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The role of reactive oxygen species in methamphetamine self-administration and dopamine release in the nucleus accumbens

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Abstract

Methamphetamine (METH) markedly increases dopamine (DA) release in the mesolimbic DA system, which plays an important role in mediating the reinforcing effects of METH. Methamphetamine-induced DA release results in the formation of reactive oxygen species (ROS), leading to oxidative damage. We have recently reported that ROS are implicated in behavior changes and DA release in the nucleus accumbens (NAc) following cocaine administration. The aim of this study was to evaluate the involvement of ROS in METH-induced locomotor activity, self-administration, and enhancement of DA release in the NAc. Systemic administration of a non-specific ROS scavenger, N-tert-butyl- α -phenylnitrone (PBN; 0, 50 and 75 mg/kg, IP), or a superoxide selective scavenger, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL; 0, 50 and 100 mg/kg, IP), attenuated METH-induced locomotor activity without affecting generalized behavior in METH naïve rats. PBN and TEMPOL significantly attenuated METH self-administration without affecting food intake. Increased oxidative stress was found in neurons, but not astrocytes, microglia or oligodendrocytes, in the NAc of METH self-administering rats. In addition, TEMPOL significantly decreased METH enhancement of DA release in the NAc. Taken together, these results suggest that enhancement of ROS in the NAc contributes to the reinforcing effect of METH.

Keywords

dopamine (DA); Methamphetamine self-administration; Nucleus accumbens; PBN; reactive oxygen species (ROS); TEMPOL

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Authors Contribution

EYJ, HYK, CHY and SCS designed the experiments, DMH, SPK, JYL, TGE, BTG, NJK and ACN performed them and analyzed the data. EYJ, DMH and SCS drafted the manuscript. EYJ, HYK, CHY and SCS were responsible for the overall direction of the project and for edits to the manuscript.

Introduction

Methamphetamine (METH) is a highly potent and addictive psychostimulant, which causes serious psychosocial problems. Methamphetamine use leads to compulsive behaviors, including repetitive taking and seeking of the drug, and a loss of control over intake. Physiologically, METH increases oxidative stress via metabolic activation. The mesocorticolimbic dopamine (DA) system projecting from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) and prefrontal cortex (PFC) plays a critical role in the reinforcing effects of METH (Koob, 1992; Rose and Grant, 2008). The reinforcing properties of METH are mediated by an elevation of extracellular DA levels in the dorsal and ventral striatum through METH's interaction with the DA transporter (DAT) and vesicular monoamine transporter 2 (VMAT2) (Volkow et al., 2007). For example, exposure to METH or amphetamine increases locomotor activity by increasing extracellular DA release in the dorsal and ventral striatum (Frankel et al., 2007; Kuczenski et al., 1991; Miller et al., 2014). In addition, repeated exposure to METH can cause neurotoxicity via formation of reactive oxygen species (ROS) in DAergic neurons (Sun et al., 2015). Others have shown that exposure to METH increased malondialdehyde, a product of lipid peroxidation by ROS in brain regions of METH-exposed rats (Horner et al., 2011) and METH users (Fitzmaurice et al., 2006). Methamphetamine significantly decreases glutathione, an antioxidant, in the NAc and PFC of chronically-treated animals (Bu et al., 2013). Recently, it has been shown that a non-specific ROS scavenger, α -phenyl-N-tert-butyl nitron (PBN), protected against METH-induced neurotoxicity (ROS formation) and against self-injurious behaviors caused by METH in mice (Kita et al., 2000). The superoxide specific scavenger 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL) has also been used for preventing mitochondrial oxidative damage and METH-induced behavioral sensitization in rats (Shiba et al., 2011). Furthermore, we and others have implicated ROS in the development of behavioral sensitization following repeated contingent and non-contingent administration of psychostimulants, such as cocaine (Jang et al., 2015; Numa et al., 2008). However, the role of ROS in the reinforcing effects of METH has not been investigated.

We hypothesized that increased ROS following chronic METH exposure is involved in reward signaling in the mesolimbic DAergic pathway, which might contribute to the reinforcing effects of METH. To evaluate this hypothesis, we investigated the effects of the non-specific ROS scavenger PBN, and the superoxide dismutase (SOD) mimetic TEMPOL on METH-induced locomotor activity using the open-field paradigm, METH self-administration, and METH-induced DA release in the NAc using fast scan cyclic voltammetry (FSCV). In addition, using immunohistochemistry, we characterized the cellular source of ROS in the NAc of rats self-administering METH.

Materials and methods

Animal Subjects

Male Sprague-Dawley rats (Daehan Animal, Seoul, Korea; Charles Laboratory, CA, USA) weighing 270–350 g were used in the locomotor activity and METH self-administration experiments and male Wistar rats weighing 250–320 g from our breeding colony at Brigham Young University were used in DA FSCV studies *in vivo*. All rats were housed in groups of

2–3 rats per cage in a temperature (21–23°C) and humidity-controlled (55–65%) environment on a reverse light/dark cycle with *ad libitum* food and water. All experimental procedures were approved by the Institutional Animal Care and Use Committees at Daegu Haany University and Brigham Young University.

Drugs and Chemicals

(+)-Methamphetamine hydrochloride (Sigma, ST. Louis, MO, USA) was dissolved in physiological saline and filtered through a syringe-mounted 0.22- μ m Millex-Ha filter unit (Millipore, MO, USA) immediately before self-administration and IV injection of METH *in vivo* as previously described (Jang et al., 2015). The ROS scavengers PBN (a non-specific ROS scavenger; Sigma) and TEMPOL (a superoxide selective scavenger; Sigma) were dissolved in physiological saline prior to use and administered intraperitoneally (IP) 10 min prior to the beginning of self-administration or IV or IP injection of METH. Injection volume of PBN or TEMPOL was 5 ml/kg for all respective doses.

Locomotor Activity

Locomotor activity was measured as described previously (Jeon et al., 2008; Zhao et al., 2005). Locomotor activity was monitored and measured with a video tracking system (Ethovision, Nodus Information Technology BV, Wageningen, Netherlands) that provided automatic measures of traveled distance (m). Each rat was placed in a square open field box made of black acrylic (40 cm X 40 cm X 45 cm) in a dimly-lit room. On the test day, rats were habituated for 1 hr in the open field and then basal activity was measured for 30 min. After recording basal activity, rats received saline, PBN (75 mg/kg, IP) or TEMPOL (100 mg/kg, IP) 10 min prior to saline or METH (1 mg/kg, IP) administration. Locomotor activity was recorded for an additional 1 hr following systemic injection of saline or TEMPOL. Data are expressed as total traveled distance (m) for 1 hr.

Food Training and Surgical Procedure of Catheters

Self-administration was carried out in operant chambers (32 X 25 X 34 cm; Med Associates, St. Albans, VT, USA) equipped on one wall with two retractable levers (one active and one inactive lever), a white house light, and a stimulus light. To facilitate the acquisition of operant responding in administration chambers, rats were subjected to mild food restriction (approximately 16 g of lab chow a day) and trained to lever-press for 45 mg food pellets (Bio-Serv, Frenchtown, NJ, USSA) on a fixed ratio (FR-1) reinforcement schedule until criterion (100 food pellets for three consecutive days) was achieved in 3 hr daily sessions, as described previously (Jang et al., 2015). Two days after the last food training, rats were implanted with a chronic catheter in the right jugular vein. The catheter was passed subcutaneously to exit the back of rat through 22 gauge tubing embedded in dental cement and secured with surgical mesh (Ethicon Inc., Somerville, NJ, USA). Following IV catheter surgery, the catheter patency was maintained by a daily flush of 0.2 ml of heparinized saline (30 U/ml), including gentamicin sulfate (0.33 mg/ml), to prevent clotting and infection. Rats were allowed at least 7 days to recover prior to experiments..

Methamphetamine Self-administration

To evaluate the effect of PBN and TEMPOL on METH self-administration, equipment and training procedures were used as previously described (Jang et al., 2015). After at least 7 days of recovery from IV catheter surgery, the rats began METH self-administration during daily 2 hr fixed ratio 1 (FR 1) schedule sessions for 5–6 days/week. To establish a stable baseline, rats were trained to self-administer METH for 2–3 weeks. Levers were allocated as active or inactive. An active lever press delivered a 0.1 ml infusion of METH (0.05 mg/kg) over 5 sec. METH infusion was paired with illumination of a cue light above the active levers, and the house light was extinguished during infusion of METH. Each infusion was followed by an additional 15 sec time-out, while the house light remained off. Lever responses on the active lever during the time-out did not result in METH infusion. We recorded active lever responses and number of METH infusions in this study. Inactive lever press responses were recorded, but had no programmed consequence. Following the establishment of a stable baseline of the METH-reinforced response (three consecutive sessions with less than a 10% variation), the rats received IP injections of saline ($n = 7$ –10), PBN (50 mg/kg, $n = 5$; or 75 mg/kg, $n = 5$) or TEMPOL (50 mg/kg, $n = 6$; or 100 mg/kg, $n = 7$) 10 min before beginning METH self-administration. Doses of PBN and TEMPOL were selected based on previous studies (Jang et al., 2015; Kim et al., 2004; Numa et al., 2008). PBN, TEMPOL and saline were counterbalanced among animals and the catheters were flushed with 0.2 ml of heparinized saline before and after self-administration.

Food Reinstatement

To evaluate the possibility that ROS scavengers, PBN or TEMPOL, cause non-specific motor impairment, the responses on each lever were measured in food-trained rats. The rats were food-restricted to maintain 85% of the initial body weight and trained to lever-press for 45 mg food pellets under a FR 1 with 1 min time-out during 2 hr daily sessions. After stable lever responding was achieved in three consecutive sessions, rats received saline ($n = 7$), PBN (75 mg/kg, $n = 5$) or TEMPOL (100 mg/kg, $n = 7$) 10 min prior to the food reinforcement test. We measured the total number of active and inactive lever responses with food pellets during the 2 hr session.

Immunofluorescence

To measure the level of oxidative stress and the type of ROS-producing cells in the NAc, METH-administering rats were perfused as previously described (Jang et al., 2015). Briefly, rat brains were removed under pentobarbital anesthesia (80 mg/kg, IP), post-fixed with 10% sucrose/4% paraformaldehyde for 2 hr and cryoprotected in 30% sucrose for at least 48 hr. Brains were then cryosectioned into 30 μ m slices. Brain tissues were incubated in blocking solution containing 4% normal goat serum and 0.1% bovine serum albumin at room temperature for 1 hr. After rinsing in 0.01M PBS, the tissues 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, 1:1000, Millipore, MA, USA), anti-rabbit NeuN (a neuronal marker, 1:1000, Millipore, MA, USA), anti-rabbit Iba-1 (a microglial marker; 1:1000, Wako, Japan), and anti-rabbit NG2 (an oligodendrocyte marker; 1:1000, Chemicon, MA, USA) overnight at 4 °C. The sections

were then processed with secondary antibodies donkey anti-mouse Alexa Fluor 594 (red; 1:1000) and donkey anti-rabbit Alexa Fluor 488 (green; 1:1000, Invitrogen, Gland Island, NY, USA). All sections were cover-slipped with a mounting medium (Vector Laboratories, Burlingame, CA, USA) and were imaged with a 20X objective using a Carl Zeiss AxioCam digital camera attached to the microscope (Carl Zeiss Axioskop, Oberkochen, Germany). The fluorescence intensities (FI) of 8-OHgin sections (Area: 30,000 μm^2) were estimated by computerized densitometry (ZEN, Oberkochen, Germany).

Fast Scan Cyclic Voltammetry

Electrically-evoked DA release in the NAc was measured by fast scan cyclic voltammetry (FSCV). A 7.0 μm 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 150~200 μm of bare fiber protruding from the end of the glass micropipette. The tip of the CFE was dipped in cyanoacrylate, allowed to dry and back-filled with 3 M KCl. The electrode potential was linearly scanned with a triangular waveform from -0.4 V to 1.3 V and back to -0.4 V versus Ag/AgCl using a scan rate of 400 V/sec. Cyclic voltammograms were recorded at the CFE every 100 msec (i.e. 10 Hz) by means of a ChemClamp voltage clamp amplifier (Dagan Corporation, Minneapolis, MN, USA). Voltammetry recordings were performed and analyzed using LabVIEW based (National Instruments, Austin, TX, USA) customized software [Demon Voltammetry (Yorgason et al., 2011)]. For *in vivo* voltammetry recordings of DA signals, rats were anesthetized with 2% 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 -8.3 mm from skull), and a capillary glass-based CFE in the NAc ($+1.6$ 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 msec pulse width) at 2 min intervals. DA levels in the NAc were monitored for a stabilization period typically lasting between 20 min and 1 hr. Once the stimulated DA response was stable for four or five successive collections, and did not vary by more than 10%, baseline measurements were taken for control and drug treatment. After a stable baseline was established, saline (5 ml/kg) or TEMPOL (100 mg/kg, IP) was injected 10 min prior to injection of METH (0.1 mg/kg, IV). DA signals were recorded in 2 min intervals for 30 min post injection. Extracellular concentrations of DA were calibrated by comparing the current at the peak oxidation potential for DA with a known concentration of DA (3 μM). For recordings in live tissue slices, the CFE was positioned ~ 100 μm from a glass stimulating electrode in the NAc core. The core was stimulated with 20 biphasic pulses at 10 Hz (4 msec pulse width) at 2 min intervals. Once the stimulated DA response was stable for four or five successive collections, and did not vary by more than 10%, baseline measurements were taken for control and drug treatment. After a stable baseline was established, compounds were bath applied to the tissue.

Preparation of Brain Slices

Coronal brain slices were prepared as previously described (Steffensen et al., 2008). Briefly, male mice were anesthetized, decapitated, and brains were quickly dissected and sectioned into 400 μm thick coronal slices in ice-cold artificial cerebrospinal fluid (ACSF), bubbled with 95% O_2 /5% CO_2 . The cutting solution consisted of (in mM): 220 Sucrose, 3 KCl, 1.25 NaH_2PO_4 , 25 NaH_2CO_3 , 12 MgSO_4 , 10 Glucose, and 0.2 CaCl_2 . Slices were immediately placed into an incubation chamber containing normal ACSF bubbled with 95% O_2 /5% CO_2 at 34–35°C consisting of (in mM): 124 NaCl, 2 KCl, 1.25 NaH_2PO_4 , 24 NaHCO_3 , 12 glucose, 1.2 MgSO_4 , 2 CaCl_2 , pH 7.3. Once slices were transferred to a recording chamber with continuous normal ACSF flow (2.0 mL/min), the temperature was maintained at 36°C throughout the experiment. Slices were allowed to stabilize for an additional 15 min before recordings. The striatum was visualized with Nikon Diaphot inverted microscopes in the transmitted light mode and the NAc core and shell were visualized by microscopic inspection under low magnification at the level of the anterior commissure. A red light filter was placed in the light path to lower the exposure of the slice to short-wavelength light.

Data Analysis

Data were analyzed using SPSS Statistic 21 (Somers, NY, USA). All data are presented as mean $\pm$ SEM (standard error of the mean) and analyzed by one-way repeated measurement analysis of variance (ANOVA), followed by *post hoc* testing using the Tukey method and Student's *t*-test, with statistical significance set at (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$. Analysis of DA reuptake kinetics was performed using LabVIEW (National Instruments, Austin, TX)-based customized software (Demon Voltammetry, (Yorgason et al., 2011)).

Results

Effects of PBN and TEMPOL on METH-induced Locomotor Activity

We evaluated the effects of PBN and TEMPOL on METH-induced locomotor activity. After recording basal activity for 30 min, rats were administered either intraperitoneal (IP) saline, PBN (75 mg/kg) or TEMPOL (100 mg/kg) 10 min prior to systemic injection of saline or METH (1 mg/kg, IP). The dose of METH was selected based on previous studies (Miller et al., 2013; Yun, 2014). A single administration of METH increased locomotor activity (expressed as total distance in m) compared to the saline-treated group (Fig. 1B; Saline 44.40 ± 6.37 m vs. METH 197.46 ± 8.09 m, $F_{(1,12)} = 4.747$, $P < 0.001$, $n = 6-8$). Both PBN and TEMPOL significantly attenuated the enhancement of locomotor activity induced by METH (Fig. 1A,B; $F_{(1,12)} = 4.747$, $P < 0.01$ and $P < 0.001$ vs. METH, PBN; 93.53 ± 18.19 m; $F_{(1,14)} = 4.600$, $P < 0.01$ and $P < 0.05$ vs. METH, TEMPOL: 137.59 ± 12.50 m, $n = 6-8$). Neither PBN nor TEMPOL administration alone affected locomotor activity in METH-naïve rats (Fig. 1C, $F_{(2,12)} = 2.119$, $P = 0.153$ vs. PBN; $P = 0.373$ vs. TEMPOL, $n = 5$).

Effects of PBN and TEMPOL on Methamphetamine Self-Administration

We evaluated the effects of ROS scavengers, PBN and TEMPOL, on METH self-administration. After stable acquisition of METH self-administration, rats were given an IP

injection of PBN (0, 50 and 75 mg/kg) 10 min prior to the beginning of METH self-administration. Lever presses and the number of METH infusions were monitored for 2 hr following IP injections of saline or PBN (Fig. 2A). Systemic administration of both 50 and 75 mg/kg PBN significantly decreased infusions of METH in a dose-dependent manner compared to saline-injected controls during the 2 hr test session (Fig. 2B,C; 50 mg/kg PBN = 18.0 ± 6.0 responses and 75 mg/kg PBN = 1.0 ± 0.6 responses; $F_{(2,17)} = 20.650$, $P < 0.001$; $n = 5-10$ per group compared to saline = 33 ± 2.8 responses). Additionally, both 50 and 75 mg/kg PBN significantly decreased active lever responses (Fig. 2D; 50 mg/kg PBN = 23.0 ± 7.6 responses and 75 mg/kg PBN = 1.0 ± 0.6 responses; $F_{(2,17)} = 18.292$, $P < 0.001$ compared to saline = 40.1 ± 3.7 responses). The inactive lever responding exhibited no significant differences among groups (Fig. 2E; 50 mg/kg PBN = 6.0 ± 4.8 responses; and 75 mg/kg PBN = 1.0 ± 0.6 responses; $F_{(2,17)} = 1.895$, $P > 0.181$ compared to saline = 0.8 ± 0.4 responses).

To verify the involvement of superoxide, the precursor of most ROS (Turrens, 2003), in mediating METH reinforcement, we performed an experiment similar to PBN, but with the specific superoxide scavenger TEMPOL. Following systemic administration of TEMPOL, 10 min prior to METH self-administration, lever presses and the number of METH infusions were monitored for 2 hr following IP injections of saline or TEMPOL (Fig. 3A). Systemic administration of TEMPOL significantly decreased the number of METH infusions at 100 mg/kg TEMPOL compared to saline-injected controls (Fig. 3B,C; 100 mg/kg TEMPOL = 15.6 ± 2.4 responses; $F_{(2,17)} = 5.106$, $P = 0.08$; $n = 6-7$ per group compared to saline = 26.8 ± 3.5 responses. Active lever responding also decreased in a dose-dependent manner (Fig. 3D; $F_{(2,17)} = 3.829$, 50 mg/kg TEMPOL = 30.8 ± 2.9 responses, $P = 0.206$; 100 mg/kg TEMPOL = 18.0 ± 3.0 responses, $P < 0.05$ compared to saline 32.4 ± 4.2). TEMPOL, at the 100 mg/kg dose level, significantly decreased active lever pressing. Inactive lever presses were not altered (Fig. 3E; 50 mg/kg TEMPOL = 2.2 ± 0.6 , $P > 0.05$ and 100 mg/kg TEMPOL = 1.6 ± 1.6 , $P > 0.05$ compared to saline 1.6 ± 0.7). We also evaluated whether PBN or TEMPOL affects generalized behavioral responses in METH naïve rats. Neither PBN (75 mg/kg, $n = 5$) nor TEMPOL (100 mg/kg, $n = 7$) altered food reinforcement (Fig. 4), compared to the saline controls ($n = 7$) ($F_{(2,16)} = 2.271$, $P = 0.135$). Taken together, these results show that scavenging ROS with PBN or TEMPOL specifically attenuated the reinforcing effects of METH self-administration without impairment generalized behavior or food reinforcement.

Oxidative Stress in Neurons in the Nucleus Accumbens

To determine ROS production in the NAc following chronic METH self-administration, we performed immunohistochemical experiments with an antibody against 8-hydroxyquinine (8-OHG), a cellular marker of oxidative stress (Kim et al., 2010; Manczak et al., 2005). Methamphetamine self-administering rats showed about a two-fold increase in number of 8-OHG positive cells (red signal) as compared with controls (Fig. 5A–C; Student's *t*-test, $P = 0.034$ vs. normal controls, $n = 3-4$). To further identify the types of cells producing ROS, we performed double immunofluorescence labeling using the following cell-type markers: NeuN (a marker for neurons; green), GFAP (a marker for astrocytes; green), Iba-1 (a marker for microglia; green), and NG2 (a marker for oligodendrocytes; green). Most 8-OHG positive

cells were co-localized with NeuN-positive neurons in the NAc (green nucleus with red circle in Fig. 5D–F), but not astrocytes (Fig. 5G–I), microglia (Fig. 5J–L) or oligodendrocytes (Fig. 5M–O), indicating that increased ROS production in the NAc of rats self-administering METH occurs almost exclusively in neurons.

Effect of TEMPOL on Electrically-evoked Dopamine Release

In our previous study, TEMPOL decreased cocaine enhancement of electrically-evoked “phasic” DA release in the NAc (Jang et al., 2015). We evaluated the effects of TEMPOL (100 mg/kg) on phasic DA release following IV METH injection in the NAc core using FSCV. Intravenous METH administration markedly increased electrically-evoked DA release with an EC_{50} of 0.1 mg/kg in the NAc core by $200.2 \pm 15.7\%$ (Fig. 6A,C; $F_{(1,24)} = 40.702$, $P < 0.001$, compared to control; $n = 13$). While 100 mg/kg TEMPOL alone did not alter DA release ($P = 0.175$ vs. baseline controls, data not shown), systemic administration of 100 mg/kg TEMPOL significantly decreased METH enhancement of DA release by $135.8 \pm 12.8\%$ compared to saline controls (Fig. 6B,D; $F_{(1,19)} = 8.202$, $P = 0.01$, $n = 8$).

Effects of METH and TEMPOL on the DAT

In order to determine if TEMPOL has a direct effect on the DAT, we analyzed DA reuptake kinetics using the time constant of DA reuptake, or tau, from FSCV recordings taken in the slice preparation. TEMPOL (1 mM) had no impact on tau (Fig. 7A,C; $F_{(1,14)} = 0.133$, $P = 0.721$) as compared to DA reuptake before TEMPOL superfusion. In contrast, METH (5 μ M), which is known to inhibit the DAT, increased tau (Fig. 7B,C; $F_{(1,20)} = 4.998$, $P = 0.037$), which reflects an increased reuptake time. There was no difference in tau between METH (5 μ M) vs METH (5 μ M) + TEMPOL (1 mM) (Fig. 7C; $F_{(1,17)} = 0.35$, $P = 0.562$).

Discussion

The present study demonstrates that systemic administration of the ROS scavengers PBN and TEMPOL results in: 1) A decrease in METH enhancement of locomotor activity without affecting generalized behavior in METH-naïve rats; 2) A decrease in self-administration of METH without affecting food reinforcement; and 3) A decrease in METH-enhanced phasic DA release without affecting the reuptake kinetics mediated by the DAT. In addition, METH increased production of ROS specifically in NAc neurons. Taken together, these findings suggest an important role of ROS in mediating the reinforcing effects of METH in the mesolimbic DA system.

Several recent reports have indicated that oxidative stress can modulate the reinforcing effects of drug abuse (Jang et al., 2015; Numa et al., 2008). Reactive oxygen species are produced through mitochondrial respiration and are critical signaling molecules in cell proliferation and survival. Activation of ROS also leads to oxidative stress via mitochondrial dysfunction, playing a critical role in the progression of a variety of neurodegenerative and psychiatric disorders. Of particular relevance to drug abuse, METH administration is associated with excessive oxidative stress. For example, acute and repeated METH administration causes mitochondrial dysfunction and oxidative stress in rat brains (Beauvais et al., 2011; Horner et al., 2011; Valvassori et al., 2010). Burrows et al. have shown that

proper mitochondrial function was inhibited 23–30% by METH in the dorsal striatum and NAc (Burrows et al., 2000). Additionally, increased oxidative stress markers (lipid and protein oxidation) have been found in the mesocorticolimbic DA system after exposure to METH (Macedo et al., 2010; Shiba et al., 2011). Increased oxidative stress leads to abnormal behavior including increased locomotor activity (da-Rosa et al., 2012a; da-Rosa et al., 2012b; Gluck et al., 2001; Numa et al., 2008; Shiba et al., 2011). We and others have shown that mitochondrial oxidative damage and behavioral changes can be produced by psychostimulants (Jang et al., 2015; Kita et al., 2000; Shiba et al., 2011). We hypothesize that oxidative stress may, at least in part, modulate the reinforcing properties of METH. In the present study, administration of PBN, a non-specific ROS scavenger, and TEMPOL, a superoxide-specific scavenger, significantly attenuated the reinforcing properties of METH. These results show a crucial role for ROS, especially superoxide, in the positive reinforcing effects of METH. Superoxide ($O_2^{\bullet-}$) is a primary ROS formed in cells and is enzymatically converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD), which itself can react with free ions to produce highly reactive and toxic hydroxyl radicals (Fenton reaction).

In the present study, we also evaluated the effects of the SOD mimetic TEMPOL on METH enhancement of DA release using *in vivo* FSCV. While METH is known to affect all monoamine systems [i.e. norepinephrine and serotonin (Kogan et al., 1976)], its reinforcing properties are mediated mostly through the mesocorticolimbic DA system. Methamphetamine increases DA transmission, which is associated with behavioral changes (Akiyama et al., 1994; Hamamura et al., 1991). Due to its structural similarity to DA, METH can enter the cell through DAT, and interacts with VMAT, causing high cytoplasmic levels of DA, which eventually overwhelms the transporter, which is referred to as reverse transport (Cho, 1990; Fleckenstein et al., 2007; Kitanaka et al., 2003). Further, excessive cytoplasmic DA levels contribute to mitochondrial dysfunction and induce oxidative stress via DA auto-oxidation, which produces ROS through the enzymatic degradation of DA. Breakdown of DA produces H_2O_2 , a precursor to hydroxyl radicals (Graham et al., 1978), DA-quinone (Miyazaki et al., 2006; Moszczynska et al., 1998), 6-hydroxydopamine (Maharaj et al., 2005), and also decreases reduced glutathione levels in the brain (Moszczynska et al., 1998). We found that METH enhanced DA release with an ED_{50} of 0.1 mg/kg, which is consistent with the behavioral reinforcing effects (Baracz et al., 2015). TEMPOL (100 mg/kg) significantly attenuated METH enhancement of DA release in the NAc, suggesting that ROS may contribute to the reinforcing effects of METH by oxidation in the NAc. In a previous study, we determined that the effects of TEMPOL are not related to interactions with the DAT (Jang et al., 2015), eliminating this potential confounding variable.

Methamphetamine induces unchecked oxidative stress in DA neurons that alters DA release, influencing the reinforcing potential of METH. A possible mechanism for this could be the oxidative stress targeting proteins involved in proper DA uptake and vesicle packaging. We examined the effect of TEMPOL with or without METH *ex vivo*. While METH dramatically increased DA release in the slice preparation and the time constant τ for DA uptake, TEMPOL did not affect DA uptake. It has been shown that repeated high doses of METH cause a decrease in VMAT2 levels and an increase in S-nitrosylated VMAT2 following METH administration (Eyerman and Yamamoto, 2007). This finding indicates that there are

lasting molecular changes due to oxidative stress, particularly superoxide, with high potential to alter proper DA release due to METH. Future studies are needed to confirm this interaction.

Methamphetamine is a particularly addictive substance, which affects both the peripheral and central nervous systems. It acts directly on DAergic neurons, bypassing typical regulation such as GABA circuitry and inhibitory feedback, and causes high levels of DA to be released from DA terminals. Attempting to restore homeostasis, DA systems overcompensate leading to abnormally low DA release during periods of abstinence. This causes a cycle of drug-seeking behavior, as the limbic system learns that the easiest and quickest way to increase DA release is by drug administration. In conclusion, both PBN and TEMPOL attenuated METH-induced locomotor activity without affecting generalized behavior in METH naïve rats. Both PBN and TEMPOL also attenuated METH self-administration without affecting food intake and increased oxidative stress was found in NAc neurons of METH self-administering rats. In addition, TEMPOL reduced METH-induced phasic DA transmission in the NAc. Taken together, our findings indicate that increased ROS in the NAc contributes to the reinforcing effects of METH and that ROS scavengers, like TEMPOL, may be effective strategies in reducing the reinforcing properties of METH.

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References

- Akiyama K, Kanzaki A, Tsuchida K, Ujike H. Methamphetamine-induced behavioral sensitization and its implications for relapse of schizophrenia. *Schizophr Res.* 1994; 12:251–257. [PubMed: 8054317]
- Baracz SJ, Everett NA, Cornish JL. The Involvement of Oxytocin in the Subthalamic Nucleus on Relapse to Methamphetamine-Seeking Behaviour. *PloS one.* 2015; 10:e0136132. [PubMed: 26284529]
- Beauvais G, Atwell K, Jayanthi S, Ladenheim B, Cadet JL. Involvement of dopamine receptors in binge methamphetamine-induced activation of endoplasmic reticulum and mitochondrial stress pathways. *PloS one.* 2011; 6:e28946. [PubMed: 22174933]
- Bu Q, Lv L, Yan G, Deng P, Wang Y, Zhou J, Yang Y, Li Y, Cen X. NMR-based metabonomic in hippocampus, nucleus accumbens and prefrontal cortex of methamphetamine-sensitized rats. *Neurotoxicology.* 2013; 36:17–23. [PubMed: 23462569]
- Burrows KB, Gudelsky G, Yamamoto BK. Rapid and transient inhibition of mitochondrial function following methamphetamine or 3,4-methylenedioxymethamphetamine administration. *European journal of pharmacology.* 2000; 398:11–18. [PubMed: 10856443]
- Cho AK. Ice: a new dosage form of an old drug. *Science.* 1990; 249:631–634. [PubMed: 17831955]
- da-Rosa DD, Valvassori SS, Steckert AV, Arent CO, Ferreira CL, Lopes-Borges J, Varela RB, Mariot E, Dal-Pizzol F, Andersen ML, Quevedo J. Differences between dextroamphetamine and methamphetamine: behavioral changes and oxidative damage in brain of Wistar rats. *J Neural Transm (Vienna).* 2012a; 119:31–38. [PubMed: 21858431]
- da-Rosa DD, Valvassori SS, Steckert AV, Ornell F, Ferreira CL, Lopes-Borges J, Varela RB, Dal-Pizzol F, Andersen ML, Quevedo J. Effects of lithium and valproate on oxidative stress and behavioral

- changes induced by administration of m-AMPH. *Psychiatry research*. 2012b; 198:521–526. [PubMed: 22429481]
- Eyerman DJ, Yamamoto BK. A rapid oxidation and persistent decrease in the vesicular monoamine transporter 2 after methamphetamine. *Journal of neurochemistry*. 2007; 103:1219–1227. [PubMed: 17683483]
- Fitzmaurice PS, Tong J, Yazdanpanah M, Liu PP, Kalasinsky KS, Kish SJ. Levels of 4-hydroxynonenal and malondialdehyde are increased in brain of human chronic users of methamphetamine. *The Journal of pharmacology and experimental therapeutics*. 2006; 319:703–709. [PubMed: 16857724]
- Fleckenstein AE, Volz TJ, Riddle EL, Gibb JW, Hanson GR. New insights into the mechanism of action of amphetamines. *Annu Rev Pharmacol Toxicol*. 2007; 47:681–698. [PubMed: 17209801]
- Frankel PS, Hoonakker AJ, Danaceau JP, Hanson GR. Mechanism of an exaggerated locomotor response to a low-dose challenge of methamphetamine. *Pharmacol Biochem Behav*. 2007; 86:511–515. [PubMed: 17303233]
- Gluck MR, Moy LY, Jayatilake E, Hogan KA, Manzano L, Sonsalla PK. Parallel increases in lipid and protein oxidative markers in several mouse brain regions after methamphetamine treatment. *Journal of neurochemistry*. 2001; 79:152–160. [PubMed: 11595767]
- Graham DG, Tiffany SM, Bell WR Jr, Gutknecht WF. Autoxidation versus covalent binding of quinones as the mechanism of toxicity of dopamine, 6-hydroxydopamine, and related compounds toward C1300 neuroblastoma cells in vitro. *Molecular pharmacology*. 1978; 14:644–653. [PubMed: 567274]
- Hamamura T, Akiyama K, Akimoto K, Kashihara K, Okumura K, Ujike H, Otsuki S. Co-administration of either a selective D1 or D2 dopamine antagonist with methamphetamine prevents methamphetamine-induced behavioral sensitization and neurochemical change, studied by in vivo intracerebral dialysis. *Brain research*. 1991; 546:40–46. [PubMed: 1677305]
- Horner KA, Gilbert YE, Cline SD. Widespread increases in malondialdehyde immunoreactivity in dopamine-rich and dopamine-poor regions of rat brain following multiple, high doses of methamphetamine. *Frontiers in systems neuroscience*. 2011; 5:27. [PubMed: 21602916]
- Jang EY, Ryu YH, Lee BH, Chang SC, Yeo MJ, Kim SH, Folsom RJ, Schilaty ND, Kim KJ, Yang CH, Steffensen SC, Kim HY. Involvement of reactive oxygen species in cocaine-taking behaviors in rats. *Addiction biology*. 2015; 20:663–675. [PubMed: 24975938]
- Jeon JP, Buono RJ, Han BG, Jang EY, Kim SC, Yang CH, Hwang M. Proteomic and behavioral analysis of response to isoliquiritigenin in brains of acute cocaine treated rats. *J Proteome Res*. 2008; 7:5094–5102. [PubMed: 19367698]
- Kim D, You B, Jo EK, Han SK, Simon MI, Lee SJ. NADPH oxidase 2-derived reactive oxygen species in spinal cord microglia contribute to peripheral nerve injury-induced neuropathic pain. *Proceedings of the National Academy of Sciences of the United States of America*. 2010; 107:14851–14856. [PubMed: 20679217]
- Kim HK, Park SK, Zhou JL, Taghialatela G, Chung K, Coggeshall RE, Chung JM. Reactive oxygen species (ROS) play an important role in a rat model of neuropathic pain. *Pain*. 2004; 111:116–124. [PubMed: 15327815]
- Kita T, Matsunari Y, Saraya T, Shimada K, O'Hara K, Kubo K, Wagner GC, Nakashima T. Evaluation of the effects of alpha-phenyl-N-tert-butyl nitron pretreatment on the neurobehavioral effects of methamphetamine. *Life sciences*. 2000; 67:1559–1571. [PubMed: 10983851]
- Kitanaka N, Kitanaka J, Takemura M. Behavioral sensitization and alteration in monoamine metabolism in mice after single versus repeated methamphetamine administration. *European journal of pharmacology*. 2003; 474:63–70. [PubMed: 12909196]
- Kogan FJ, Nichols WK, Gibb JW. Influence of methamphetamine on nigral and striatal tyrosine hydroxylase activity and on striatal dopamine levels. *European journal of pharmacology*. 1976; 36:363–371. [PubMed: 6286]
- Koob GF. Drugs of abuse: anatomy, pharmacology and function of reward pathways. *Trends in pharmacological sciences*. 1992; 13:177–184. [PubMed: 1604710]
- Kuczenski R, Segal DS, Aizenstein ML. Amphetamine, cocaine, and fencamfamine: relationship between locomotor and stereotypy response profiles and caudate and accumbens dopamine dynamics. *J Neurosci*. 1991; 11:2703–2712. [PubMed: 1715389]

- Macedo DS, Vasconcelos SM, Andrade-Neto M, Belchior LD, Honorio JE Junior, Goncalves DO, Fonteles MM, Silva MI, Aguiar LM, Viana GS, de Sousa FC. Cocaine-induced status epilepticus and death generate oxidative stress in prefrontal cortex and striatum of mice. *Neurochemistry international*. 2010; 56:183–187. [PubMed: 19822180]
- Maharaj H, Sukhdev Maharaj D, Scheepers M, Mokokong R, Daya S. L-DOPA administration enhances 6-hydroxydopamine generation. *Brain research*. 2005; 1063:180–186. [PubMed: 16257392]
- Manczak M, Jung Y, Park BS, Partovi D, Reddy PH. Time-course of mitochondrial gene expressions in mice brains: implications for mitochondrial dysfunction, oxidative damage, and cytochrome c in aging. *Journal of neurochemistry*. 2005; 92:494–504. [PubMed: 15659220]
- Miller DK, Oelrichs CE, Sage AS, Sun GY, Simonyi A. Repeated resveratrol treatment attenuates methamphetamine-induced hyperactivity and [3H]dopamine overflow in rodents. *Neurosci Lett*. 2013; 554:53–58. [PubMed: 24012682]
- Miller DK, Oelrichs CE, Sun GY, Simonyi A. Subchronic apocynin treatment attenuates methamphetamine-induced dopamine release and hyperactivity in rats. *Life Sci*. 2014; 98:6–11. [PubMed: 24398042]
- Miyazaki I, Asanuma M, Diaz-Corrales FJ, Fukuda M, Kitaichi K, Miyoshi K, Ogawa N. Methamphetamine-induced dopaminergic neurotoxicity is regulated by quinone-formation-related molecules. *FASEB journal: official publication of the Federation of American Societies for Experimental Biology*. 2006; 20:571–573. [PubMed: 16403784]
- Moszczynska A, Turenne S, Kish SJ. Rat striatal levels of the antioxidant glutathione are decreased following binge administration of methamphetamine. *Neuroscience letters*. 1998; 255:49–52. [PubMed: 9839724]
- Numa R, Kohen R, Poltyrev T, Yaka R. Tempol diminishes cocaine-induced oxidative damage and attenuates the development and expression of behavioral sensitization. *Neuroscience*. 2008; 155:649–658. [PubMed: 18619523]
- Rose ME, Grant JE. Pharmacotherapy for methamphetamine dependence: a review of the pathophysiology of methamphetamine addiction and the theoretical basis and efficacy of pharmacotherapeutic interventions. *Ann Clin Psychiatry*. 2008; 20:145–155. [PubMed: 18633741]
- Shiba T, Yamato M, Kudo W, Watanabe T, Utsumi H, Yamada K. In vivo imaging of mitochondrial function in methamphetamine-treated rats. *NeuroImage*. 2011; 57:866–872. [PubMed: 21624473]
- Steffensen SC, Taylor SR, Horton ML, Barber EN, Lyle LT, Stobbs SH, Allison DW. Cocaine disinhibits dopamine neurons in the ventral tegmental area via use-dependent blockade of GABA neuron voltage-sensitive sodium channels. *Eur J Neurosci*. 2008; 28:2028–2040. [PubMed: 19046384]
- Sun D, Yue Q, Guo W, Li T, Zhang J, Li G, Liu Z, Sun J. Neuroprotection of resveratrol against neurotoxicity induced by methamphetamine in mouse mesencephalic dopaminergic neurons. *Biofactors*. 2015; 41:252–260. [PubMed: 26212417]
- Turrens JF. Mitochondrial formation of reactive oxygen species. *The Journal of physiology*. 2003; 552:335–344. [PubMed: 14561818]
- Valvassori SS, Rezin GT, Ferreira CL, Moretti M, Goncalves CL, Cardoso MR, Streck EL, Kapczinski F, Quevedo J. Effects of mood stabilizers on mitochondrial respiratory chain activity in brain of rats treated with d-amphetamine. *J Psychiatr Res*. 2010; 44:903–909. [PubMed: 20334877]
- Volkow ND, Fowler JS, Wang GJ, Swanson JM, Telang F. Dopamine in drug abuse and addiction: results of imaging studies and treatment implications. *Arch Neurol*. 2007; 64:1575–1579. [PubMed: 17998440]
- Yorgason JT, Espana RA, Jones SR. Demon voltammetry and analysis software: analysis of cocaine-induced alterations in dopamine signaling using multiple kinetic measures. *Journal of neuroscience methods*. 2011; 202:158–164. [PubMed: 21392532]
- Yun J. L-tetrahydropalmatine inhibits methamphetamine-induced locomotor activity via regulation of 5-HT neuronal activity and dopamine D3 receptor expression. *Phytomedicine*. 2014; 21:1287–1291. [PubMed: 25172791]

Zhao RJ, Koo BS, Kim GW, Jang EY, Lee JR, Kim MR, Kim SC, Kwon YK, Kim KJ, Huh TL, Kim DH, Shim I, Yang CH. The essential oil from *Angelica gigas* NAKAI suppresses nicotine sensitization. *Biol Pharm Bull.* 2005; 28:2323–2326. [PubMed: 16327174]

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Figure 1. Effect of PBN and TEMPOL on methamphetamine-induced locomotor activity
 After basal activity for 30 min, rats were given saline, PBN (75 mg/kg, IP) or TEMPOL (100 mg/kg, IP) 10 min prior to systemic injection of saline or METH (1 mg/kg). **A:** Time course of distance traveled (expressed in m) after systemic injection of saline or METH. **B:** Total distance for 1 hr following systemic injection saline or METH. **C:** Total distance for 1 hr following PBN or TEMPOL administration alone in METH naïve rats. Asterisks **, *** represent significance levels $P < 0.01$ and $P < 0.001$, respectively, compared to Saline + Saline, and hashtags ##, ### represent $P < 0.01$ and $P < 0.001$ compared to Saline+METH. Values in parenthesis indicate n values.

Figure 2. Effect of pretreatment with PBN, a non-specific ROS scavenger, on methamphetamine self-administration (METH SA) in rats

Rats were given a non-specific ROS scavenger PBN (50 or 75 mg/kg, IP) or saline 10 min prior to METH SA. **A:** A representative example of METH-infused SA pattern for 2 hr after injection of saline or PBN. Tick marks indicate an infusion of METH. **B:** Time course analysis of number of METH infusions during the 2 hr session after systemic injection of PBN at 50 mg/kg and 75 mg/kg compared to saline. **C–E:** Total number of responses on number of infusions (C), active lever (D), and inactive lever (E) in 2 hr-sessions of METH SA. PBN significantly reduced infusion of METH and active lever responding for METH at dose of both 50 mg/kg and 75 mg/kg, but not inactive lever responding. Asterisks *, **, ***

represent significance levels $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, compared to saline. Values in parenthesis indicate n values.

compared to saline. Asterisk * represents significance level $P < 0.05$ compared to saline. Values in parentheses indicate n values.

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Figure 4. Effects of PBN and TEMPOL on food reinforcement in methamphetamine naïve rats
 Figure shows total number of responses on active lever (left) and inactive lever (right) during a 2 hr session of food pellet SA. When either PBN (75 mg/kg, IP), TEMPOL (100 mg/kg, IP) or saline was injected before beginning of food self-administration, there was no significant difference between saline and PBN or TEMPOL groups in total number of responses on active lever and inactive lever during the 2 hr session. 'Food pellets' on y-axis represents mean number of lever presses for food pellets over 2 hr. Values in parentheses indicate n values.

Figure 5. Increased oxidative stress in neurons of the nucleus accumbens in rats self-administering methamphetamine

A,B: Immunohistochemical staining of 8-hydroxyguanine (8-OHG; red), an oxidative stress marker, in the NAc of normal (Normal; A) or METH self-administering (METH SA; B) rats. **C:** A significant increase in fluorescence intensity of 8-OHG (8-OHG FI on y-axis) was found in the NAc of METH SA rats (C), compared to that of controls (A; * $P < 0.05$). **D–O:** These images show double-immunostaining in the NAc for 8-OHG (red) with NeuN (neurons; green) and merge (F), GFAP (astrocytes; green) with merge (I), Iba-1 (microglia; green) with merge (L), and NG2 (oligodendrocytes; green) with merge (O). Most 8-OHG positive cells were double-labeled with NeuN (F). Note double-labeled cells of green nucleus (NeuN) surrounded by red cytoplasm (8-OHG) in neurons (F), but not other cells (I, L, O). AC= anterior commissure. $n = 3-4$ per group. Scale bar = 50 μm.

Figure 6. TEMPOL decreases methamphetamine enhancement of phasic DA release in the nucleus accumbens *in vivo*

A, B: Representative time course of DA release (peak height) in the NAc in an anesthetized rat demonstrating enhancement of DA release produced by IV METH following IP injection of saline (A) or 100 mg/kg TEMPOL (B). Insets show superimposed DA FSCV signals obtained 30 min after administration of METH in the presence of saline (A) TEMPOL (B). **C:** Effects of METH on DA release in the NAc *in vivo* (dose-response). **D:** Summary of saline and 100 mg/kg TEMPOL on the peak amplitude of phasic DA release in the NAc core evoked by 0.1 mg/kg METH. TEMPOL (100 mg/kg) significantly decreased METH

enhancement of DA release. Asterisks **,*** represent significance levels $P < 0.01$, and $P < 0.001$, respectively, compared to saline. Values in parentheses represent n values.

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Figure 7. Effects of methamphetamine and TEMPOL on the DAT

(A) Representative FSCV recording showing of phasic DA release evoked by local stimulation in the slice with and without TEMPOL. TEMPOL did not appear to affect reuptake kinetics, as measured by the time constant or tau. (B) Representative tracing of a DA spike with and without METH. Methamphetamine dramatically increased tau. (C) Quantitative representation of pooled data for tau measurements. Methamphetamine (alone or in the presence of TEMPOL) increased the tau compared to controls. TEMPOL alone had no effect on tau and did not alter METH enhancement of tau. Asterisk * represents significance levels $P < 0.05$ compared to saline. Values in parentheses represent n values.