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Varenicline Provokes Impulsive Action by Stimulating $\alpha 4\beta 2$ Nicotinic Acetylcholine Receptors in the Infralimbic Cortex in a Nicotine Exposure Status-Dependent Manner

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Abstract

Higher impulsivity is a risk factor for criminal involvement and drug addiction. Because nicotine administration enhances impulsivity, the effects of stop-smoking aids stimulating nicotinic acetylcholine receptors (nAChRs) on impulsivity must be determined in different conditions. Our goals were 1) to confirm the relationship between varenicline, a stop-smoking aid and $\alpha 4\beta 2$ nAChR partial agonist, and impulsivity, 2) to elucidate the mechanisms underlying the effects of varenicline, 3) to examine whether a low dose of varenicline that does not evoke impulsive action could block the stimulating effects of nicotine on impulsive action, 4) to determine whether the route of administration could modulate the effects of varenicline on impulsive action, and 5) to determine whether the effects of varenicline on impulsivity could be altered by smoking status. We used a 3-choice serial reaction time task to assess impulsivity and other cognitive functions in rats. Our findings are as follows: 1) acute subcutaneous (s.c.) injection of varenicline evoked impulsive action in a dose-dependent manner; 2) the effects of varenicline on impulsivity were blocked by the microinjection of dihydro- β -erythroidine, a $\alpha 4\beta 2$ nAChRs antagonist, into the infralimbic cortex; 3) the low dose of varenicline did not attenuate the effects of nicotine on impulsive action at all; 4) oral administration of varenicline evoked impulsive action in a similar manner to s.c. injection; and 5) the stimulating effects of varenicline on impulsive action were not

observed in rats that received nicotine infusion for 8 days or nicotine-abstinent rats after discontinuing infusion. Additionally, we found that oral varenicline administration enhanced attentional function whether nicotine was infused or not. Thus, although varenicline administration could be harmless to heavy smokers or ex-smokers, it could be difficult for non-smokers with respect to impulsivity, whereas it may be beneficial with respect to attentional function.

1. Introduction

Tobacco smoking is responsible for 12% of all deaths among adults aged ≥ 30 years (WHO, 2012). Tobacco smoking/nicotine induces not only health problems, such as lung cancer, but also impulsive behavior (Ohmura et al., 2005; Tsutsui-Kimura et al., 2009; 2010). Smoking/nicotine-enhanced impulsivity may cause serious problems because higher impulsivity is a risk factor for drug addiction (Diergaarde et al., 2008; Economidou et al., 2009) and criminal involvement (Babinski et al., 1999). Thus, much effort has been made to develop stop-smoking aids though few drugs are clinically-available so far (Cahill et al., 2013; Perkins, 2014).

Of these drugs, varenicline (trade name: Chantix/Champix) is a quite effective stop-smoking aid (Cahill et al., 2013). However, it is an $\alpha 4\beta 2$ nicotinic acetylcholine receptor (nAChRs) partial agonist (Mihalak et al., 2006) and may provoke impulsive behavior because $\alpha 4\beta 2$ nAChRs are responsible for nicotine-induced impulsive action (Tsutsui-Kimura et al., 2010). Indeed, a previous study demonstrated that intraperitoneal injection (i.p.) of varenicline evoked impulsive action in rats (Wouda et al., 2011) while inconsistent results have been reported in human studies examining the effects of orally-taken varenicline on impulsivity (Austin et al., 2014; Mocking et al., 2013; Rhodes et al., 2012).

It is difficult to reconcile these contradictory findings because there were many differences among these previous studies in experimental conditions such as species, smoking status, route of drug administration, and task paradigm for assessing impulsivity (Austin et al., 2014; Mocking et al., 2013; Rhodes et al., 2012; Wouda et al., 2011). Previous studies have shown that higher impulsivity is a risk factor for several problems (Babinski et al., 1999; Diergaarde et al., 2008; Economidou et al., 2009), and it is a concern whether varenicline can be used for other issues such as alcohol addiction (Litten et al., 2013). Therefore, to clarify the relationship between varenicline and impulsivity is important and it should be repeatedly confirmed in similar experimental conditions with small differences. In the present study, we first replicated the previous study (Wouda et al., 2011) using nicotine-naïve rats and a similar task paradigm but subcutaneous injection (s.c.) of varenicline (Experiment 1).

To obtain a clue to understand the side effect, the mechanisms by which varenicline stimulates impulsive action must be elucidated. We examined the mechanisms of action focusing on $\alpha 4\beta 2$ nAChRs in the infralimbic cortex (Experiment 2), since we have previously reported that nicotine evoked impulsive action through the stimulation of $\alpha 4\beta 2$ nAChRs in the infralimbic cortex which is a ventral part of the medial prefrontal cortex, but not in the prelimbic cortex which is a more dorsal part (Tsutsui-Kimura et al.,

2010).

Although higher doses of varenicline could induce impulsive action, a low dose of varenicline that does not evoke impulsive action might block the stimulating effects of nicotine on impulsive action because varenicline is an $\alpha 4\beta 2$ nAChR partial agonist with low efficacy (Mihalak et al., 2006). We tested the hypothesis by using acute nicotine and varenicline administration (Experiment 3).

Because it is known that the effects of orally-taken drugs are relatively mild (Volkow et al., 2004), a difference in the route of administration might explain the inconsistent results between animal studies using i.p. or s.c. and human studies using oral administration (p.o.). Thus, we used p.o. administration instead of s.c. injection to determine whether the route of administration could modulate the effects of varenicline on impulsive action (Experiment 4).

Furthermore, it is of interest whether the effects of varenicline on impulsivity could be altered by smoking status. If the effects disappear in smokers and/or ex-smokers, it would mean that varenicline is a safe drug as long as it is used for smokers and/or ex-smokers. We tested the possibility by using rats that received nicotine infusion for 8 days (Experiment 4) and nicotine-abstinent rats after discontinuing infusion (Experiment 5).

Additionally, we examined the effects of varenicline not only on impulsivity, but also on attentional functions, because nAChRs have been promising targets for developing cognitive enhancers (Potter et al., 2014) and because nicotine-induced cognitive benefits might motivate smoking (Russell et al., 1974). However, previous studies have not shown the consistent effects of nicotine or varenicline on attentional functions (Hahn et al., 2002; Mocking et al., 2013; Rhodes et al., 2012; Tsutsui-Kimura et al., 2009; 2010; Wouda et al., 2011). Therefore we assessed attentional functions in different experimental conditions of this study (Experiments 1-5).

2. Methods

2.1. Animals

Fifty-nine 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 p.m. to 7 a.m.) at approximately 21°C and a relative humidity of 40–50%. Rats received 3-choice serial reaction time task (3-CSRTT) training and were allocated to one or two of six experimental conditions: 8 rats were used for acute varenicline administration (Experiment 1), 9 rats were used for acute varenicline administration and $\alpha 4\beta 2$ nAChR antagonist microinjection (Experiment 2), 15 rats were used for acute varenicline and nicotine administration (Experiment 3), 19 rats were used for acute

varenicline administration during continuous nicotine administration (Experiments 4), 9 rats used in Experiment 4 were used again for repeated varenicline administration during nicotine withdrawal (Experiment 5), and 8 rats were used for food consumption test (Experiment 6). When the rats were 9 weeks old (270–290 g), we started to restrict their food intake so as to maintain their body weights at 85% of those under free-feeding conditions. The daily food of rats in the home cage was purchased from CLEA JAPAN, Inc. (CE-2; Tokyo, Japan) and was given after their daily sessions. Their food intake in the home cage was 8–16 g in the training period and 8–12 g (plus 2–3 g of reward pellets consumed in the operant box, see also 3-CSRTT in the experimental period). Water was available *ad libitum*. The treatment of animals complied with the NIH Animal Care Guidelines and 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. *Impulsivity Assessment (3-CSRTT)*

To assess impulsive action, we employed a 3-CSRTT (Tsutsui-Kimura et al., 2009; 2010), which is a simple version of the 5-choice serial reaction time task (5-CSRTT) (Carli et al., 1983). This task is performed in an operant chamber containing a horizontal array of three holes. A light in the aperture of one of the three holes is briefly

flashed in a pseudorandom order. Animals are required to pay attention to these holes to correctly make a nose-poke response into the flashed hole to get a food pellet. Therefore, accuracy in this task is regarded as an index of attentional function. Responses that occur before the presentation of the stimulus light are described as premature responses and result in a 5-s time-out period. These responses are regarded as a form of impulsive-like action and a failure in impulse control (Robbins, 2002). Thus, premature responses reflect one of the simple forms of impulsive action in rodents, and hence, 3-CSRTT is suitable for examining the effects of drugs on impulsive action.

The apparatus, training procedure, and task sequence employed in 3-CSRTT 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 5 s of limited hold was recorded as a correct response and was rewarded by the immediate delivery of a palatable food pellet (45-mg each, dustless precision pellets, Bio-serv, Frenchtown, NJ, USA) to the food magazine. Nose poking into another hole was recorded as an incorrect response. When a rat failed to nose poke

within the time limit, it was recorded as an omission. After the delivery and collection of the food pellet by a 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 illumination of 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 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 or six sessions per week.

At the beginning of the training schedule, the stimulus duration was 30 s. Depending on individual performances, it 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 (as a percentage of correct responses) and <20 omissions in a session, the stimulus duration was reduced in the next session.

We used seven behavioral parameters, as described below.

(a) Premature responses (counts per session)

(b) Accuracy (percentage of correct responses): $[\text{correct responses}/(\text{correct and incorrect responses})] \times 100$

(c) Omissions (counts per session)

(d) Perseverative responses (counts per session)

(e) Responses during time-out (counts per session)

(f) Correct response latency(s): the mean time between stimulus onset and nose poke to the correct hole

(g) Reward latency (s): the mean time between reward delivery and nose poke to the food magazine

We can interpret the results of measures of primary interest (i.e. (a) and (b)) more precisely by analyzing the other measures too (Robbins, 2002).

Training was completed and moved on to test phase when the animal reached the target phase (stimulus duration: 1 s) and showed stable performance. During the test phase, the stimulus duration was fixed at 1 s regardless of performance.

2.3. Experiment 1: the effects of acute varenicline injection on impulsive action

To determine the effects of varenicline on impulsivity at several doses, we injected varenicline (0, 0.0075, 0.075, and 0.75 mg/kg, s.c.) to eight rats 60 min before the testing session. A previous study showed that a s.c. injection of varenicline (0.75 mg/kg)

did not significantly increase dopamine release in the rat nucleus accumbens (Ericson et al., 2009). Dopamine release in the nucleus accumbens has been associated with rewarding effects of nicotine (Biala et al., 2010) and enhanced impulsivity (Cole and Robbins, 1989). Thus we excluded the possibility that varenicline-induced dopamine release in the nucleus accumbens could affect our results. We did not use higher doses of varenicline (>0.75 mg/kg) because higher doses induced sedation in our preliminary study. Drug treatments were carried out with a Latin square design. Drugs were administered on Tuesdays and Fridays.

2.3.1. Drugs

Varenicline tartrate was purchased from Sigma-Aldrich (St. Louis, MO, USA). Varenicline tartrate was dissolved in saline. Acute injections were administered s.c. in a volume of 1 ml/kg. In this article, we expressed the doses as a salt.

2.4. Experiment 2: the mechanisms of action of varenicline-induced impulsive action

To elucidate the mechanisms of action underlying varenicline-induced impulsive action, we injected varenicline (0 and 0.075 mg/kg, s.c.) into eight rats 60 min before the testing session and microinjected dihydro- β -erythroidine (DH β E; 0 and 6 μ g/site), a preferential $\alpha 4\beta 2$ nAChR antagonist, 10 min before the testing session. We did not use 0.75 mg/kg of varenicline because the dose still induced sedation in some of the rats in

Experiment 1. Drug treatments were carried out with a Latin square design. Each drug session was conducted with more than a 2-day interval.

2.4.1. Surgery

After completing the training, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and fixed in a stereotaxic frame (Narishige, Tokyo, Japan). Double guide cannulas (C232G-1.5-SPC, Plastics One Inc., VA, USA) were bilaterally implanted with coordinates 3.2 mm anterior to the bregma, 0.75 mm lateral to the midline, and 2.0 mm ventral to the dura (Paxinos and Watson 1997). Dummy cannulas (C232DC-SPC, Plastics One Inc.) were inserted, which penetrated to the tip of the guide cannulas. After surgery, the rats were housed individually and allowed to recover for 4 days prior to retraining.

2.4.2. Drugs

Varenicline tartrate and DH β E were purchased from Sigma-Aldrich. Varenicline tartrate was dissolved in saline, while DH β E was dissolved in 0.01-M phosphate buffered saline (PBS; pH = 7.4).

2.4.3. Drug microinjection procedure

Rats were gently restrained and 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 were made to extend beyond the guide cannulas by 2.0 mm. DH β E (0 and 6 μ g in 0.5 μ l PBS per side) was infused at a rate of 0.5 μ l/min and in an injection volume of 0.5 μ l per side into the infralimbic cortex. The solution was infused over a period of 1 min at a constant flow rate using a microinjection pump (CMA100, Carnegie Medicine, Sweden). The injection cannula was left in place for 1 min after injection to allow for diffusion.

2.4.4. Verification of Cannula Placements

After the completion of the above behavioral experiments, rats were anesthetized with urethane (2 g/kg, i.p.) 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 μ m thickness on a cryostat and mounted onto slides. After drying, the sections were stained with toluidine blue and cannula placements were verified under a microscope according to the atlas (Paxinos and Watson, 1997). Only data from rats with correct injection needle placements were included in the statistical analysis.

2.5. Experiment 3: the effects of acute varenicline injection combined with acute nicotine injection on impulsive action

To examine whether a low dose of varenicline that does not evoke impulsive action could block the stimulating effects of nicotine on impulsive action, we injected varenicline (0, 0.0075, 0.075, and 0.75 mg/kg, s.c.) to 15 rats 60 min before the testing session and injected nicotine (0 and 0.2 mg/kg, s.c.) 10 min before the testing session. The dose of nicotine was the same as that used in our previous studies, which demonstrated that nicotine evoked impulsive action (Tsutsui-Kimura et al., 2009; 2010). Drug treatments were performed with a Latin square design. Drugs were administered on Tuesdays and Fridays.

2.5.1. Drugs

Varenicline tartrate and (–)-nicotine bitartrate salt was purchased from Sigma-Aldrich. Varenicline was dissolved in saline, whereas nicotine was dissolved in 0.01 M PBS.

2.6. Experiment 4: the effects of oral varenicline administration on impulsive action during continuous nicotine administration

To examine the interaction between varenicline and nicotine exposure status, we administered varenicline (0.075 mg/kg, p.o.) to 19 rats 60 min before the testing session after 8 days of continuous nicotine (n = 12) or sodium tartrate (control solution, n = 7)

administration with osmotic minipumps. The rats were gently held, and varenicline was administered via the esophagus with a gastric sonde needle. In Experiment 4, p.o. administration instead of s.c. injection was used to determine whether the route of administration could modulate the effects of varenicline on impulsive action because varenicline is taken p.o. in clinical settings. Rats performed the 3-CSRTT every single day during the 8 days of continuous nicotine or sodium tartrate.

2.6.1. Drugs

Varenicline tartrate was dissolved in distilled water at a volume of 3 ml/kg. (–)-Nicotine bitartrate salt was dissolved in saline. Equimolar sodium tartrate dissolved in saline was used as the control solution.

2.6.2. Osmotic minipump implantations

For chronic nicotine administration, osmotic minipumps (Model 2ML2, Durect Corporation, Cupertino, CA, USA) for drug infusion were surgically implanted s.c. between the scapulae under halothane anesthesia. The pumps were filled with either control solution or nicotine. The nicotine concentration was adjusted to deliver a dose of 9 mg/kg/day of nicotine salt (3.16 mg/kg/day nicotine base). Nicotine or the control solution was infused via implanted pumps at a rate of 5 µl/h for 8 days. The blood concentrations resulting from this dose in rats are comparable to those measured in

heavy smokers (Benowitz et al., 1982; LeSage et al., 2002).

2.7. Experiment 5: the effects of repeated oral varenicline administration on impulsive action after stopping continuous nicotine administration

To further examine the interaction between varenicline and nicotine exposure status, we continued to administer varenicline (0.075 mg/kg, p.o.) to 9 of 12 nicotine-infused rats in Experiment 4, 60 min before the testing on 3-CSRTT. This was done once per day for 7 days after stopping continuous nicotine administration with osmotic minipumps. Rats performed the 3-CSRTT every day during and after continuous nicotine or sodium tartrate.

2.7.1. Osmotic minipump removals

Osmotic minipumps for nicotine infusion (9 mg/kg/day of nicotine salt) were implanted as described in Experiment 4 and were surgically removed under halothane anesthesia 8 days after implantation. This dose and duration has been enough to induce nicotine withdrawal after pump removal (Malin et al., 1992; Ohmura et al., 2011a; Ohmura et al., 2011b; Ohmura et al., 2011c).

2.8. Experiment 6: the effects of oral varenicline administration on appetite

Omissions and reward latency in the 3-CSRTT are a measure of appetite/motivation, but they are indirect measures. Since the 3-CSRTT is a food-motivated task and varenicline

reduced omissions under conditions of increased attentional load in a previous study (Wouda et al., 2011), we conducted a simple food consumption test for 30 min (Ohmura et al., 2012a) to directly assess appetite and discriminate between drug effects on performance in the 3-CSRTT and motivation for food. A food-restricted rat was allowed to eat food pellets in the food magazine where 15 g of food pellets were placed. Eight rats received varenicline (0.075 mg/kg, p.o.) or distilled water (3 ml/kg) administration 60 min before the food consumption test. Half of the rats received varenicline administration first and water administration a week later, and the other half of the rats received water administration first and varenicline administration a week later. The rats received 3-CSRTT training on weekdays and food consumption test on Saturday.

2.9. Data analysis

Seven behavioral measures were analyzed (see 3-CSRTT). Each measure was analyzed separately by analysis of variance (ANOVA). If Mauchly's sphericity test was significant, Greenhouse–Geisser correction was used. Multiple comparisons with Bonferroni's correction were also conducted in cases where ANOVA found a significant main effect except for the case where only two levels were used. In Experiment 4, the average of 7 consecutive days before varenicline administration was used for the comparison (cf. Fig. 4a). In Experiment 5, the average of 3 consecutive days before

pump implantation (baseline), the average of 7 consecutive days before varenicline administration (nicotine), and the average of 7 consecutive days during repeated varenicline administration after pump removal (withdrawal) were used for comparison (cf. Fig. 5a). To test the statistical significance of differences between two conditions in Experiment 6, paired t tests were used. The alpha level was set at 0.05 for all statistical procedures. All statistical analyses were conducted using SPSS (version 23.0).

3. Results

Raw data are available in DiB article (Ohmura et al., 2016).

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

Figs. 1a–g show the effects of varenicline on premature responses, accuracy (percent correct responses), omissions, perseverative responses, responses during time-out, correct response latency, and reward latency. Repeated one-factor ANOVA revealed a significant dose effect on premature responses (with Greenhouse–Geisser correction, $F_{1.279, 8.952} = 14.04$, $p = 0.003$) and correct response latency ($F_{3, 21} = 4.44$, $p = 0.014$) but not on accuracy [$F_{3, 21} = 0.86$, not significant (NS)], omissions ($F_{3, 21} = 2.60$, NS), perseverative responses ($F_{3, 21} = 1.56$, NS), responses during time-out ($F_{3, 21} = 0.49$, NS), or reward latency ($F_{3, 21} = 0.77$, NS).

Multiple comparisons using Bonferroni's correction revealed that 0.075 or 0.75

mg/kg dose of varenicline significantly increased the number of premature responses compared with the vehicle ($p = 0.014$ and $p = 0.015$, respectively). Multiple comparisons using Bonferroni's correction for correct response latency did not indicate any significant pairwise differences between the dose conditions.

3.2. Experiment 2: the mechanisms of action of varenicline-induced impulsive action

Fig. 2a shows the locations of the cannula tips in the infralimbic cortex region of rats that were included in the present study. Out of nine implanted rats, one rat had to be excluded because the cannulas were located outside the target region, resulting in $n = 8$. A representative photomicrograph is shown in Supplementary Fig. S1. Fig. 2b shows the effect of microinjection of DH β E into the infralimbic cortex on systemic varenicline-induced impulsive action. Two-factor ANOVA with the dose of varenicline and the dose of DH β E as within subject factors revealed a significant interaction ($F_{1,7} = 6.66$, $p = 0.036$), indicating that microinjection of DH β E into the infralimbic cortex blocked varenicline-induced impulsive action. Figs. 2c–h show the effects of varenicline and DH β E on accuracy (percent correct responses), omissions, perseverative responses, correct response latency, and reward latency. There was no significant main effect or an interaction effect on those parameters ($F_{s\ 1,7} < 4.64$, NS).

Additionally, we conducted a correlation analysis between the number of premature

responses at vehicle and the increase rate by varenicline (0.075 mg/kg) administration by using the data of Experiments 1 and 2 because some previous studies have demonstrated that some drugs exert the effects in high-impulsive rats only (Ansquer et al., 2014; Moreno et al., 2013). However, there was no significant relationship between them (Supplementary Fig. S2; $r = -0.21$, product-moment correlation coefficients, NS). As far as the effects of varenicline on impulsive action are concerned, the effects would not depend on baseline levels of impulsive action.

3.3. Experiment 3: the effects of acute varenicline injection combined with acute nicotine injection on impulsive action

Figs. 3a–g show the effects of varenicline combined with nicotine on performance in 3-CSRTT. Repeated one-factor ANOVA revealed a significant main effect on premature responses ($F_{4, 56} = 8.21, p < 0.001$), correct response latency (with Greenhouse–Geisser correction, $F_{1.543, 21.604} = 4.22, p = 0.037$), and reward latency ($F_{4, 56} = 3.40, p = 0.015$), but not on accuracy ($F_{4, 56} = 0.43$, NS), omissions (with Greenhouse–Geisser correction, $F_{2.352, 32.926} = 2.82$, NS), perseverative responses ($F_{4, 56} = 0.64$, NS), or responses during time-out (with Greenhouse–Geisser correction, $F_{2.463, 34.479} = 0.82$, NS). Multiple comparisons with Bonferroni’s correction for premature responses revealed that nicotine alone and nicotine with varenicline 0.0075, 0.075, or 0.75 mg/kg

significantly increased the number of premature responses compared with the vehicle ($p = 0.017, 0.007, 0.003$, and 0.005 , respectively). Multiple comparisons for correct response latency revealed that nicotine with varenicline 0.0075 mg/kg significantly reduced the correct response latency compared with the vehicle ($p = 0.030$). Multiple comparisons for reward latency did not indicate any significant pairwise differences between the conditions.

3.4. Experiment 4: the effects of oral varenicline administration on impulsive action during continuous nicotine administration

Fig. 4b shows the effects of varenicline on premature responses under continuous nicotine infusion. Two-factor ANOVA with the effect of varenicline as the within-subject factor and the type of implanted pump as the between-subject factor revealed a significant interaction ($F_{1,17} = 8.21, p = 0.011$), indicating that continuous nicotine infusion blocked varenicline-induced impulsive action. Figs. 4c, d, and g show the effects of varenicline under continuous nicotine infusion on accuracy (percent correct responses), omissions, and correct response latency. Two-factor ANOVA for these parameters revealed a significant main effect of varenicline (accuracy, $F_{1,17} = 12.26, p = 0.003$; omissions, $F_{1,17} = 17.50, p = 0.001$; correct response latency, $F_{1,17} = 8.22, p = 0.011$) without interaction, indicating that varenicline increased accuracy and

decreased omissions/correct response latency, independent of the type of implanted pump. For the other parameters (Figs. 4e, f, and h), there was no significant main effect or an interaction effect on them ($F_{s\ 1, 17} < 3.40$, NS).

3.5. Experiment 5: the effects of repeated oral varenicline administration on impulsive action after stopping continuous nicotine administration

Fig. 5b shows the effects of repeated varenicline on premature responses after stopping continuous nicotine infusion. One-factor repeated measures ANOVA did not find a significant effect of experimental phase (baseline, nicotine, or withdrawal) on premature responses (with Greenhouse–Geisser correction, $F_{1.179, 9.436} = 0.47$, NS), indicating that repeated varenicline administration during the withdrawal phase did not alter impulsive action. As shown in Figs. 5c and f, there were significant main effects of the experimental phase on accuracy ($F_{2, 16} < 4.22$, $p = 0.034$) and responses during time-out ($F_{2, 16} < 3.96$, $p = 0.04$). However, multiple comparisons for these parameters did not indicate any significant pairwise differences between the experimental phases. For the other parameters (Figs. 5d, e, g, and h), there were no significant main effects or interaction effects on them ($F_{s\ 1, 17} < 3.54$, NS).

3.6. Experiment 6: the effects of oral varenicline administration on appetite

Fig. 6 shows the effects of acute oral varenicline administration on the amount of

food consumption. Varenicline did not alter the amount of food consumption ($t_7 = 0.30$, NS), indicating that varenicline administration did not affect appetite.

4. Discussion

4.1. Experiment 1: the effects of acute varenicline injection on impulsive action

We demonstrated that s.c. varenicline administration significantly and selectively provoked impulsive action in a dose-dependent manner (Fig. 1). Although varenicline treatment also had a main effect on correct response latency, multiple comparisons did not reach statistical significance. Other parameters in 3-CSRTT were not affected by varenicline administration. These results are mostly consistent with those obtained by (Wouda et al., 2011); we successfully replicated their results. The effective dose range in our results was different from that in their results; however, this dose depends on several factors, including the degree of food restriction and the route of administration. We used subcutaneous injection in the present study while intraperitoneal injection was used in (Wouda et al., 2011).

4.2. Experiment 2: the mechanisms of action of varenicline-induced impulsive action

Although varenicline has agonistic properties for $\alpha 7$, $\alpha 3\beta 4$, $\alpha 3\beta 2$, and $\alpha 6$, in addition to $\alpha 4\beta 2$ (Mihalak et al., 2006), bilateral microinjection of DH β E, a preferential $\alpha 4\beta 2$ nAChR antagonist, into the infralimbic cortex completely blocked varenicline-induced

impulsive action (Fig. 2). These results indicate that varenicline evokes impulsive action through the stimulation of $\alpha 4\beta 2$ nAChRs in the infralimbic cortex. These results are congruent with previous results regarding nicotine-induced impulsive action (Tsutsui-Kimura et al., 2010); varenicline could enhance impulsivity through the same mechanism as nicotine-induced impulsive action. Although we cannot completely exclude the possibility that varenicline could evoke impulsive action through a different mechanism, at least it is unlikely that varenicline-induced dopamine release in the nucleus accumbens was responsible for the present results though dopamine release in the nucleus accumbens has been associated with enhanced impulsivity (Cole and Robbins, 1989). It is because a previous study showed that 0.75 mg/kg of varenicline did not significantly increase dopamine release in the rat nucleus accumbens (Ericson et al., 2009) and we used the same or smaller doses in the present study.

4.3. Experiment 3: the effects of acute varenicline injection combined with acute nicotine injection on impulsive action

Varenicline failed to block acute nicotine-induced impulsive action (Fig. 3) though varenicline is an $\alpha 4\beta 2$ nAChR partial agonist. It should be noted that the low dose (0.0075 mg/kg) of varenicline that did not increase premature responses failed to block the effects of nicotine. Varenicline can attenuate nicotine-induced dopamine release in

the nucleus accumbens (Ericson et al., 2009) and block nicotine-induced conditioned place preference (Biala et al., 2010), indicating that varenicline could reduce the rewarding effects of nicotine. However, since varenicline could not block nicotine-induced impulsiveness, it is likely that the neural mechanisms underlying these effects are independent of those underlying nicotine/varenicline-induced impulsive action.

4.4. Experiment 4 and 5: the effects of oral varenicline administration on impulsive action during continuous nicotine administration/after stopping continuous nicotine administration

The stimulating effects of varenicline on impulsive action would not depend on the route of administration because either oral or subcutaneous administration of varenicline evoked impulsive action (Figs. 1 and 4). Given that varenicline is taken p.o. in clinical settings, varenicline prescription may need added cautions in terms of impulsivity.

Although previous studies have demonstrated that repeated injection or continuous infusion of nicotine increased impulsive choice (Dallery and Locey, 2005; Kayir et al., 2014), continuous nicotine infusion in the present study did not stimulate impulsive action (Fig. 4b). A possible explanation for this discrepancy is that the impulsive “choice” and impulsive “action” are controlled by similar but different neural

mechanisms as suggested before (Dalley et al., 2011; Ohmura et al., 2012b). Indeed, a previous study showed that continuous nicotine infusion evoked impulsive action, but only transiently (Semenova et al., 2007). Because we used the average of premature response during continuous nicotine infusion for the comparison (Fig. 4a), it is no wonder that the significant effects of continuous nicotine infusion on impulsive action were not detected. Restricting analysis to premature responses on a day after the start of nicotine infusion, a trend of evoked impulsive action was observed, consistent with a previous study (Semenova et al., 2007), though it was not statistically significant (Supplementary Fig. S3).

Interestingly, the stimulating effects of varenicline on impulsive action were not observed in chronic nicotine-infused or nicotine-abstinent rats after discontinuing infusion (Figs. 4 and 5). It is speculated that chronic nicotine administration desensitized $\alpha 4\beta 2$ nAChRs (Wang and Sun, 2005) and thereby mitigated the stimulating effects of varenicline on impulsive action. Because the effects of varenicline disappeared even after stopping nicotine administration (Fig. 5), it is likely that the desensitization of $\alpha 4\beta 2$ nAChRs in the infralimbic cortex did not recover at least 1 week after abstinence. However, a previous study demonstrated that chronic nicotine infusion-induced desensitization of nAChRs in the hypothalamic paraventricular and

supraoptic nuclei did not continue after 72-h withdrawal (Salminen et al., 2000). When we restricted the analysis to the latter half of the nicotine abstinence period, there was no significant effect of varenicline on impulsive action (cf. Supplementary Fig. S3). The mechanisms by which the stimulating effects of varenicline on impulsive action disappeared even after stopping chronic nicotine administration need to be clarified in future studies. One possibility is that repeated varenicline administration maintained the desensitization of $\alpha 4\beta 2$ nAChRs in the infralimbic cortex. Our preliminary results showed that acute varenicline administration 8 days after stopping nicotine infusion stimulated impulsive action, supporting this possibility (Supplementary Fig. S5).

Additionally, varenicline administration may be problematic to non-smokers and perhaps light smokers, although researchers have pursued the possibility that varenicline can be useful for other problems such as alcohol and opioid addiction (Biala et al., 2010; Litten et al., 2013). However, varenicline administration would be harmless in terms of impulsivity to heavy smokers, ex-smokers (at least for a while), or subjects with desensitization of $\alpha 4\beta 2$ nAChRs in the prefrontal cortex for any reason.

4.5. Comparisons to human studies

In human studies, acute varenicline administration weakly disrupted inhibitory control in smokers (Austin et al., 2014), and repeated varenicline administration did not

affect impulsive behavior in smokers (Mocking et al., 2013; Rhodes et al., 2012). These results are almost consistent with our present results indicating that varenicline would not enhance impulsivity in smokers. However, a previous study showed that repeated varenicline administration did not affect impulsive behavior even in non-smokers (Mocking et al., 2013), while animal studies have demonstrated that acute varenicline consistently evokes impulsive action (Wouda et al., 2011). These inconsistent results might be due to the differences between acute and repeated administration. That is, repeated varenicline might desensitize $\alpha 4\beta 2$ nAChRs and thereby dampen the effects of varenicline on impulsivity. However it is not sure whether repeated varenicline could desensitize $\alpha 4\beta 2$ nAChRs though our results imply it (see discussion regarding Experiment 4 and 5). Thus our present results could partly reconcile contradictory findings in animal and human studies.

Alternatively, the reason why these previous studies were incongruent to each other or to animal studies is that varenicline provokes only a certain type of impulsive behavior. Because impulsive behavior is a multifaceted concept (Dalley et al., 2011; Ohmura et al., 2012b), the results of human studies using a stop-signal task (Austin et al., 2014; Rhodes et al., 2012) could reflect a different aspect of impulsive behavior from that assessed by 3-CSRTT/5-CSRTT in rats (Dalley et al., 2011; Ohmura et al.,

2012b). Human studies using a recently-developed analogue of the rodent serial reaction time task (Voon et al., 2014) are required to address this issue.

4.6. Additional Findings (attentional function)

Additionally, we found that acute or repeated oral varenicline administration increased accuracy, a measure of attentional function, in 3-CSRTT (Figs. 4c and 5c), while the enhancing effect was not observed when subcutaneous administration was employed (Figs. 1–3). The reasons for this need to be clarified in future studies because the route of administration often changes the effects of drugs (Volkow et al., 2004).

More intriguingly, in contrast to impulsive action, the enhancing effect appeared to be independent of nicotine exposure status (Figs. 4c and 5c). Although speculative, the enhancing effect might be due to receptors other than $\alpha 4\beta 2$ nAChRs if varenicline desensitizes $\alpha 4\beta 2$ nAChRs as nicotine does.

It should be also noted that repeated varenicline administration does not seem to dampen the enhancing effect of varenicline on attentional function over time (Supplementary Fig. S3) and, rather, it might gradually enhance the effects of varenicline over time on the measures partly related to attentional function such as omission and correct response latency (Supplementary Figs. S3 and S4). Moreover, repeated varenicline exposure tended to reduce the number of responses during time-out

(Fig. 5f). It is possible that repeated varenicline administration enhanced sustained attention, resulting in reduced responses irrelevant to the task. However, as for Experiment 5, we cannot exclude the possibility that the effects of time on performance were confounded. Further studies are required to clarify this issue.

4.7. Conclusions

In summary, our results demonstrate that varenicline enhances impulsivity through the same mechanism ($\alpha 4\beta 2$ nAChRs in the infralimbic cortex) as nicotine-induced impulsive action. The stimulating effects of varenicline on impulsivity would depend on smoking status, while the enhancing effects of varenicline on attentional function could be independent of smoking status. Although further studies are needed, these results could provide guidance to the safe use of varenicline and the development of new stop-smoking aids.

Conflicts of interest

None.

Acknowledgments

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Titles and Legends to Figures

Fig. 1. Effects of acute varenicline injection (a-g) on three-choice serial reaction time task (3-CSRTT) performance. We injected varenicline (0, 0.0075, 0.075, and 0.75 mg/kg, s.c.) to 8 rats 60 min before the testing session. The bars represent the mean, and the lines represent the SEM. * $p < 0.05$ (with Bonferroni's correction, compared with the vehicle), ⁺ $p < 0.05$ (with one-way ANOVA, a main effect).

Fig. 2. Effect of microinjection of DH β E into the infralimbic cortex on systemic varenicline-induced changes of 3-CSRTT performance (a–g). (a) Schematic diagrams showing cannula tips in the infralimbic cortex (closed circles), +3.7, +3.2, or +2.7 mm anterior to the bregma. PL: prelimbic cortex, MO: medial orbital cortex, IL: infralimbic cortex, DP: dorsal peduncular cortex (Paxinos and Watson, 1997). Open circles indicate incorrect cannula tips of animals that were excluded from statistical analysis. Eight rats were included in statistical analysis. Figures adapted from (Paxinos and Watson 1997). (b–g) Rats received either systemic saline or varenicline (0.075 mg/kg, s.c.) and microinjection of vehicle (0.01 M PBS) or DH β E (6 μ g per side) into the infralimbic cortex. The bars represent the mean, and the lines represent the SEM. ⁺ $p < 0.05$ (a significant interaction between dose of varenicline and the dose of DH β E).

Fig. 3. Effects of acute varenicline injection combined with acute nicotine injection

(a–g) on 3-CSRTT performance. We injected varenicline (0, 0.0075, 0.075, and 0.75 mg/kg, s.c.) to 15 rats 60 min before the testing session and nicotine (0 and 0.2 mg/kg, s.c.) 10 min before the testing session. The bars represent the mean, and the lines represent the SEM. * $p < 0.05$ (with Bonferroni's correction, compared with the vehicle), ⁺ $p < 0.05$ (with one-way ANOVA, a main effect).

Fig. 4. Effects of oral varenicline administration on 3-CSRTT performance during continuous nicotine administration (a–h). (a) We orally administered varenicline (0.075 mg/kg) to 19 rats 60 min before the testing session after 8-day continuous sodium tartrate (n = 7) or nicotine (n = 12) administration with osmotic minipumps (9 mg/kg/day). The average of 7 consecutive days before varenicline administration was used for the comparison as indicated by dotted lines. (b–h) The bars represent the mean, and the lines represent the SEM. ⁺ $p < 0.05$ (a significant interaction between the effect of varenicline and the type of implanted pump), * $p < 0.05$ (a significant main effect of varenicline).

Fig. 5. Effects of repeated oral varenicline administration on 3-CSRTT performance after stopping continuous nicotine administration (a–h). (a) We administered varenicline (0.075 mg/kg, p.o.) to 9 rats used in Experiment 4 (Fig 4) 60 min before the testing on the 3-CSRTT, once per day for 7 days after stopping

continuous nicotine administration (9 mg/kg/day) with osmotic minipumps. The average performances during the period surrounded by dotted lines were used for the comparison. **(b–h)** The bars represent the mean, and the lines represent the SEM. * $p < 0.05$ (a significant main effect of experimental phase).

Fig. 6. Effects of oral varenicline administration on the amount of food consumption. We administered water or varenicline (0.075 mg/kg, p.o.) to 8 rats 60 min before the testing. No significant effects of 0.075 mg/kg varenicline were observed. The bars represent the mean, and the lines represent the SEM.

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Supporting Information

Supplementary Figure S1. Cannula placements in the infralimbic cortex

(Experiments 2). A representative photomicrograph shows the coronal section of a typical site of injection cannulae.

Supplementary Figure S2. Scatter plot of the number of premature responses at vehicle and the increase rate of premature responses by varenicline (0.075 mg/kg)

administration (Experiments 1 and 2). Each increase rate was calculated by
$$\left(\frac{\text{premature responses at 0.075mg/kg of varenicline}}{\text{premature responses at vehicle}} - 1 \right) \times 100.$$

Supplementary Figure S3. Time course of main parameters in the 3-CSRTT

during Experiment 5. Nine rats used in Experiment 4 (Fig 4) were used again and received repeated oral administration of varenicline (0.075 mg/kg, p.o.) 60 min before the testing on the 3-CSRTT, once per day for 7 days after stopping continuous nicotine administration (9 mg/kg/day) with osmotic minipumps. The bars represent the mean, and the lines represent the SEM.

Supplementary Figure S4. Time course of other parameters in the 3-CSRTT

during Experiment 5. Nine rats used in Experiment 4 (Fig 4) were used again and received repeated oral administration of varenicline (0.075 mg/kg, p.o.) 60 min before

the testing on the 3-CSRTT, once per day for 7 days after stopping continuous nicotine administration (9 mg/kg/day) with osmotic minipumps. The bars represent the mean, and the lines represent the SEM.

Supplementary Figure S5. The effects of varenicline on performance in the 3-CSRTT after discontinuing nicotine infusion. The average performances during the period surrounded by dotted lines were used for the comparison. The bars represent the mean, and the lines represent the SEM. * $p < 0.05$.

Figure 1

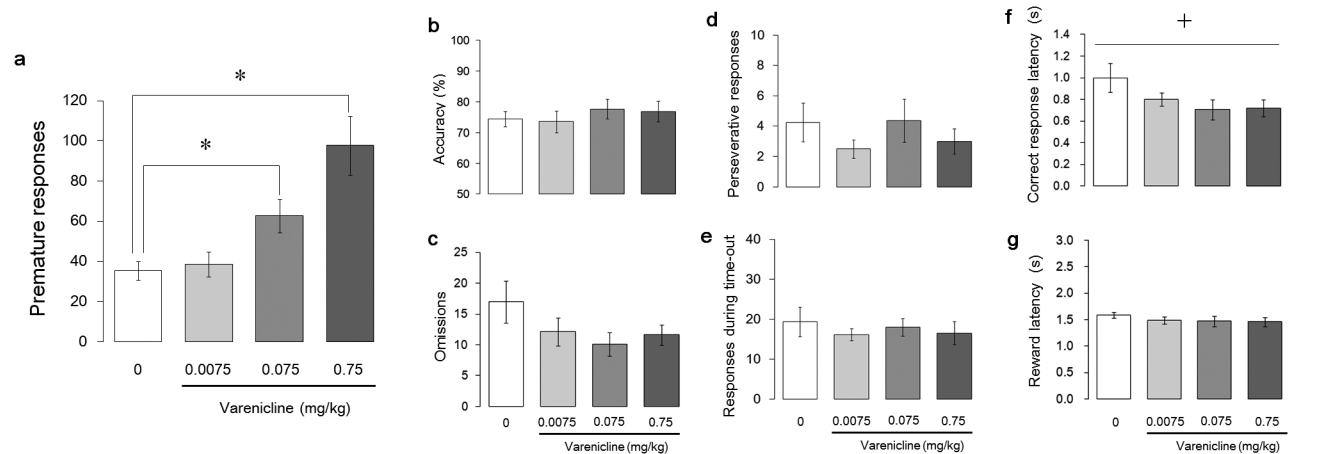
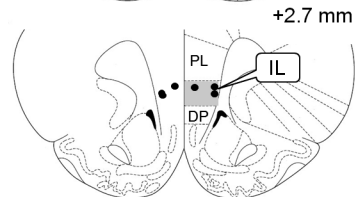
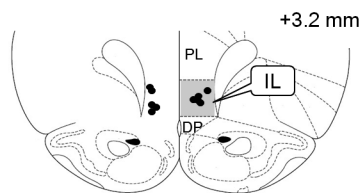
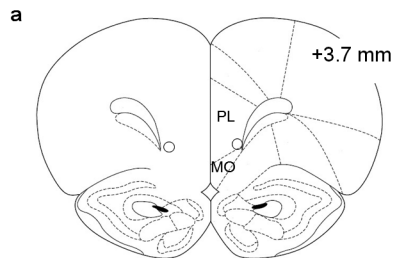
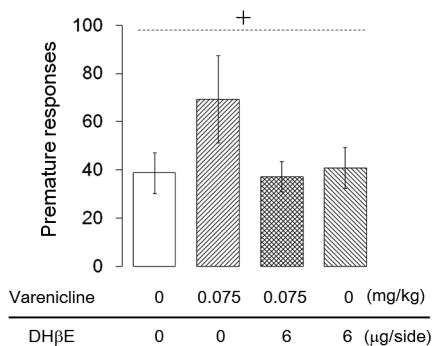


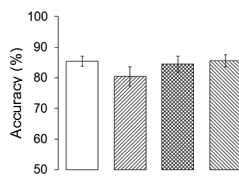
Figure 2



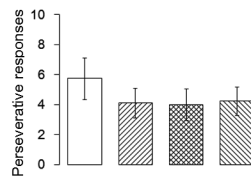
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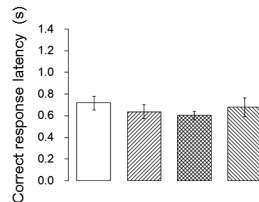
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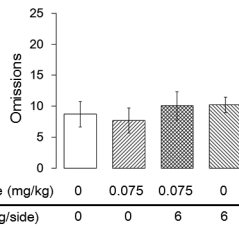
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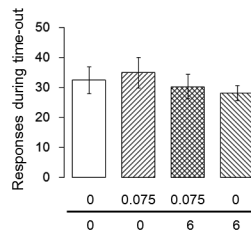
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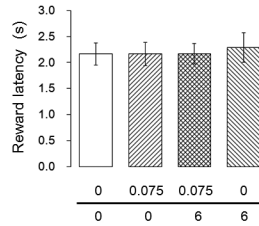


Figure 3

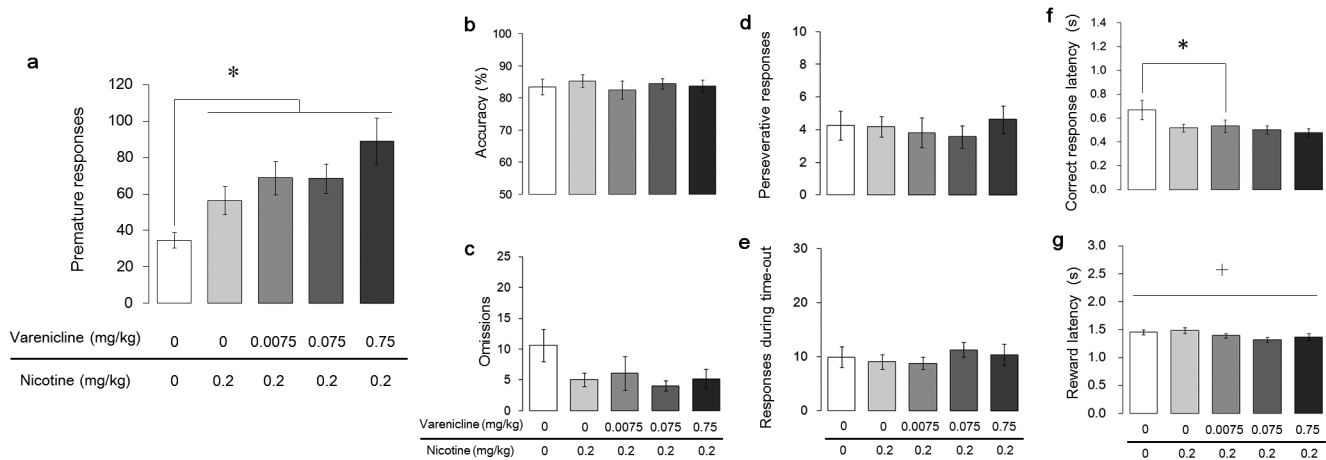


Figure 4

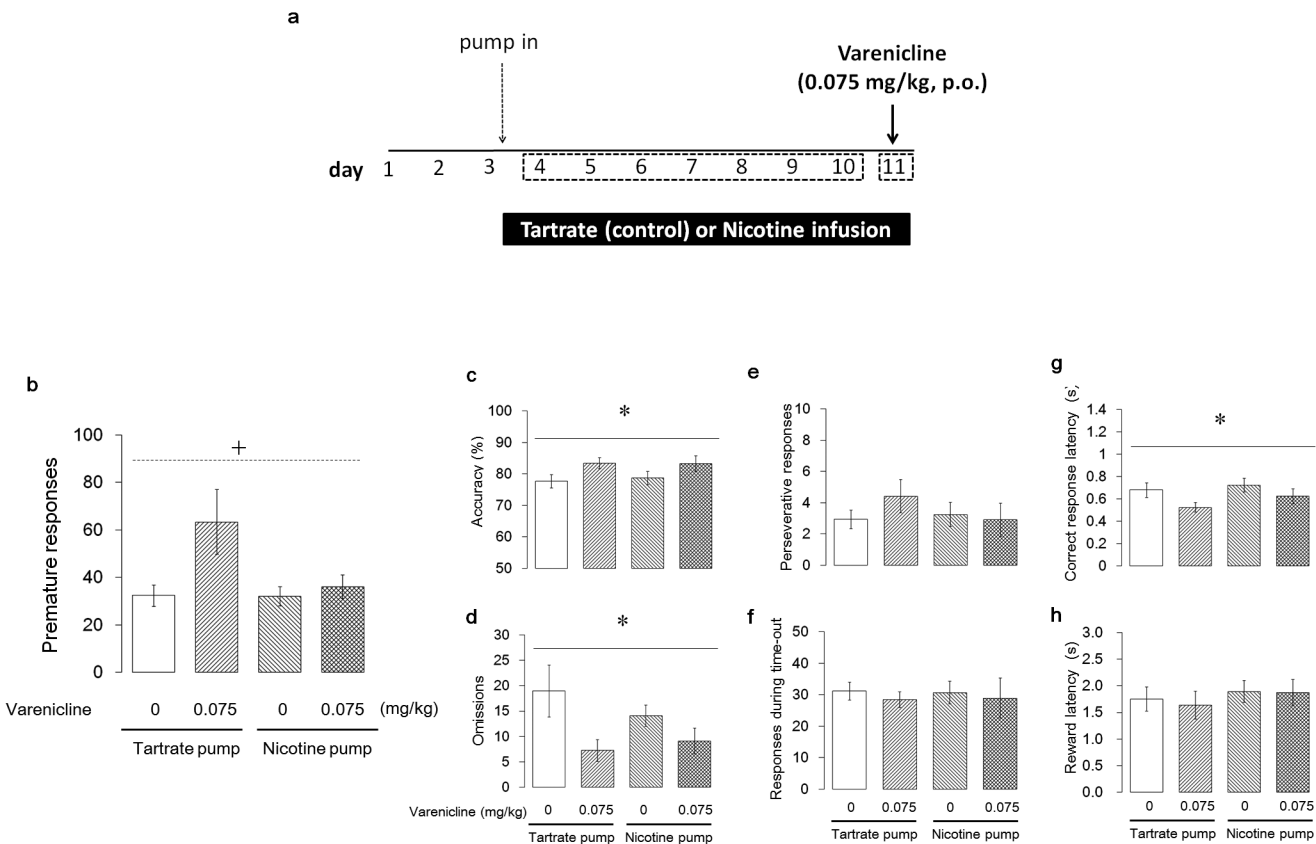
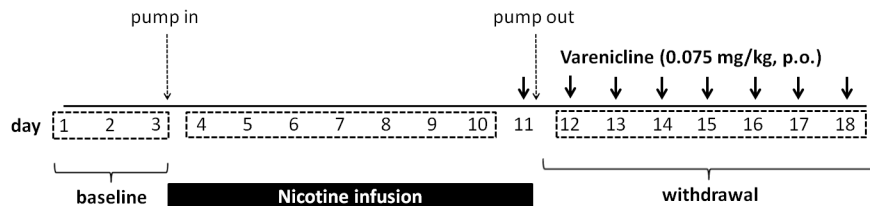
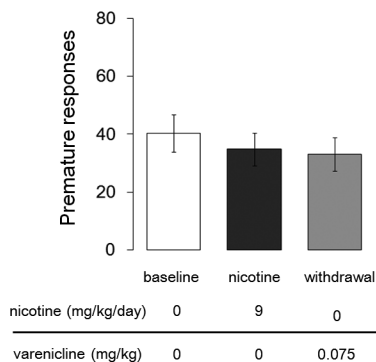


Figure 5

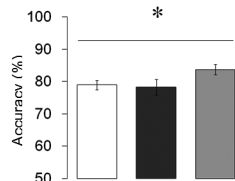
a



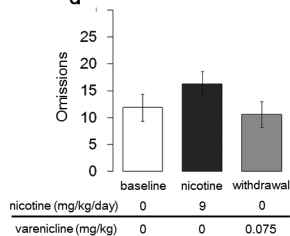
b



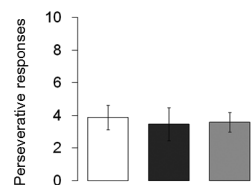
c



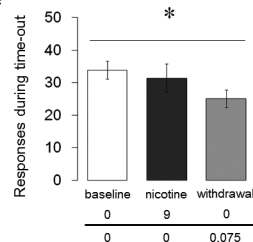
d



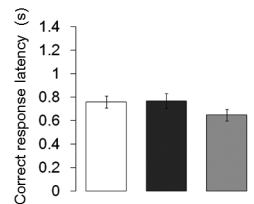
e



f



g



h

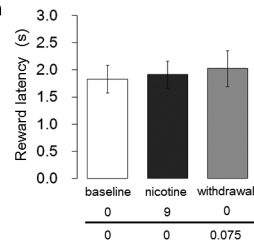


Figure 6

