



OPEN The superoxide dismutase mimetic TEMPOL modulates nicotine-induced hyperlocomotor activity and nicotine-taking behavior in male rats

Kyung Oh Jeon^{1,2}, Jordan T. Yorgason³, Lauren Ford³, Joel T. Woolley³, Seong-Hoon Park¹, Byoung-Seok Lee¹, Eun Kyeong Lee¹, Jaerang Rho² & Eun Young Jang¹✉

Reactive oxygen species (ROS) have been implicated in behaviors induced by acute or repeated cocaine or methamphetamine administration in rodents. In the present study, we investigated the involvement of ROS in behavioral changes induced by nicotine administration and dopamine (DA) transmission changes in the nucleus accumbens (NAc) of rats. Rats were given repeated saline or nicotine (0.4 mg/kg) administration once daily for seven days, and the induction of hyperlocomotor activity, and oxidative stress marker expression induced by the increase in ROS production in the NAc were measured on day 7. We also tested the effect of ROS scavengers on repeated nicotine-induced hyperlocomotor activity and nicotine self-administration, and DA levels in the NAc. Repeated nicotine administration induced hyperlocomotor activity and decreased the expression of oxidative stress markers, such as superoxide dismutase-1 and glutathione peroxidase 1/2, by elevating ROS production in the NAc. Pretreatment with the nonspecific ROS scavenger PBN and the superoxide-selective scavenger TEMPOL significantly attenuated nicotine-induced hyperlocomotor activity without impairing motor function in nicotine-naïve rats on day 7. In addition, in intravenous nicotine self-administration study, TEMPOL significantly reduced nicotine-taking behavior without affecting food intake in nicotine-naïve rats. Furthermore, TEMPOL pretreatment prevented nicotine effects on stimulated DA release in the NAc, which was associated with nicotine-induced behavioral changes. Taken together, these findings suggest that increased ROS production in the NAc contributes to the neuropharmacological properties of nicotine.

Keywords Dopamine, Hyperlocomotor, Nicotine, Nucleus accumbens, Oxidative stress, ROS, Self-administration

Nicotine is the main psychoactive substance in tobacco and exerts addictive properties, such as psychomotor, rewarding, and reinforcing effects, by enhancing dopaminergic transmission via the stimulation of nicotinic acetylcholine receptors (nAChRs) in the brain. In general, the rewarding and reinforcing effects of nicotine are mediated by increased dopamine (DA) levels in the nucleus accumbens (NAc)^{1,2}.

Oxidative stress caused by reactive oxygen species (ROS) is a key factor in neurodegenerative diseases^{3–5}. Recently, psychoactive substances such as cocaine, methamphetamine, and nicotine have been shown to increase ROS production, which leads to oxidative stress in the brain^{6–8}. For example, acute or repeated cocaine administration induced oxidative stress in the NAc^{6,9} and acute amphetamine administration also resulted in increased levels of 8-hydroxyguanosine (8-OHG), a marker of oxidative stress, in the NAc¹⁰. In addition, repeated nicotine administration also resulted in oxidative stress, as indicated by altered superoxide dismutase (SOD), glutathione (GSH), and malondialdehyde (MDA) levels in the brain regions^{8,11,12}. In another study, repeated administration of nicotine altered the levels of oxidative stress markers including MDA levels and the activities

¹Center for Convergence Toxicology Research, Korea Institute of Toxicology, Daejeon 34114, Republic of Korea.

²Department of Microbiology and Molecular Biology, Chungnam National University, Daejeon 34134, Republic of Korea. ³Department of Cell Biology and Physiology, 3019 LSB, Brigham Young University, Provo, UT 84602, USA.

✉email: eunyoung.jang@kitox.re.kr; jangey80@gmail.com

of SOD and catalase in the NAc of rats⁸, where play a critical role in the reinforcing effects of psychostimulants¹³. More importantly, according to growing evidence, increased ROS production in the brain regions is involved in the changes of addictive behaviors induced by psychostimulants such as cocaine or methamphetamine. In support of this finding, in previous studies, we and others demonstrated that ROS participates in cocaine- or methamphetamine-induced hyperlocomotor activity and the reinforcing effects of cocaine or methamphetamine by regulating dopaminergic transmission in the NAc of rodents^{6,7,9,14}. The previous studies showed that oxidative stress was detected in the NAc of rats that self-administered cocaine or methamphetamine, and the ROS scavengers PBN and TEMPOL inhibited the acute cocaine- or methamphetamine-induced increase in DA levels in the NAc^{6,7}. In addition, Zegers-Delgado et al. demonstrated that ROS modulate locomotor activity and extracellular DA levels in the NAc via phosphorylation of the DA transporter (DAT) in acute amphetamine-treated rats¹⁰. These findings suggest that increased ROS production in the NAc may contribute to the reinforcing effects of nicotine via modulation of the dopaminergic system.

Thus, we hypothesized that the increase in ROS levels induced by repeated administration of nicotine may be involved in pharmacological effects such as psychomotor or reinforcing effect of nicotine in the mesolimbic dopaminergic system. To investigate this hypothesis, we examined whether ROS scavengers attenuate nicotine-induced hyperlocomotor activity, i.v. nicotine self-administration, and NAc DA release in rats.

Results

Repeated nicotine administration induced hyperlocomotor activity and oxidative stress by increasing ROS production in the NAc of rats

To test whether repeated administration of nicotine increases locomotor activity and whether this increase in locomotor activity is associated with oxidative stress in the NAc, we measured hyperlocomotor activity after repeated administration of saline or nicotine and evaluated ROS production and the expression of oxidative stress markers in the NAc of rats repeatedly administered saline or nicotine. The experimental protocol is illustrated in Fig. 1A. As shown in Fig. 1B, compared with repeated saline administration, repeated nicotine administration significantly increased hyperlocomotor activity (Fig. 1B; two-way, day, $F_{(3,32)} = 3.508$, $P = 0.0263$; group, $F_{(1,32)} = 55.99$, $P < 0.0001$; interaction, $F_{(3,32)} = 3.986$, $P = 0.016$). In addition, locomotor activity was significantly higher on day 7 than on day 1 in the nicotine administration group ($P < 0.01$ vs. day 1). In temporal analysis, we found that compared with saline, nicotine significantly increased locomotor activity at both time points

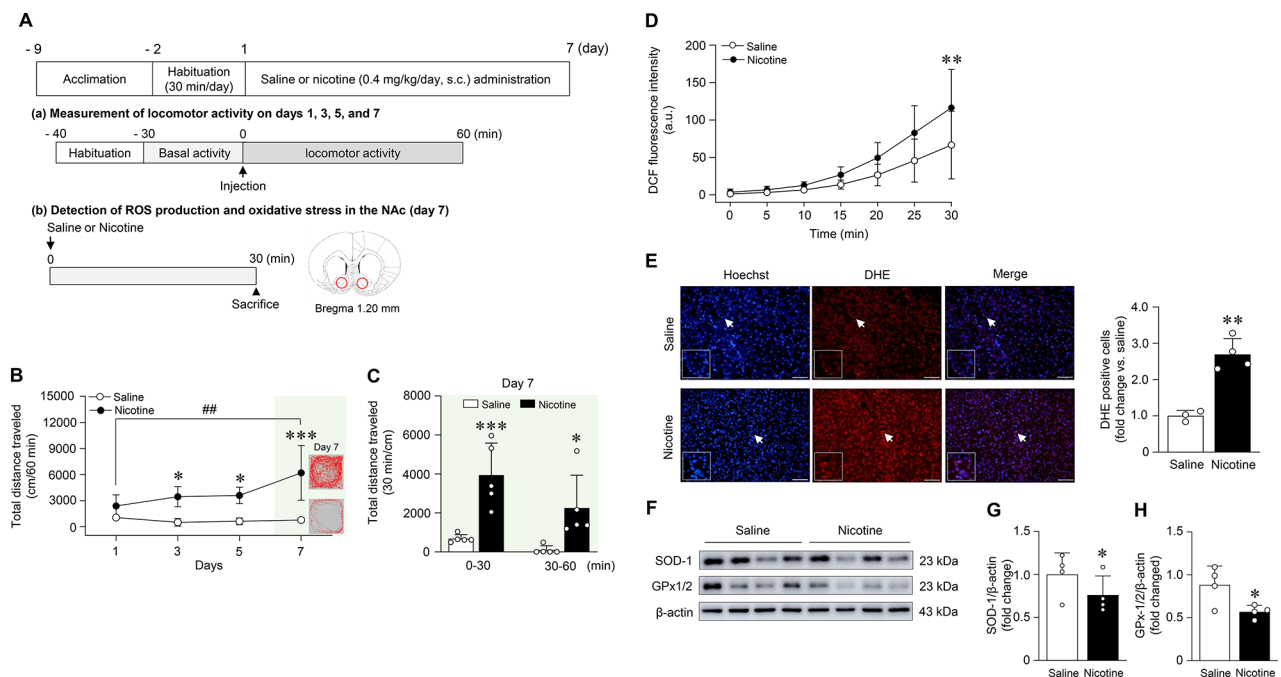


Fig. 1. Effect of repeated administration of nicotine on the induction of hyperlocomotor activity and oxidative stress via the generation of ROS in the NAc of rats. (A). Experimental timeline for the measurement of locomotor activity (a) and the assessment of ROS production and oxidative stress marker expression in the NAc of rats (b). B, C. Mean total distance traveled on days 1, 3, 5, and 7 and representative locomotor trajectories (red lines in each gray rectangle) on day 7 (B), and mean total distance traveled at each time period after saline or nicotine administration on day 7 (C). $*P < 0.05$, $***P < 0.001$ vs. the saline group, $##P < 0.01$ vs. the nicotine group on day 1. $n = 5$ /group. D, E. Measurement of ROS levels (D) and representative images of ROS production using DHE staining with Hoechst in the NAc of rats repeatedly administered saline or nicotine on day 7 (E). $*P < 0.05$ and $**P < 0.01$ vs. the saline group. Bar = 100 μ m. $n = 3 \sim 4$ /group. F–H. Representative blots of oxidative stress markers (F), SOD-1 (G) and GPx-1/2 (H) in the NAc of rats repeatedly administered saline or nicotine on day 7. $*P < 0.05$ vs. the saline group. $n = 4$ /group. All the values are presented as the mean $\pm$ SD.

(0–30 min and 30–60 min; Fig. 1C; two-way, interaction, $F_{(8,81)} = 4.279$, $P = 0.0003$; treatment, $F_{(8,81)} = 10.40$, $P < 0.0001$; group, $F_{(1,81)} = 53.44$, $P < 0.0001$). In addition, repeated nicotine administration increased ROS production (Fig. 1D: DCF-DA assay, two-way; group, $F_{(6,49)} = 11.44$, $P < 0.0001$; time, $F_{(2,49)} = 27.95$, $P < 0.0001$; interaction, $F_{(12,49)} = 4.264$, $P = 0.0001$; Fig. 1E: DHE assay, t test, $P = 0.0015$, $t = 6.315$, $df = 5$) and decreased the expression levels of oxidative stress markers such as SOD-1 (Fig. 1F,G); t test, $P = 0.0227$, $t = 3.042$, $df = 6$) and GPx-1/2 (Fig. 1F,H); t test, $P = 0.0365$, $t = 2.682$, $df = 6$) in the NAc at 30 min after nicotine administration compared with that of saline control group on day 7.

PBN attenuated nicotine-induced hyperlocomotor activity

In our previous study, the nonselective ROS scavenger PBN significantly attenuated acute methamphetamine-induced hyperlocomotor activity in rats⁷. To determine whether ROS contribute to repeated nicotine administration-induced hyperlocomotor activity, we evaluated the effect of PBN on repeated nicotine administration-induced hyperlocomotor activity using the open-field test. The timeline is illustrated in Fig. 2A. While locomotor activity was increased in the repeated nicotine administration group compared to the repeated saline administration group on day 7, pretreatment with PBN significantly attenuated repeated nicotine administration-induced hyperlocomotor activity in a dose-dependent manner (Fig. 2B,C); one-way, $F_{(4,33)} = 26.54$, $P < 0.0001$). When the effect of PBN at different time points was analyzed, we found that 75 mg/kg PBN significantly attenuated the nicotine-induced increase in locomotor activity at a first 30-min period (0–30 min) (Fig. 2D, two-way: time, $F_{(1,66)} = 27.75$, $P < 0.0001$; group, $F_{(4,66)} = 37.07$, $P < 0.0001$; interaction, $F_{(4,66)} = 2.170$, $P = 0.0819$; $P < 0.0001$ vs. Sal + Nic). Pretreatment with 75 mg/kg PBN administration alone did not affect locomotor activity in nicotine-naïve rats on day 7 (Fig. 2C, $P = 0.9537$ vs. Sal + Sal).

TEMPOL attenuated nicotine-induced hyperlocomotor activity

The SOD mimetic TEMPOL also attenuates amphetamine- or methamphetamine-induced locomotor activity in rodents^{7,10}. The timeline is illustrated in Fig. 3A. As shown in Fig. 3B,C), TEMPOL significantly attenuated repeated nicotine administration-induced hyperlocomotor activity in a dose-dependent manner on day 7 (Fig. 3B,C); one-way, $F_{(4,26)} = 20.6$, $P < 0.0001$; $P < 0.025$ vs. Sal + Nic). When the effect of TEMPOL at different time points was analyzed, we found that TEMPOL significantly decreased nicotine-induced hyperlocomotor activity at the first 30-min period (Fig. 3D, two-way: time, $F_{(1,52)} = 1.192$, $P = 0.2799$; group, $F_{(4,52)} = 33.23$, $P < 0.0001$; interaction, $F_{(4,52)} = 1.889$, $P = 0.1263$; $P < 0.0001$ vs. Sal + Nic). Pretreatment with 50 mg/kg TEMPOL administration alone did not affect locomotor activity in the nicotine-naïve rats on day 7 (Fig. 3C, $P = 0.9837$ vs. Sal + Sal).

TEMPOL decreased nicotine self-administration without affecting food intake

In our previous studies, TEMPOL attenuated the reinforcing effect of cocaine or methamphetamine in i.v. cocaine and methamphetamine self-administration studies^{6,7}. In addition, based on the results of the locomotor activity test in the present study, we evaluated the involvement of superoxide, the precursor of most ROS¹⁵, in mediating the reinforcing effect of nicotine in a nicotine self-administration study. As shown in Fig. 4, compared with saline treatment, systemic injection of 50 mg/kg TEMPOL significantly attenuated the number of nicotine infusions (Fig. 4B & 4C, t test, $t = 3.514$, $df = 10$, $P = 0.0056$) and active lever presses (Fig. 4D, t test, $t = 2.837$, $df = 10$, $P = 0.0176$), but there was no significant difference in the number of inactive lever presses between the two groups (Fig. 4E, t test, $t = 1.832$, $df = 10$, $P = 0.096$). We also tested whether TEMPOL affects generalized behavioral responses in nicotine-naïve rats by performing food reinforcement test. Compared with saline administration, TEMPOL did not affect food reinforcement (Fig. 5A: earned food pellets, t test, $t = 2.135$, $df = 10$, $P = 0.0586$; Fig. 5B, active lever presses, t test, $t = 1.266$, $df = 10$, $P = 0.2342$; Fig. 5C, inactive lever presses, t test, $t = 1.964$, $df = 10$, $P = 0.0799$).

TEMPOL reversed nicotine-mediated DA changes in the NAc

In prior work, acute bath application of nicotine resulted in reduced terminal DA release in the NAc^{16–18}. Furthermore, nAChRs on DA terminals are prone to rapid desensitization^{18,19}. Therefore, we examined the effects of TEMPOL on the acute effects of nicotine on NAc DA terminals in naïve (saline)- and nicotine-treated rats (Fig. 6). Rats were treated with saline or nicotine (Fig. 6A; 0.4 mg/kg, s.c., 6 day), and brain slices (obtained on day 7) were perfused with ACSF or ACSF + TEMPOL (3 mM) before nicotine (100 nM) application. Evoked DA release was observed in the NAc via FSCV recordings and was reduced by nicotine (Fig. 6B example traces and colorplots from a saline control rat). In the saline-treated rats, bath application of nicotine (100 nM) resulted in a reduction in evoked DA release, which was not affected by TEMPOL (3 mM) pre-application (Fig. 6C, one-way; $F_{(2,22)} = 12.44$, $P = 0.0002$). In the nicotine-treated rats, bath application of nicotine increased the variability of DA signals, but did not consistently reduce DA signals, indicating desensitization of nAChR. In contrast, in TEMPOL pre-application experiments, evoked DA signals were reduced by nicotine, suggesting that TEMPOL restores nAChR sensitivity in nicotine treated rats (Fig. 6D, one-way; $F_{(2,26)} = 9.212$, $P = 0.0009$). In supplementary Fig. 3, pretreatment with TEMPOL significantly attenuated TH (a rate-limiting enzyme in DA synthesis) mRNA level in the VTA compared to pretreatment with saline in nicotine-treated rats on day 7 (Supplementary Fig. 3; t test, $t = 2.838$, $df = 8$, $P = 0.0219$).

Discussion

In general, activation of dopaminergic neurotransmission in the NAc is closely associated with the effects of psychoactive drugs, including their psychomotor, rewarding, and reinforcing effects²⁰. For example, repeated nicotine administration increases DA release in the NAc via the activation of nAChRs, and increased DA levels are associated with the induction of hyperlocomotor activity in rats^{8,21}. Recently, ROS-induced oxidative stress was

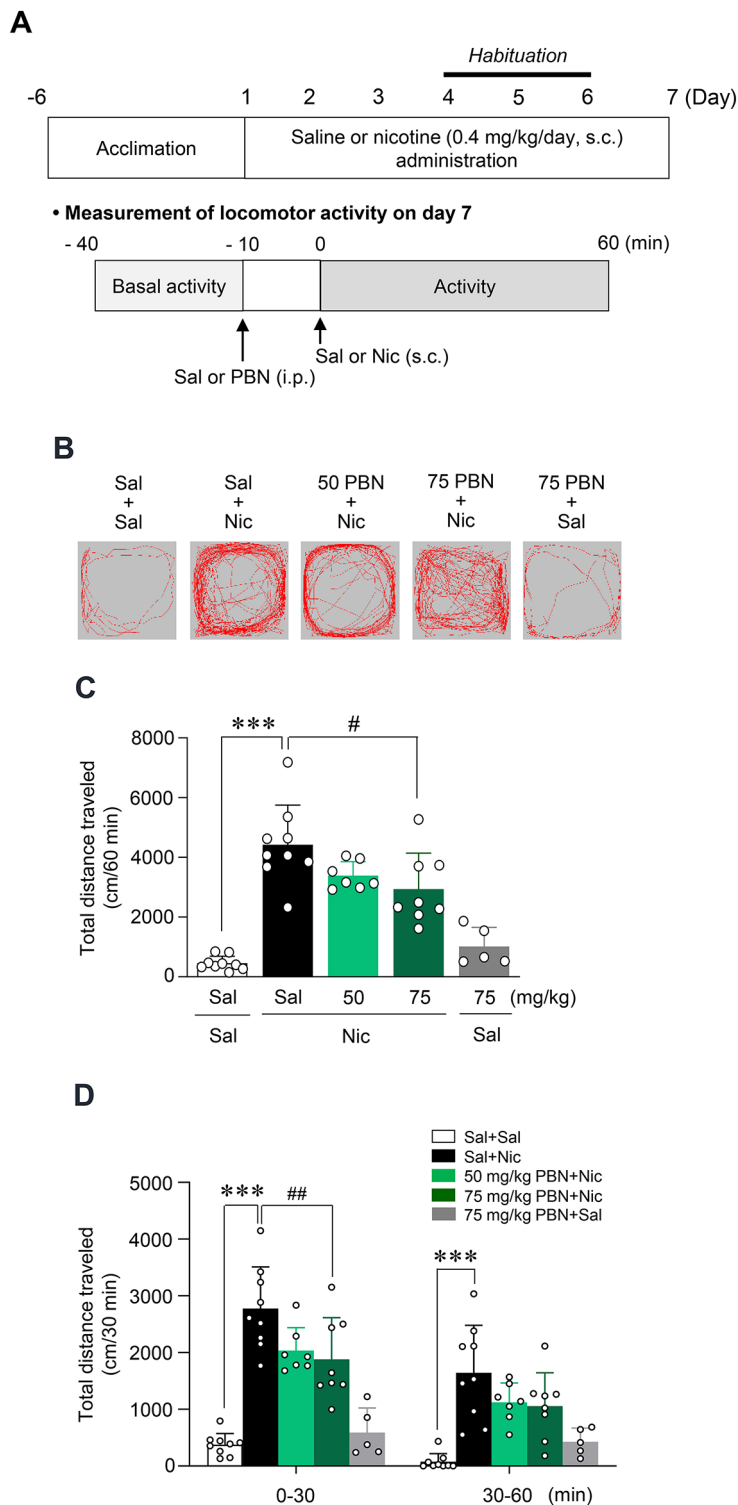


Fig. 2. Effect of PBN, a nonspecific ROS scavenger, on nicotine-induced hyperlocomotor activity in rats. **(A).** Experimental schedule. Saline or PBN (50 or 75 mg/kg, i.p.) was injected 10 min before saline or nicotine (0.4 mg/kg, s.c.) administration on day 7. **B, C.** Representative locomotor trajectories (red lines in each gray rectangle, **(B)**) and mean total distance traveled over 60 min after systemic injection of saline or nicotine on day 7 **(C)**. **(D).** Temporal analysis of the total distance traveled at different times after saline or nicotine administration on day 7. *** $P < 0.001$ vs. the Sal + Sal group, # $P < 0.05$ or ## $P < 0.01$ vs. the Sal + Nic group. Sal = saline, Nic = nicotine. $N = 5-9$ /group. All the values are presented as the mean $\pm$ SD.

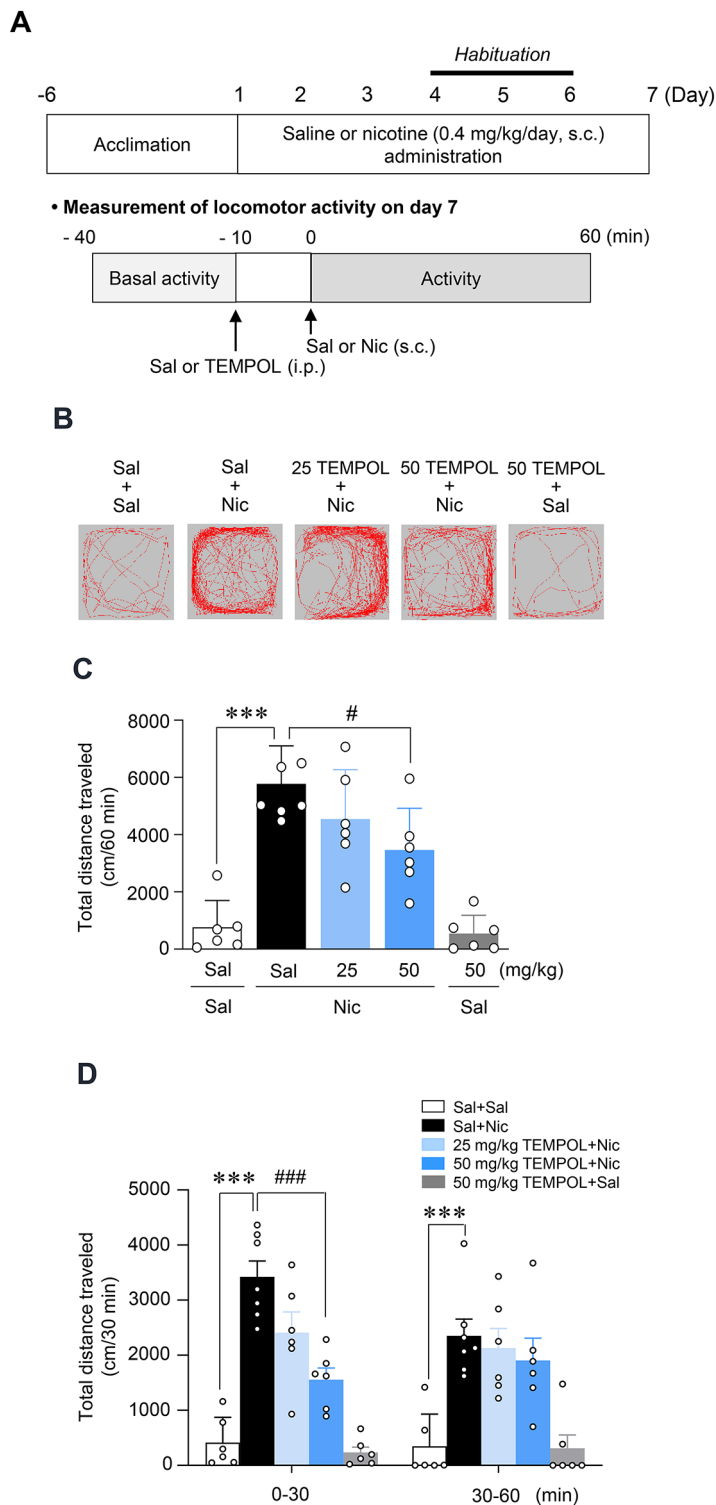


Fig. 3. Effect of pretreatment with TEMPOL, a SOD mimetic, on nicotine-induced hyperlocomotor activity in rats. **(A).** Experimental schedule. Saline or TEMPOL (25 or 50 mg/kg, i.p.) was injected 10 min before saline or nicotine (0.4 mg/kg, s.c.) administration on day 7. **(B,C).** Representative locomotor trajectories (red lines in each gray rectangle, **B**) and mean total distance traveled over 60 min after systemic injection of saline or nicotine on day 7 (**C**). **(D).** Temporal analysis of the total distance traveled at different times after saline or nicotine administration on day 7. *** $P < 0.001$ vs. the Sal + Sal group, # $P < 0.05$ or ### $P < 0.001$ vs. the Sal + Nic group. Sal = saline, Nic = nicotine. $n = 6-7$ /group. All the values are presented as the mean $\pm$ SD.

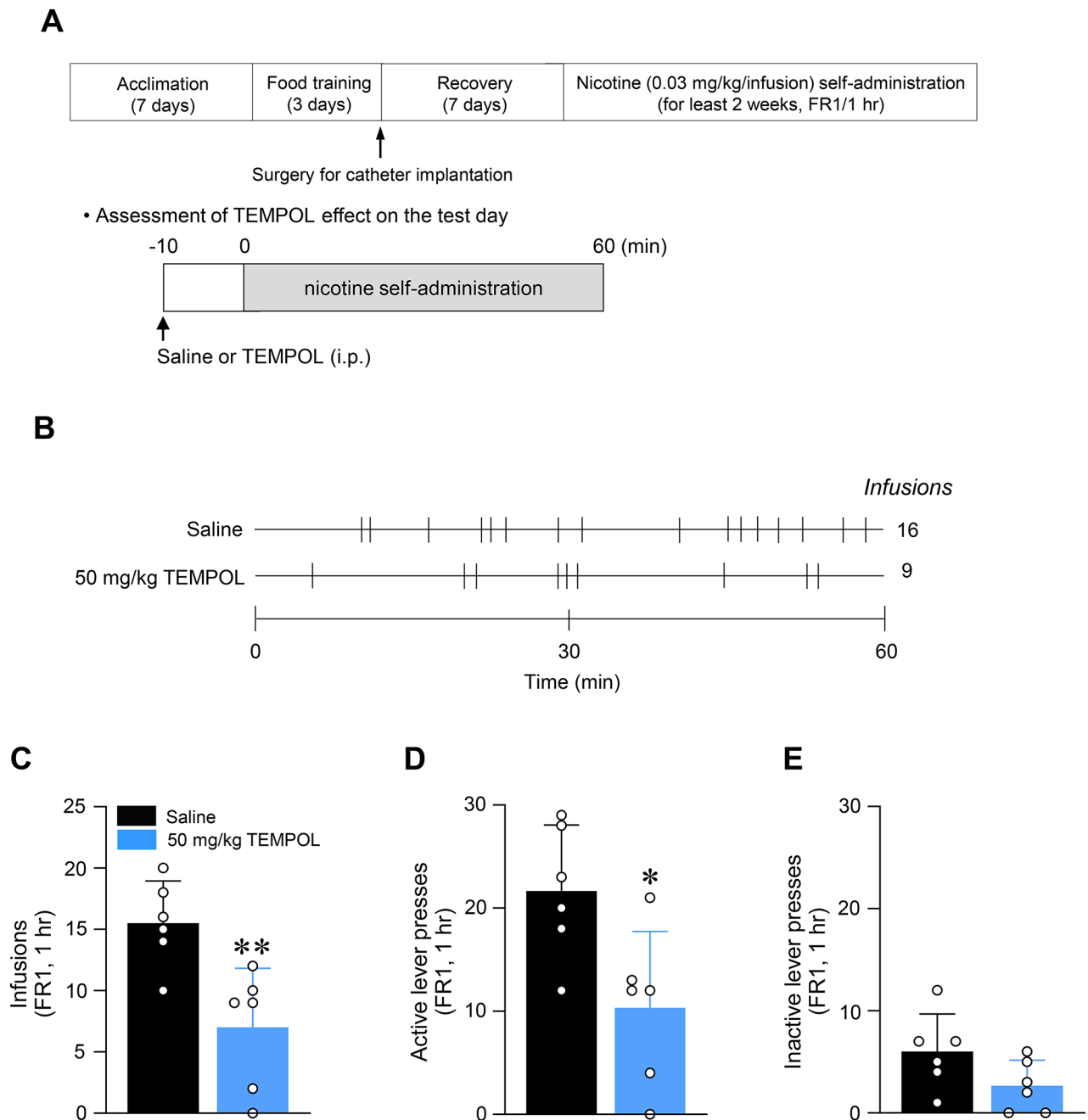


Fig. 4. Effect of TEMPOL on nicotine self-administration in rats. **(A).** Experimental schedule. Saline or TEMPOL (50 mg/kg, i.p.) was injected 10 min before the beginning of the nicotine self-administration test on the test day. **(B).** Representative pattern of nicotine infusion during a 1 h session of the nicotine self-administration test after pretreatment with saline or TEMPOL. **C-E.** Mean number of infusions (**C**) and active (**D**) and inactive (**E**) lever presses over 1 h of the nicotine self-administration test after pretreatment with saline or 50 mg/kg TEMPOL. * $P < 0.05$ or ** $P < 0.01$ vs. the saline control group. $n = 6/\text{group}$. All the values are presented as the mean $\pm$ SD.

implicated in cell damage in a variety of brain regions, including the NAc, and damage to PC12 cells repeatedly exposed to drugs of abuse such as cocaine and (meth)amphetamine^{14,22–24}. According to a growing number of studies, psychoactive drugs such as cocaine and methamphetamine induce oxidative stress in the NAc, which contributes to drug-induced behavioral changes^{9,14}. In support of this, in previous studies by our group and others, oxidative stress was detected in the NAc of rats that self-administered cocaine or methamphetamine and those acutely treated with cocaine^{6,7,10,14}. Furthermore, nicotine induces oxidative stress via the generation of ROS²⁵, which are capable of initiating and promoting cellular oxidative damage, leading to the development of DNA damage, cancer, and brain dysfunction as well as the progression of neurodegenerative diseases^{4,5,26}. Oxidative stress is characterized by decreased activity of antioxidant enzymes such as SOD, GPx, glutathione

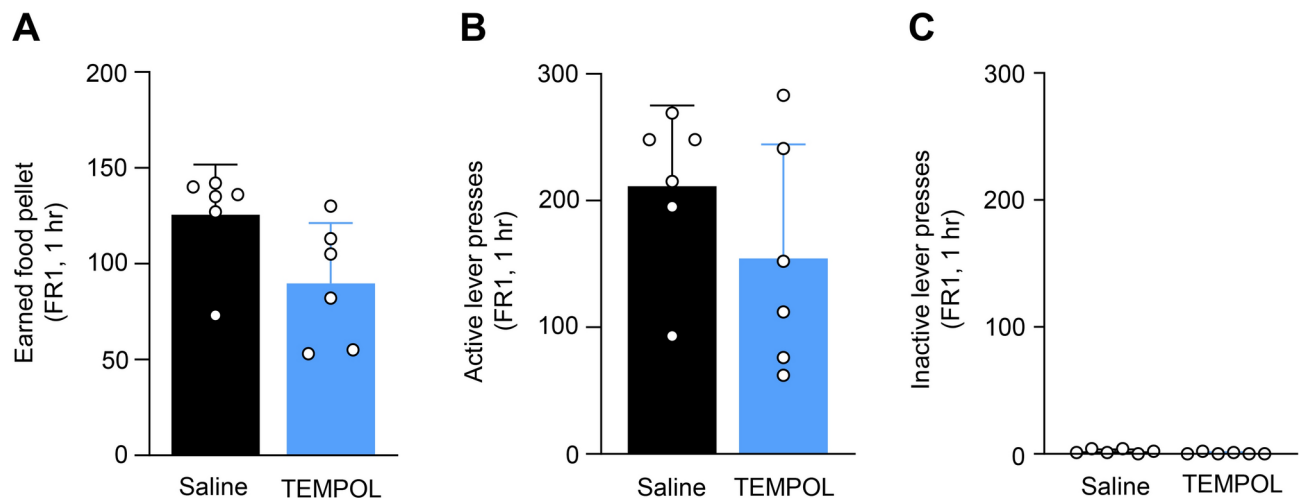


Fig. 5. Effect of TEMPOL in the food reinforcement test. (A–C). Effect of TEMPOL on the mean number of earned food pellets (A) and mean number of active (B) and inactive (C) lever presses over a 1 h session of the food reinforcement test. Saline or TEMPOL (50 mg/kg, i.p.) was injected 10 min before the food reinforcement test on the test day. $n = 6/\text{group}$. All the values are presented as the mean $\pm$ SD.

reductase, and catalase^{8,27}. In 2017, Zhao et al. reported that nicotine challenge increased the concentration of MDA (a marker of lipid peroxidation) and decreased the activity of antioxidant enzymes, such as SOD and catalase, in the NAc of rats, indicating the induction of oxidative stress accompanied by nicotine-induced locomotor sensitization⁸. Consistent with the findings of previous studies, our study showed that repeated administration of nicotine induced hyperlocomotor activity in rats. In addition, repeated administration of nicotine increased ROS production in the NAc, as shown by the DCF-DA assay and DHE staining, and induced oxidative stress by reducing the levels of antioxidant proteins, such as SOD-1 and GPx, indicating increased ROS production in the NAc⁸. These results indicate that repeated nicotine-induced hyperlocomotor activity is associated with increased oxidative stress in the NAc.

Several previous studies have shown that oxidative stress can modulate the reinforcing effect of drugs, such as cocaine^{6,14}, and the neurobehavioral effects of methamphetamine²⁸. We and others have shown that ROS scavengers attenuate the psychomotor or reinforcing effects of psychostimulants. In support of this, PBN, a nonspecific ROS scavenger, was found to prevent the reinforcing effect of cocaine or methamphetamine in a self-administration study and acute methamphetamine exposure-induced locomotor activity in rats^{6,7}. TEMPOL, a superoxide-specific scavenger, was also found to diminish cocaine- or amphetamine-induced oxidative stress in the NAc and prefrontal cortex, behavioral sensitization and acute locomotor activity^{10,14}. In another study, Qi et al. demonstrated that while microinjection of TEMPOL, a SOD mimetic, into the NAc blocked the expression of morphine-conditioned place preference, microinfusion of t-BOOH, a ROS donor, into the NAc rescued the impairment of morphine CPP produced by the mGluR5 antagonist 3-((2-methyl-1,3-thiazol-4-yl)ethyl)pyridine (MTEP)²⁹. Consistent with the findings of these previous studies, in the present study, systemic injection of PBN or TEMPOL attenuated repeated nicotine administration-induced hyperlocomotor activity without affecting motor impairment of in naïve rats. In addition, pretreatment of TEMPOL significantly reversed the reinforcing effect of nicotine in the nicotine self-administration study without affecting food reinforcement, suggesting that the drug-induced increase in oxidative stress in the NAc plays a crucial role in behavioral responses to drugs of abuse. Superoxide ($\text{O}_2^{\cdot-}$) is a key ROS that is generated in cells and is enzymatically converted to hydrogen peroxide (H_2O_2) by SOD, which itself can react with free ions to produce highly reactive and toxic hydroxyl radicals (Fenton reaction).

As mentioned above, psychoactive drugs such as cocaine and nicotine induce DA release in the reward system, especially in the NAc, which is associated with behavioral responses to drugs of abuse^{30,31}. ROS may modulate DA system in the mesolimbic DA system, projecting from the VTA to the NAc. In our slice FSCV studies on NAc DA release, we found that TEMPOL restored acute nicotine sensitivity in nicotine-treated rats, which decreased DA release in the NAc. We did not observe TEMPOL induced changes in acute nicotine sensitivity, suggesting that nicotine sensitivity is modulated by several factors, and that superoxide effects are more involved in prolonged nicotine effects on nAChRs than on acute effects. In our preliminary study, while repeated administration of nicotine increased the level of TH mRNA, a rate-limiting enzyme in DA synthesis, in the VTA, TEMPOL attenuated the increase in TH mRNA levels in the VTA by repeated nicotine administration, resulting in a reduction in DA levels in the NAc (Supplementary Fig. 3). Thus, we speculate that TEMPOL has multiple effects to influence NAc DA levels and sensitivity to nicotine, which include VTA TH regulatory effects, and effects of nicotine on ROS formation and downstream effects on nAChR function. Finally, the present study has the experimental limitations. Even though we investigated the effect of PBN or TEMPOL in nicotine-induced addictive behavior and accumbal DA levels, we did not rule out the effect of PBN or TEMPOL on increased ROS levels in the NAc of nicotine-treated rats. Regarding this, in our previous study, we tested PBN or TEMPOL effect on ROS levels produced by acute cocaine by detecting of 8-hydroxyguanine (8-OHG), a marker

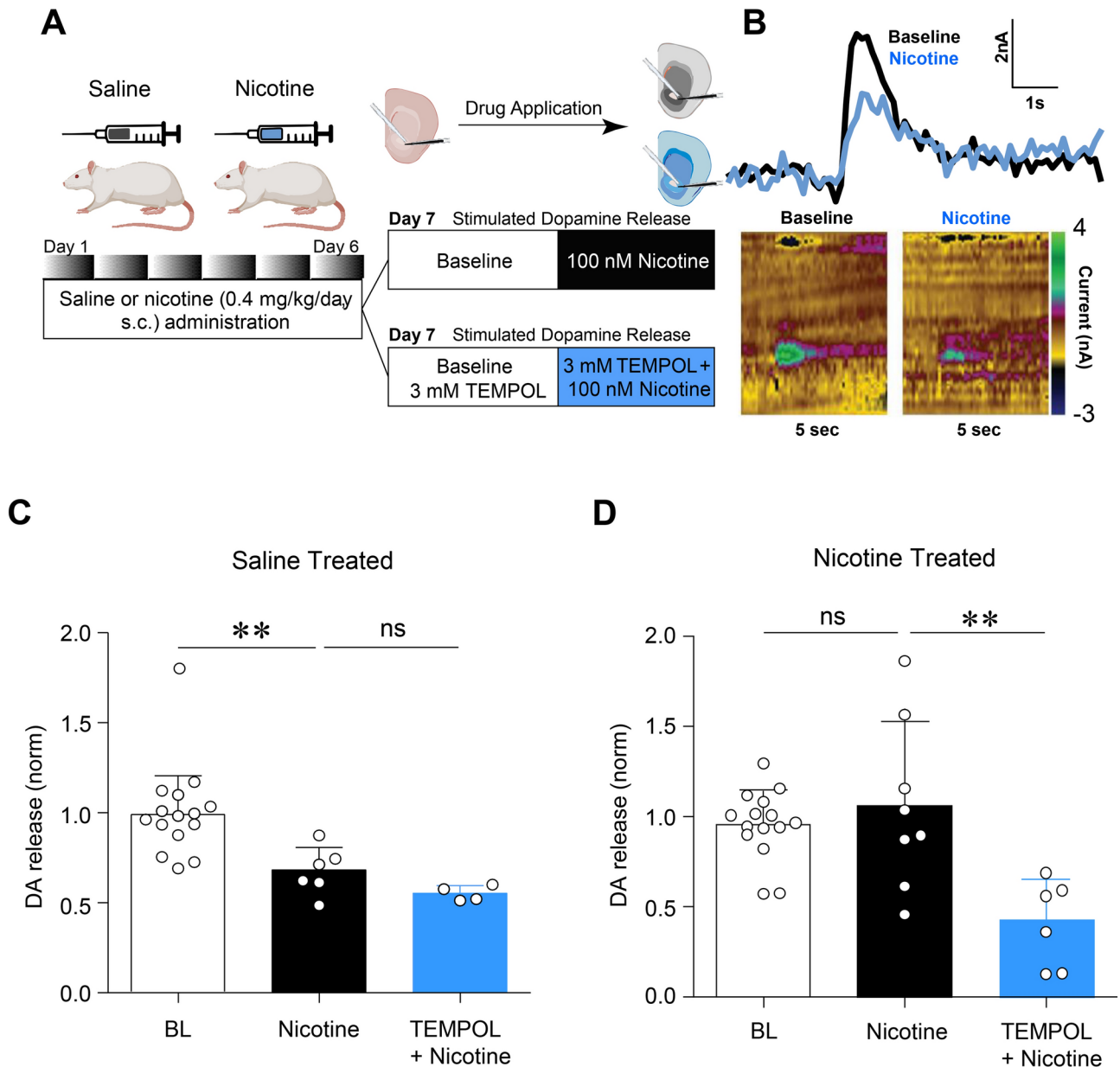


Fig. 6. Effect of TEMPOL on nicotine mediated DA inhibition in the NAc. **(A).** Experimental timeline for nicotine injections in vivo and brain slice recordings. **(B).** Example current traces and colorplots from fast scan cyclic voltammetry experiments. Acute nicotine (100 nM) bath application reduces evoked NAc DA release in a saline injected rat. ** $P < 0.01$ vs. BL. **(C).** In the saline injected rats, DA release was reduced by nicotine bath application regardless of presence of TEMPOL (3 mM). **(D).** The nicotine injected rats had increased sensitivity to nicotine-induced DA reductions if pretreated with TEMPOL. ** $P < 0.01$ vs. Nicotine. BL = baseline and ns = no significance. Individual dots (o) represent experiments from brain slices in 4 – 8 rats. Bar plots are presented as the mean $\pm$ SD. $n = 4$ –8/group.

of oxidative stress, expression in the NAc of rats⁶. Our previous result showed that while the acute cocaine administration increased 8-OHG expression in the NAc, this increased 8-OHG expression was suppressed in PBN- or TEMPOL-pretreated rats, indicating PBN or TEMPOL effectively attenuates ROS levels produced by cocaine exposure⁶. Thus, we speculate that PBN or TEMPOL may also decrease nicotine-induced ROS levels in the NAc. In addition, in the present study, while we tested the effect of TEMPOL with systemic injection on nicotine-induced behaviors, we did not perform the direct effect of TEMPOL in behavioral changes by nicotine after microinjection of TEMPOL into the NAc. However, in our previous study, microinfusion of TEMPOL into the NAc inhibited cocaine taking-behavior in cocaine self-administration study of rats⁶. Based on our previous finding, we speculate that intra-accumbal TEMPOL also may attenuate nicotine-induced hyperlocomotor activity or nicotine self-administration via modulating NAc dopaminergic system. Thus, future studies are needed to confirm the intra-accumbal TEMPOL effect on neuropharmacological properties of nicotine.

Conclusion

The present study showed that repeated administration of nicotine induced hyperlocomotor activity and oxidative stress by increasing ROS production in the NAc of rats. In addition, PBN, a nonspecific ROS scavenger, and TEMPOL, a superoxide-specific scavenger, significantly attenuated repeated nicotine-induced hyperlocomotor activity without causing motor impairment in nicotine-naïve rats, and TEMPOL decreased nicotine self-administration without affecting food intake. Finally, TEMPOL reversed nicotine-mediated DA release in the NAc in the FSCV study, indicating nicotine related ROS modulation of the dopaminergic system. Taken together, these findings suggest that ROS contribute to repeated nicotine-induced hyperlocomotor activity and the reinforcing effect of nicotine by regulating the dopaminergic system in rats.

Material and methods

Animals

Adult male Sprague-Dawley rats (Orient Bio., Inc., Seongnam, South Korea) weighing 250–300 g were used in all the experiments. The rats were housed individually for self-administration experiments or in groups of 2 animals for other experiments at a controlled temperature (21–23 °C) and humidity (45–55%) on a 12-h light–dark cycle (lights on at 08:00 AM). The rats had ad libitum access to food and water except in the self-administration study. During the self-administration experiment, rats received restricted access to food (13–15 g/day) and free access to water during the food training period to increase the number of lever presses. All the experimental procedures were approved by the Institutional Animal Care and Use Committee of the Korea Institute of Toxicology (No. IAC-21-01-288 and IAC-21-01-378) and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. This study is in accordance with ARRIVE guidelines.

Drugs

(-)-Nicotine tartrate salt (Cayman Chemical Company, Michigan, USA) was dissolved in 0.9% sterile saline and filtered through a syringe-mounted 0.22- μ m Minisart Single-use filter unit (Sartorius Stedim Biotech, Goettingen, Germany) for the self-administration study. Nicotine was injected subcutaneously (s.c.) at a dose of 0.4 mg/kg for the open-field test, measurement of ROS generation in the NAc, and measurement of tyrosine hydroxylase (TH) mRNA levels in the VTA, and 0.03 mg/kg/infusion of nicotine was intravenously (i.v.) infused through the right jugular vein for the self-administration experiment. N-tert-butyl- α -phenylnitrone (PBN; a nonspecific ROS scavenger; Sigma-Aldrich, Missouri, USA)^{32,33}, and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL; a superoxide dismutase mimetic; Sigma-Aldrich)^{33,34} were dissolved in 0.9% sterile saline and administered intraperitoneally (i.p.) 10 min before all behavioral assessments and measurement of TH levels in the VTA. All the drug solutions were prepared immediately before treatment. The PBN or TEMPOL dose was selected based on the results of a preliminary study and our previous studies^{6,7}.

Measurement of ROS levels in the NAc

DCF-DA assay

To measure intracellular ROS levels, the rats were anesthetized with sodium pentobarbital (80 mg/kg, i.p.), and their brains were then removed at 30 min after the final saline or nicotine administration on day 7 (Fig. 1A–(b)). Bilateral NAc tissue was collected by a steel borer (1 mm inner diameter) at coordinates selected according to a rat brain atlas³⁵. Isolated NAc tissues were transferred to RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with protease and phosphatase inhibitor cocktails (Thermo Fisher Scientific), homogenized, and then incubated on ice for 1 h. After incubation, the lysates were centrifuged at 12,000 g for 30 min at 4 °C. The pellet was discarded, and the supernatant was centrifuged again at 12,000 g rpm for 30 min at 4 °C. The concentration of the solubilized proteins in the supernatant fraction was determined using a BCA assay kit (Thermo Fisher Scientific). Then, 10 μ g of lysate was mixed with 1.25 μ M 2',7'-dichlorofluorescein diacetate (50 μ L, H₂DCF-DA, Sigma Aldrich, St. Louis, USA) to a final volume of 250 μ L. The fluorescence intensity was measured every 5 min for 30 min using a fluorescence microplate reader (GloMax[®] Explorer Multimode, Promega, Wisconsin, USA) at an excitation wavelength of 475 nm and an emission wavelength of 530 nm.

DHE staining

For measurement of ROS (superoxide) production in the NAc of nicotine-treated rats, rats from a separate group were perfused with 4% paraformaldehyde (PFA) under sodium pentobarbital (80 mg/kg, i.p.) anesthesia 30 min after saline or nicotine administration on day 7 (Fig. 1A–(b)). The brains were isolated and postfixed in 4% PFA overnight at 4 °C, transferred to 30% sucrose solution, and protected for at least 48 h. The brains were then cut into 30- μ m-thick coronal slices using a cryostat (Leica Biosystems, Hesse, Germany), and three tissues per rat were subjected to the dihydroethidium (DHE) (Invitrogen, Massachusetts, USA) assay. Briefly, the brain sections were washed twice in 1X phosphate-buffered saline (PBS) for 10 min and placed on glass slides. Ten micromolar DHE solution was dropped onto the tissues, which were subsequently incubated in the dark at 37 °C for 30 min. After incubation, the stained brain sections were washed three times with 1X PBS and mounted with Hoechst 33,342 (Invitrogen), and cover-slipped. The stained sections were imaged using an Olympus BX53 fluorescence microscope (Olympus, Tokyo, Japan) with cellSens imaging software. The number of cells co-localized with Hoechst was counted by blinded observers from images captured with 20X objectives using ImageJ software (National Institutes of Health, Bethesda, MD, USA) and normalized to saline control group.

Western blotting

To examine whether repeated administration of nicotine induces oxidative stress by increasing ROS production in the NAc, bilateral NAc tissues were collected from a separate group of rats 30 min after the administration

of saline (1 mL/kg, s.c.) or nicotine (0.4 mg/kg, s.c.) on day 7 (Fig. 1A–b). Western blotting was performed as described previously³⁶. The NAc tissue was homogenized with lysis buffer (Thermo Fisher Scientific) containing protease inhibitor cocktail (Thermo Fisher Scientific) and centrifuged at 12,000 g for 30 min at 4 °C. Protein concentrations were determined using BCA protein assay (Thermo Fisher Scientific). Equal amounts of protein were separated by 10–12% SDS-PAGE, and the separated proteins were subsequently transferred to a polyvinylidene membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 5% skim milk for 1 h and incubated with rabbit anti-SOD-1 (1:2000; Cat. No. 37385; Cell Signaling Technology, Massachusetts, USA), mouse anti-glutathione peroxidase (GPx)-1/2 (1:1000; Cat. No. sc-133160; Santa Cruz Biotechnology, Texas, USA), and mouse anti- β -actin (1:2000; Cat. No. A5316; Sigma-Aldrich, Missouri, USA) overnight at 4 °C. The next day, the membrane was washed with Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST) three times for 10 min and then incubated with goat anti-rabbit (1:2000, Cat No. 656120, Invitrogen), and goat anti-mouse (1:2000, Cat No. 31430, Invitrogen) secondary antibodies for 2 h at room temperature. Immunoreactive protein bands were detected using enhanced chemiluminescence reagents (Thermo Fisher Scientific). The protein band density was quantified using ImageJ software (National Institutes of Health), and the β -actin band was used for normalization. The western blotting experiment was replicated two times (Supplementary Fig. 1). Additionally, in previous studies, SOD1, but not SOD2 or SOD3, was used as an antioxidant mediator in the NAc or striatum of psychostimulant-injected animals including nicotine^{37–39}. Based on this, in the present study, we used only SOD1 as a marker of oxidative stress.

Measurement of locomotor activity

A locomotor activity test was conducted in an open field arena (43 cm × 43 cm × 30 cm; ENV-515S; Med Associates, Georgia, VT, USA) in a sound-attenuating cubicle (SAC-283422-NIR, Med Associates). The rat trajectory and total distance traveled were recorded using a monochrome video camera (VID-CAM-MONO-1, Med Associates) and analyzed with video tracking software (EthoVision[®] XT, Noldus, Wageningen, Netherlands). The animals were habituated to the open field arena without any treatment for at least 3 days (over 30 min/day) to avoid the effect of the environment on behavior. In the first experiment, to determine whether repeated administration of nicotine results in hyperlocomotor activity, the rats were administered saline (1 mL/kg) or nicotine (0.4 mg/kg, s.c.) for 7 days, and locomotor activity was measured every other day (days 1, 3, 5, and 7). On the day that locomotor activity was recorded, the rats were allowed to habituate to the open field arena for 10 min, and subsequently basal activity was measured for 30 min. After recording of basal activity, the rats were systemically injected with saline (1 mL/kg, s.c.) or nicotine (0.4 mg/kg, s.c.), and the distance traveled and their trajectories were recorded for an additional 60 min. On days 2, 4, and 6, the rats were treated with saline or nicotine in their home cage without recording locomotor activity (Fig. 1A–a)). In the second experiment, to determine whether the ROS scavengers PBN and TEMPOL block nicotine-induced hyperlocomotor activity, the rats were repeatedly administered saline or nicotine for 6 days in their home cage. On days 4–6, the rats were habituated for more than 30 min to the open field arena before systemic injection of saline or nicotine to avoid the effects of the environment on their behavior on day 7. On day 7, the rats were habituated for 10 min to the open field arena, and basal activity was measured for 30 min. After basal activity was recorded, the rats were given an injection of saline, PBN (50 or 75 mg/kg, i.p.), or TEMPOL (25 or 50 mg/kg, i.p.) 10 min before systemic injection of saline or nicotine. The distance traveled and their trajectories were then recorded for an additional 60 min (Fig. 2A). The data are expressed as the total distance traveled (centimeters, cm) over 60 min.

Food training and intravenous catheter implantation

Food training and i.v. catheter implantations were performed as described previously^{31,40}. Briefly, food training and nicotine self-administration were performed in operant chambers (35 × 25 × 34 cm; Med Associates, St. Albans, VT, USA) equipped with two levers (an active and inactive lever) on one wall, a white house light, and a cue light above each lever. To facilitate the acquisition of operant behavior in the chambers, the rats were trained to press a lever to receive 45 mg of food pellets (Bio-Serv, Frenchtown, NJ) under a fixed ratio (FR) 1 schedule of reinforcement during a 1 h session. During the food training period, the rats were subjected to mild food restriction (approximately 16 g of lab chow a day). When the rats achieved the acquisition criterion (80 food pellets/1 h for 3 consecutive days), a catheter (inner diameter: 0.02 inch, outer diameter: 0.03 inch; Dow Corning, Midland, MI) was implanted into the right jugular vein under anesthesia with sodium pentobarbital (50 mg/kg, i.p.). The catheter was passed subcutaneously through 22-gauge tubing embedded in dental cement and secured with surgical mesh (Ethicon, Inc., Somerville, NJ, USA) to exit the back of the rat. After i.v. catheter implantation, catheter patency was maintained by a daily flush with 0.2 mL of heparinized saline (30 U/mL) containing gentamycin sulfate (0.33 mg/mL) to prevent clotting and infection. The rats were allowed to recover for at least 7 days before beginning the nicotine self-administration study.

Nicotine self-administration

For determination of the effect of TEMPOL on the reinforcing effect of nicotine, nicotine self-administration experiments were performed as described previously^{21,31,41}. After recovery from i.v. catheter implantation, the rats were exposed to nicotine (0.03 mg/kg/infusion) on an FR1 schedule during daily 1-h sessions for at least 2 weeks. During self-administration, a press of the active lever (drug-paired lever) resulted in the i.v. delivery of 0.1 mL of nicotine for 4.1 s. During drug infusion, the house light was turned off, and the cue light above the active lever was turned on. The house light remained on throughout the session, except during the infusion of drugs and time-out (TO) periods. Each infusion was followed by an additional 20 s TO period. During the TO period, active lever-pressing responses were recorded, but the responses did not result in nicotine delivery. Inactive lever presses were also recorded but had no consequence. During the nicotine self-administration training, if the rats met the criterion for operant behavior (stable baseline: three consecutive sessions with

less than a 10% variation in the number of active lever presses and infusions; Supplementary Fig. 2), the rats were given a systemic injection of saline or 50 mg/kg TEMPOL 10 min before the beginning of nicotine self-administration on the test day. The dose of TEMPOL was selected based on the results of the locomotor activity test in the present study. Pretreatment with saline or TEMPOL was counterbalanced among the animals.

Food reinforcement test

To test the possibility that TEMPOL impairs general motor function, we performed a food reinforcement test as described previously^{6,7}. The rats were food restricted to maintain 85% of their initial body weight and trained to press a lever to receive 45 mg of food pellets (FR1 schedule, TO: 20 s) during a 1 h daily session. When the rats achieved a stable pattern of active lever pressing to earn food pellets (less than 10% variation in the total number of active lever presses and earned food pellets for 3 consecutive days), saline or 50 mg/kg TEMPOL was injected 10 min before the beginning of the food reinforcement test. Active lever presses resulted in the delivery of 45 mg of food pellet followed by a 20-s TO period. During the TO period, active lever-pressing responses were recorded, but did not result in the delivery of food pellets. Inactive lever presses were also recorded but had no consequence.

Brain slice preparation and fast scan cyclic voltammetry recordings

In a separate group, the rats received saline or nicotine (0.4 mg/kg, s.c.) for 6 days. On day 7, brain slices were obtained as described previously⁴². Briefly, rats were anesthetized (2–4% isoflurane), and the brain were extracted and glued onto a vibrotome cutting stage (Leica VT1200S, Leica Biosystems, Deer Park, IL). Brains were sectioned in room temperature artificial cerebral spinal fluid (ACSF; in mM: 126 NaCl, 2.5 KCl, 1.2 NaH₂PO₄, 21.4 NaHCO₃, 11 glucose, 1.2 MgCl₂, 2.4 CaCl₂) and bubbled with 95% O₂/5% CO₂. Coronal NAc slices (220 µm thick) were placed in an incubation chamber containing ACSF perfused with 95% O₂/5% CO₂ for at least 30 min. After 30 min, brain slices were then placed in a recording tissue chamber with ACSF continuously flowing at physiological temperatures (~37 °C).

For voltammetry recordings, a 7.0 µm diameter carbon fiber was inserted into borosilicate capillary tubing (1.0 mm i.d.; A-M Systems, Sequim, WA) under negative pressure and subsequently pulled on a vertical pipette puller (Narishige, East Meadow, NY). The carbon fiber electrode (CFE) was then cut under microscopic control with 70–100 µm of bare fiber protruding from the end of the glass micropipette. The CFE was backfilled with 3 M KCl. Dopamine release was evoked every 2 min by a 4-ms pulse (300 µA, Isoflex). The electrode potential was linearly scanned as a triangular waveform from –0.4 to 1.2 V and back to –0.4 V vs Ag/AgCl using a scan rate of 400 V/s. Cyclic voltammograms were recorded at the carbon fiber electrode every 100 ms (i.e., 10 Hz) by means of a ChemClamp voltage-clamp amplifier (Dagan Corporation, Minneapolis, MN) or customized potentiostats. Voltammetry recordings were performed and analyzed using LabVIEW (National Instruments, Austin, TX)-based customized software [Demon Voltammetry,⁴³]. Dopamine release was monitored for a stabilization period lasting typically ~1 h. Once the stimulated DA response was stable for 4–5 successive collections and did not vary by more than 10%, baseline measurements were taken and recorded during bath application of either nicotine (100 nM) and/or TEMPOL (3 mM). Recordings were obtained for at least 15 min until the signal stabilized. Slices bathed in TEMPOL were then recorded for another 15 min with the addition of nicotine. The magnitude of electrically-evoked DA release (peak height) was measured for determining pharmacological effects. All FSCV data were analyzed using Demon Voltammetry and Analysis software written in LabVIEW (National Instruments, Austin, TX)⁴³.

Data analysis

Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA). The data are presented as the mean ± standard deviation (SD). For the locomotor activity test (Fig. 1B) and DCF-DA assay (Fig. 1D), the significance of the difference between the groups was determined by two-way analysis of variance (ANOVA). For the DHE assay, western blotting, nicotine self-administration study, and food reinforcement test, the significance of the difference between the groups was determined by a two-tailed unpaired *t* test. For the effect of PBN or TEMPOL on hyperlocomotor activity and FSCV experiments on day 7, the data were analyzed by one-way or two-way ANOVA, followed by testing using the Tukey's post hoc test. Statistical significance was set at *P* < 0.05 (* and #), *P* < 0.01 (** and ##), and *P* < 0.001 (***) and ###).

Data availability

The data will be made available from the corresponding author upon reasonable request.

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Author contributions

Jeon Kyung Oh: Methodology, data curation, visualization, and writing – original draft preparation. Jordan T Yorgason, Lauren Ford, Joel Woolley, and Eun Kyeong Lee: Methodology, data curation, visualization, and writing—original draft and review. Seung-Hoon Park and Byoung-Seok Lee—writing—review, funding acquisition. Jaerang Rho: writing—review. Eun Young Jang: investigation, conceptualization, supervision, visualization, funding acquisition, and writing – review & editing.

Declarations

Competing interests

The authors declare no competing interests.

Ethics approval and consent to participate

All procedures regarding the handling and use of animals in the present study were conducted as approved by the Institutional Animal Care and Use Committee (IACUC) of the Korea Institute of Toxicology (KIT; No. 2112–0049 and 2112–0055).

Additional information

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Correspondence and requests for materials should be addressed to E.Y.J.

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