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Cocaine-Induced Adaptations in Cellular Redox Balance Contributes to Enduring Behavioral Plasticity

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Impaired glutamate homeostasis in the nucleus accumbens has been linked to cocaine relapse in animal models, and results in part from cocaine-induced downregulation of the cystine–glutamate exchanger. In addition to regulating extracellular glutamate, the uptake of cystine by the exchanger is a rate-limiting step in the synthesis of glutathione (GSH). GSH is critical for balancing cellular redox in response to oxidative stress. Cocaine administration induces oxidative stress, and we first determined if downregulated cystine–glutamate exchange alters redox homeostasis in rats withdrawn from daily cocaine injections and then challenged with acute cocaine. Among the daily cocaine-induced changes in redox homeostasis were an increase in protein *S*-glutathionylation and a decrease in expression of GSH-*S*-transferase pi (GSTpi). To mimic reduced GSTpi, a genetic mouse model of GSTpi deletion or pharmacological inhibition of GSTpi by administering ketoprofen during daily cocaine administration was used. The capacity of cocaine to induce conditioned place preference or locomotor sensitization was augmented, indicating that reducing GSTpi may contribute to cocaine-induced behavioral neuroplasticity. Conversely, an acute cocaine challenge after withdrawal from daily cocaine elicited a marked increase in accumbens GSTpi, and the expression of behavioral sensitization to a cocaine challenge injection was inhibited by ketoprofen pretreatment; supporting a protective effect by the acute cocaine-induced rise in GSTpi. Together, these data indicate that cocaine-induced oxidative stress induces changes in GSTpi that contribute to cocaine-induced behavioral plasticity.
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INTRODUCTION

Glutathione (GSH) is the most abundant non-protein cellular antioxidant, which together with its corresponding disulfide, GSH disulfide (GSSG), modulates intracellular redox status (Mieyal *et al*, 2008; Townsend, 2007). Both oxidative and nitrosative stresses lead to changes in the ratio of GSH–GSSG and thereby cause posttranslational modifications on redox-sensitive cysteine residues that include *S*-glutathionylation, *S*-nitrosylation, or *S*-oxidation of cellular proteins. Importantly, *S*-glutathionylation of proteins is a significant signaling event in the cellular response to oxidative stress. For example, *S*-glutathionylation of c-Jun and c-Jun aminoterminal kinase (JNK) is a necessary antecedent for protein phosphorylation and subsequent gene regulation by these proteins (Klatt and Lamas, 2002) and *S*-glutathionylation of actin promotes the formation of G-actin, thereby affecting cell morphology (Fiaschi *et al*, 2006). In addition, *S*-glutathionylation regulates many proteins involved in endoplasmic reticulum (ER) stress and the unfolded protein response that accompanies oxidative stress (Townsend, 2007). A number of neurodegenerative diseases and psychiatric disorders have been linked to abnormal GSH levels, ER stress, and accompanying *S*-glutathionylation of proteins, such as Parkinson's disease (Chinta *et al*, 2006; Sian *et al*, 1994), Alzheimer's disease (Di Domenico *et al*, 2009; Zhu *et al*, 2006), Huntington's disease (Klepac *et al*, 2007), and schizophrenia (Radonjic *et al*, 2010; Raffa *et al*, 2009; Steullet *et al*, 2006).

GSH cannot cross cell membranes and therefore needs to be synthesized *de novo*. Neurons are able to synthesize GSH through the intracellular constitutive amino acids, glutamate, cysteine, and glycine, or the dipeptide cysteinylglycine that is supplied by astrocytes (Dringen *et al*, 1999). In addition, astrocytes are able to exchange glutamate for cystine (cyteine disulfide) through the cystine–glutamate exchanger, system Xc-, which allows cystine to be used for astrocytic GSH synthesis (O'Connor *et al*, 1995). Cystine–glutamate exchange (Xc-) is downregulated by daily cocaine

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or nicotine administration, and the ensuing decrease in release of non-synaptic glutamate via system Xc- contributes to relapse vulnerability in animal models of addiction (Baker *et al*, 2003; Knackstedt *et al*, 2009; Madayag *et al*, 2007). Although research has focused on system Xc- mediated release of glutamate, it is possible that by downregulating system Xc- daily cocaine may also affect cellular redox via decreasing substrate for *de novo* GSH synthesis.

There are limited data on the capacity of daily cocaine to alter tissue levels of *S*-glutathionylation, protein thiol conjugation, and redox-sensitive enzymes, such as GSH reductase and glutaredoxin. Previous studies found an increase in GSH reductase in the spleen of Wistar rats after both acute and daily cocaine treatment (Pacifici *et al*, 2003), and while numerous studies on cocaine-induced hepatotoxicity have measured GSH reductase in the liver or spleen of animals (Devi and Chan, 1997; Labib *et al*, 2002a, b; Pacifici *et al*, 2003; Visalli *et al*, 2004), few have evaluated cocaine effects on brain GSH. Daily cocaine administration reduces GSH levels in the hippocampus, and restoration of GSH was associated with normalizing cocaine-induced memory impairments (Muriach *et al*, 2010). Also, an acute cocaine overdose in mice produces an elevation in GSH in the prefrontal cortex and striatum (Macedo *et al*, 2010). Although direct measures of GSH and GSH metabolic enzymes are largely absent from the cocaine literature, other measures of oxidative stress, such as lipid peroxidation and superoxide dismutase levels, indicate that acute and daily cocaine induce oxidative stress in the brain (Dietrich *et al*, 2005; Kovacic, 2005; Numa *et al*, 2008). Here, we determined the redox potential, levels of modified protein thiols and protein levels of GSH reductase, glutaredoxin, and GSH-*S*-transferase pi (GSTpi) in the nucleus accumbens of cocaine-withdrawn rats before and after a subsequent acute cocaine injection. Cocaine-withdrawn animals were used for comparison with daily saline-treated controls in order to focus on the enduring neurochemical changes that might underlie long-lasting cocaine-induced behaviors, such as behavioral sensitization. We observed a marked affect by acute and daily cocaine on GSTpi and employed GSTpi knockout (GST*P1P2*-KO) mice and the GST inhibitor ketoprofen to evaluate how cocaine-induced changes in GSTpi might affect measures of cocaine reward (conditioned place preference (CPP)) and cocaine-induced locomotor behavior.

SUBJECTS AND METHODS

Animals and Housing

Male rats (200–250 g) were purchased from Charles River Laboratories and housed with food and water provided ad libitum using a 12 h light–dark cycle with the light cycle starting at 0600 hours. All experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. On days 1 and 7 rats were injected with cocaine (15 mg/kg, i.p. on days 1 and 7; 30 mg/kg, i.p. on days 2–6), while control rats received saline injections on all days. This protocol typically elicits a sensitized locomotor response to cocaine when animals are tested with acute cocaine (15 mg/kg, i.p.) after a 3-week withdrawal period. The lower dose of cocaine administered on days 1, 7, and 28 elicits a submaximal locomotor response without significant stereotypies that permits measurement of both a reduction and augmentation in behavior (Kalivas *et al*, 1988). In these neurochemical experiments, at 3 weeks after withdrawal on day 28 half of the animals were decapitated following an i.p. needle stick (ie, time = 0 min), or they were administered a cocaine challenge (15 mg/kg, i.p.) and decapitated 45 min later.

Male mice were used for behavioral studies. The generation of mice harboring a GST*P1P2* gene deletion is described in detail elsewhere (Henderson *et al*, 1998), and these mice have no major phenotype except some myeloproliferative effects and increased sensitivity to some carcinogens (Gate *et al*, 2005). Importantly, the GST*P1P2* KO mice have no change in the expression of other GST isoforms, including GSTmu and GSTalpha (Kitteringham *et al*, 2003). Genotypic comparisons were between KO and their respective WT littermates. Mice were bred, genotyped, and after weaning housed in groups of three in an AALAC-approved animal facility (lights on, 0800 hours; 25 °C). All testing was conducted during the light cycle. Food and water were available *ad libitum*. All experiments were approved by the Institutional Animal Care and Use Committee of the Medical University of South Carolina and conducted in accordance with the National Institutes of Health (NIH) Principles of Laboratory Animal Care (1985).

ThioGlo Assay

Free sulfhydryls in the nucleus accumbens were measured as previously described (Townsend *et al*, 2008, 2009). Briefly, 100 μg of protein from homogenized brain tissue lysate was passed through BioSpin6 (Bio-Rad, Hercules, CA) micro-column that retains both GSH and GSSG. Then 20 μl of eluent was added to 2 ml of 40 mM PB (pH = 7.4) in a quartz cuvette of a PTI QM-8 spectrofluorometer (PTI, Birmingham, NJ) under constant stirring at 37 °C. The emission (513 nm, excitation at 379 nm) of each sample was recorded for 1 min (background) before and 2 min after an addition of 5 μM (final concentration) of ThioGlo-1 (Calbiochem). Each sample fluorescence saturation value corresponds to a concentration of free thiols. At the end of each experiment, 1 μM reduced GSH was added to the sample to ensure that saturation was not associated with the concentration of ThioGlo-1.

Redox Potential

All solvents and water were HPLC grade and were purchased from Fisher Scientific (USA). The internal standard (IS), Glu-Val-Phe, GSH, and GSSG were purchased from Sigma (St Louis, MO). The IS, GSSG, and GSH were dissolved in 20 mm formic acid at 10 mg/ml and solutions were prepared by diluting stock solutions with HPLC grade water. A GSSG and GSH standard curve (including the IS) ranging from 0 to ~40 μg/ml and 0 to 80 μg/ml were generated with $R^2 = 0.99$. An Acquity UPLC coupled to a Quattro Premier XE mass spectrometer (Waters, Milford, MA) was used to measure GSH and GSSG concentrations. Chromatographic separation was performed on an Acquity

UPLC HSS C18 2.1 × 100 mm (1.8 μm) column preceded by an Acquity UPLC HSS C18 (1.8 μm) pre-column. The mobile phases were 20 mM formic acid (pH 2.2) (A) and 100% acetonitrile (B). The 7.50 min gradient was programmed as follow: 0 min, 98% A; 2 min, 98% A; 2.50 min, 50% A; 4.75 min 50% A; 4.90 min 98% A; and 7.50 min, 98% A. The flow rate was 0.2 ml/min. The mass spectrometer was operated in positive ion mode with capillary voltage 4.25 kV, source temperature 120 °C, desolvation temperature 400 °C and nitrogen gas flow at 1000 l/h. Data acquisition was performed using MassLynx 4.1 and quantification using QuanLynx 4.1 (Waters). The multiple reaction monitoring transitions were as follow: IS m/z 394 → 165.8, GSSG m/z 612.9 → 354.95, and GSH m/z 307.9 → 178.7. The cone voltages were 23 V, 35 V, and 25 V, and the collision energy 13 V, 22 V, and 12 V, respectively. The redox potential (E_h) of thiol/disulfide levels in the nucleus accumbens was calculated from the GSSG and GSH concentrations using the Nernst equation, $E_h = 7.4: -264 + 30\log(GSSG)/(GSH)^2$ (Jones *et al*, 2000; Uys *et al*, 2010). The values are expressed in mV ± SEM.

Western Blotting

In a separate group of rats, the accumbens was dissected from the brain after the animals were killed by decapitation to measure GSH reductase, GSTpi, glutaredoxin, and thioredoxin protein levels. Briefly, the tissue was homogenized in RIPA buffer with HALT protease and phosphatase inhibitors (Pierce, Rockford, IL) and centrifuged for 10 min at 12 000 *g*. Protein concentration was determined by the BCA assay. Samples were denatured, reduced in sample buffer, and loaded on a 10% Criterion XT Bis-Tris gel with MOPS running buffer (Bio-Rad). The proteins were transferred onto a PVDF membrane with an iBlot dry blotting system (Invitrogen, Carlsbad, CA), blocked in 5% milk in TBS-Tween, and incubated overnight at 4 °C in rabbit anti-GSH reductase (Abcam, 1 : 1000), rabbit anti-glutaredoxin 1 (Abcam, 1 : 250), rabbit anti-GSTpi (MBL, 1 : 1000), rabbit anti-xCT (Shih *et al*, 2006; Szumlinski *et al*, 2004; 1 : 500), or rabbit anti-thioredoxin (Cell Signaling, 1 : 1000). After extensive washing in TBS-Tween (3 × 10 min), the membranes were probed with goat anti-rabbit HRP-conjugated antibodies (Millipore, 1 : 10 000). The membranes were incubated in SuperSignal West Pico chemiluminescent substrate (Pierce) and exposed to ECL Hyperfilm (GE Healthcare, Pittsburgh, PA). Protein bands were quantified using Quantity One densitometry software (Bio-Rad) and normalized against levels of GAPDH or calnexin (Cell Signaling, 1 : 1000).

Behavioral Testing

Cocaine reward was assessed using a place conditioning procedure and apparatus similar to that described previously (Szumlinski *et al*, 2004). Briefly, place conditioning proceeded in the following four sequential phases: habituation, pre-conditioning test (pre-test), conditioning, and post-conditioning test (post-test). Each test lasted for 15 min. During the habituation, pre-test, and post-test the mouse was allowed to explore both compartments of the apparatus. The time spent in each compartment during the pre-test was recorded to establish compartment preferences. Cocaine conditioning was conducted over a 6-day period. Mice were brought into the conditioning room and were injected with cocaine (15 mg/kg, i.p.) and placed in the conditioning chamber. Mice received a saline injection in their preferred compartment and a cocaine injection in the non-preferred compartment on alternate days. A post-test was conducted the day following the last conditioning session (day 8).

An additional experiment was conducted in different mice to evaluate the effect GSTpi gene deletion on spontaneous locomotor activity in a novel open field, and on the acute and sensitized locomotor effect of cocaine. Locomotor activity was first recorded for 120 min after placing the animals in a novel open field photocell apparatus that contained eight photocells to record movement and software to estimate distance traveled by the animal based on consecutive breaking of adjacent photobeams *vs* breaking of the same photobeam (plexiglas activity chambers; 22 × 43 × 33 cm; Accuscan, Columbus, OH). Animals were then given seven daily injections of cocaine (15 mg/kg, i.p.; days 1–7) in the photocell apparatus, and after 3 weeks of withdrawal in the home cage the mice were returned to the photocell apparatus for a final injection of cocaine (15 mg/kg, i.p.; day 28) to assess locomotor sensitization.

In a separate experiments, the cocaine-induced locomotor sensitization protocol was repeated except mice WT were pretreated with the GST inhibitor ketoprofen (40 mg/kg, i.p.; Osbild *et al*, 2008) or DMSO/saline 10 min before to injecting cocaine on the first 7 days of daily cocaine administration (15 mg/kg, i.p.; days 1–7). As before, after 3 weeks of withdrawal all animals were returned to the photocell apparatus and administered cocaine (15 mg/kg, i.p.; day 28) without a ketoprofen pretreatment. Alternatively, WT mice were administered daily cocaine (15 mg/kg, i.p.; days 1–7) without ketoprofen or DMSO/saline pretreatment but were pretreated with either ketoprofen (40 mg/kg, i.p.) or vehicle 10 min before the final cocaine challenge injection (15 mg/kg, i.p.) on day 28. For this experiment involving acute treatment with ketoprofen, we employed a cross-over design. Accordingly, 1 week after the first cocaine challenge (day 28) on day 35 of the study animals were injected with the opposite pretreatment 10 min before administering cocaine and locomotor activity, and then behavior was monitored for 60 min.

Data Analysis

The mean and SEM were computed for each treatment group. Statistically significant differences between control and treatment groups were detected by a two-way ANOVA with a Bonferonni *post hoc* for multiple comparisons using GraphPad Prism. If only two groups were compared either a Student's *t*-test or a Mann–Whitney *U*-test was performed.

RESULTS

Cocaine-Induced Locomotor Sensitization

Figure 1 shows the motor response to cocaine and saline injections in animals used for the neurochemical studies.

Daily cocaine treatment resulted in significant sensitization of locomotor activity comparing the first (day 1) to the last (day 7) daily injection (two-way ANOVA with repeated measures over days; effect of daily saline *vs* cocaine $F(1,54)=18.55$, $p<0.001$, effect of day $F(1,54)=34.98$, $p<0.001$, interaction $F(1,54)=16.54$, $p<0.001$). On day 28, half of the animals were injected with cocaine (15 mg/kg, i.p.) and motor activity monitored for 45 min before decapitation for neurochemical measurements, and the other half were decapitated at time = 0, without a cocaine injection. Animals pretreated with daily cocaine demonstrated a significantly greater motor response over 45 min after acute cocaine than animals pretreated with daily saline (Figure 1b; two-tailed Student'*t*-test $(26)=2.51$, $p=0.018$).

Acute Cocaine Increases Redox Potential (E_h) in Accumbens

The redox state (E_h) can be expressed in terms of a voltage, where the reducing capacity is defined as the size of the redox buffering system (Schafer and Buettner, 2001). Given that GSH is the most abundant non-protein cellular thiol and redox buffer (Lopez-Mirabal and Winther, 2008), the redox potential can be calculated using GSH and GSSG concentrations through the Nernst equation (Jones *et al*, 2000; Schafer and Buettner, 2001; Uys *et al*, 2010). The E_h of thiol/disulfide levels in the nucleus accumbens was calculated from the GSSG and GSH concentrations (see Supplementary Table S1 for raw values). An increased redox potential was noted ($F(1,21)=9.25$, $p=0.006$) in the accumbens at 45 min after acute cocaine exposure in both the daily saline and cocaine pretreated animals. However, the redox potential of these daily treatment groups did not differ at baseline, nor did the effect on redox potential measured at 45 min after acute cocaine differ significantly between groups (Figure 2a).

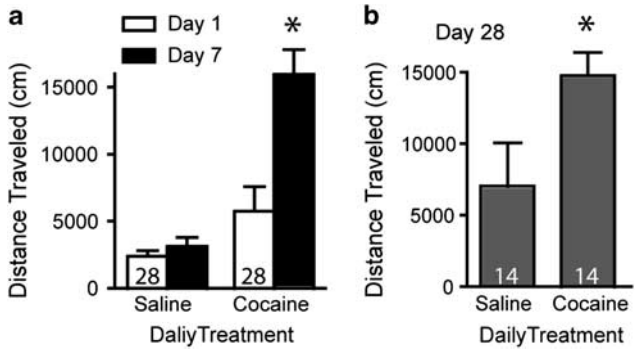

Figure 1 Daily cocaine administration produced locomotor sensitization. (a) Rats were treated with daily cocaine or saline for 7 days, and the locomotor response was increased in the daily cocaine group on day 7 compared with day 1. Data are shown as means ± SEM cm over 90 min after saline or cocaine administration during the first and last daily injection trial. $^*p<0.05$, comparing day 1 *vs* day 7 within each daily treatment group using a Bonferroni *post hoc* test. (b) After 3 weeks of no drug treatment, animals were challenged with cocaine (15 mg/kg, i.p.) and motor activity monitored for 45 min before obtaining brain tissue for neurochemical analysis. The daily cocaine group showed a greater locomotor response than the daily saline group. Data are shown a means ± SEM cm traveled over 45 min after cocaine administration. *N* is shown in the bars. $^*p<0.05$ using a two-tailed Student's *t*-test.

Free Sulfhydryl Levels

The elevated redox potential could result from lower cellular levels of GSH as a consequence of GSH utilization for *S*-glutathionylation of proteins and the subsequent formation of GSSG (see Figure 5). Free sulfhydryl levels are an indirect indication of the amount of GSH conjugated with cysteine residues in cellular proteins. A two-way ANOVA revealed that daily cocaine treatment reduced free sulfhydryls by daily cocaine treatment ($F(1,68)=8.68$, $p=0.04$). Also, acute cocaine reduced free sulfhydryls in both daily treatment groups ($F(1,68)=4.87$, $p=0.031$), but no interaction between the acute and daily treatments was measured (Figure 2b). These data indicate that both daily and acute cocaine increase protein *S*-glutathionylation, and that the effects are additive.

Levels of GSH-Related Proteins

The reduction in free sulfhydryl levels by acute and daily cocaine could arise from cocaine-induced alterations in GSH-metabolizing proteins that regulate the *S*-thionylation, *S*-nitrosylation, or *S*-oxidation of cysteine residues on proteins (see Figure 5 for illustration of enzyme regulation of GSH–GSSG). Glutaredoxin is responsible for de-glutathionylation (Mieyal *et al*, 2008), while thioredoxin is a protein denitrosylation catalyst (Tannenbaum and White, 2006). A two-way ANOVA revealed that glutaredoxin levels were increased by acute cocaine at 45 min after injection ($F(1,15)=18.96$, $p<0.001$). However, there was no effect of daily treatment or an interaction between daily acute treatments, indicating that the levels of glutaredoxin were not altered after withdrawal from daily cocaine (Figure 3a). In contrast, for thioredoxin no significant effect of acute cocaine, daily treatment or an interaction between acute and daily treatments was found; indicating that neither acute nor daily cocaine altered the levels of thioredoxin (Figure 3b). GSH reductase was measured because it catalyzes the conversion of GSSG back to GSH, thereby restoring the redox potential and interfering with the *S*-glutathionylation of proteins (Townsend, 2007). However, a two-way ANOVA revealed no effect by acute or daily cocaine (Figure 3c). Finally, GSTpi catalyzes the *S*-glutathionylation of cysteine residues on cellular

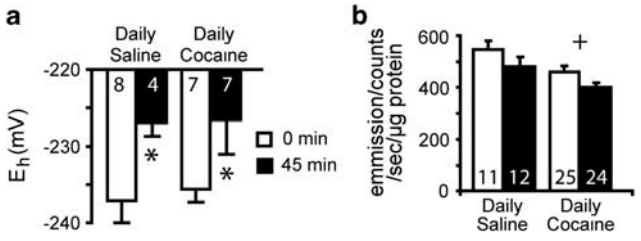

Figure 2 Effect of acute and daily cocaine on redox potential and free sulfhydryls in rat nucleus accumbens. (a) Redox potential was reduced by acute cocaine in both daily treatment groups. (b) Free sulfhydryls were reduced by acute cocaine only in the daily cocaine-withdrawn animals. *N* is shown in the bars. $^*p<0.05$, comparing 45 min after acute cocaine with pre-injection levels within each daily treatment group. $^+p<0.05$, comparing pre-injection redox levels between daily cocaine and saline groups.

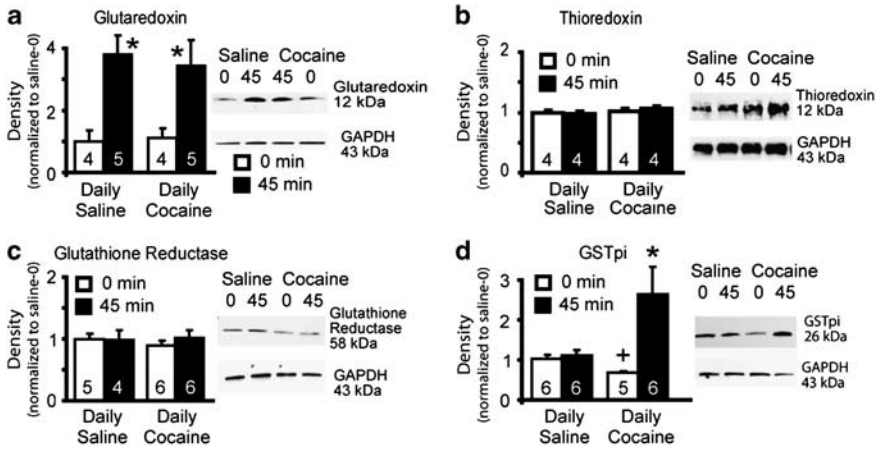

Figure 3 Effect of acute and daily cocaine on GSH-related proteins in rat nucleus accumbens. (a) Glutaredoxin was increased by acute cocaine in both daily treatment groups, but there was no effect of daily treatment. (b) No effect of acute or daily cocaine on the levels of thioredoxin. (c) No effect of acute or daily cocaine on the levels of GSH reductase. (d) GSTpi was significantly reduced by daily cocaine, but after acute cocaine injection levels were markedly increased. *N* is shown in the bars. $^{*}p<0.05$, comparing 45 min after acute cocaine with pre-injection levels within each daily treatment group using a Bonferonni *post hoc*, $^{+}p<0.05$, comparing daily cocaine to daily saline at 0 min using a Bonferonni *post hoc*.

proteins (Townsend *et al*, 2009). Figure 3d shows that there was a significant reduction in basal levels (ie, time = 0 min) of GSTpi following daily cocaine (acute treatment $F(1,19) = 5.98$, $p = 0.024$). Also, there was a marked increase after acute cocaine only in daily cocaine-withdrawn animals (interaction between acute and daily treatment $F(1,19) = 4.78$, $p = 0.042$). As a result of the marked changes in GSTpi in the accumbens, we examined GSTpi levels in the prefrontal cortex. All four treatment groups showed equivalent levels of GSTpi in the prefrontal cortex, indicating that this effect may be relatively selective for the accumbens ($n = 6$ in all groups; saline, time $0 = 100 \pm 16.9$; saline, time $45 = 118.4 \pm 9.2$; cocaine, time $0 = 121.7 \pm 5.4$; cocaine, time $45 = 113.6 \pm 8.4$).

Cocaine-Induced Changes in GSTpi Contribute to the Behavioral Effects of Cocaine

In order to assess the functional significance of the lower levels of GSTpi after withdrawal form daily cocaine, we examined the effects of acute and daily cocaine administration in mice harboring a gene deletion of GST*P1P2* (GST*P1P2*-KO), or in mice pretreated with the GST antagonist ketoprofen (Osbild *et al*, 2008). Figure 4a shows that a treatment regimen of cocaine that did not induce CPP in WT mice produced significant preference for the cocaine-paired side of the apparatus GST*P1P2*-KO mice (Student's *t*-test, $t(11) = 2.38$, $p = 0.037$). When animals were placed into a novel open-field no difference in locomotor activity was measured between genotypes (Figure 4b). However, a subsequent injection of cocaine (15 mg/kg, i.p.) elicited a greater locomotor response in the GST*P1P2*-KO during the first 10 min after injection compared with WT mice (Figure 4c; two-way ANOVA with repeated measures over time, genotype $F(3,320) = 9.62$, $p < 0.001$, time $F(10,320) = 87.61$, $p < 0.001$, interaction $F(30,320) = 3.76$, $p < 0.001$). All mice were then given another seven daily injections of cocaine, and after 3 weeks of withdrawal (day 28) challenged with another cocaine injection (15 mg/kg, i.p.). Figure 4c shows that the sensitized motor response on day 28 in the GST*P1P2*-KO was greater than in the WT mice between 30 and 40 min after cocaine administration. Also, while sensitization was significant only during the first 10 min after injection in WT (ie, comparing day 1 with day 28), the sensitized response in the GST*P1P2*-KO mice was manifested for the first 50 min after injection. Finally, GSH synthesis is regulated in part by availability of intracellular cystine arising from active cystine–glutamate exchange (McBean, 2002). The exchanger is a heterodimer, with xCT being the catalytic subunit, and levels of xCT are reduced after chronic cocaine (Kalivas, 2009). It has previously been shown that GST*P1P2* deletion does not alter the expression of other GST isoforms (Kitteringham *et al*, 2003). Similarly, we found that the level of xCT in nucleus accumbens membranes was not altered in GST*P1P2*-KO (106.7 ± 9.2, $n = 5$) compared with WT (100 ± 10.1, $n = 6$) mice (see Supplementary Figure S1).

With constitutive gene deletion it is not possible to know if the augmented sensitized motor response to cocaine in KO mice resulted from promoting the development of sensitized behavior during daily injections of cocaine, or from preventing the acute increase in GSTpi produced by a cocaine challenge in animals withdrawn from daily cocaine. Ketoprofen is an antagonist of GSTpi, and to pharmacologically evaluate the potential involvement of GSTpi in the development of sensitization animals we administered ketoprofen 10 min before each daily cocaine injection, but not before the challenge injection of cocaine made 3 weeks after discontinuing daily cocaine. Pretreatment on day 1 produced a trend toward reducing the acute motor response to cocaine (Figure 4d). However, at 3 weeks of withdrawal after 7 daily cocaine injections, the animals treated with ketoprofen before each daily cocaine injection on days 1–7 showed a larger sensitized motor response from 10 to 15 min after injection than animals administered vehicle before each daily cocaine injection (note that on day 28 animals were administered only cocaine). Moreover, comparing day 1 with 28 in control animals

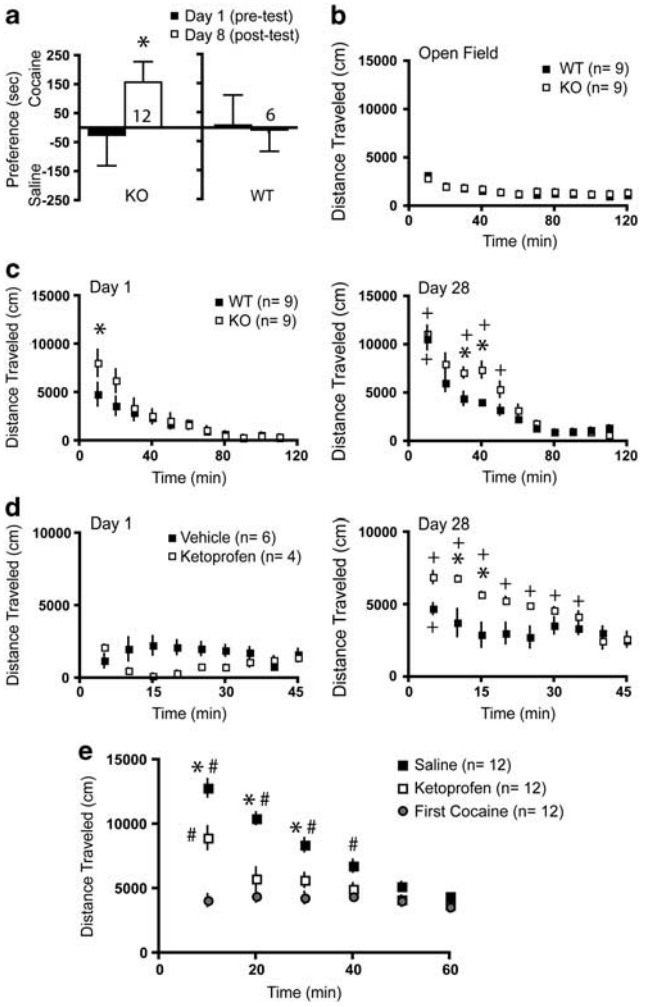

Figure 4 Reducing GSTpi function promotes cocaine-induced conditioned place preference and behavioral sensitization. (a) Genetic deletion of GSTP1P2 facilitated preference for the cocaine-paired side of the apparatus. Data are shown as difference in time spent between the saline- and cocaine-paired sides of the apparatus. *N* is shown in the bars. $^{*}p<0.05$ comparing pre-test to post-test. (b) GSTP1P2-KO and WT animals show equivalent motor responses in a novel open field. (c) GSTP1P2-KO animals show enhanced sensitization compared with WT mice. (d) Ketoprofen potentiated cocaine sensitization. (e) Acute ketoprofen reduces the sensitized motor response to cocaine a cocaine challenge administered on day 28 (ie, at 3 weeks after the last daily cocaine injection) $^{*}p<0.05$, comparing between WT and KO, or vehicle to ketoprofen using a Bonferroni *post hoc*, $^{+}p<0.05$, comparing between days 1 and 28 within each genotype or vehicle and ketoprofen treatment group using a Bonferonni *post hoc*. $^{\#}p<0.05$, comparing vehicle or ketoprofen on day 28 to the first cocaine injection (panel e) using a Bonferonni *post hoc*.

revealed that sensitization was measured only during the first 5 min after injection, while in the animals treated with ketoprofen during daily cocaine administration showed a sensitized motor response during the first 35 min after injection. These data were statistically analyzed using a two-way ANOVA with repeated measures over time, drug $F(3,24)=50.98$, $p<0.001$, time $F(8,30)=7.16$, $p<0.001$, interaction $F(24,128)=12.07$, $p<0.001$.

In the final experiment, 3 weeks after the last daily cocaine injection animals were pretreated with ketoprofen or vehicle 10 min before a cocaine injection, and the effect of acute GSTpi blockade on the expression of cocaine sensitization determined. Figure 4e shows that while both vehicle and ketoprofen pretreated animals showed locomotor sensitization compared with the first injection of cocaine, the sensitized motor response in the vehicle animals was significant greater than in the ketoprofen animals during the first 30 min after cocaine administration. These data were statistically analyzed using a two-way ANOVA with repeated measures over time, drug $F(2,155)=26.47$, $p<0.001$, time $F(5,155)=26.19$, $p<0.001$, interaction $F(10,155)=14.28$, $p<0.001$.

DISCUSSION

The downregulation of system Xc- by daily cocaine reduces the extracellular levels of glutamate in the nucleus accumbens that is associated with a number of enduring changes in glutamatergic synaptic transmission thought to promote cocaine-seeking in animals models of addiction (Kalivas, 2009). Given that the uptake of cystine through system Xc- is a rate-limiting step in glial synthesis of GSH (Griffith, 1999; O'Connor *et al*, 1995) and the importance of GSH in maintaining cellular redox potential (Lopez-Mirabal and Winther, 2008), it was surprising that withdrawal from daily cocaine did not alter accumbens redox potential (considered the most general index of oxidative stress (Jones *et al*, 2000)). However, it has been suggested that changes in cellular protein thiol status could be a more accurate indicator of redox status, because protein thiols represent a larger potential redox pool than free GSH (Hansen *et al*, 2009). Importantly, withdrawal from daily cocaine reduced the level of free sulfhydryls, consistent with an increase in protein *S*-glutathionylation. Proteins catalyzing the de-glutathionylation or de-nitrosylation of proteins, glutaredoxin and thioredoxin, respectively, were unchanged after daily cocaine. Furthermore, the level of the primary catalyst for glutathionylating proteins, GSTpi, was reduced by daily cocaine implying a diminished capacity for the forward reaction of glutathionylation (Figure 5). One possible explanation for the seemingly contradictory effects of daily cocaine on protein glutathionylation and levels of GSTpi in the accumbens is that after 3 weeks of withdrawal this balance of GSH-metabolizing proteins and protein glutathionylation creates a compensatory homeostasis preserving cellular redox potential. Thus, after withdrawal from daily cocaine there is increased *S*-glutathionylation of endogenous proteins that may be preserving cellular redox potential in spite of the cocaine-induced reduction in system Xc- to maintain GSH levels.

Acute Cocaine Increased Redox Potential

In contrast with daily cocaine-withdrawn animals, an acute cocaine challenge markedly increased the redox potential to an equivalent extent in both the saline and cocaine daily treatment groups, indicating that cocaine-induced changes in overall cellular redox are not affected by daily cocaine. The increase in redox potential by acute cocaine is consistent with previously reported measures of increased oxidative stress after cocaine administration (Dietrich *et al*,

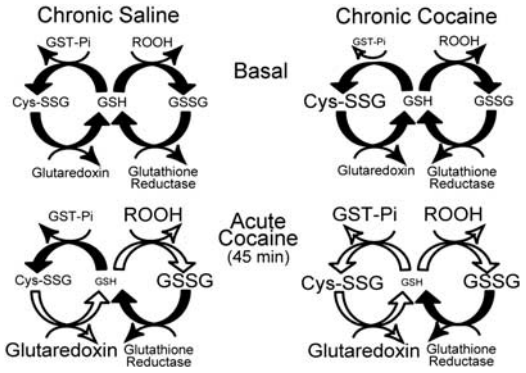

Figure 5 Model illustrating changes in redox potential, protein thionylation (Cys-SSG) and GSH-related proteins after acute and daily cocaine. In the absence of an acute cocaine challenge (Basal), withdrawal from daily cocaine reduced GSTpi and increased *S*-glutathionylation (Cyss-SSG), which did not significantly impact redox potential in the nucleus accumbens. At 45 min after acute cocaine the redox potential of the accumbens was reduced regardless of daily treatment, which is reflected by decreased GSH and increased GSSG resulting in part from an increase in glutaredoxin-mediated de-glutathionylation of proteins. In addition, in daily cocaine subjects, GSTpi was dramatically increased by acute cocaine, leading to a further increase in protein *S*-glutathionylation (increased Cys-SSG). Although not reflected by significant further reductions in redox potential in daily cocaine animals, the increase in GSTpi and Cys-SSG implies less GSH availability in the event of augmented oxidative stress, but also poses increased signaling via protein *S*-glutathionylation that can protect cells from oxidative stress (Townsend, 2007; Townsend *et al*, 2009). Reduced protein levels and metabolic processing are indicated in blue and smaller text, increased protein and metabolic function is indicated in red and larger text.

2005; Numa *et al*, 2008). The oxidative increase in redox potential was accompanied by an adaptive increase in glutaredoxin, which would be expected to increase free GSH via de-glutathionylating proteins. In contrast, acute cocaine reduced levels of free sulfhydryl groups, implying increased amounts of protein *S*-glutathionylation. In the daily cocaine-withdrawn animals, increased protein *S*-glutathionylation likely arises from the marked elevation in GSTpi after acute cocaine administration that occurred only in daily cocaine-withdrawn animals.

Cocaine Changes GSTpi Expression

Of all the measurements made in this study, GSTpi expression was most affected by exposure to acute and daily cocaine, posing the protein as a key homeostatic adaptation produced by daily cocaine treatment that could contribute to the enduring neuroplasticity underlying the long-term behavioral changes associated with cocaine addiction. This protein is known to be a cytosolic phase II detoxifying enzyme, and a recent study provided evidence for GSTpi in catalyzing *S*-glutathionylation of proteins and leading to a decrease in free protein sulfhydryl thiols similar to the results obtained in this study (Townsend *et al*, 2009). Interestingly, GSTpi is enriched in the striatum and dopamine neuron-containing regions of the ventral mesencephalon (Castro-Caldas *et al*, 2009). Accordingly, GST*P1P2*-KO mice show an enhanced sensitivity to MPTP-induced dopamine depletion (Smeyne *et al*, 2007), and overexpression of three allelic GSTpi variants provides protection against rotenone-induced dopaminergic cell loss (Shi *et al*, 2009). In addition, human polymorphisms in GSTpi have been shown to increase susceptibility to Parkinson's disease (Vilar *et al*, 2007). Importantly, the high activity GSTpi polymorphism (Ile105Val) differed between male cocaine users and controls (Guindalini *et al*, 2005). To investigate the role of GSTpi in cocaine-mediated behavioral plasticity, we administered daily cocaine to GST*P1P2*-KO mice and measured locomotor sensitization and CPP. GST*P1P2*-KO mice showed an enhanced response in both of these examples of enduring cocaine-induced behavioral plasticity. Also, pharmacological inhibition of GST by systemic ketoprofen produced a similar augmentation in behavioral sensitization to cocaine. As both behaviors are thought to involve overlapping, but in part distinct, cocaine-induced plasticity in brain circuitry (Tzschentke, 2007; Vanderschuren and Kalivas, 2000), this indicates that daily cocaine-induced reductions in GSTpi may be an adaptation that confers general susceptibility to enduring cocaine-induced behavioral changes. Along these lines, it is possible that the marked elevation in GSTpi protein levels after an acute cocaine challenge only in daily cocaine-withdrawn animals may be compensatory and serves to limit the expression of cocaine-induced sensitization. For example, elevated GSTpi could enhance protein *S*-glutathionylation and thereby protect cysteine residues from further oxidation by irreversible modifications (eg, sulfination and sulfonation) that can lead to protein degradation (Mallis *et al*, 2002). This possibility was borne out by finding that acute inhibition of GSTpi before the sensitization trial potentiated cocaine-induced locomotor activity.

Potential Role of Cocaine-Induced Protein *S*-Glutathionylation

Given the importance of *S*-glutathionylation for cell signaling in response to oxidative stress, and the finding herein and elsewhere indicating that cocaine produces oxidative stress, it is possible that some of the many changes in cell signaling proteins that have been reported after acute and daily cocaine administration may be related to protein *S*-glutathionylation. For example, a number of proteins known to be affected by cocaine, including actin, JNK, nuclear kinase kappaB, and cyclic AMP-dependent protein kinase (Hyman *et al*, 2006; Kalivas and O'Brien, 2008), are also regulated by *S*-glutathionylation (Fiaschi *et al*, 2006; Humphries *et al*, 2005; Klatt and Lamas, 2002; Reynaert *et al*, 2006). Indeed, kinases such as JNK and ASK1 are susceptible to direct inhibition by GSTpi or thioredoxin, respectively (Adler *et al*, 1999). *S*-glutathionylation of JNK breaks the protein–protein interactions necessary to keep the GSTp–JNK complex together. This activates JNK and stimulates downstream pathways associated with c-jun activation. Thus, one plausible interpretation of our data is that cocaine-induced oxidative stress may be a key precipitant of neuroadaptations induced by cocaine via signaling associated with increased *S*-glutathionylation. These could ultimately contribute to addictive behaviors and may in part account for the emerging findings that in animal models, treatment with antioxidants can inhibit

drug-induced behaviors such as sensitization, CPP, and reinstated drug-seeking (Achat-Mendes *et al*, 2007; Moussawi *et al*, 2009; Muriach *et al*, 2010; Numa *et al*, 2008).

System Xc- and Cocaine-Induced Changes in Redox

The finding that constitutive genetic deletion of GST*P1P2* did not alter accumbens levels of xCT, the catalytic subunit of system Xc- (Shih *et al*, 2006), is not supportive of a linkage between the previously reported cocaine-induced downregulation of system Xc- (Baker *et al*, 2003) and the changes produced in GST*P1P2* and cellular redox shown herein. Downregulation of xCT reduces extracellular glutamatergic tone on synaptic mGluRs and this is thought to contribute to the vulnerability to reinstate cocaine-seeking (Kalivas, 2009). Moreover, system Xc- is rate limiting in supplying cystine substrate for GSH synthesis Xc- (McBean, 2002) and the increase in glutationylated protein may serve as a reserve source of GSH (Hansen *et al*, 2009); making it possible that the cocaine-induced regulation of GST*P1P2* might be related to the reduction in system Xc-. While an interaction may still be possible, deletion of GST*P1P2* did not regulate xCT levels; although the constitutive nature of the genetic deletion leaves open the possibility that developmental compensation may have masked an interaction between system Xc- and GST*P1P2*. Clearly, further experimentation into a mechanistic linkage between these two cocaine-induced protein adaptations in necessary to conclude a lack of interaction.

CONCLUSIONS

Our data demonstrate that in addition to reducing the activity of system Xc-, daily cocaine administration produces enduring alterations in some aspects of cellular redox, specifically an apparent increase in protein *S*-glutathionylation and a decrease in GSTpi. When the cocaine-induced decrease in GSTpi was modeled either by genetic deletion or by pharmacological inhibition of GSTpi, the capacity of daily cocaine to induce CPP or locomotor sensitization was enhanced, indicating that the reduction in GSTpi may promote the actions of cocaine to induce neuroplasticity. Moreover, the marked rebound in GSTpi by acute cocaine in animals withdrawn from daily cocaine appeared compensatory because preventing increased GSTpi activity with ketoprofen potentiated locomotor sensitization. The combination of increased *S*-glutathionylation and dynamic changes in GSTpi and the apparent role of GSTpi in locomotor sensitization and CPP poses the possibility that adaptations in cellular redox potential may contribute to the glutamatergic neuroplasticity thought to strongly contribute to cocaine addiction (Kalivas, 2009; Kauer and Malenka, 2007). However, the cellular mechanisms that may link cocaine-induced alterations in GSTpi with glutamatergic transmission remain to be determined.

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DISCLOSURE

The authors declare no conflict of interest.

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