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Involvement of reactive oxygen species in cocaine-taking behaviors in rats

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Abstract

Reactive oxygen species (ROS) have been implicated in the development of behavioral sensitization following repeated cocaine exposure. We hypothesized that increased ROS following cocaine exposure would act as signaling molecules in the mesolimbic dopamine (DA) system, which might play an important role in mediating the reinforcing effects of cocaine. The aim of this study was to evaluate cocaine enhancement of brain metabolic activity and the effects of ROS scavengers on cocaine self-administration behavior, cocaine-induced ROS production in the nucleus accumbens (NAc) and cocaine enhancement of DA release in the NAc. Metabolic neural activity monitored by temperature and oxidative stress were increased in NAc following cocaine exposure. Systemic administration of the ROS scavenger N-tert-butyl- α -phenylnitrone (PBN) or 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL), either pre- or post-treatment, significantly decreased cocaine self-administration without affecting food intake. Infusion of TEMPOL into the NAc inhibited cocaine self-administration. Increased oxidative stress was found mainly on neurons, but not astrocytes, microglia or oligodendrocytes, in NAc of rats self-administering cocaine. TEMPOL significantly attenuated cocaine-induced enhancement of DA release in the NAc, compared to saline controls. TEMPOL had no effect on the enhancement of DA release produced by the DA transporter inhibitor GBR12909. Taken together, these findings suggest that enhancement of ROS production in NAc neurons contributes to the reinforcing effect of cocaine.

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Authors Contribution

EYJ, SCC, MJY, RJF, SHK and NDS performed the experiments. EYJ and HYK drafted the manuscript. EYJ, YHR, BHL and KJK analyzed the data. HYK, CHY and SCS were responsible for the study concept and design and for edits to the manuscript. All authors critically reviewed content and approved final version for publication.

Keywords

Cocaine self-administration; NAc; reactive oxygen species; reinforcing effect; TEMPOL

INTRODUCTION

The mesocorticolimbic dopamine (DA) system originating in the ventral tegmental area (VTA) and projecting to the nucleus accumbens (NAc) and prefrontal cortex (PFC) plays a critical role in the reinforcing effects of cocaine (Koob *et al.* 1994). Cocaine has considerable addictive liability, as characterized by the pattern of compulsive self-administration at the expense of life-prolonging behaviors (Johanson & Fischman 1989). Cocaine's locomotor and reinforcing properties are mediated primarily by enhancement of extracellular DA levels via inhibition of the DA transporter (DAT) in several regions of the brain (Rocha *et al.* 1998). However, cocaine's peripheral sympathomimetic and sodium channel blocking effects have also been implicated in its reinforcing properties (Kiyatkin & Brown 2006; Steffensen *et al.* 2006). Both contingent and non-contingent cocaine administration are accompanied by increases in brain temperature, which is a reliable indicator of metabolic neural activation (Kiyatkin & Brown 2003, 2006). The enhanced metabolism can increase generation of reactive oxygen species (ROS) (Sohal & Allen 1985; Brookes *et al.* 2004). In support of this, increased levels of ROS such as lipid peroxide and hydrogen peroxide are found in the brain reward system including striatum and frontal cortex in rats treated with cocaine (Dietrich *et al.* 2005; Bashkatova *et al.* 2006). Most of the previous studies have emphasized the putative role of oxidative stress as a crucial mediator for cocaine-induced neuronal cell death in brain reward systems (Kovacic 2005b). Recently, several lines of studies suggest that oxidative stress of the brain reward system is associated with cocaine psychomotor responses (Numa *et al.* 2008; Uys *et al.* 2011). Treatment with the superoxide dismutase (SOD) mimetic 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL) can attenuate oxidative stress in the PFC and NAc as well as the development of behavioral sensitization induced by repeated exposure to cocaine (Numa *et al.* 2008). Uys *et al.* (2011) have demonstrated that increased oxidative stress following cocaine exposure contributes to cocaine-induced behaviors via modulation of glutathione redox status. Indeed, evidence is mounting that ROS may function as a cell signaling molecule which influences synaptic plasticity, intracellular signaling and neuromodulation (Kishida & Klann 2007; Kim *et al.* 2011). We and others have shown that ROS scavengers block long term potentiation (LTP) in brain (Knapp & Klann 2002) and spinal cord (Kim *et al.* 2011), and increased mitochondrial ROS regulate Ca^{2+} -dependent protein kinases, including protein kinase C (PKC), Ca^{2+} -calmodulin-dependent kinase II (CaMKII), protein kinase A (PKA) and extracellular signal-related kinase (ERK) (Hongpaisan, Winters & Andrews 2004; Kim *et al.* 2009, 2011), which are critical for synaptic plasticity involved in memory, pain and addiction (Hyman, Malenka & Nestler 2006; Kim *et al.* 2011). However, the role of ROS in the reinforcing effects of drugs of abuse, and cocaine-induced changes in DA transmission, needs to be elucidated.

Increased ROS following cocaine exposure may be involved in reward signaling in the mesolimbic DAergic pathway associated with the reinforcing effects of cocaine. To test this,

we evaluated, in rats, cocaine enhancement of brain temperature and ROS generation, the effects of ROS scavengers on cocaine self-administration, and cocaine enhancement of DA release in the NAc. We also characterized the cellular source of ROS and the effects of ROS scavengers on ROS production in the NAc.

MATERIALS AND METHODS

Animal subject

Male Sprague-Dawley rats (Daehan Animal, Seoul, Korea; Charles River Laboratory, Hollister, CA, USA) weighing 280–350 g were used in all experiments. They were individually housed in a temperature (21–23°C) and humidity-controlled (55–65%) environment on a 12-hour light-dark cycle with *ad libitum* food and water. All procedures were approved by the Institutional Animal Care and Use Committees at Daegu Haany University and Brigham Young University. Each group consisted of five to eight rats, unless stated otherwise.

Drugs and chemicals

Cocaine hydrochloride (Macfarlan Smith Ltd., Edin-burgh, UK) was dissolved in saline and filtered through a syringe-mounted 0.45- μ m Millex-HA filter unit (Millipore, MO, USA) immediately before self-administration experiments. Lidocaine hydrochloride (Sigma, St. Louis, MO, USA) and GBR12909 (Tocris Bioscience, Ellisville, MO, USA) were also dissolved in filtered saline before intravenous (i.v.) injection. For systemic injection of ROS scavengers, PBN [a non-specific ROS scavenger (Dhainaut *et al.* 2000); Sigma] and TEMPOL [a SOD mimetic (Wilcox & Pearlman 2008); Sigma] were dissolved in physiological saline prior to use and administered intraperitoneally (i.p.) 10 minutes prior to the start of self-administration or i.v. injection of cocaine or GBR12929.

Measurement of NAc temperature

Temperature changes in brain following cocaine administration were monitored as described previously (Kiyatkin & Brown 2004) with slight modifications. In brief, animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and placed in a stereotaxic apparatus. An access hole was drilled through the skull over the NAc (stereotaxic coordinates: +1.7 mm anterior, +0.8 mm lateral to bregma, –7 mm from skull) and a thermocouple needle microprobe (NJ-07013, WPI, Sarasota, FL, USA) was slowly lowered to the desired target depth. A second thermocouple probe was inserted in the rectum to measure body temperature. Temperature was recorded every 5 minutes for 150 minutes using a digital thermometer (DT-3891F, CEM, Shenzhen, China). Measurements were conducted at room temperature (22–24°C) and during body warming by a heating pad set at 36.5°C. After basal recording of 20–30 minutes, cocaine (15 mg/kg, i.p.) was injected and the recordings continued for another 2 hours.

Self-administration training and surgical implantation of i.v. catheters

To facilitate the acquisition of operant responding in self-administration chambers (Med Associates, St. Albans, VT, USA), rats were subjected to mild food restriction and trained to lever-press for 45 mg food pellets (Bio-Serv, Frenchtown, NJ, USA) on a fixed ratio (FR-1)

reinforcement schedule until criterion (100 food pellets for 3 consecutive days) had been achieved in 3-hour daily sessions. After the operant training, the rats were allowed free access to food and water for at least 2 days and then implanted with chronic i.v. jugular catheters. The catheter was passed subcutaneously to exit the back of animal through 22 gauge tubing embedded in dental cement and secured with surgical mesh (Ethicon Inc., Somerville, NJ, USA). The catheters were flushed once daily with 0.2 ml of heparinized (30 U/ml) saline to prevent clotting and gentamicin sulfate (0.33 mg/ml) to prevent infection.

Cocaine self-administration procedure

After 7 days of recovery from surgery, the rats began cocaine self-administration on a continuous FR-1 reinforcement schedule during daily 2-hour test sessions for 6–7 days/week. Each active lever press caused a 0.1 ml infusion of 0.25 mg/kg over 5 seconds. During the cocaine infusion, a cue light above the active lever was illuminated, and the house light was extinguished. Each infusion was followed by an additional 10 seconds timeout, when the house light remained off. Responses on the active lever during the time-out did not result in cocaine infusion. Inactive lever press responses were recorded, but had no programmed consequence. Following the establishment of stable baseline in cocaine-reinforced responding (three consecutive sessions with less than a 10% variation), the rats were given i.p. injections of ROS scavengers, PBN (0, 50 and 100 mg/kg) or TEMPOL (0, 50 and 100 mg/kg), 10 minutes before (pre-treatment) or 60 minutes (post-TEMPOL) after initiation of self-administration, which continued up to 2 hours after injection. These doses of PBN and TEMPOL were selected based on previous studies (Kim *et al.* 2004; Numa *et al.* 2008). Drug treatments were counterbalanced among animals.

Guide cannula implantation for microinjection of TEMPOL into the NAc

Following the test of i.p. PBN and TEMPOL effects in cocaine-taking behavior (about 4–5 weeks after jugular vein catheterization), the rats were subjected to a second surgery for cannula implantation into NAc. Under pentobarbital anesthesia (50 mg/kg, i.p.), each animal was positioned in a stereotaxic apparatus and implanted bilaterally with 26 gauge stainless steel dual-guide cannulae (Plastics One, Roanoke, VA, USA) into the NAc (stereotaxic coordinates: +1.7 mm anterior, ± 0.8 mm lateral to bregma, –6.0 mm from skull). After at least 3 days of recovery, the rats were trained to self-administer cocaine. After establishment of a stable responding, the rats were given a microinjection of either TEMPOL (400 μ g in 1 μ l saline, 1 μ l/side) or saline (1 μ l/side) bilaterally into the NAc over a 10-minute period (0.1 μ l/minute) through the implanted guide cannula before beginning cocaine self-administration.

Food reinforcement test

To examine the influence of TEMPOL on food-reinforced responding, the responses on each lever were measured in food-trained rats. In brief, rats were food restricted to maintain 85% of initial body weight and trained to lever-press for 45 mg food pellets (FR-1 schedule; 1 minute time-out) during 2-hour daily sessions. When rats had acquired a stable pattern of lever responding (less than 10% variation in total number of food pellets for 3 consecutive days), TEMPOL (100 mg/kg) or saline was injected i.p. 10 minutes prior to the food

reinforcement test and the total number of active and inactive lever responses with food pellets delivery were measured during the 2-hour session.

Immunohistochemistry for 8-OHG, GFAP, NeuN, Iba-1 and NG2

To identify the level of oxidative stress and the type of ROS-producing cells in the NAc, the brains of cocaine self-administered or acute cocaine-treated rats were removed under pentobarbital anesthesia (80 mg/kg, i.p.), post-fixed with 4% paraformaldehyde, cryoprotected in 30% sucrose, and cryosectioned into 30 μ m in slices. The sections were incubated with primary antibody for anti-mouse 8-hydroxyguanosine (8-OHG; a cellular marker of oxidative damage; 1:400, Abcam, MA, USA), anti-rabbit GFAP (an astrocyte marker, 1:2000, Millipore, MA, USA), anti-rabbit NeuN (a neuronal marker, 1:2000, Millipore, MA, USA), anti-rabbit Iba-1 (a microglial marker; 1:2000, Wako, Japan) and anti-rabbit NG2 (an oligodendrocyte marker; 1:2000, Chemicon, MA, USA). The sections were then processed with secondary antibodies, donkey anti-mouse Alexa Fluor 488 (green; 1:200) and donkey anti-rabbit Alexa Fluor 594 (red; 1:200, Invitrogen, Gland Island, NY, USA). Tissue sections were imaged with a 20X objective using a confocal fluorescent imaging system (Leica TSC-SP5-II, Buffalo Grove, IL, USA) attached to a DM 6000-CFS upright microscope. Furthermore, the fluorescence intensities (FIs) of 8-OHG in each section were estimated by computerized densitometry (i-solution, IMT, Daejeon, Korea).

Fast scan cyclic voltammetry

Phasic DA release in the NAc was measured by fast scan cyclic voltammetry (FSCV) *in vivo*. A 7.0 μ m diameter carbon fiber was inserted into borosilicate glass capillary tubing (1.2 mm o.d., A-M Systems, Sequim, WA, USA) under negative pressure and subsequently pulled on a vertical pipette puller (Narishige, East Meadow, NY, USA). The carbon fiber electrode (CFE) was then cut under microscopic control with 150 μ m of bare fiber protruding from the end of the glass micropipette. The CFE was back-filled with 3 M KCl. The electrode potential was linearly scanned with a triangular waveform from -0.4 to 1.2 V and back to -0.4 V versus Ag/AgCl using a scan rate of 400 V/second. Cyclic voltammograms were recorded at the CFE every 100 milliseconds (i.e. 10 Hz) by means of a ChemClamp voltage clamp amplifier (Dagan Corporation, Minneapolis, MN, USA). Voltammetric recordings were performed and analyzed using LabVIEW-based (National Instruments, Austin, TX, USA) customized software [Demon Voltammetry (Yorgason, Espana & Jones 2011)]. For *in vivo* voltammetric recordings of DA signals, rats were anesthetized with isoflurane and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). Bipolar, coated stainless steel electrodes were stereotaxically implanted into the medial forebrain bundle (MFB; -2.5 mm posterior, $+1.9$ mm lateral from bregma, -8.0 to -8.5 mm from skull), and a capillary glass-based CFE in the NAc core ($+1.6$ mm anterior, $+1.9$ mm lateral from bregma, -6.5 to -8.0 mm from skull). The MFB was stimulated with 60 monophasic pulses at 60 Hz (4 milliseconds pulse width) at 2-minute intervals. DA levels were monitored for a stabilization period typically lasting 20 minutes to 1 hour. Once the stimulated DA response was stable for five successive collections, and did not vary by more than 10%, baseline measurements were taken for control and drug treatment. After baseline was established, saline (5 ml/kg) and TEMPOL (50 or 100 mg/kg) were injected 10 minutes prior to injections of cocaine (0.1, 0.25, 0.5 and 1.0 mg/kg, i.v.) or

GBR12909 (a DAT inhibitor, 1.0 mg/kg, i.v.). In order to test the effects of TEMPOL on DA release, four different doses of cocaine were sequentially administered i.v. at 30-minute intervals. EC₅₀ values were then extrapolated from the dose–response curve. DA signals were evoked at 2-minute intervals for 30 minutes post injection. The area of cocaine effect was determined with a 30-minute rectangular integration on the peak amplitude time series plots. Extracellular concentrations of DA were calibrated by comparing the current at the peak oxidation potential for DA with a known concentration of DA (3 μ M).

Data analysis

All data are presented as mean $\pm$ SEM (standard error of the mean) and analyzed by one- or two-way repeated-measurement analysis of variance (ANOVA), followed by *post hoc* testing using the Tukey method or Student's *t*-test, where appropriate. Statistical significance was considered at $P < 0.05$. All statistics were calculated with IBM SPSS Statistics 21 (Armonk, NY, USA).

RESULTS

Effect of acute cocaine on brain temperature

To determine if cocaine exposure enhances brain metabolic activity that might promote formation of ROS (Brookes *et al.* 2004), the temperature changes in NAc following acute cocaine were evaluated. Acute injection of cocaine (15 mg/kg, i.p.; $n = 5$) gradually increased temperature up to $0.99 \pm 0.27^\circ\text{C}$ and $0.11 \pm 0.27^\circ\text{C}$ in NAc and body over 120 minutes after injection, respectively (Fig. 1a). Significant elevations in brain-body differentials were seen 40 minutes after cocaine injection, compared to the values before injection (Fig. 1b; Tukey posttests after ANOVA, $*P < 0.05$), suggesting enhanced metabolic brain activity by cocaine.

Effects of PBN and TEMPOL on cocaine self-administration

To explore the involvement of ROS in cocaine reinforcement, the effect of a non-specific ROS scavenger PBN on cocaine self-administration was tested. Following establishment of baseline, rats were given an i.p. injection of PBN (0, 50 and 100 mg/kg) 10 minutes before cocaine self-administration (pre-treatment) and monitored up to 2 hours after injection. During the 2-hour test session, the active lever presses following systemic injection of PBN decreased in a dose-dependent manner (Fig. 2a). A slight inhibition of active lever responding at a dose of 50 mg/kg was observed after injection, while the significant inhibition at a 100 mg/kg dose of PBN was sustained up to 2 hours (Tukey posttests after ANOVA; $F_{(2,16)} = 5.808$, $P < 0.05$ versus saline controls, $n = 5\text{--}6$ each). In this study, it is noted that four of the six animals injected with 100 mg/kg PBN showed long pauses of approximately 60 minutes in cocaine responding (Fig. 2c and d). On the other hand, the numbers of inactive lever presses were minimal across tests and showed no differences among groups (Fig. 2b).

To verify the involvement of superoxide, the precursor of most ROS (Turrens 2003), in mediating cocaine reinforcement, we repeated the experiment by using the SOD mimetic TEMPOL. When TEMPOL was injected 10 minutes prior to the beginning of cocaine self-

administration, the numbers of active lever presses were reduced dose dependently, with significance at 100 mg/kg TEMPOL (Tukey posttests after ANOVA; $F_{(2,16)} = 4.538$, $P < 0.05$ versus saline controls; TEMPOL 28.6 ± 9.3 versus saline 65.8 ± 4.5 , $n = 5-7$; Fig. 3a and d), while inactive lever presses were not altered (Fig. 3b). In this study, of the seven animals treated with 100 mg/kg TEMPOL, four animals showed long pauses in self-administration for approximately 60 minutes after injection, while the other three animals pressed the active lever less than 8 times per 10 minutes (Fig. 3c and d). To further evaluate the mediation of superoxide radicals during the maintenance phase of cocaine self-administration, 100 mg/kg TEMPOL was administered during self-administration (60 minutes after the start of self-administration; post-TEMPOL) and the lever presses were compared before and after i.p. injection of TEMPOL. Post-TEMPOL (100 mg/kg, i.p.) resulted in a decrease of active lever presses, compared to the saline-treated group (TEMPOL 24.6 ± 2.8 versus saline 34.2 ± 1.6 ; Student's t -test, $F_{(1,9)} = 8.9$, $P = 0.045$ versus saline controls, $n = 5$ each; post-drugs in Fig. 4a) without affecting inactive lever presses in each group (post-drugs in Fig. 4b). The significant suppression of active lever responding was found 10 minutes after injection of TEMPOL (Student's t -test; $F_{(1,9)} = 21.5122$, $P = 0.002$) and recovered back to the saline-treated level 20 minutes after injection of TEMPOL (Fig. 4d), suggesting the critical involvement of ROS, especially superoxide radicals, in cocaine reinforcement. Since the NAc is implicated in the reinforcing effects of cocaine (Pierce & Kumaresan 2006), the effect of NAc microinjection of TEMPOL on cocaine self-administration was further investigated. As the maximum solubility of TEMPOL was 400 μg in 1 μl PEG, doses of 400, 300, 200 and 100 μg TEMPOL were injected bilaterally into the NAc at a volume of 1 μl in cocaine self-administering rats (1–3 rats per each) and the number of active/inactive lever presses was evaluated. Furthermore, potential side effects including seizure, abnormal gait and slowed movement were also examined. Because the inhibition of self-administration by TEMPOL tended to show dose dependence without any side effects, a maximal dose of 400 μg was chosen for NAc microinjection (data not shown). When TEMPOL (400 $\mu\text{g}/\text{site}$, 1 μl) was delivered to the bilateral NAc via the guide cannula prior to cocaine self-administration, active lever pressing, but not inactive lever pressing, up to 60 minutes after injection was significantly reduced, compared to saline-treated rats (Student's t -test, $F_{(1,13)} = 6.5703$, $P = 0.042$ versus saline controls, $n = 7$ each; Fig. 5a, b and d). As observed with systemic injection of TEMPOL, the pause in cocaine self-administration pattern was also seen following NAc injection of TEMPOL (Fig. 5c). To test if TEMPOL affects generalized behavioral responses, the effects of TEMPOL on food self-administration were explored using cocaine-naïve rats. As shown in Fig. 6, systemic injection of TEMPOL (100 mg/kg) did not alter food reinforcement, compared to the saline controls. Taken together, these results indicate that scavenging ROS with TEMPOL specifically reduced the reinforcing effects of cocaine self-administration without disrupting operant performance or food reinforcement.

Identification of the types of cells producing ROS

To determine whether ROS production is increased in the NAc following chronic cocaine self-administration, tissues were immunostained with an antibody against 8-hydroxyguanine (8-OHG), a cellular marker of oxidative stress (Kim *et al.* 2010). There were very few 8-OHG+ cells in the NAc of control rats, while cocaine self-administering rats showed a

marked increase in numbers of 8-OHG+ cells (green) and about a fourfold increase relative to control in the average fluorescence intensity (FI) (Student's *t*-test, $P = 0.001$ versus saline controls, $n = 3-4$; Fig. 7a–c). To further identify the types of cells producing ROS, double immunofluorescence labeling was performed using cell type markers to identify neurons (NeuN; red), microglia (Iba-1; red), oligodendrocytes (NG2; red) and astrocytes (GFAP; red). Most 8-OHG+ cells (green signal) were double-labeled with NeuN-positive neurons in the NAc (red nucleus with green circle in Fig. 7d), but not microglia, astrocytes or oligodendrocytes (Fig. 7e–g). The data indicate that increased ROS production in the NAc of cocaine self-administered rats occurs mainly in neurons.

To determine whether ROS scavengers would be able to decrease ROS levels in the NAc, rats ($n = 22$) were randomized into four groups: (1) normal ($n = 5$); (2) cocaine ($n = 5$); (3) TEMPOL ($n = 6$); and (4) PBN ($n = 6$). In TEMPOL and PBN groups, rats were given either TEMPOL (100 mg/kg, i.p.) or PBN (100 mg/kg, i.p.) 10 minutes before an acute injection of cocaine (15 mg/kg, i.p.). Forty minutes after cocaine injection, the brains were taken out and processed for 8-OHG immunohistochemistry in the NAc. Acute cocaine-treated rats (Fig. 8b) showed a significant increase relative to control (Fig. 8a) in the average FI in NAc, while the increase of 8-OHG expression following cocaine injection was suppressed in PBN (Fig. 8c) or TEMPOL (Fig. 8d) groups, indicating TEMPOL or PBN effectively reduces ROS levels produced by cocaine exposure (Fig. 8e).

Electrically stimulated DA responses

We also evaluated the effects of TEMPOL on cocaine enhancement of DA release using FSCV. Intravenous administration of cocaine at 30-minute intervals consistently and reproducibly enhanced DA release in the NAc core across the dose levels tested (0.1–1.0 mg/kg; Fig. 9a). There was no acute tolerance or sensitization to a repeated dose of cocaine at the same dose level (data not shown). Intraperitoneal administration of 100 mg/kg TEMPOL reduced cocaine enhancement of DA release (Fig. 9b) compared to saline controls. TEMPOL significantly reduced both the peak amplitude (Fig. 9c) and area (Fig. 9d) of cocaine enhancement of DA release in the NAc core *in vivo* at the 100 mg/kg dose level (Amplitude: Tukey posttests after ANOVA; $F_{(2,19)} = 4.520$, $P = 0.02$ at 0.25 mg/kg, $F_{(2,18)} = 5.225$, $P = 0.014$ at 0.5 mg/kg and $F_{(2,16)} = 4.934$, $P = 0.018$ at 1.0 mg/kg dose of cocaine versus saline controls, $n = 5-7$ each. Area: $F_{(2,16)} = 4.011$, $P = 0.033$ at 1.0 mg/kg dose of cocaine versus saline controls; $n = 5-7$ each), but not at the 50 mg/kg dose level. To evaluate if cocaine's sodium channel blocking properties might contribute to its DA release enhancing effects, we evaluated the effects of i.v. lidocaine (1 mg/kg), a dose that blocks VTA gamma-aminobutyric acid (GABA) neuron spiking (Steffensen *et al.* 2008). Lidocaine had no effect on DA release (Student's *t*-test, $P = 0.496$ versus saline alone, $n = 4$; data not shown). To evaluate if TEMPOL was lowering cocaine enhancement of DA release via action on the DAT, we evaluated the effects of TEMPOL on DA release produced by the DAT inhibitor GBR12909 (Fig. 10). Compared to saline controls, TEMPOL did not significantly affect the marked and prolonged enhancement of DA release produced by GBR12909 (Student's *t*-test, $P = 0.363$ versus saline controls, $n = 6-7$).

DISCUSSION

Systemic injection of PBN and TEMPOL attenuated cocaine self-administration without affecting food intake or responding, suggesting that cocaine's reinforcing effects are mediated, at least in part, via activation of ROS. Injection of TEMPOL into the NAc during cocaine self-administration produced an immediate and dramatic, but transient, reduction of cocaine responding, and increased production of ROS in NAc neurons was also found in the rats self-administering cocaine, indicating the critical role of ROS formation in the mesolimbic DA system.

Although ROS are normally produced through mitochondrial respiration and activation of intracellular enzymes in living cells, it is known that the increase of metabolic activity under certain conditions promotes ROS generation. Interest in the role of ROS as crucial signaling molecules in various pathological conditions is growing (Brookes *et al.* 2004). Of particular relevance to addiction, acute or repeated cocaine administration increases ROS in DA terminal regions in the NAc, PFC and striatum (Dietrich *et al.* 2005; Bashkatova *et al.* 2006).

Cocaine increased temperature (Fig. 1) and ROS production (Fig. 8) in NAc suggest that the enhanced brain metabolic activity following cocaine exposure is accompanied by increased production of ROS. The increased ROS following acute and chronic cocaine exposure are known to be toxic mediators that produce functional and structural damage to DAergic systems (Pomierny-Chamiolo *et al.* 2013). Recently, novel concepts have emerged that increased ROS at low concentrations in the brain reward system may be correlated with cocaine psychomotor responses (Kovacic 2005a,b; Numa *et al.* 2011). In support of this, Numa *et al.* (2008) reported that in rats exposed to passive cocaine, lowering oxidative damage with TEMPOL in NAc and PFC can prevent cocaine-induced behavioral sensitization.

In the present study, cocaine-reinforced responding was effectively reversed or prevented by systemic PBN, a non-specific ROS scavenger (Dhainaut *et al.* 2000), and TEMPOL, a superoxide-specific scavenger (Wilcox & Pearlman 2008), as well as NAc microinjection of TEMPOL. This evidence supports a crucial role for ROS, especially superoxide radicals, in the reinforcing effects of cocaine. Superoxide radicals can be produced from intra-cellular compartments in response to increased DA, or cocaine metabolism and its oxidative metabolites (Kovacic 2005a,b; Bashkatova *et al.* 2006; Bhattacharya *et al.* 2009). Immunohistochemistry in the NAc of rats self-administering cocaine revealed that neurons were the main cellular source of ROS, indicating that ROS produced by cocaine exposure is localized to intracellular compartments in neurons. Among intracellular organelles, mitochondria are a major source of intracellular ROS, primarily the superoxide radicals generated by impaired respiratory chains (Brookes *et al.* 2004). Impaired mitochondrial respiration and increased mitochondrial ROS generation have been found in animals treated with cocaine (Leon-Velarde, Huicho & Monge 1992; Boess *et al.* 2000). Increased ROS generation in NAc neurons and reduced cocaine self-administration by intra-NAc injection of the SOD mimetic TEMPOL suggests that cocaine reinforcement is associated with increased production of mitochondrial superoxide in neurons of the NAc. We, and others, have shown that increased mitochondrial superoxide in neurons enhances activation of

intracellular signaling cascades such as PKA, PKC, CaMKII and ERK (Hongpaisan *et al.* 2004; Kim *et al.* 2011). Upregulation of these protein kinases in the NAc is also known to be essential for the reinforcement properties of cocaine (Lee & Messing 2008). It is possible that mitochondrial superoxide generation in NAc neurons during cocaine self-administration upregulates protein kinases, and thus increases cocaine reinforcement. The mechanism linking increased ROS in brain reward circuits to cocaine reinforcement needs to be further explored.

ROS scavengers also suppressed cocaine self-administration without altering inactive lever responses or food self-administration, indicating specific reduction of cocaine reinforcement by ROS scavengers, rather than non-specific actions on response performance. Reduction of cocaine self-administration by ROS scavengers, PBN and TEMPOL, was accounted for by periods of non-responding at the beginning of self-administration, or long inter-infusion pauses, rather than decreased rate of responding during the test sessions. It has been suggested that animals tend to show long pauses after self-administration of a high dose of cocaine, treatment with GABA agonists (e.g. baclofen), motor depression (e.g. sedation or anesthesia) or adverse side effects (Brebner, Phelan & Roberts 2000b), although the underlying mechanisms are unclear. The doses of PBN and TEMPOL tested in the present study have been shown to have no adverse effects, such as sedation, anesthesia and motor deficit in other studies (Kim *et al.* 2004; Numa *et al.* 2008). GABA_B agonists such as baclofen are reported to produce long pauses in cocaine self-administration patterns (Brebner, Phelan & Roberts 2000a), which is similar to the findings shown by systemic ROS scavengers in the present study. Our previous study showed that excessive level of ROS in spinal dorsal horn neurons selectively inhibited release of GABA without affecting glutamatergic transmission or glycine-mediated inhibitory transmission, and the reduced GABA release recovered when ROS were scavenged with PBN (Yowtak *et al.* 2011). Cocaine has been shown to inhibit NAc GABAergic neuronal firings or GABA transmission during cocaine self-administration (Peoples *et al.* 2004; Li *et al.* 2009). In general, GABA neurons are believed by many to modulate DA neurons via GABA_B receptors expressed on VTA DA neurons and GABA_B receptors play a modulatory role in the reinforcing effects of cocaine (Filip & Frankowska 2008). Taken together, ROS scavengers may enhance GABA transmission in the NAc which reverses the inhibition of NAc GABA release during cocaine self-administration, thereby producing long pauses in self-administration through activation of GABA receptors, similar to the GABA_B agonist baclofen.

Enhanced DA transmission is associated with the behavioral response to cocaine (Vetulani 2001). It was suggested that excessive DA oxidation in the brain results in a massive production of compounds such as DA-quinones and 6-hydroxyDA which enhances oxidative damage to proteins, lipids and DNA (Smythies & Galzigna 1998). We found that cocaine enhanced DA release *in vivo* with an EC₅₀ of approximately 0.25 mg/kg, which is similar to what others have reported (Li *et al.* 2013). This is consistent with its rewarding concentrations. There was no change in the amplitude or area of cocaine enhancement of DA release with repeated administration of the same dose level of cocaine, suggesting that no tolerance or desensitization accrued to multiple doses at the dosing intervals tested. Cocaine enhancement of DA release was significantly reduced by the ROS scavenger TEMPOL, suggesting that some of cocaine's activating properties on DA release are mediated by

oxidation at terminals in the NAc. However, other high-affinity targets for cocaine include voltage-sensitive sodium channels (VSSCs) (Gawin & Ellinwood 1988). It is well established that local anesthetics, including cocaine, are use-dependent blockers of VSSCs (O'Leary & Chahine 2002). Although cocaine's affinity for VSSCs is lower than that for monoamine transporters [$IC_{50} = 14\text{--}17\text{ }\mu\text{M}$ (Gifford & Johnson 1992)], peak brain (2–6 minutes) cocaine levels of 2, 6, 9 and 26 μM can be obtained from single i.v. reinforcing doses of 0.1, 0.25, 0.5 and 1 mg/kg, respectively (Fowler *et al.* 1998), similar to the levels used in this study. The reinforcing properties of cocaine might involve combined or opposing effects at both the DAT and VSSCs (Kiyatkin & Brown 2006). Indeed, we have previously reported that VTA GABA neurons, which presumably regulate DA neuron activity, are sensitive to cocaine's VSSC blocking properties at levels of cocaine that are self-administered (Steffensen *et al.* 2008). In order to rule out a role for VSSCs in cocaine enhancement of DA release, we tested the effects of the VSSC blocker lidocaine, which does not have any DAT inhibiting properties. Lidocaine was without effects on DA release. Furthermore, in order to determine if TEMPOL was affecting cocaine's DAT inhibiting properties, we tested the effects of the specific DAT inhibitor GBR12909. While GBR12909 markedly enhanced DA release, TEMPOL did not affect the ability of GBR12909 to enhance DA release. These findings suggest that TEMPOL is reducing cocaine enhancement of DA release via its ROS effects, and not via an action on the DAT or VSSCs.

In conclusion, scavenging ROS with PBN or TEMPOL reduced cocaine self-administration without disrupting food intake. Cocaine self-administration was suppressed by intra-NAc infusion of TEMPOL and an increase in oxidative stress was found in NAc neurons. TEMPOL also decreased cocaine-induced extracellular DA level in the NAc without involving the DAT or VSSCs. Taken together, these findings provide compelling evidence that increased ROS in the NAc contributes to the reinforcing effects of cocaine.

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Figure 1.

Cocaine administration increased brain temperature, an indicator of neural metabolic activity. (a) Time course analysis of absolute brain (NAc) and body (rectal) temperature in cocaine-treated rats. When administered as a single injection of cocaine (15 mg/kg, i.p.), brain and body temperature gradually increased over 2 hours. This increase was more pronounced in the brain than in the body. (b) Time course analysis of brain-body temperature differences every 5 minutes. $n = 5$ per group. One-way repeated ANOVA test followed by Tukey posttests, $*P < 0.05$

Figure 2.

Pretreatment with the non-specific ROS scavenger PBN prevented cocaine self-administration (SA). Rats were given a non-specific ROS scavenger PBN (50 or 100 mg/kg, i.p.) or saline 10 minutes prior to cocaine SA. (a, b) Total number of responses on active lever (a) and inactive lever (b) in 2-hour sessions of cocaine SA. PBN at a dose of 100 mg/kg significantly reduced active lever responding, but not inactive lever responding. (c) A representative example of cocaine SA pattern for 60 minutes after injection of PBN or saline. Hash marks represent active lever responses that infuse cocaine. A long pause in hash marks after PBN injection is seen. (d) Time course analysis of active lever responses during

2-hour sessions after systemic injection of PBN. $*P < 0.05$, compared to saline controls; $n = 5-7$ per group. The y-axes show mean number of active lever presses for 2 hours (a; active lever), inactive lever presses for 2 hours (b; inactive lever) and active lever presses every 10 minutes (d; active lever) during cocaine SA, respectively

Figure 3.

Pretreatment with the SOD mimetic TEMPOL attenuated cocaine self-administration (SA). TEMPOL (a superoxide dismutase mimetic, 50 or 100 mg/kg, i.p.) or saline was injected 10 minutes before each test session. (a, b) Total number of responses on active lever (a) and inactive lever (b) in 2-hour sessions of cocaine SA. Systemic injection of TEMPOL at a dose of 100 mg/kg significantly attenuated active lever responding, compared to saline controls. (c) A representative example of cocaine SA pattern for 60 minutes after injection of TEMPOL or saline. Hash marks represent active lever responses that infuse cocaine. Note the long pause in hash marks after TEMPOL injection. (d) Time course analysis of active

lever responses during 2-hour sessions after systemic injection of TEMPOL. $*P < 0.05$, compared to saline group; $n = 5-7$ per group. The y-axes show mean number of active lever presses for 2 hours (a; active lever), inactive lever presses for 2 hours (b; inactive lever) and active lever presses every 10 minutes (d; active lever) during cocaine SA, respectively

Figure 4.

Post-treatment of TEMPOL reversed cocaine self-administration (SA). TEMPOL (100 mg/kg) or saline was injected i.p. 1 hour after start of cocaine SA. (a, b) Number of responses on active lever (a) and inactive lever (b) per hour in 2-hour sessions of cocaine. Active lever presses were significantly reduced after TEMPOL injection (61–120 minutes), compared to saline controls. (c) A representative example of cocaine SA pattern for 60 minutes after injection of TEMPOL or saline. Hash marks represent active lever responses that infuse cocaine. (d) Time course analysis of active lever responses during 2-hour sessions. Injection of TEMPOL (arrow on 60 minutes) during cocaine SA produced an immediate, but transient, reversal of cocaine-taking behaviors. * $P < 0.05$, compared to saline group; $n = 5$

per group. The y-axes show mean number of active lever presses for 60 minutes (a; active lever), inactive lever presses for 60 minutes (b; inactive lever) and active lever presses every 10 minutes (d; active lever) during cocaine SA, respectively

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Figure 5.

Microinjection of TEMPOL into the nucleus accumbens suppressed cocaine self-administration (SA). Rats received microinjection of TEMPOL (400 μ g) into the nucleus accumbens before start of test sessions. (a, b) Total number of responses on active lever (a) and inactive lever (b) in 2-hour sessions of cocaine. (c) A representative example of cocaine SA pattern for 60 minutes after injection of TEMPOL or saline into the NAc. Hash marks represent active lever responses that infuse cocaine. (d) Time course analysis of active lever responses during 2-hour sessions after systemic injection of TEMPOL. * $P < 0.05$, compared to saline controls; $n = 7-8$ per group. The y-axes show mean number of active lever presses

for 60 minutes (a; active lever), inactive lever presses for 60 minutes (b; inactive lever) and active lever presses every 10 minutes (d; active lever) during cocaine SA, respectively

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Figure 6.

TEMPOL did not affect food reinforcement in cocaine-naïve rats. Figure reveals total number of responses on active lever (left) and inactive lever (right) in 2-hour sessions. When TEMPOL (100 mg/kg) or saline was injected i.p. before beginning of food self-administration, there were no significant differences between saline and TEMPOL groups in total number of responses on active lever and inactive lever in 2-hour sessions. $n = 6$ per group. The 'Food pellets' on y-axes represents mean number of lever presses for food pellets for 2 hours

Figure 7.

Increased oxidative stress in neurons of the nucleus accumbens in rats self-administering cocaine. (a, b) Immunohistochemical staining of 8-hydroxyguanine (8-OHG; green), an oxidative stress marker, in the NAc of cocaine self-administering (cocaine SA; b) or normal (normal; a) rats. (c) A significant increase in fluorescence intensity of 8-OHG (8-OHG FI on y-axis) was found in the NAc of cocaine self-administering rats (b), compared to that of normal rats (a; $*P < 0.05$). (d–g) These images show double immunostaining in the NAc for 8-OHG (green) with NeuN (neuron; red, d), GFAP (astrocytes; red, e), Iba-1 (microglia; red, f) and NG2 (oligodendrocytes; red, g). Most 8-OHG positive cells were double-labeled with NeuN (d). Note double-labeled cells of red nucleus (NeuN) surrounded by green cytoplasm (8-OHG) in neurons (d), but not other cells (e–g). Bar = 100 μm

Figure 8.

The ROS scavengers, PBN and TEMPOL, reduced increased oxidative stress by acute cocaine in the NAc. (a–d) These images show immunohistochemical staining of 8-hydroxyguanine (8-OHG), an oxidative stress marker, in the NAc. While saline did not elicit expression of 8-OHG (a), it significantly increased expression of 8-OHG (8-OHG FI) in the NAc of acute cocaine group (b). Both PBN (100 mg/kg, i.p.; c) and TEMPOL (100 mg/kg, i.p.; d) reduced expression of 8-OHG in the NAc by acute cocaine. (e) Expression of 8-OHG was enhanced significantly by cocaine and reduced by both PBN and TEMPOL. Bar = 100 μ m, $n = 5$ –6 per group. # $P < 0.05$ versus saline controls; * $P < 0.05$ versus cocaine groups

Figure 9.

TEMPOL reduced cocaine enhancement of DA release in the nucleus accumbens, as measured by FSCV *in vivo*. (a) Representative time course of DA release in the NAc core in an anesthetized rat demonstrating the marked enhancement of DA release by i.v. cocaine (0.1–1.0 mg/kg). Inset shows superimposed DA FSCV signals obtained with saline and 2 minutes after administration of cocaine. Calibration bars are nA and seconds. (b) Representative time course of DA release in the NAc in an anesthetized rat demonstrating reduction of DA release produced by i.v. cocaine following i.p. injection of 100 mg/kg TEMPOL. Inset shows superimposed DA FSCV signals obtained 2 minutes after administration of cocaine in the presence of TEMPOL. (c) Summary of saline and TEMPOL effects (50 and 100 mg/kg) on the peak amplitude of DA release in the NAc core evoked by 0.1, 0.25, 0.5 and 1.0 mg/kg doses of cocaine. TEMPOL (100 mg/kg) significantly decreased cocaine enhancement of DA release amplitude. (d) Summary of saline and TEMPOL effects on the area of cocaine enhancement of DA release in the NAc core.

TEMPOL (100 mg/kg) significantly reduced cocaine enhancement of DA release area. The y-axis in (d) displays area under curve after stimulation. * $P < 0.05$ versus saline; $n = 5-7$ per group

Figure 10.

TEMPOL had no effect on GBR12909 enhancement of DA release in the nucleus accumbens, as measured by FSCV *in vivo*. (a) Representative time course of DA release in the NAc core in an anesthetized rat demonstrating the marked and prolonged enhancement of DA levels by i.v. administration of the DAT inhibitor GBR12909 (1.0 mg/kg). Inset shows superimposed DA signals obtained with saline and 2 minutes after administration of GBR12909. Calibration bars are nA and seconds. (b) Representative time course of DA release in the NAc in an anesthetized rat demonstrating DA release produced by i.v. GBR12909 following i.p. injection of 100 mg/kg TEMPOL. Inset shows superimposed DA signals obtained 2 minutes after administration of GBR12909 in the presence of TEMPOL. (c) Summary of saline and TEMPOL effect on the peak amplitude of GBR12909 enhancement of DA release in the NAc core. TEMPOL had no effect on GBR12909 enhancement of DA release amplitude in the NAc core. $n = 6-7$ per group. Data for control are averaged with three consecutive values before treatment with saline or TEMPOL. For TEMPOL and saline groups, three consecutive values 15 minutes after GBR12909 are averaged, respectively, and the values are expressed as percentages of control