

Self-Administration of Cotinine in Wistar Rats: Comparisons to Nicotine

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ABSTRACT

Nicotine is the major addictive component in tobacco. Cotinine is the major metabolite of nicotine and a weak agonist for nicotinic acetylcholine receptors (nAChRs). Nicotine supports self-administration in rodents. However, it remains undetermined whether cotinine can be self-administered. This study aimed to characterize cotinine self-administration in rats, to compare effects of cotinine to those of nicotine, and to determine potential involvement of nAChRs in cotinine's effects. Adult Wistar rats were trained to self-administer cotinine or nicotine (0.0075, 0.015, 0.03, or 0.06 mg/kg per infusion) under fixed-ratio (FR) and progressive-ratio (PR) schedules. Blood nicotine and cotinine levels were determined after the last FR session. Effects of mecamylamine, a nonselective nAChR antagonist, and varenicline, a partial agonist for $\alpha 4\beta 2^*$ nAChRs, on cotinine and nicotine self-administration were determined. Rats readily acquired cotinine self-administration, responded more on active lever, and increased motivation to self-administer cotinine when the reinforcement requirement increased. Blood cotinine levels ranged from 77 to 792 ng/ml. Nicotine induced more infusions at lower doses during FR schedules and greater breakpoints at higher doses during the PR schedule than

cotinine. There was no difference in cotinine self-administration between male and female rats. Mecamylamine and varenicline attenuated nicotine but not cotinine self-administration. These results indicate that cotinine was self-administered by rats. These effects of cotinine were less robust than nicotine and exhibited no sex difference. nAChRs appeared to be differentially involved in self-administration of nicotine and cotinine. These results suggest cotinine may play a role in the development of nicotine use and misuse.

SIGNIFICANCE STATEMENT

Nicotine addiction is a serious public health problem. Cotinine is the major metabolite of nicotine, but its involvement in nicotine reinforcement remains elusive. Our findings indicate that cotinine, at doses producing clinically relevant blood cotinine levels, supported intravenous self-administration in rats. Cotinine self-administration was less robust than nicotine. Mecamylamine and varenicline attenuated nicotine but not cotinine self-administration. These results suggest cotinine may play a role in the development of nicotine use and misuse.

Introduction

Cigarette smoking persists as a leading public health issue despite a significant decline in prevalence in recent decades. In 2018, approximately 17% of Americans age 12 and older were current smokers (Substance Abuse and Mental Health Services Administration (SAMHSA), 2019). Electronic cigarette use is gaining popularity, especially among youth, with nearly 5 million middle and high school students in the United States as current users in 2019 (Cullen et al., 2019). Smoking causes harm to nearly every organ of the body and claims ~480,000 deaths per year, making it the leading cause of preventable death in the United States [U.S. Department of Health and Human Services (HHS), 2014].

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Nicotine is the major addictive component in cigarettes. It activates nicotinic acetylcholine receptors (nAChRs) to produce its effects (De Biasi and Dani, 2011). Pharmacotherapies, including nicotine replacement therapy, varenicline (a partial agonist for $\alpha 4\beta 2^*$ nAChRs), and bupropion (a monoamine reuptake inhibitor and a nAChR antagonist) target effects of nicotine to aid in smoking cessation (Prochaska and Benowitz, 2016). Despite their clinical benefits, these medications only provide limited effectiveness that diminishes over time. Sustained abstinence rates for these three medications were approximately 40% at 3 months, 25% at 6 months, and below 20% at 12 months (Aubin et al., 2014; Rosen et al., 2018). Therefore, there is critical need for further understanding of mechanisms underlying smoking to develop more effective therapies.

There have been considerable efforts in studying non-nicotine constituents that may contribute to the addictiveness of smoking. Several cigarette components were found to support self-administration by themselves and/or enhance nicotine self-administration. These include nornicotine

ABBREVIATIONS: FR, fixed ratio; COT, cotinine; NIC, nicotine; nAChR, nicotinic acetylcholine receptor; PR, progressive ratio.

(a minor tobacco alkaloid and a metabolite of nicotine), acetaldehyde (a major byproduct of smoking), a cocktail of five minor tobacco alkaloids, menthol (a flavoring additive to cigarettes), and nor-harmine (a tobacco constituent and monoamine oxidase inhibitor) (Bardo et al., 1999; Belluzzi et al., 2005; Clemens et al., 2009; Arnold et al., 2014; Biswas et al., 2016). These components may contribute to the reinforcing effects of smoking and facilitate continuous use.

Cotinine is the major metabolite of nicotine. In total, 70%–80% of nicotine is converted to cotinine through the liver enzyme CYP2A6 (Benowitz and Jacob, 1994). Cotinine has a longer half-life (15–19 vs. 1 to 2 hours) and accumulates to higher blood levels (250–900 vs. 10–50 ng/ml) than nicotine. Cotinine is commonly used as a biomarker for tobacco use and a probe for CYP2A6 activity (Hukkanen et al., 2005). Cotinine readily crosses the blood-brain barrier (Riah et al., 1998) and acts as a weak agonist for nAChRs, with potency being several orders of magnitude less than nicotine (Abood et al., 1983; Anderson and Arneric 1994). Accumulating evidence indicates that cotinine produces its own effects. In animals, cotinine changed food-maintained behaviors (Risner et al., 1985; Goldberg et al., 1989), altered monoamine neurotransmitter levels (Fuxe et al., 1979; Dvoskin et al., 1999), and substituted for discriminative stimulus effects of nicotine (Rosecrans and Chance, 1977; Takada et al., 1989). Recent studies indicate that cotinine enhanced attention, learning, and memory in animal models of cognitive impairment, improved prepulse inhibition of the acoustic startle reflex, and was beneficial in animal models of Alzheimer disease and schizophrenia (Terry et al., 2005, 2012; Buccafusco and Terry, 2009; Echeverria et al., 2011; Patel et al., 2014; Grizzell et al., 2017). Although cotinine did not appear to alter somatic signs of nicotine withdrawal in mice (Elhassan et al., 2017), cotinine altered irritability and desire to smoke in acute abstinent smokers (Benowitz et al., 1983). Cotinine by itself did not induce withdrawal symptoms (Hatsukami et al., 1997), but instead, it altered subjective states related to smoking withdrawal (Keenan et al., 1994) and interacted with nicotine patches to modulate smoking withdrawal symptoms (Hatsukami et al., 1998).

It has remained undetermined whether cotinine could support self-administration. A preliminary study from our laboratory demonstrated that cotinine could be self-administered intravenously by rats under a low fixed-ratio (FR) reinforcement schedule. Rats responded more on the cotinine-associated lever than the nonassociated lever. Therefore, the current study aimed to further characterize the dose-effect relation of cotinine self-administration in rats under various reinforcement schedules. Since cotinine is a weak agonist for nAChRs, the potential involvement of nAChRs in cotinine self-administration was investigated. We also compared the effects of cotinine to nicotine, its parent compound. The hypotheses to be tested were whether 1) cotinine would be self-administered by rats, 2) cotinine self-administration would be less robust than nicotine, and 3) nAChRs would be involved in cotinine self-administration.

Materials and Methods

Animals. Young adult male and female (starting at ~7 to 8 weeks old) Wistar rats (Envigo, Indianapolis, IN) were housed in pairs upon arrival on a reversed 12-hour light/dark cycle in rooms controlled for

temperature and humidity. Rats were acclimated for approximately 1 week before catheterization surgery and were individually housed after surgery. Food and water were available ad libitum, except during self-administration testing. Experiments were performed during the dark phase. Protocols used were approved by the respective Institutional Animal Care and Use Committee at Indiana University School of Medicine and Pennsylvania State University College of Medicine. All experiments were performed in accordance with the principles outlined in the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011).

Intravenous Catheterization. After the acclimation period, rats were surgically implanted with a catheter into the jugular vein following a procedure detailed previously (Berg et al., 2014). Briefly, rats were anesthetized with 2% to 3% isoflurane inhalation. Silastic tubing (i.d. \times o.d. = 0.51 \times 0.94 mm; Dow Silicones Corporation, Midland, MI) was inserted into the right jugular vein using aseptic techniques, and the remaining portion of the catheter coursed subcutaneously over the shoulder to exit the back of the rat via a 22-gauge cannula (Plastics One, Roanoke, VA). Bupivacaine (0.5%) and carprofen (5 mg/kg) were applied as analgesia during surgery. Catheters were flushed daily with ~0.5 ml heparinized saline (20 IU/ml; McKesson, Livonia, MI) containing 0.13 mg/ml gentamicin sulfate (McKesson). Rats were checked once a week after the Friday session for catheter patency with intravenous administration of ~0.1 ml of 10 mg/ml methohexital sodium (Par Pharmaceutical, Chestnut Ridge, NY). Rats with failed catheters were excluded from further experiment and analysis.

Intravenous Self-Administration of Cotinine and Nicotine in Male Rats. Self-administration was conducted in standard chambers equipped with two levers, a cue light, and a house light (Med Associates Inc., St. Albans, VT) following procedures previously described (Donny et al., 1999; Berg et al., 2014). A light food restriction procedure was introduced to maintain rats at ~85% body weight (with two to three full bricks per day of standard rat chow) to promote exploratory behavior. Responses on the active lever led to an intravenous infusion of either saline, (–)-nicotine hydrogen tartrate salt (Sigma, St. Louis, MO), or (–)-cotinine (Sigma) at 0.0075, 0.015, 0.03, or 0.06 mg base/kg per infusion. Doses of cotinine were determined based on our preliminary study showing that cotinine at 0.03 mg/kg per infusion was self-administered by alcohol-preferring (P) rats. Infusions were delivered over 3 seconds, during which the house light was turned off and the cue light was turned on. The infusion was followed by a 17-second time-out period with both the cue light and the house light turned off. Lever presses during the infusion and time-out periods were recorded but produced no further infusions. Responses on the inactive lever were recorded with no programmed consequences. Daily sessions were conducted on weekdays. At the beginning of each session, a passive infusion was delivered by the experimenter. Self-administration started with an FR1 schedule of reinforcement for 3 weeks, was moved to an FR2 schedule for 1 week, and was then switched to a progressive-ratio (PR) schedule for 1 week. The PR schedule involved a step increase of three active responses after a previous infusion, which resulted in a sequence of required responses per infusion: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30... FR sessions were 2 hours, and PR sessions were 4 hours in duration. Tail blood was collected immediately after the last FR2 session for analysis of blood nicotine and cotinine levels. There were 22 rats excluded from analysis as a result of failed catheters. These include four rats each from the saline group, the 0.0075NIC group, and the 0.015NIC group; three rats from the 0.03NIC group; two rats each from the 0.015COT group and 0.03COT group; and one rat each from the 0.0075COT group, 0.06NIC group, and 0.06COT group.

Intravenous Self-Administration of Cotinine in Male and Female Rats. Rats of 9 to 10 weeks of age began the training to self-administer cotinine at 0.03 mg/kg per infusion on an FR1 schedule for 3 weeks, an FR2 schedule for 1 week, and a PR schedule for 1 day on the Monday of week 5. Self-administration continued with cotinine reduced to 0.015 mg/kg per infusion on an FR2 schedule during the 4

remaining days of week 5 and on a PR schedule for 1 day on the Monday of week 6. Afterward, cotinine was increased to 0.06 mg/kg per infusion on an FR2 schedule during the rest of week 6 and a PR schedule on the Monday of week 7. Given a significant correlation between breakpoints during the first session and those averaged across all five sessions in the above experiment (Fig. 3D), only one PR session was conducted in this experiment to reduce the number of sessions to avoid premature loss of catheter patency. Tail blood was collected after the last FR2 session at each dose for analysis of blood cotinine levels. There were eight rats excluded from analysis as a result of failed catheters, with four rats from each sex.

Effects of Mecamylamine and Varenicline on Cotinine and Nicotine Self-Administration in Male Rats. Rats were trained to self-administer cotinine or nicotine at 0.03 mg/kg per infusion on an FR1 schedule for approximately 3 weeks. Rats were then divided to receive treatment with either mecamylamine, a nonselective nAChR antagonist, or varenicline, a partial $\alpha 4\beta 2^*$ nAChR agonist. Mecamylamine (0, 0.75, 1.5, and 3.0 mg/kg) or varenicline (0, 0.5, 1.0, and 2.0 mg/kg) treatments were administered subcutaneously at 1 ml/kg approximately 30 minutes prior to operant sessions using a within-subject design. Nontreatment sessions between treatments were included to allow responses back to baseline levels. Mecamylamine (Corrigall and Coen, 1989; DeNoble and Mele, 2006) and varenicline (Rollema et al., 2007; O'Connor et al., 2010) at these doses have been shown to reduce nicotine self-administration in rats. There were 12 rats excluded from analysis as a result of failed catheters, with three rats from each group.

Blood Nicotine and Cotinine Levels. Tail blood samples were processed to extract cotinine and nicotine and were analyzed with high-performance liquid chromatography coupled with UV detection following procedures previously described (Page-Sharp et al., 2003; Katner et al., 2015). Briefly, blood samples were centrifuged at 4000 rpm for 10 minutes, and 100 μ l of plasma was transferred to a 1.5-ml centrifuge tube. Plasma samples were alkalized with 100 μ l of 5 M NaOH/1.1 M NaCl solution and extracted with 650 μ l of dichloromethane by vigorous vortex for 10 minutes. After centrifuging at 4000 rpm for 10 minutes, the bottom organic layer was carefully transferred into a new 1.5-ml centrifuge tube. Samples were added with 20 μ l 0.1 M HCl, vortexed vigorously for 5 minutes, and evaporated to dryness in a Savant SpeedVac. Residues were reconstituted in 60 μ l of 10% methanol. In total, 50 μ l of samples was loaded on a Zorbax SB-C8 column (2.1 \times 100 mm, 3.5 μ m; Agilent Technology, Santa Clara, CA) with a mobile phase (30 mM K_2HPO_4 , 30 mM citric acid, 15% acetonitrile, 0.5% trimethylamine, pH 6.7) flowing at 0.2 ml/min. Nicotine and cotinine were detected with a Shimadzu SPD-20A UV detector at 260 nm (Columbia, MD). Quantification was determined using EZChrom software (Agilent Technologies).

Statistical Analysis. Data were expressed as means \pm S.E.M. Time-course data were analyzed with mixed ANOVAs with repeated measures on session followed by multiple comparisons with Bonferroni correction. One-way ANOVAs were used to analyze data on averaged infusions and breakpoints and blood nicotine or cotinine levels, followed by Tukey's *b* post hoc analysis. Linear regression was used to analyze the correlation between breakpoints in the first PR session and those averaged across all five PR sessions. Student's *t* tests were used to analyze the amount of cotinine infusion and blood cotinine levels between male and female rats and direct comparisons between nicotine and cotinine at the same dose. The significance level was set at $P < 0.05$.

Results

Cotinine and Nicotine Self-Administration in Male Rats. Repeated measures ANOVA revealed significant effects of session, treatment, lever, and session \times treatment \times lever interaction (all *F* values > 1.3 , all *P* values < 0.01) for lever responses across FR1 and FR2 sessions (Fig. 1B). Rats

reliably responded more on the active lever than the inactive lever approximately 1 week after self-administration of nicotine and cotinine. Nicotine induced significantly more active responses than cotinine during most sessions at 0.0075 mg/kg per infusion and during four FR2 sessions at 0.015 mg/kg per infusion.

Significant effects of session, treatment, and session \times treatment interaction (all *F* values > 1.6 , all *P* values < 0.001) were revealed on number of infusions across FR sessions (Fig. 1C). Nicotine at all doses, and cotinine at 0.015–0.06 mg/kg per infusion, induced more infusions than saline, mostly starting from the second week of self-administration. Nicotine induced more infusions than cotinine during most sessions at 0.0075 mg/kg per infusion and during FR2 sessions at 0.015 mg/kg per infusion.

Significant effects of treatment on an average number of infusions were detected for both FR1 and FR2 schedules (top panels in Fig. 2, B and C; *F* values > 4.4 , *P* values < 0.004). Number of infusions for saline, cotinine, and nicotine were 10, 23–34, and 34–48 per session, respectively, during the last five FR1 sessions and were 7, 15–29, and 34–47 per session, respectively, across FR2 sessions. During the FR2 schedule, more infusions were obtained for cotinine at 0.06 than at 0.0075 mg/kg per infusion. Comparisons between cotinine and nicotine at the same doses revealed that nicotine induced more infusions than cotinine at 0.0075 mg/kg per infusion during the FR1 schedules and at 0.0075 and 0.015 mg/kg per infusion during the FR2 schedule.

The intake of nicotine and cotinine increased dose-dependently during both FR1 and FR2 schedules (bottom panels in Fig. 2, B and C; *F* values > 24.1 , *P* values < 0.001). Cotinine and nicotine intakes were 0.2–2.0 and 0.4–2.0 mg/kg per session, respectively, during the FR1 schedule and 0.1–1.7 and 0.3–2.0 mg/kg, respectively, during the FR2 schedule. For both nicotine and cotinine, there was significantly greater intake at 0.03 than at 0.0075, and at 0.06 mg/kg per infusion than all other doses.

Cotinine self-administration resulted in dose-dependent increases of blood cotinine levels (Table 1; $F_{3, 43} = 7.4$, $P = 0.000$), which ranged from 77 to 792 ng/ml. Higher levels were seen from 0.03 to 0.06 mg/kg per infusion compared with those from 0.0075 and/or 0.015 mg/kg per infusion. No nicotine was detected in these rats. Nicotine self-administration resulted in dose-dependent increases of blood nicotine ($F_{3, 40} = 4.5$, $P = 0.009$) and cotinine ($F_{3, 40} = 14.7$, $P = 0.000$) levels (Table 1). Blood nicotine levels ranged from 10–46 ng/ml, with higher levels from 0.06 than those from 0.0075 to 0.015 mg/kg per infusion. Blood cotinine levels ranged from 96 to 431 ng/ml, with greater levels from 0.03 than 0.0075 mg/kg per infusion, and from 0.06 mg/kg per infusion than all other doses.

During PR sessions, significant effects of session, treatment, and session \times treatment interaction were found on breakpoints (Fig. 3B; all *F* values > 2.0 , all *P* values < 0.01). Cotinine induced greater breakpoints than saline during session 1 at 0.0075–0.015 mg/kg per infusion and all sessions at 0.03 and 0.06 mg/kg per infusion. Nicotine induced greater breakpoints than saline during sessions 1 and 2 at 0.0075 mg/kg per infusion, sessions 1 and 3–5 at 0.015 mg/kg, and all sessions at 0.03–0.06 mg/kg per infusion. Nicotine induced greater breakpoints than cotinine during sessions 2 and 3 at 0.03 mg/kg per infusion and session 2 at 0.06 mg/kg per infusion.

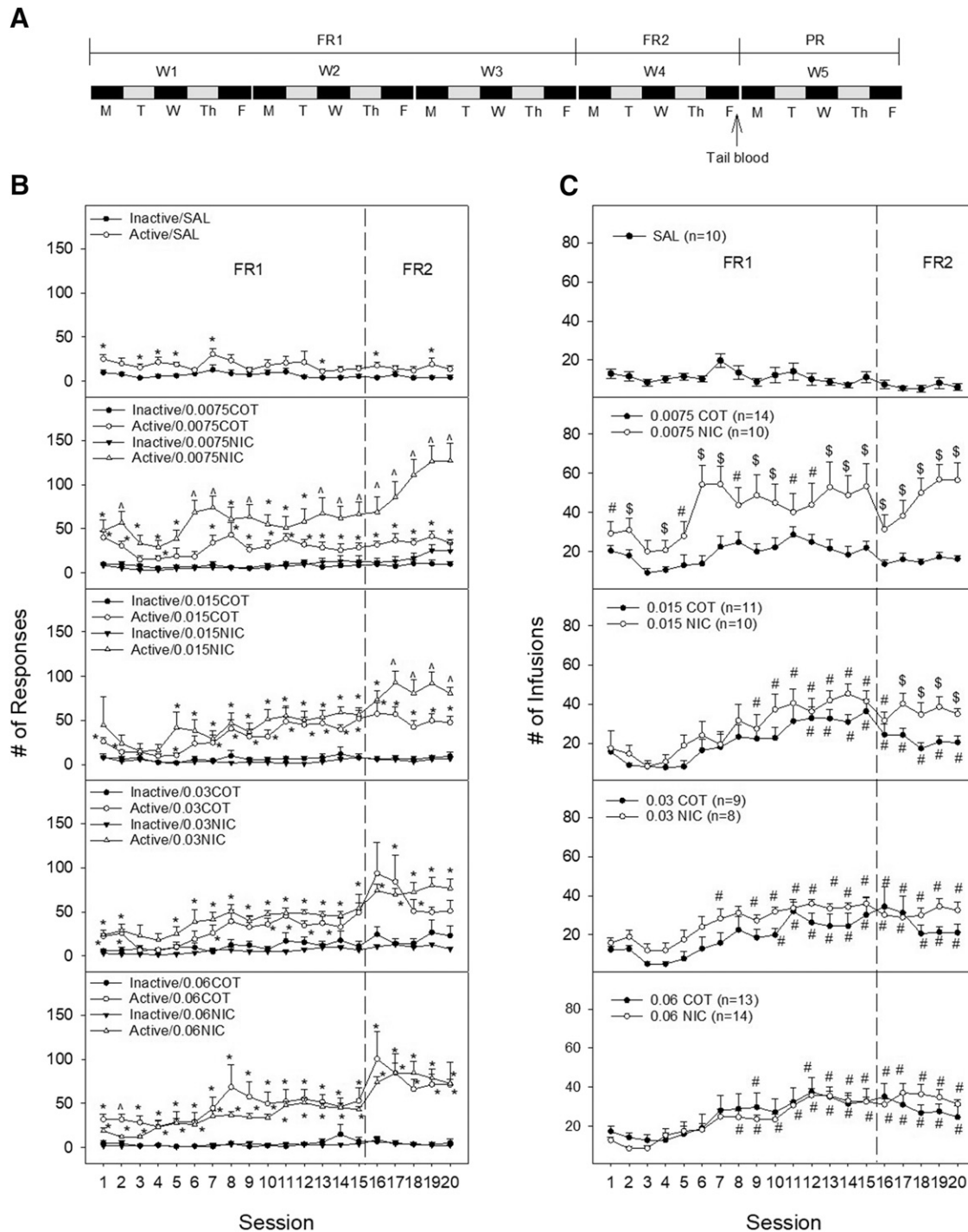


Fig. 1. Intravenous self-administration of saline (SAL), COT, or NIC in male Wistar rats under both FR1 and FR2 schedules. (A) The timeline of self-administration. (B) Lever responses across sessions. *, significantly greater responses than those on inactive lever; ^, significantly greater active responses for NIC than for COT at the same dose. (C) Number of infusions across sessions. #, significantly greater than the SAL group; \$, significantly greater than the SAL group and the COT group at the same dose.

COT and NIC induced greater breakpoints than saline across all PR sessions (Fig. 3C; F values >4.1 , P values <0.01). Breakpoints for cotinine ranged from 7 to 10 per session, with greater breakpoints than saline (4 per session) at 0.03 and 0.06 mg/kg per infusion. Average breakpoints for nicotine were 7–13, with greater breakpoints than saline at 0.015–0.06 mg/kg per infusion. Comparisons between nicotine and cotinine at the same doses revealed that nicotine induced

greater breakpoints than cotinine at 0.03–0.06 mg/kg per infusion.

Linear regression analysis revealed a significant correlation ($F_{1, 94} = 125.7$, $P = 0.000$) between breakpoints during the first PR session and average breakpoints across all five PR sessions (Fig. 3D). The correlation coefficient (R) value was 0.76, suggesting the performance during the first session could predict performance during the following sessions.

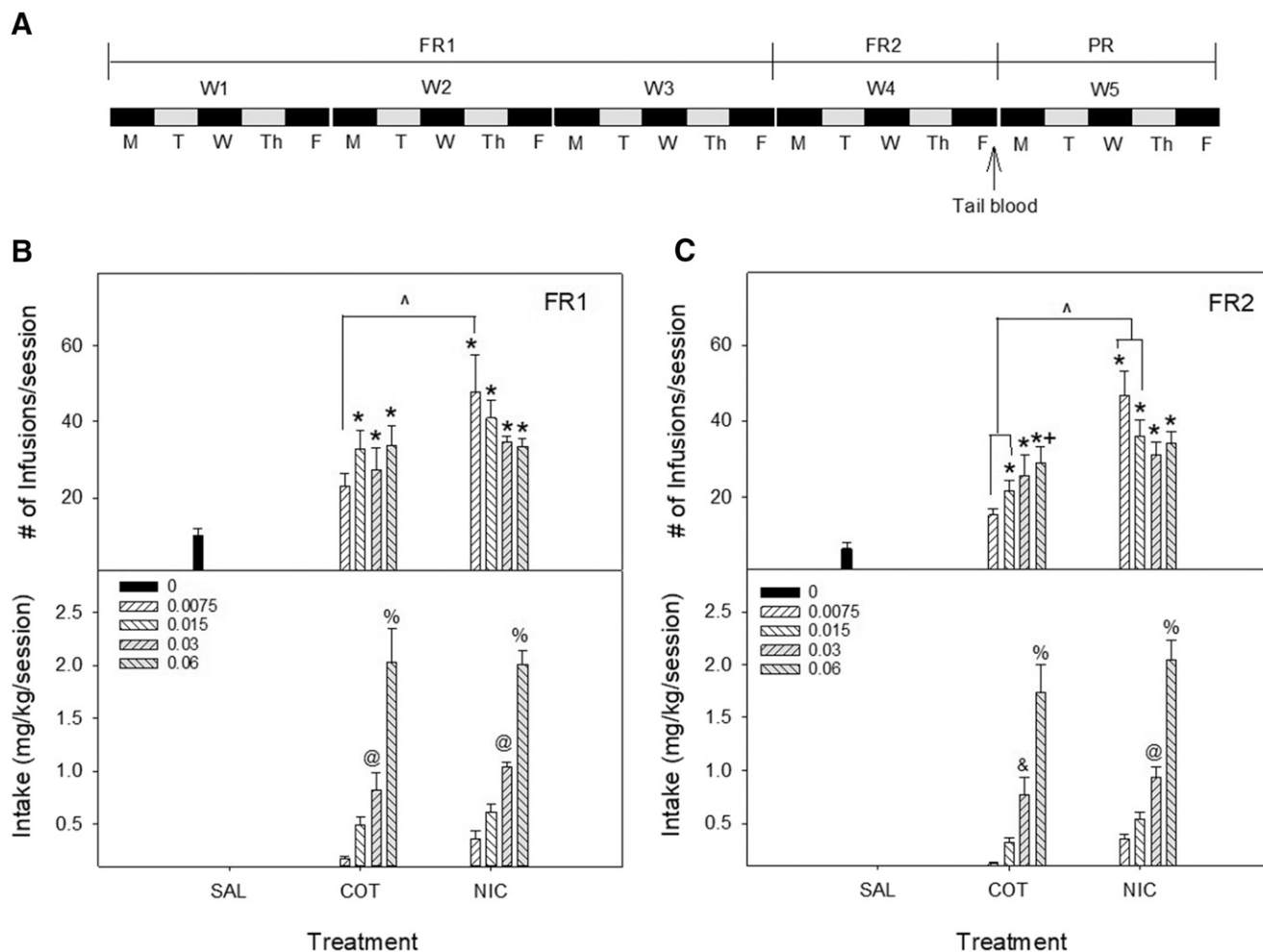


Fig. 2. Average number of infusions and amount of intake of saline (SAL), COT, and NIC during the last five FR1 sessions (B) and across all FR2 sessions (C). (A) The timeline of self-administration. For the number of infusions [top panels in (B and C)]: *, significantly greater than the SAL group; +, significantly greater than the 0.0075 COT group; ^, significantly different between COT and NIC groups. For the amount of intake [bottom panels in (B and C)]: @, significantly greater than the 0.0075 group; &, significantly greater than the 0.0075 and 0.015 COT groups; %, significantly greater than all other groups.

Cotinine Self-Administration in Male and Female Rats. During the FR1 schedule, no significant sex difference in number of infusions (Fig. 4B top panel; sex: $F_{1, 22} = 0.4$, $P = 0.85$) or lever responses (Fig. 4B bottom panel; sex: $F_{1, 44} = 2.0$, $P = 0.17$) were revealed.

No significant effect of concentration, sex, or concentration \times sex (all F values < 1.4 , all P values > 0.21) was detected for dose-response effects during FR2 sessions (Fig. 4C top panel) or breakpoints during the PR schedule (Fig. 4C bottom panel).

TABLE 1

Intake levels during and blood levels after the last FR2 session in rats self-administering nicotine and cotinine at various doses

Because of poor resolution during high performance liquid chromatography analysis, one nicotine value was missing from one rat in the 0.03NIC group.

Drug	Milligrams per Kilogram per Infusion	Intake (n)	COT (n)	NIC (n)
		mg/kg	ng/ml	ng/ml
COT	0.0075	0.13 ± 0.01 (13)	77 ± 10 (13)	—
	0.015	0.31 ± 0.05 (10)	199 ± 26 (10)	—
	0.03	0.66 ± 0.14 (9) ^a	449 ± 185 (9) ^b	—
	0.06	1.54 ± 0.37 (12) ^c	792 ± 172 (12) ^a	—
NIC	0.0075	0.42 ± 0.06 (10)	96 ± 11 (10)	10 ± 6 (10)
	0.015	0.54 ± 0.06 (9)	137 ± 18 (9)	17 ± 5 (9)
	0.03	0.98 ± 0.13 (8) ^b	228 ± 46 (8) ^b	24 ± 10 (7)
	0.06	1.88 ± 0.12 (14) ^c	431 ± 54 (14) ^c	46 ± 10 (14) ^a

^aSignificantly greater than 0.0075 and 0.015 groups.

^bSignificantly greater than the 0.0075 group.

^cSignificantly greater than all other groups.

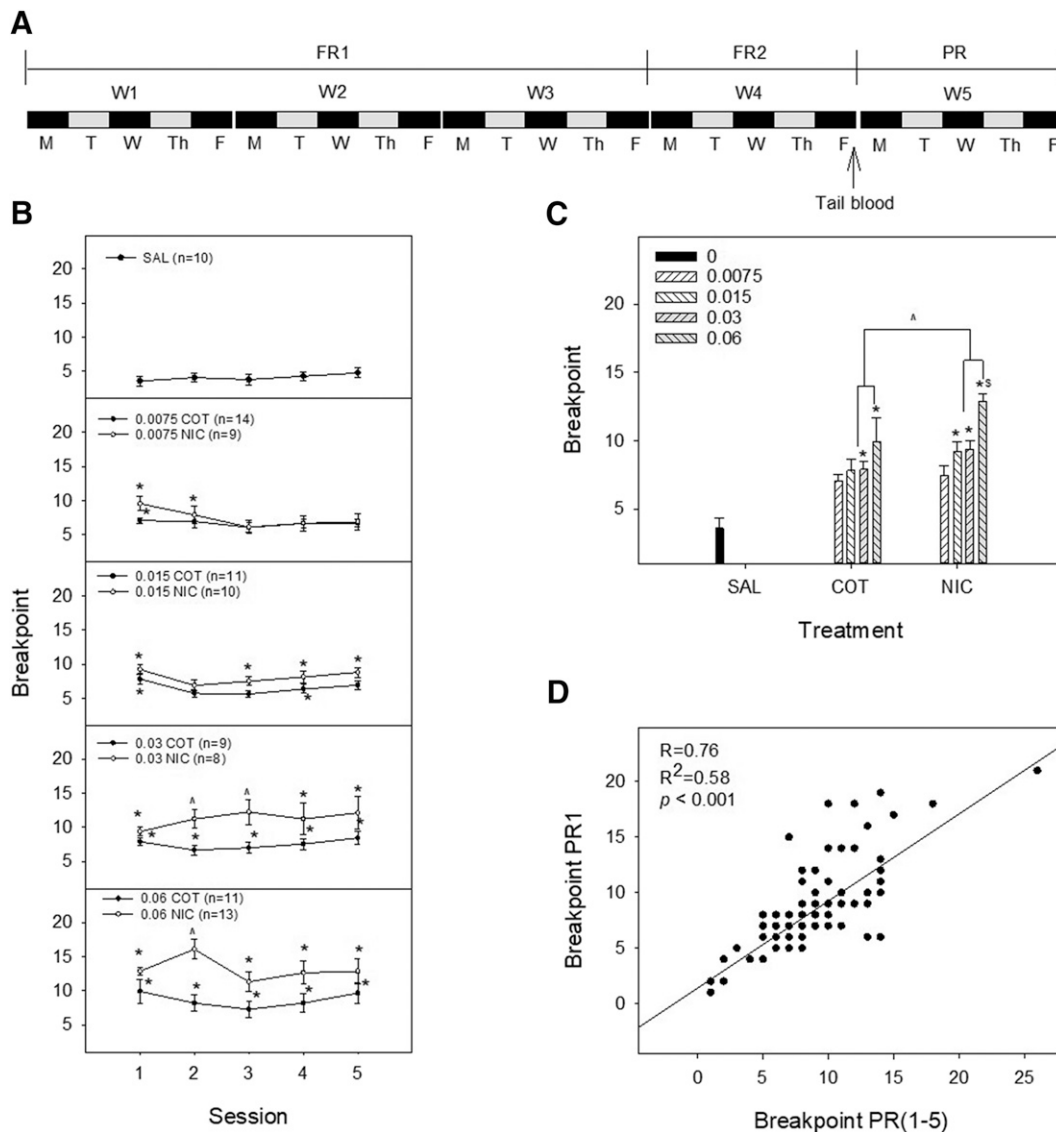


Fig. 3. Breakpoints expressed as the total number of infusions during PR sessions for self-administration of saline (SAL), NIC, or COT. (A) The timeline of self-administration. (B) Time-course change of breakpoints across sessions. *, significantly greater than SAL; ^, significantly greater than SAL and the same dose of COT. (C) Average breakpoints across all PR sessions. *, significantly greater than SAL; \$, significantly greater than NIC at 0.0075 and 0.015 mg/kg; ^, significantly different between COT and NIC at the same dose. (D) Correlation between breakpoints during the first PR session and breakpoints across all five PR sessions.

No significant differences existed between male and female rats in either the amount of cotinine intake or blood cotinine levels after the last FR2 session (Table 2; all P values >0.05).

Effects of Varenicline and Mecamylamine on Nicotine and Cotinine Self-Administration. Varenicline (Fig. 5A) reduced the number of infusions ($F_{4, 45} = 8.6$, $P = 0.000$) and active responses ($F_{4, 45} = 7.5$, $P = 0.000$), but not inactive responses ($F_{4, 45} = 0.8$, $P = 0.54$), during nicotine self-administration. Similarly, mecamylamine (Fig. 5B) significantly reduced the number of infusions ($F_{4, 30} = 5.4$, $P = 0.002$) and active responses ($F_{4, 30} = 3.8$, $P = 0.013$), but not inactive responses ($F_{4, 30} = 0.9$, $P = 0.5$), during nicotine self-administration. However, neither varenicline nor mecamylamine significantly altered the number of infusions or active or inactive responses (all F values <1.4 , all P values >0.1) during cotinine self-administration.

Discussion

Our results indicate that rats acquired intravenous self-administration of cotinine, responded more on the active lever, and increased motivation for cotinine self-administration upon gradual increase of the reinforcement requirement. These results suggest that cotinine may be reinforcing in rats. Nicotine induced more infusions at lower doses under the FR schedule and greater breakpoints at higher doses under the PR schedule than cotinine, suggesting that rats may be more sensitive and more responsive to the self-administration of nicotine than cotinine. Mecamylamine and varenicline reduced nicotine but not cotinine self-administration, suggesting that nAChRs may be differentially involved in mediating self-administration of cotinine and nicotine. Taken together, these results indicate that cotinine supports self-administration in rats, suggesting that cotinine may play a role in the

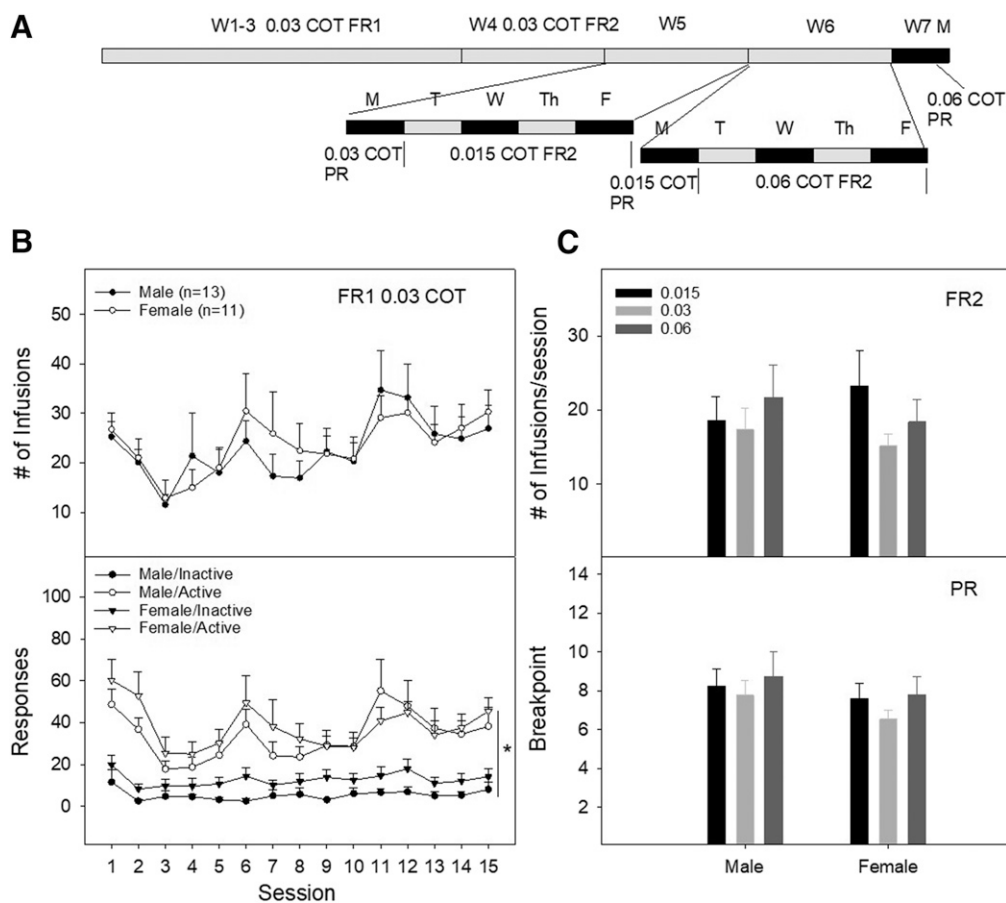


Fig. 4. Self-administration of COT in both male and female Wistar rats. (A) The timeline of self-administration. (B) Number of infusions (top) and lever responses (bottom) across FR1 sessions at 0.03 mg/kg per infusion. (C) Dose-response effects of number of infusions averaged across the last two FR2 sessions (top) and breakpoints during the PR session (bottom).

development of nicotine reinforcement. Self-administration of cotinine and nicotine produced blood nicotine and/or cotinine levels comparable to levels seen in human smokers (Hukkanen et al., 2005), suggesting that these models provide good translational relevance for future studies examining the potential role of cotinine in smoking.

The current results are consistent with several studies showing that cotinine substituted for nicotine-like discriminative stimulus effects. In rats trained on nicotine, intraventricular administration of cotinine generalized to the discriminative stimulus effects of nicotine, with 16 μ g cotinine generalizing to an equal amount of nicotine (Rosecrans and Chance, 1977). Later studies demonstrated that systemic cotinine nearly completely substituted for the discriminative stimulus effects of nicotine in both rats and squirrel monkeys, with cotinine being 1000- to 2000-fold less potent than nicotine

(Goldberg et al., 1989; Takada et al., 1989). It remains unknown what accounted for the difference in cotinine potency among these studies. Procedural differences were noticed and may have contributed to the difference in potency, e.g., central versus systemic administration, nicotine training on a variable-interval schedule of 15 seconds versus a tandem variable-interval and fixed-ratio 10 schedule of 1 minute. Goldberg et al. (1989) speculated that nicotine as an impurity in cotinine samples might have contributed to the observed effects of cotinine given the huge difference in potency values between nicotine and cotinine in their studies. It is noted that no pharmacological data or blood nicotine data were provided to further support their speculation. They also found that the same cotinine samples produced differential effects on food-reinforced behavior than nicotine, and mecamylamine blocked nicotine but not cotinine effects in the same study. Such

TABLE 2

Cotinine intake during, and blood cotinine levels after, the last FR2 session at each dose between male and female Wistar rats.

COT	Intake			Blood COT		
	Male (n)	Female (n)	P	Male (n)	Female (n)	P
	<i>mg/kg</i>			<i>ng/ml</i>		
0.015	0.30 \pm 0.06 (12)	0.34 \pm 0.08 (11)	0.68	136 \pm 33 (12)	165 \pm 34 (10)	0.55
0.03	0.55 \pm 0.10 (13)	0.49 \pm 0.06 (11)	0.60	271 \pm 91 (13)	256 \pm 49 (11)	0.60
0.06	1.34 \pm 0.33 (12)	0.92 \pm 0.20 (10)	0.31	542 \pm 161 (10)	584 \pm 249 (7)	0.88

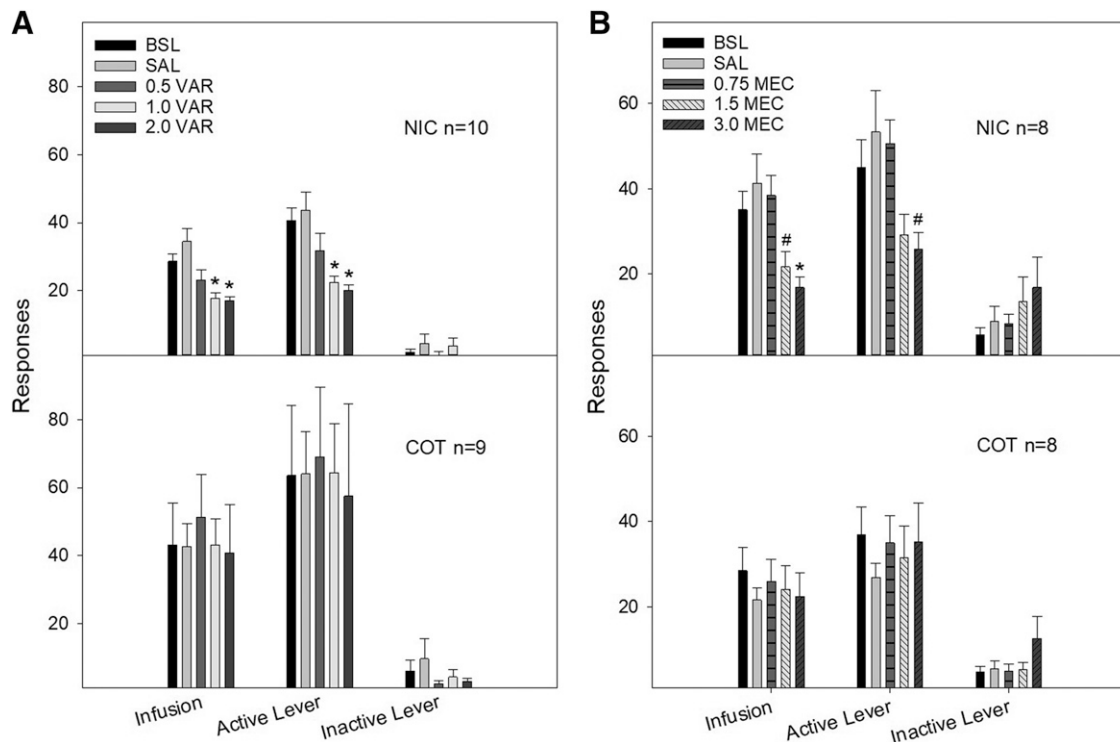


Fig. 5. Effects of systemic administration of varenicline (VAR; Panel A) or mecamylamine (MEC; Panel B) on COT self-administration at 0.03 mg/kg per infusion. *, significantly lower than the baseline (BSL) condition and the saline (SAL) treatment. #, significantly lower than the SAL treatment.

findings seem to argue against their speculation. Therefore, it remains unknown what mechanisms underlie the discriminative stimulus effects of cotinine. On the other hand, a recent study did not find cotinine substitution for the discriminative stimulus effects of epibatidine in nonhuman primates, adding another layer of complexity to this issue (Desai et al., 2016).

Receptor mechanisms underlying cotinine self-administration remain unknown. It was unexpected that both mecamylamine and varenicline reduced nicotine but not cotinine self-administration. Several studies also compared the involvement of nAChRs in effects of cotinine and nicotine. Goldberg et al. (1989) reported that mecamylamine attenuated effects of nicotine but not cotinine on food-reinforced responding in rats. Riah et al. (1999) demonstrated that the ganglionic nAChR antagonist hexamethonium reduced nicotine toxicity but enhanced cotinine toxicity in mice. Abbruscato et al. (2002) showed that the $\alpha 7$ nAChR antagonist α -bungarotoxin attenuated the effects of nicotine but not cotinine on the permeability of cultured bovine brain microvessel endothelial cells. The current finding is in line with these studies and suggests that nAChRs may be differentially involved in mediating certain effects of nicotine and cotinine. Furthermore, cotinine and nicotine produced differential effects on cortical serotonin uptake (Fuxe et al., 1979), food-related operant behaviors (Risner et al., 1985; Goldberg et al., 1989), and brain microvascular permeability (Abbruscato et al., 2002), which further suggest differential actions/mechanisms between nicotine and cotinine. On the other hand, mecamylamine was shown to reduce cotinine-induced dopamine release in vitro (Dwoskin et al., 1999; Oliver et al., 2007). The lack of consensus regarding nAChR mechanisms underlying effects of cotinine and nicotine is intriguing and warrants further studies.

Different binding profiles were noted between cotinine and nicotine. The IC_{50} values for displacing [3H]nicotine binding were 0.3–200 nM for nicotine and 2–100 mM for cotinine (Abood et al., 1983; Riah et al., 1999). The K_i values for displacing [3H]nicotine and [3H]cytisine binding (presumably $\beta 2^*$ nAChRs) were at 0.58 and 0.6 nM for nicotine and at more than 1 mM and 200 μ M for cotinine (Anderson and Arneric, 1994). IC_{50} values for displacing [^{125}I] α -bungarotoxin binding (presumably $\alpha 7$ nAChRs) were 10 μ M for nicotine but 1 mM for cotinine (Riah et al., 1999). Cotinine at 1 mM produced only 1% of activation on $\alpha 7$ nAChRs (Briggs and McKenna, 1998). These studies suggest that cotinine is much less potent than nicotine in binding to nAChRs. Furthermore, cotinine at physiologic concentrations (0.1–1 μ M) did not inhibit [3H]cytisine binding (Sziráki et al., 1999). Cotinine at 10 μ M had no agonistic or antagonistic activity on $\alpha 4\beta 2^*$ or $\alpha 7$ nAChRs (Terry et al., 2015). In the current study, blood cotinine levels were 200–800 ng/ml (~ 1.1 – 4.5 μ M) after reliable cotinine self-administration (Table 1). Therefore, it may be unlikely that cotinine could act mainly through nAChRs to induce self-administration.

A recent study indicated a lack of activity of cotinine on more than 70 molecular targets, including receptors and transporters of major neurotransmitters, ion channels, and intracellular signaling transducers and enzymes (Terry et al., 2015). Interestingly, a study identified a 40-kDa cotinine-binding protein that showed ~ 1000 -fold greater affinity for cotinine than nicotine in rat brain (Riah et al., 2000). The property of this putative cotinine receptor remains to be further characterized. Therefore, future studies are warranted to identify molecular targets of cotinine's action.

Self-administration of nicotine exhibited sex differences in rats. In general, female rats tended to be more sensitive to

nicotine reinforcement and to produce more nicotine infusions than male rats (Donny et al., 2000; Rezvani et al., 2008; Lynch, 2009). Elevated susceptibility to tobacco smoking has also been reported in women compared with men in developed countries (O'Dell and Torres, 2014). The current study did not find sex differences in cotinine self-administration and blood cotinine levels, suggesting that cotinine may not contribute to sex differences in nicotine reinforcement. However, it is noted that male rats were tested in a majority of experiments, and female rats were understudied in the current study. Future studies with more female rats tested will be needed for a more definitive conclusion.

Although cotinine induced self-administration in rats, its potential role in nicotine self-administration remains elusive. Nicotine at higher doses (0.03–0.06 mg/kg per infusion) produced blood cotinine levels comparable to those in rats self-administering cotinine at 0.015–0.03 mg/kg per infusion. If cotinine is contributing to nicotine self-administration at these blood levels, one would expect to see more nicotine infusions than cotinine. However, our results indicate no difference in the number of infusions between cotinine and nicotine. A ceiling effect may have prevented observation of any difference, as studies suggest that nicotine at doses around 0.03 mg/kg per infusion may be close to optimal doses for self-administration (Corrigall and Coen, 1989; Donny et al., 1995). Interestingly, a recent study reported that a novel CYP2A6 inhibitor decreased nicotine self-administration (Chen et al., 2020). Increased bioavailability of nicotine may have contributed to these effects. However, nicotine and cotinine are covariate, i.e., CYP2A6 inhibition will simultaneously increase nicotine and decrease cotinine levels. Therefore, it is difficult to exclude a potential contribution of cotinine reduction in reduced nicotine self-administration after CYP2A6 inhibition.

In addition, it is expected that pharmacokinetics of cotinine after cotinine self-administration would be different than that after nicotine self-administration. Blood cotinine levels should arise immediately and quickly reach peak levels after cotinine self-infusion as a result of slow metabolism. However, in nicotine self-administration, cotinine will be gradually formed and released after nicotine metabolism, and blood cotinine levels will continue to rise until all nicotine is metabolized. It has been suggested that slow increases in blood levels of a drug of abuse, e.g., nicotine, will result in low abuse liability (Hukkanen et al., 2005). Therefore, the potential role of cotinine in nicotine self-administration may be more complicated than what current results may have suggested. Future studies should investigate how cotinine may alter nicotine reinforcement. This is even more interesting given some recent findings with other metabolites of nicotine. For example, nornicotine is a metabolite of nicotine and supported self-administration in rats. However, pretreatment with nornicotine reduced nicotine self-administration (Caine et al., 2014).

In summary, the current study indicates that cotinine was self-administered intravenously by rats. The effects of cotinine were less robust than those of nicotine, exhibited no sex difference, and did not appear to involve nAChRs. These findings provide a foundation for future research to investigate the potential role of cotinine in nicotine self-administration and reinforcement and to study molecular and cellular mechanisms underlying these effects of cotinine.

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Authorship Contributions

Participated in research design: Ding.

Conducted experiments: Ding, Gao, Sentir, Tan.

Performed data analysis: Ding, Tan.

Wrote or contributed to the writing of the manuscript: Ding, Tan.

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