseverative responses} / \text{correct responses}] \times 100$; (e) Responses during time-out (count per session); (f) Correct response latency (s): the mean time between stimulus onset and nose poke to

the correct hole; and (g) Reward latency (s): the mean time between reward delivery and nose poke into the food magazine. The measure of primary interest (premature response) is more precisely analyzed by including these other 6 measurements as well (Robbins, 2002).

Training was completed when the animal reached the target phase (stimulus duration, 1 s) and showed stable performance. After completion of the training, the stimulus duration was fixed at 1 s regardless of performance.

2.4. Experiment 1: The effects of acute duloxetine injection on impulsive action

To determine the effects of duloxetine on impulsive action at several doses, we injected duloxetine (0, 0.3, 1.0, and 3.0 mg/kg) intraperitoneally into 16 rats 60 min before the 3-choice serial reaction time task testing session. We did not use higher doses of duloxetine (> 3.0 mg/kg) because higher doses induced sedation in our preliminary study. Drug treatments were carried out using a Latin square design. Drugs were administered on Tuesdays and Fridays.

2.5. Experiment 2: Extracellular levels of dopamine in the medial prefrontal cortex and nucleus accumbens after acute administration of duloxetine

To determine whether a dose of duloxetine exerting anti-impulsive effects increases extracellular dopamine levels in the medial prefrontal cortex but not in the nucleus accumbens, we conducted *in vivo* microdialysis in rats that had been food-restricted for the 3-choice serial reaction time task training.

2.5.1. Surgery

After completing the 3-choice serial reaction time task training, rats were anesthetized with sodium pentobarbital (50 mg/kg) intraperitoneally and fixed in a stereotaxic frame (Narishige, Tokyo, Japan). A guide cannula (AG-4 for the medial prefrontal cortex; AG-8 for the nucleus accumbens, Eicom Co., Kyoto, Japan) was unilaterally implanted into the medial prefrontal cortex (3.2 mm anterior to the bregma, 0.6 mm lateral to the midline, and 3.0 mm ventral to the dura) or the nucleus accumbens (1.6 mm anterior to the bregma, 1.4 mm lateral to the midline, and 5.9 mm ventral to the dura). A dummy cannula (AD-4 or AD-8, Eicom Co.) was also inserted, penetrating to

the tip of the guide cannula. The cannula placement (left or right) was counterbalanced for each brain region. After surgery, the rats were housed individually and allowed to recover for 4 days before retraining.

2.5.2. Microdialysis and drug treatment procedures

After retraining of the 3-choice serial reaction time task (> 4 sessions), rats were subjected to 3 sham handling procedures before the training sessions. On the day of testing, rats were gently restrained, and dummy cannulas were removed. A dialysis probe (2 mm long and 0.22 mm in outer diameter) (Eicom Co.) was inserted through the guide cannula. The probe was perfused with artificial cerebrospinal fluid (2.7 mM KCl, 140 mM NaCl, 1.2 mM CaCl₂, 1.0 mM MgCl₂, 0.3 mM NaH₂PO₄, and 1.7 mM Na₂HPO₄; pH 7.2) at a flow rate of 2 µl/min. Each rat was placed in a plastic observational cage (30 × 30 × 35 cm³), and allowed to move freely. Samples were collected every 15 min, and 15 µl of each collected sample was injected into a high-performance liquid chromatography (HPLC) system to measure the extracellular levels of serotonin and dopamine. After the dopamine and serotonin levels stabilized (±10% or less), 3 baseline samples were collected. Stabilization of dopamine and serotonin levels took at least 2 h. We administered saline followed by duloxetine (3.0 mg/kg)

intraperitoneally during *in vivo* microdialysis. The drug was administered at least 2 h after the saline injections.

2.5.3. Dopamine and Serotonin Analysis

Dialysate dopamine and serotonin concentrations were measured using HPLC (Eicompak PP-ODS II 4.6 mm i.d. × 30 mm, Eicom) with electrochemical detection (ECD-300, Eicom) as described previously (Matsumoto et al., 2008; Matsumoto et al., 2005; Ohmura et al., 2010; Yoshioka et al., 1995). The mobile phase (2.1 mM sodium 1-decansulfonate, 0.1 mM EDTA-2Na/0.1 M phosphate buffer (pH 5.4), and 2% (v/v) methanol) was pumped at a rate of 500 µl/min. Data are expressed as the percentage of the baseline value, which was calculated as the average of 3 consecutive dialysates before drug injections. The area under the curve (AUC) for dopamine and serotonin levels during the 60–105 min period was calculated because 3-choice serial reaction time task was conducted within the period in Experiment 1.

2.5.4. Verification of Probe Placements

After completion of the microdialysis experiments, the rats were deeply anesthetized with urethane (2 g/kg) intraperitoneally and transcardially perfused via the left ventricle with 0.9% saline followed by 10% formalin solution (Sigma-Aldrich).

After removal from the skull, brains were immersed overnight in the same fixative at 4°C, placed in 0.1 M PB containing 30% sucrose at 4°C, and sectioned at 50-µm thickness using a cryostat and mounted onto slides. After drying, the sections were stained with toluidine blue, and probe placements were verified under a microscope according to the atlas (Paxinos 2008). Only data from rats with correct microdialysis probe placement were included in the statistical analysis.

2.6. Experiment 3: Mechanisms of action underlying duloxetine-enhanced control of impulsive action

To determine whether the neural mechanisms underlying the anti-impulsive effects of duloxetine support our hypothesis (Ohmura et al., 2012), we microinjected SCH23390, a dopamine D₁-like receptor antagonist, into the infralimbic cortex of duloxetine-treated rats.

2.6.1. Surgery

After completing the 3-choice serial reaction time task training, rats were anesthetized with sodium pentobarbital (50 mg/kg) intraperitoneally and fixed in a

stereotaxic frame (Narishige, Tokyo, Japan). Double guide cannulas (C232G-1.5-SPC, Plastics One Inc., VA, USA) were bilaterally implanted (3.2 mm anterior to the bregma, 0.75 mm lateral to the midline, and 2.0 mm ventral to the dura) (Paxinos, 2008). Dummy cannulas (C232DC-SPC, Plastics One Inc.) were inserted, penetrating to the tip of the guide cannulas. After surgery, the rats were housed individually and allowed to recover for 4 days before retraining.

2.6.2. Drug treatment procedure

After the performance of 3-choice serial reaction time task recovered, rats were subjected to 3 sham handling procedures before the training sessions. On the day of testing, rats were gently restrained, and the dummy cannulas were removed and replaced with double injection cannulas (C313I-SPC, Plastics One Inc.) attached to a polyethylene tube. The tips of the injection cannulas extended beyond the guide cannulas by 2.0 mm. SCH23390 (0 or 3.0 ng in 0.5 μ l PBS/side) was infused at a rate of 0.5 μ l/min into the infralimbic cortex. The solution was infused over a period of 1 min at constant flow using a microinjection pump (CMA100, Carnegie Medicine, Sweden), and the injection cannula was left in place to allow diffusion for 1 min after injection.

Fifty min before the microinjection of PBS or SCH23390, the rats were

administered saline or duloxetine (3.0 mg/kg) intraperitoneally. Behavioral testing was conducted 10 min after the injection of PBS or SCH23390. Drug treatments were carried out using a Latin square design. The drug was administered at intervals of more than 2 days.

2.6.3. Verification of Cannula Placement

After the completion of behavioral experiments, cannula placement was verified as described in Experiment 2. Only data from rats with correct injection needle placement were included in the statistical analysis.

2.7. Experiment 4: Mechanisms of action underlying atomoxetine-enhanced control of impulsive action

To further determine whether the neural mechanisms underlying the anti-impulsive effects of atomoxetine also support our hypothesis (Ohmura et al., 2012), we microinjected SCH23390, a dopamine D₁-like receptor antagonist, into the infralimbic cortex of atomoxetine-treated rats as described in the experiment 3. The same procedures as Experiment 3 were used except that atomoxetine was used instead of duloxetine. Twenty min before the microinjection of PBS or SCH23390, the rats were administered saline or atomoxetine (1.0 mg/kg) intraperitoneally. We used this dose (1.0

mg/kg) because 1.0 mg/kg was the most effective dose in our previous study (Tsutsui-Kimura et al., 2009).

2.8. Data analysis

Seven behavioral measures were analyzed (see **2.3. 3-choice serial reaction time task**). Each measure was analyzed separately by analysis of variance (ANOVA). If Mauchly's sphericity test was significant, Greenhouse-Geisser correction was used. In the Experiment 3 and 4, the behavioral measures were analyzed separately using a two-factor ANOVA. In cases in which there was a significant interaction, it was followed by a one-factor ANOVA for each level. Multiple comparisons with Bonferroni's correction were also conducted in cases where ANOVA revealed a significant main effect except for the case where only two levels were used. For microdialysis data, the AUC of dopamine or serotonin levels was analyzed separately by one-factor repeated-measures ANOVA (see **2.5.3. Dopamine and Serotonin Analysis**). The alpha level was set at 0.05 for all statistical procedures. All statistical analyses were conducted using SPSS (version 23.0).

3. Results

3.1. Experiment 1: the effects of acute duloxetine injection on impulsive action

Duloxetine administration selectively reduced the number of premature responses in a dose-dependent manner (Fig. 1A). One-factor repeated-measures ANOVA revealed a significant dose effect on premature responses (Fig. 1A) ($F_{3,45} = 12.19$; $P < 0.001$) but not on accuracy (Fig. 1B) ($F_{3,45} = 0.82$; not significant [NS]), omissions (Fig. 1C) ($F_{3,45} = 2.08$; NS), percentage of perseverative responses (Fig. 1D) ($F_{3,45} = 0.70$; NS), responses during time-out (Fig. 1E) ($F_{3,45} = 1.32$; NS), correct response latency (Fig. 1F) (with Greenhouse–Geisser correction, $F_{1.916, 28.740} = 0.77$; NS), or reward latency (Fig. 1G) (with Greenhouse–Geisser correction, $F_{1.171, 17.569} = 1.79$; NS). A multiple comparison with Bonferroni's correction revealed that the 3.0 mg/kg dose of duloxetine decreased the number of premature responses compared to the vehicle ($P = 0.004$) or the 0.3 mg/kg dose ($P < 0.001$).

3.2. Experiment 2: Extracellular levels of dopamine and serotonin in the medial prefrontal cortex and nucleus accumbens after acute administration of duloxetine

The target locations of the dialysis probes in the medial prefrontal cortex and

nucleus accumbens are shown in Figs. 2A and 3A. Of the 14 implanted rats, 2 were excluded because the probes were located outside the target region ($n = 12$). Dialysis probes in the medial prefrontal cortex were placed mainly in the ventral region.

3.2.1. Effect of duloxetine on dopamine and serotonin concentration in the medial prefrontal cortex and nucleus accumbens

The dose of duloxetine exerting anti-impulsive effects (3.0 mg/kg) significantly increased the extracellular dopamine levels in the medial prefrontal cortex ($F_{1,5} = 24.75$; $P = 0.004$) (Figs. 2B and C) without affecting dopamine levels in the nucleus accumbens ($F_{1,5} = 0.22$; NS) (Figs. 3B and 3C). In contrast, the dose of duloxetine significantly increased the extracellular serotonin levels both in the medial prefrontal cortex ($F_{1,5} = 27.17$; $P = 0.003$) (Figs. 2D and 2E) and in the nucleus accumbens ($F_{1,5} = 7.44$; $P = 0.041$) (Figs. 3D and 3E).

3.3. Experiment 3: Mechanisms of action underlying duloxetine-enhanced control of impulsive action

3.3.1. Histological analysis

The target locations of the cannula tips in the infralimbic cortex region are

shown in Fig. 4A. Of the 21 implanted rats, 1 was excluded because the cannulas were located outside the target region ($n = 20$).

3.3.2. Effect of microinjections of a dopamine D₁-like receptor antagonist into the infralimbic cortex on duloxetine-enhanced control of impulsive action

Figs. 4B-H show the effects of intra-infralimbic cortex injections of SCH23390 on the duloxetine-enhanced control of impulsive action. Two-factor ANOVA with the dose of duloxetine and the dose of SCH23390 as within subject factors revealed a significant main effect of duloxetine on impulsive action (Fig. 4B) ($F_{3,57} = 30.354, P < 0.001$), and a significant interaction on impulsive action ($F_{3,57} = 5.958, P = 0.025$). In addition, two-factor ANOVA revealed a significant main effect of duloxetine on correct latency (Fig. 4G) ($F_{3,57} = 6.173, P = 0.022$), and a significant interaction on percentage of perseverative responses (Fig. 4E) ($F_{3,57} = 5.219, P = 0.034$) and on reward latency (Fig. 4H) ($F_{3,57} = 4.695, P = 0.043$). There was no significant main effect or an interaction effect on accuracy (Fig. 4C), omissions (Fig. 4D), and responses during time-out (Fig. 4F) ($F_{3,57} < 3.7, \text{NS}$).

Following one-factor ANOVA showed that systemic duloxetine significantly

decreased the number of premature responses in PBS-injected group ($P < 0.001$), while the anti-impulsive effects of duloxetine disappeared in SCH23390-injected group ($P = 0.199$). Moreover, systemic duloxetine reduced the percentage of perseverative responses in SCH23390-injected group ($P = 0.037$), while there was no effect of duloxetine in PBS-injected group ($P = 0.249$). One-factor ANOVA for reward latency in each level did not indicate any significant differences.

3.4. Experiment 4: Mechanisms of action underlying atomoxetine-enhanced control of impulsive action

3.4.1. Histological analysis

Fig. 5A shows the locations of the cannula tips in the infralimbic cortex region of rats that were included in the present study. Of the 12 implanted rats, none was excluded, resulting in $n = 12$.

3.4.2. Effect of microinjections of a dopamine D₁-like receptor antagonist into the infralimbic cortex on atomoxetine-enhanced control of impulsive action

Figs. 5B-H show the effects of intra-infralimbic cortex injections of SCH23390

on the atomoxetine-enhanced control of impulsive action. Two-factor ANOVA with the dose of atomoxetine and the dose of SCH23390 as within subject factors revealed a significant main effect of atomoxetine on impulsive action (Fig. 5B) ($F_{3,33} = 7.373$, $P = 0.020$) and a significant interaction ($F_{3,33} = 14.608$, $P = 0.003$). There was no significant main effect or an interaction on accuracy (Fig. 5C), omissions (Fig. 5D), percentage of perseverative responses (Fig. 5E), responses during time-out (Fig. 5F), correct response latency (Fig. 5G), and reward response latency (Fig. 5H) ($F_{3,33} < 4.1$, NS).

Following one-factor ANOVA showed that systemic atomoxetine significantly decreased the number of premature responses in PBS-injected group ($P < 0.001$), while the anti-impulsive effects of atomoxetine disappeared in SCH23390-injected group ($P = 0.448$).

4. Discussion

In experiment 1, the administration of duloxetine selectively and dose-dependently decreased the number of premature responses during the 3-choice serial reaction time task without changing other indices of cognitive function such as attentional function, motivation/appetite, compulsive behavior, or motor function (Fig.

1). Thus, the decrease in premature responses is not caused by a decrease in motivation/appetite or increased attentional function (Dalley et al., 2008). Moreover, the suppressive effects of duloxetine on the nose-poke response appeared only during the ITI, as the number of responses during time-out was not affected by duloxetine administration (Fig. 1E), indicating that the decrease in premature responses was not the result of overall suppression of nose-poking. These results suggest that duloxetine, as well as other agents that block noradrenaline transporters, is a potential therapeutic agent for impulsivity-related disorders (Liu et al., 2009; Robinson et al., 2008b; Tsutsui-Kimura et al., 2009).

The results of experiment 2 showed that 3.0 mg/kg duloxetine, which was effective for impulse control in experiment 1, dramatically increased the extracellular concentration of dopamine in the medial prefrontal cortex (Figs. 2B and 2C) but not in the nucleus accumbens (Figs. 3B and 3C). In contrast, the extracellular serotonin levels increased in both the regions (Figs. 2D, 2E, 3D, and 3E). These results are consistent with the theory that dopamine reuptake in the frontal cortex depends on the noradrenaline transporter, while that in the striatum mainly depends on the dopamine transporter (Moron et al., 2002). Previous studies have indicated that dopaminergic

functions in the medial prefrontal cortex play a pivotal role in inhibiting impulsive behavior (Sokolowski and Salamone, 1994) while those in the nucleus accumbens facilitate it (Cole and Robbins, 1989; Pattij et al., 2007). The region-dependent effects of duloxetine on dopamine levels thus are favorable to controlling impulsivity.

We cannot completely exclude the possibility that the effects of duloxetine on dopamine/serotonin levels were somehow affected by the order of injection because we did not counterbalance the order of drug injection in experiment 2: we always injected saline first and followed by duloxetine. However it is unlikely that a previous saline injection significantly affected the effects of duloxetine because saline injection had no effect on dopamine/serotonin levels (Figs. 2 and 3) and because our preliminary results showed that repeated saline injection had no effects on these levels either (data not shown).

Microinjection of SCH23390, a selective dopamine D₁-like receptor antagonist, into the infralimbic cortex reversed the duloxetine-enhanced control of impulsive action (Fig. 4B). This result is consistent with a previous study using another serotonin and noradrenaline reuptake inhibitor milnacipran (Tsutsui-Kimura et al., 2013); however, some differences were also observed.

In the experiment 3, there was a main effect of duloxetine on correct response latency, while the effect was not statistically significant in experiment 1. Correct response latency is an index of multiple functions: attentional function, motivation, and motor function, but other parameters reflecting these functions were not altered by drug injections (Fig. 4). Thus, it is difficult to interpret the change in correct response latency.

Unexpectedly, duloxetine decreased the percentage of perseverative responses only when SCH23390 was injected simultaneously (Fig. 4E). Because this result is inconsistent with those of milnacipran (Tsutsui-Kimura et al., 2013) and atomoxetine (Fig. 5E), this effect might be specific for duloxetine, rather than for noradrenaline reuptake inhibitors. Although to address this issue more will be beyond the scope of this study, it might be interesting to examine whether this complicated effect could be replicated in animal models of obsessive-compulsive disorder.

Furthermore, by using an established anti-impulsive drug atomoxetine, we successfully replicated the blockade of the anti-impulsive effects of noradrenaline reuptake inhibitors by intra-infralimbic cortex injections of dopamine D₁-like receptor antagonist. These results strongly suggest that the same mechanisms would underlie the anti-impulsive effects of this kind of drugs. Therefore, the present study made the

strategy more promising to develop anti-impulsive drugs.

We did not address the possibility that the prelimbic cortex, which is a more dorsal part of the medial prefrontal cortex, or dopamine D₂-like receptor could partly be involved in the suppression of impulsive action. However, previous studies, including our own work, have demonstrated the infralimbic cortex, but not the prelimbic cortex, played a pivotal role in controlling impulsive action (Tsutsui-Kimura et al., 2010) (Chudasama et al., 2003; Murphy et al., 2012). Moreover, we have already demonstrated that dopamine D₁-like receptors, but not the dopamine D₂-like receptors, are critical for the impulse control (Tsutsui-Kimura et al., 2013). Thus, it is likely that dopamine D₁-like receptors in the infralimbic cortex play the main role in suppressing impulsive action.

Because duloxetine is a serotonin and noradrenaline reuptake inhibitor, it increases the extracellular levels of serotonin and noradrenaline as well as dopamine (cf. Figs. 2D, 2E, 3D, and 3E) (Gobert et al., 1997; Kihara and Ikeda, 1995). However, it is unlikely that increased noradrenaline in the medial prefrontal cortex suppressed impulsive action in the present study because a previous study demonstrated that lack of noradrenaline in neocortex did not affect impulsivity (Cole and Robbins, 1992).

However, increased noradrenaline in the nucleus accumbens might suppress impulsive action (Economidou et al., 2012). It is also less likely that increased serotonin levels in the nucleus accumbens and medial prefrontal cortex affected impulsive action in the present study because a previous study showed that neither depletion of serotonin in the medial prefrontal cortex nor nucleus accumbens altered impulsive action (Fletcher et al., 2009). Moreover, previous studies have indicated the opposing roles of serotonin 5-HT_{2A} and 5-HT_{2C} receptors in the nucleus accumbens and medial prefrontal cortex with respect to impulsive action (Anastasio et al., 2015; Robinson et al., 2008a). Thus, increased serotonin in these regions would stimulate both types of receptors, resulting in the cancellation of each other's effects. Thus, it is likely that increased dopamine in the medial prefrontal cortex played the main role in suppressing impulsive action in the present study. However, we cannot exclude the possibility that increased serotonin and/or noradrenaline in brain regions that were not examined in this study affected impulsive action.

It should be also noted that impulsive action, which is assessed by the 3-choice serial reaction time task, is only one aspect of the impulsive behavior. Impulsive behavior could be divided into four subordinate concepts: "reflection impulsivity",

“impulsive action”, “impulsive choice”, and “risky behavior” (for a review, (Ohmura et al., 2012). Further studies would be needed to discuss the effects and mechanisms of action of noradrenaline reuptake inhibitors on other aspects of impulsive behavior.

Taken together, our results suggest that noradrenaline reuptake inhibitors, duloxetine and atomoxetine, likely increase the control of impulsivity in rats by increasing dopamine levels in the medial prefrontal cortex and stimulating the dopamine D₁-like receptors in the infralimbic cortex. These results are consistent with our previous suggestion (Ohmura et al., 2012), indicating that our hypothesis could be used as a guide to developing anti-impulsivity drugs. While we focused on the dopaminergic systems in the medial prefrontal cortex and nucleus accumbens in the present study, it should be noted that other neurotransmitters and brain regions not considered here are also involved in impulsive action. For example, previous studies have shown that cholinergic (Ohmura et al., 2017; Tsutsui-Kimura et al., 2010) and glutamatergic (Tsutsui-Kimura et al., 2016; Tsutsui-Kimura et al., 2015) systems in the medial prefrontal cortex play essential roles in regulating impulsive action. Several studies have demonstrated that other brain regions, including the ventral hippocampus (Abela et al., 2013), the habenula (Lecourtier and Kelly, 2005), and the subthalamic nucleus

(Baunez and Robbins, 1997), are involved in controlling impulsive action. Moreover, even if the neural mechanisms of impulsivity have been largely elucidated, other unknown mechanisms regulating impulsive action may remain (Dalley and Robbins, 2017). Therefore, our strategy would be useful but not fully inclusive. Further studies clarifying neurochemical/pharmacological profiles in the neural networks regulating impulsivity will be used to revise and update our strategy, facilitating the discovery and development of therapeutic drugs for deficits in impulse control.

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Titles and Legends to Figures

Fig. 1. Effects of duloxetine on impulsive action and other 3-choice serial reaction time task parameters. Rats received systemic duloxetine (0, 0.3, 1.0, or 3.0 mg/kg) intraperitoneally. The bars represent the mean, and the lines represent the S.E.M. * $P < 0.05$, treatment with vehicle or duloxetine (0.3 mg/kg) versus duloxetine (3.0 mg/kg).

Fig. 2. Probe placement and the effects of duloxetine on dopamine and serotonin levels in the medial prefrontal cortex. Schematic diagrams show the placement of

dialysis probes in the medial prefrontal cortex (mPFC) (A). The time course of changes in the extracellular dopamine (B) and serotonin (D) levels. Open circles (n = 6) represent the data when the rats received an intraperitoneal (i.p.) injection of saline (5 ml/kg), and filled circles (n = 6) represent the data when the rats received an i.p. injection of duloxetine (3.0 mg/kg) (B, D). The areas under the curve (AUC) of extracellular dopamine and serotonin levels during 60–105 min were standardized (C, E). The formula is as follows: $AUC_{60-105min} = \{\sum_{n=4}^6 [(\%_{15n} + \%_{15(n+1)})/2 \times 15]\} / [(105 - 60) \times 100]$. The extracellular levels of both dopamine and serotonin increased in the mPFC (B, C, D, and E). * $P < 0.05$. We administered saline followed by duloxetine (3.0 mg/kg, i.p.) during *in vivo* microdialysis and the duloxetine was administered at least 2 h after the saline injection.

Fig. 3. Probe placement and the effects of duloxetine on dopamine and serotonin levels in the nucleus accumbens. Schematic diagrams show the placement of dialysis probes in the nucleus accumbens (NAc) (A). The time course of changes in the extracellular dopamine (B) and serotonin (D) levels. Open circles (n = 6) represent the data when the rats received an i.p. injection of saline (5 ml/kg), and filled circles (n = 6)

represent the data when the rats received an i.p. injection of duloxetine (3.0 mg/kg) (B, D). The AUC of extracellular dopamine and serotonin levels during 60–105 min was standardized (C, E) as described in Fig. 2 legend. The extracellular levels of dopamine did not increase in the NAc (B, C) in contrast to the mPFC (Figs. 2B and 2C), while those of serotonin increased in the NAc (D, E) as in the mPFC (Figs. 2D and 2E). $*P < 0.05$. We administered saline followed by duloxetine (3.0 mg/kg, i.p.) as described in Fig. 2 legend.

Fig. 4. Cannula placement and the effect of dopamine D₁-like receptor antagonist

with duloxetine on 3-choice serial reaction time task performance. Schematic

diagram shows the placements of cannula tips in the infralimbic cortex (A). PL, prelimbic cortex; IL, infralimbic cortex; DP, dorsal peduncular cortex (Paxinos, 2008).

The rats received systemic duloxetine (0 or 3.0 mg/kg) and intra-IL SCH23390 (0 or 3.0 ng/side). The bars represent the mean, and the lines represent the S.E.M. $*P < 0.05$ (a significant main effect of duloxetine), $^{+}P < 0.05$ (a significant interaction between dose of duloxetine and the dose of SCH23390).

Fig. 5. Cannula placement and the effect of dopamine D₁-like receptor antagonist with atomoxetine on 3-choice serial reaction time task performance. Schematic diagram shows the placements of cannula tips in the infralimbic cortex (A). PL, prelimbic cortex; IL, infralimbic cortex; DP, dorsal peduncular cortex (Paxinos, 2008). The rats received systemic atomoxetine (0 or 1.0 mg/kg) and intra-IL SCH23390 (0 or 3.0 ng/side). The bars represent the mean, and the lines represent the S.E.M. * $P < 0.05$ (a significant main effect of atomoxetine), + $P < 0.05$ (a significant interaction between atomoxetine and SCH23390).

Supporting information

Data Set. Raw data for each figure. Each excel file corresponds to the data of each experiment and figure.

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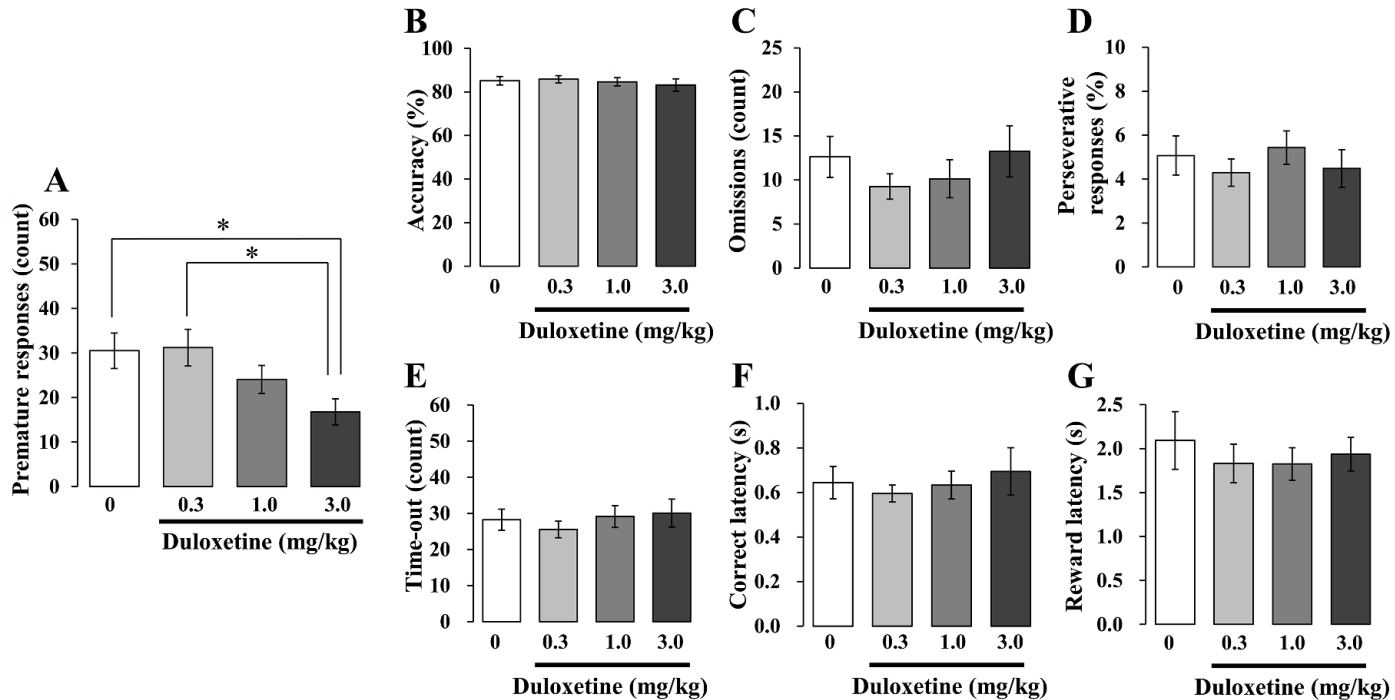


Figure 1

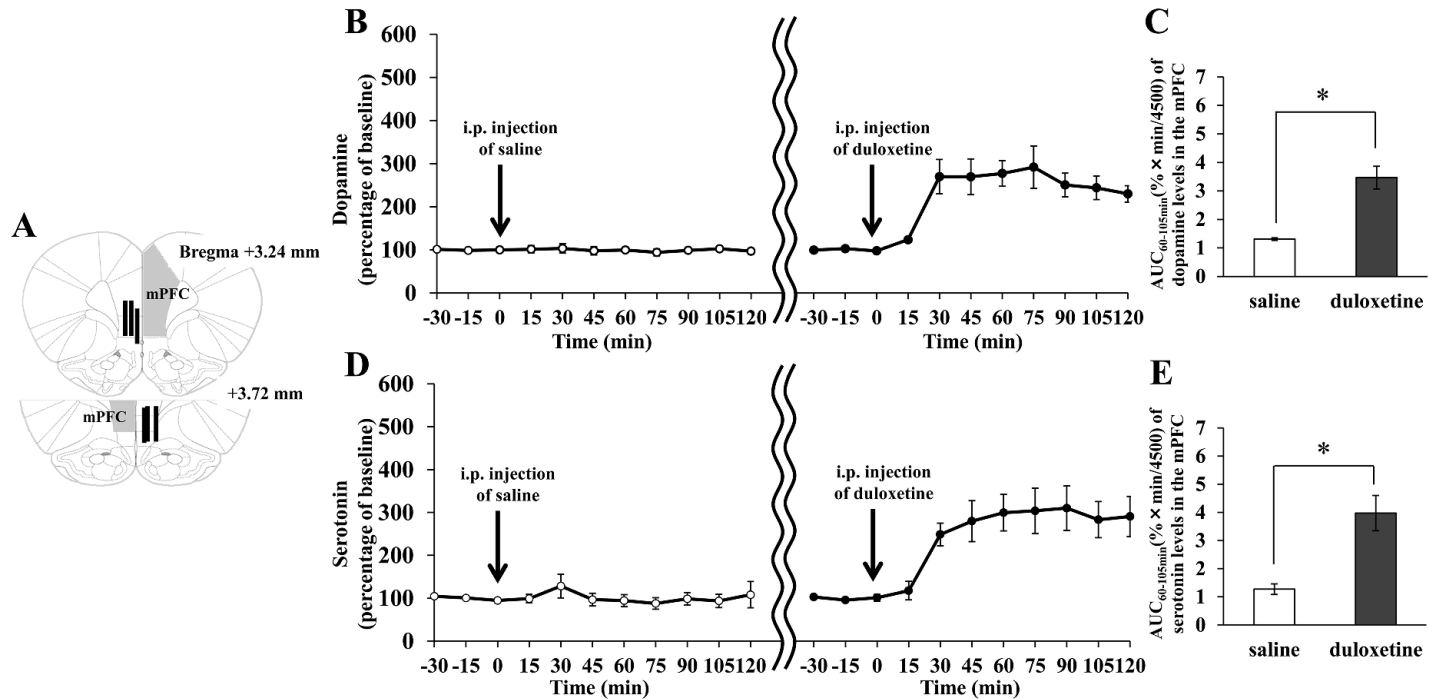


Figure 2

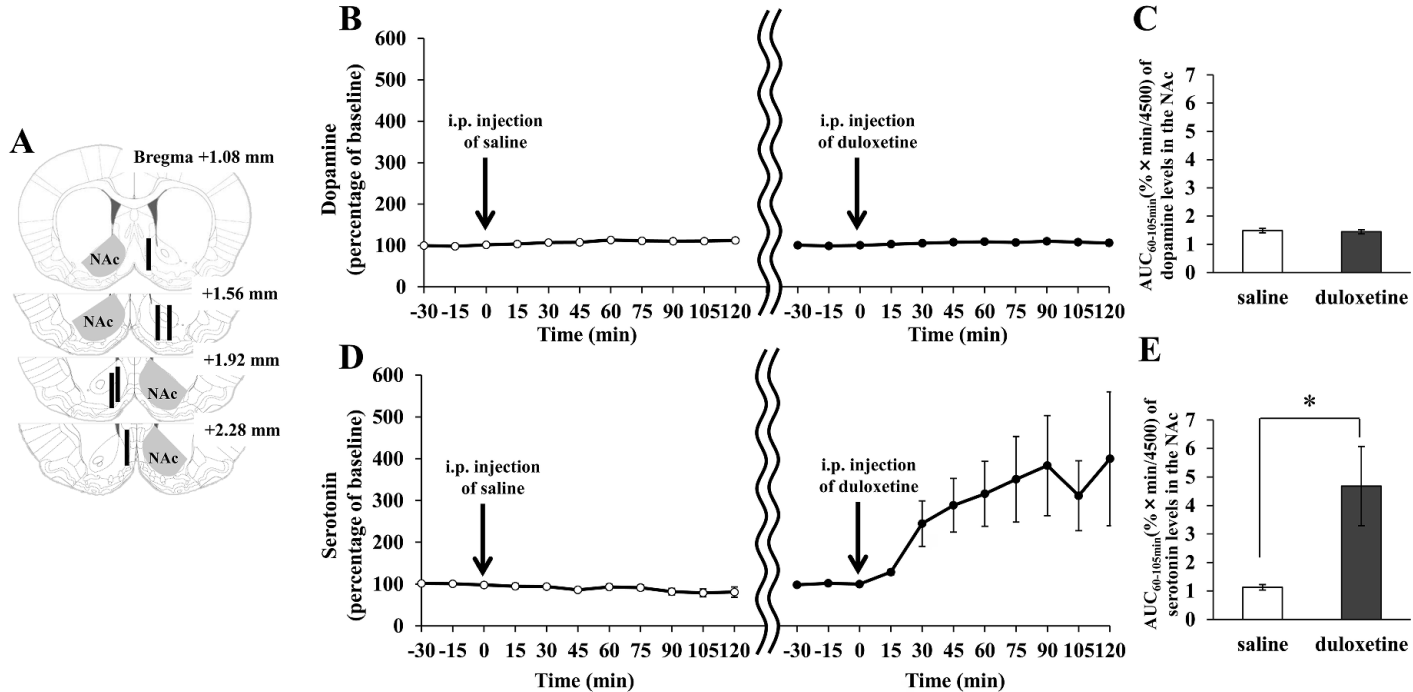


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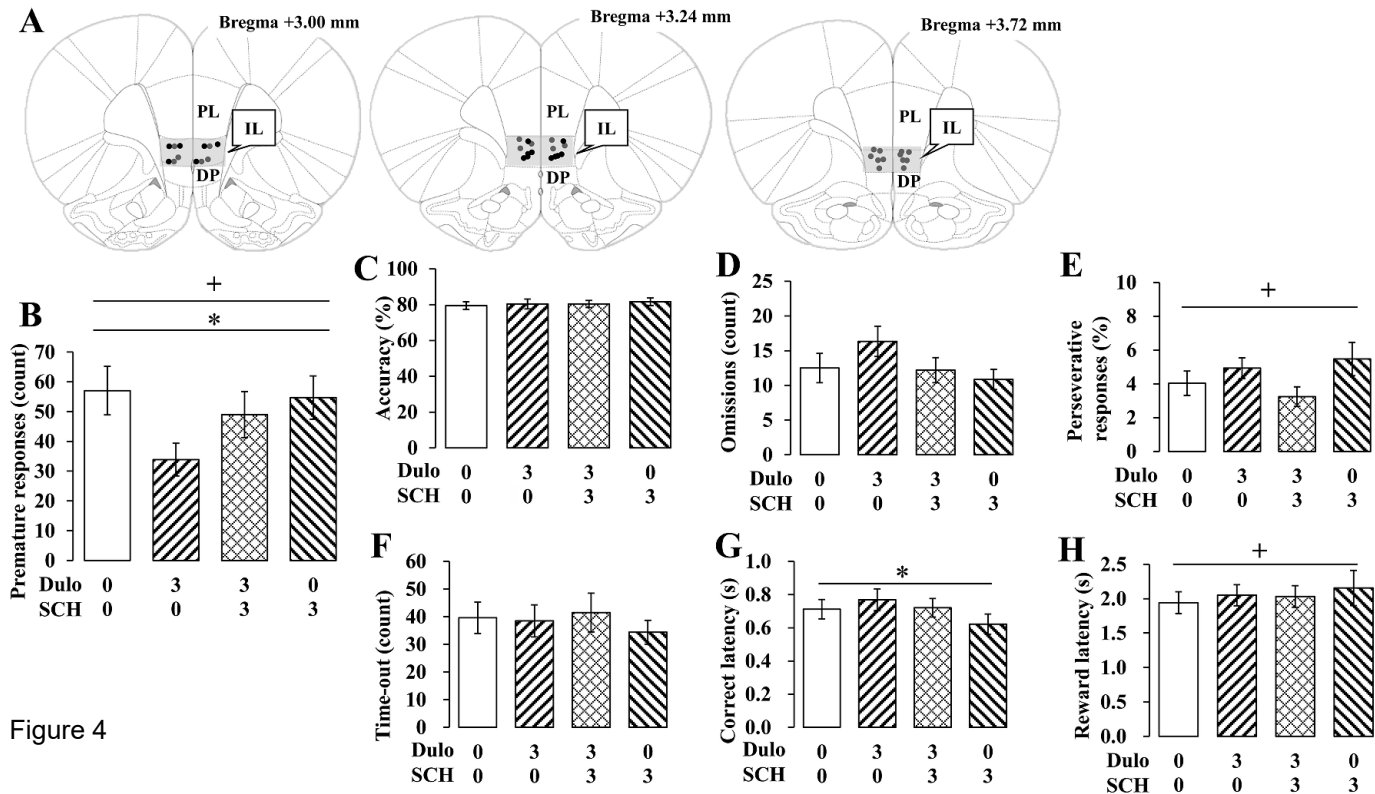


Figure 4

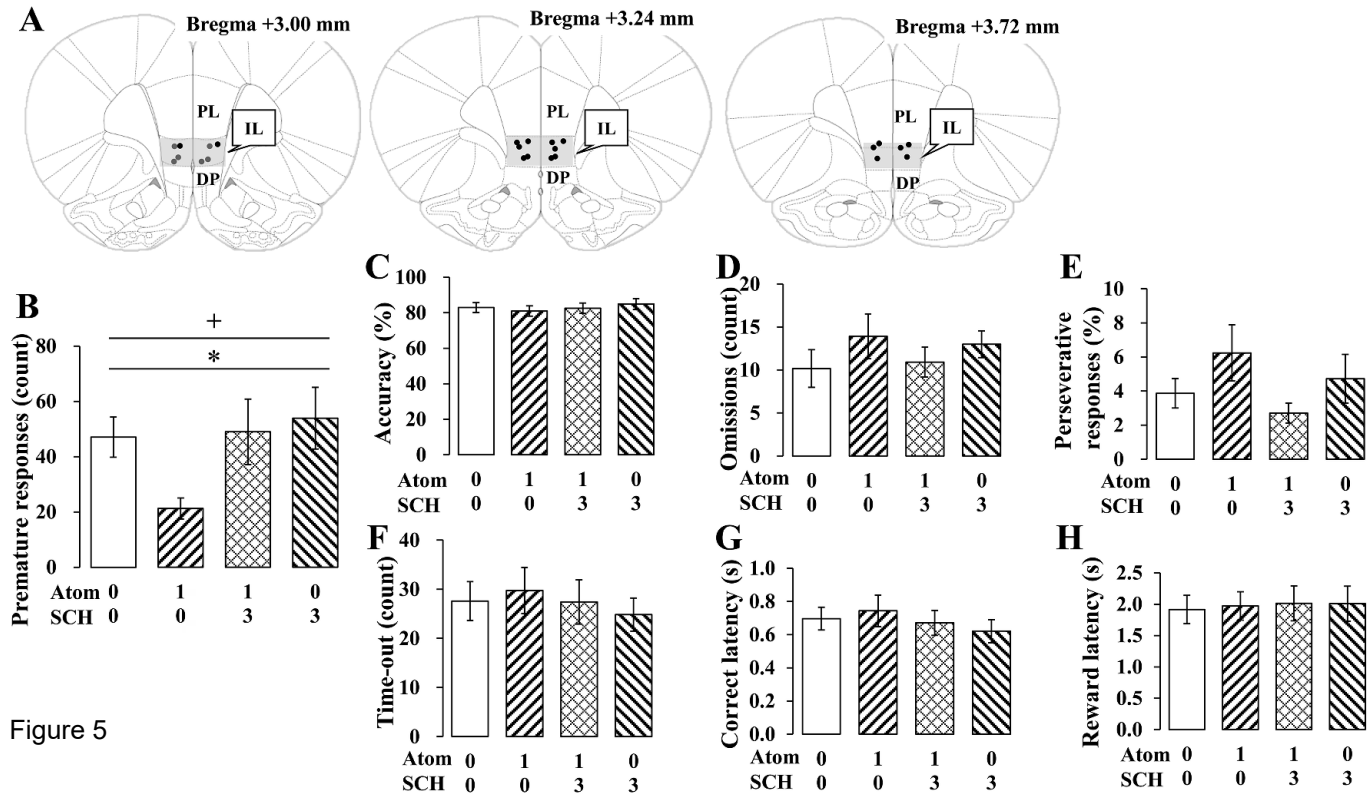


Figure 5