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Neuroadaptations in cystine-glutamate exchange underlie cocaine relapse

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Repeated cocaine treatment and withdrawal produces changes in brain function thought to be involved in relapse to drug use. Withdrawal from repeated cocaine reduced *in vivo* extracellular glutamate in the nucleus accumbens of rats by decreasing the exchange of extracellular cystine for intracellular glutamate. *In vivo* restoration of cystine/glutamate exchange by intracranial perfusion of cystine or systemically administered *N*-acetylcysteine normalized the levels of glutamate in cocaine-treated subjects. To determine if the reduction in nonvesicular glutamate release is a mediator of relapse, we examined cocaine-primed reinstatement of drug seeking after cocaine self-administration was stopped. Reinstatement was prevented by stimulating cystine/glutamate exchange with *N*-acetylcysteine and restoring extracellular glutamate. Thus, withdrawal from repeated cocaine increases susceptibility to relapse in part by reducing cystine/glutamate exchange, and restoring exchanger activity prevents cocaine-primed drug seeking.

Addiction to cocaine is marked by a transition from recreational drug use to a compulsive pattern in which addiction-related behaviors such as craving and paranoia emerge[1,2]. The reinstatement of drug-seeking behavior in rats is commonly used to model relapse in human addicts[3], and a variety of data indicate that corticolimbic circuitry, including the glutamatergic projection from the prefrontal cortex to the nucleus accumbens, mediates reinstatement in experimental animals and craving in cocaine addicts[1,4,5]. Moreover, glutamate release in the nucleus accumbens is critical for the expression of drug-seeking behavior elicited by systemic or intra-cranial administration of cocaine[6–8].

Although increased glutamate transmission in the nucleus accumbens is a mediator of drug-seeking behavior, withdrawal from repeated cocaine treatment reduces basal extracellular glutamate levels in the nucleus accumbens of rats[9–11]. *In vivo* basal levels of extrasynaptic glutamate are maintained primarily by Na^+-independent anionic amino acid transporters that exchange extracellular cystine for intracellular glutamate, and the contribution by synaptically released glutamate is minimal[12,13]. The cystine/glutamate exchanger comprises two separate proteins: a light chain, xCT, that is unique to the exchanger and a heavy chain, 4F2, that is common to many amino acid transporters[14,15]. The exchanger is ubiquitous in cells in the brain and probably has a protective function in oxidative stress to provide cysteine for glutathione synthesis[16,17], as it is concentrated in the ventricular ependyma and circumventricular organs[18]. Activity of the exchanger also modulates neurotransmission by providing glutamatergic tone on metabotropic glutamate receptors (mGluRs)[12] and can cause excitatory neurotoxicity[16].

Cystine/glutamate exchange maintains extracellular glutamate concentrations and potentially modulates excitatory neurotransmission[12,16]. In addition, withdrawal from repeated cocaine reduces extracellular glutamate, and glutamate transmission is known to be important in drug-seeking behavior[7–10]. These findings raise the question of whether the cystine/glutamate exchanger mediates cocaine-primed drug seeking. We therefore designed experiments to test two hypotheses: (i) diminished activity of cystine-glutamate exchangers after cocaine treatment underlies the persistent reduction in basal extracellular glutamate levels observed in the nucleus accumbens following withdrawal from repeated cocaine and (ii) pharmacological restoration of exchanger activity prevents cocaine-primed reinstatement of drug seeking in rats trained to self-administer cocaine. Both hypotheses were verified by our results.

RESULTS

Repeated cocaine decreases extracellular glutamate

The concentration of extracellular glutamate in the nucleus accumbens, prefrontal cortex and striatum was measured after 3 weeks of withdrawal from experimenter-delivered cocaine (15–30 mg per kg of body weight, delivered intraperitoneally (i.p.) for 7 days) using no-net flux *in vivo* microdialysis (**Fig. 1a**). Cocaine-treated rats showed lower basal glutamate levels in the nucleus accumbens relative to controls (saline, 5.60 ± 1.00 μM (mean ± s.e.m.); cocaine, 2.89 ± 0.34 μM). Basal glutamate levels were not diminished in the striatum (saline, 2.39 ± 0.40 μM; cocaine, 2.49 ± 0.40 μM) or the prefrontal cortex (saline, 1.16 ± 0.49 μM; cocaine, 1.11 ± 0.24 μM). The slopes of regression lines indicated that glutamate clearance did not differ between saline- and cocaine-treated rats in the nucleus accumbens (saline, 0.535 ± 0.0189 μM; cocaine, 0.542 ± 0.029 μM), striatum (saline, 0.465 ± 0.020 μM; cocaine, 0.424 ± 0.027 μM) or prefrontal cortex (saline, 0.437 ± 0.017 μM; cocaine, 0.448 ± 0.016 μM).

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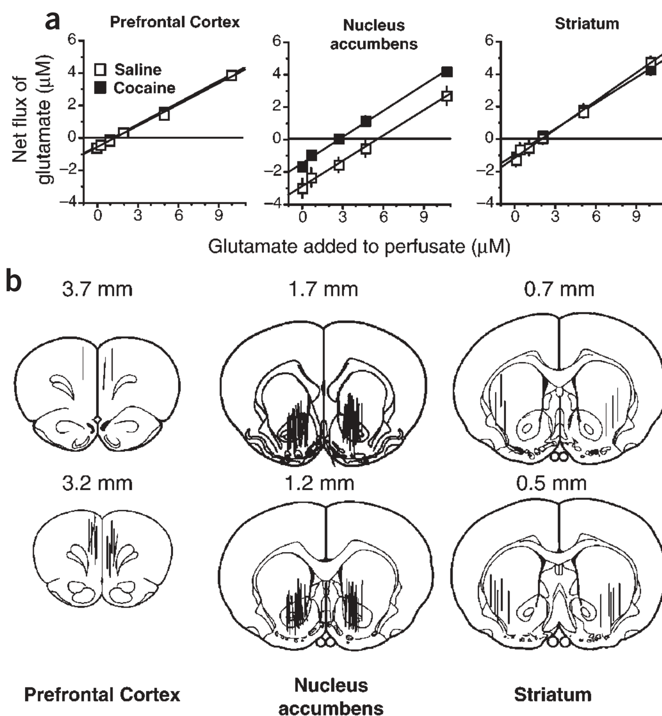

Figure 1 Basal levels of extracellular glutamate were lower in the nucleus accumbens, but not in the striatum or prefrontal cortex, after 3 weeks of withdrawal from experimenter-delivered repeated cocaine administration (15–30 mg/kg, i.p. for 7 d). (**a**) Plots from no-net flux *in vivo* microdialysis experiments showing basal glutamate levels ($y = 0$) and clearance of glutamate from the probe site (slope) in the nucleus accumbens, striatum and prefrontal cortex. Data reflect the mean ± s.e.m. ($n = 6$–8 per group). (**b**) Location of microdialysis probes aimed at the prefrontal cortex, nucleus accumbens and striatum with the numbers indicating distance from bregma[40].

The location of the probes targeting the prefrontal cortex was largely limited to the dorsal prefrontal cortex with a few extending into infralimbic cortex (**Fig. 1b**). Within the nucleus accumbens, the probes were almost entirely located in the core, with a few penetrating the ventral aspect of the shell. Within the striatum, the probes were primarily located in the ventrolateral portion.

Repeated cocaine decreases cystine-glutamate exchange

The kinetics of [^{35}S]cystine uptake by cystine-glutamate exchangers were estimated using a Lineweaver-Burke plot in *ex vivo* tissue obtained from the nucleus accumbens after 3 weeks of withdrawal from daily cocaine or saline injections (**Fig. 2a**). The affinity of cystine transport (K_m, $-1/x$-intercept) was higher in the nucleus accumbens of cocaine-treated rats (4.24 ± 0.23 μM) than in tissue from control rats (2.11 ± 0.23 μM). This increase was observed in the absence of a significant difference in velocity of cystine transport (V_{max}, $1/y$-intercept) between saline- and cocaine-treated rats. The lack of effect by repeated cocaine administration on mRNA levels of xCT is consistent with the equivalent V_{max} of cystine uptake between saline controls and cocaine-treated rats ($n = 6$ per group; percent change in mRNA from control (mean ± s.e.m.): saline, 100 ± 5%; cocaine, 95 ± 3%).

Because cystine/glutamate exchange is not energy-dependent, the K_m is determined primarily by substrate concentration (that is, intra- and extracellular cystine and glutamate)[19]. Although the extracellular cystine concentration was experimenter-controlled in the *in vitro* study (**Fig. 2**), it is possible that the *in vivo* concentration of extracellular cystine differed between cocaine and saline subjects. A no-net flux microdialysis estimate of *in vivo* extracellular cystine levels in the nucleus accumbens revealed that basal extracellular cystine ($y = 0$) did not differ between saline controls (0.131 ± 0.052 μM) and cocaine-withdrawn rats (0.194 ± 0.075 μM; **Fig. 2b**). Cystine clearance (indicated by the slope of the regression lines) also did not significantly differ between saline-treated (0.484 ± 0.011) and cocaine-treated rats (0.444 ± 0.031).

Manipulating the exchanger normalizes extracellular glutamate

We measured the contribution of cystine/glutamate exchange to the difference in extracellular glutamate between saline- and cocaine-treated rats by reverse-dialyzing the cystine/glutamate exchange inhibitor (S)-4-carboxyphenylglycine (CPG)[20]. Reverse dialysis of CPG into the nucleus accumbens significantly reduced extracellular glutamate levels in control subjects to the level measured in cocaine-withdrawn rats (3 weeks after discontinuing 7 days of daily injections; **Fig. 3a**). In contrast, CPG was without effect in the cocaine-withdrawn subjects. Thus, blockade of cystine/glutamate exchange mimicked the effect of withdrawal from repeated cocaine on extracellular glutamate. In the converse experiment, cystine/glutamate exchange was stimulated by providing the extracellular substrate cystine by reverse dialysis[19]. Activation of the exchanger by reverse dialysis of cystine into the nucleus accumbens restored the reduced levels of extracellular glutamate in cocaine subjects to the levels measured in control animals without altering extracellular glutamate levels in control subjects (**Fig. 3b**).

N-acetylcysteine is a cysteine pro-drug that can be systemically administered to deliver large amounts of cysteine to the brain[21,22]. Similar to cystine administered directly into the nucleus accumbens via reverse dialysis (**Fig. 3b**), systemic administration of *N*-acetylcysteine elevated extracellular glutamate in animals that had been treated with

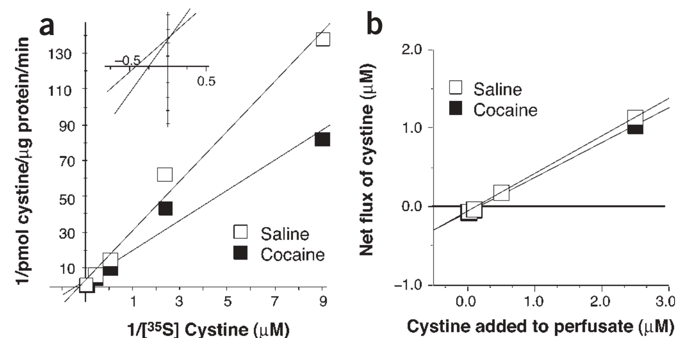

Figure 2 Three weeks withdrawal from repeated cocaine (× 7 days) treatment produced a significant increase in the K_m of cystine/glutamate exchange without altering V_{max}. (**a**) V_{max} and K_m of [^{35}S]cystine uptake in nucleus accumbens tissue slices were estimated using the Lineweaver-Burke plot and are evident as $1/y$-intercept and $-1/x$-intercept, respectively. Inset, a magnified view of the regression lines intersecting each axis. There was a significant difference in the K_m obtained from tissue slices harvested from saline- and cocaine-treated rats ($t_4 = 4.52$, $P < 0.05$) in the absence of a significant change in V_{max} ($t_4 = 0.12$, $P > 0.05$). (**b**) The no-net flux *in vivo* microdialysis technique was used to determine basal cystine levels ($y = 0$) in the nucleus accumbens from rats withdrawn from saline and cocaine treatment. Two-tailed Student's *t*-tests revealed no difference in basal cystine levels ($t_{14} = 0.325$, $P > 0.05$) or cystine clearance ($t_{14} = 0.975$, $P > 0.05$) between cocaine ($n = 8$) and saline ($n = 8$) treated rats.

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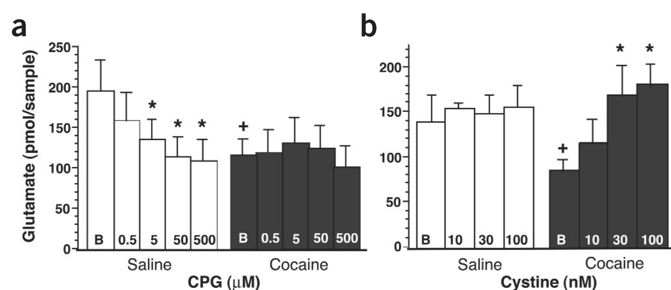

Figure 3 Cystine/glutamate exchange accounted for reduced extracellular glutamate in the nucleus accumbens following 3 weeks withdrawal from repeated cocaine treatment (× 7 days). (**a**) *In vivo* microdialysis was used to sample extracellular glutamate in the nucleus accumbens before and after infusion of the cystine/glutamate exchange blocker CPG in rats withdrawn from saline ($n = 7$) or cocaine treatment ($n = 7$). Data are presented as mean ± s.e.m. pmol/sample glutamate. A two-way ANOVA indicated a significant interaction between CPG concentration and cocaine treatment ($F_{4,48} = 5.19$, $P < 0.05$). (**b**) *In vivo* microdialysis was used to sample extracellular glutamate before and after infusion of cystine in rats withdrawn from saline ($n = 7$) and cocaine treatment ($n = 6$). A two-way ANOVA indicated a significant interaction between cystine concentration and cocaine treatment ($F_{3,33} = 4.71$, $P < 0.05$). $^+P < 0.05$, difference from saline treated controls (Fisher LSD *post hoc* analysis). $^*P < 0.05$ difference from baseline (Fisher PLSD *post hoc* analysis).

repeated cocaine injections and withdrawn for 3 weeks (**Fig. 4b**). There was no effect in the repeated saline treatment group (**Fig. 4a**). Reverse dialysis of the cystine/glutamate exchange antagonist CPG into the nucleus accumbens blocked the elevation in extracellular glutamate (**Fig. 4c**), indicating that *N*-acetylcysteine is acting by increasing cystine/glutamate exchange.

Restoring glutamate prevents cocaine increases in glutamate

After discontinuing repeated cocaine administration, a subsequent cocaine injection elevates extracellular glutamate in the nucleus accumbens[9,11,23], whereas a cocaine injection in control animals is without effect, except at very high doses[24]. Normalizing the level of extracellular glutamate in cocaine-withdrawn rats by infusing cystine into the nucleus accumbens prevented the increase in extracellular glutamate normally produced by a cocaine priming injection (**Fig. 5**). In this experiment, subjects were treated for one week with daily noncontingent cocaine or saline injections, and 3 weeks later microdialysis for glutamate was conducted in the nucleus accumbens. After collecting baseline samples, cystine or control buffer was infused through the probe for 1 h followed by an i.p. injection of saline, and 1 h later by an i.p. injection of cocaine. In the cocaine-treated rats that received prior infusion with control buffer, a cocaine priming injection elicited a significant increase in extracellular glutamate (**Fig. 5b**). In contrast, cystine infusion elevated extracellular glutamate levels in the cocaine group, and a subsequent injection of cocaine did not cause further elevations (**Fig. 5b**). In the repeated saline treatment group (**Fig. 5a**), neither cystine infusion nor the acute cocaine injection altered extracellular glutamate.

Restoring glutamate prevents cocaine-primed drug seeking

The reinstatement of drug-seeking behavior in rats extinguished from intravenous self-administration of cocaine is a widely used animal model of drug relapse[3]. Reinstatement of cocaine-seeking behavior (active lever pressing) can be initiated by a number of priming stimuli, including a noncontingent injection of cocaine. Pretreatment with *N*-acetylcysteine 4 h before administering a cocaine priming injection (10 mg/kg, i.p.) produced a dose-dependent reduction in cocaine-primed reinstatement (**Fig. 6a**). A similar effect was observed after pretreatment with another systemically active cysteine pro-drug (–)-2-oxothiazolidine-4-carboxylic acid (OTC)[25]. As in animals injected with repeated cocaine (**Fig. 5**), in subjects trained to self-administer cocaine, an acute cocaine injection significantly elevated extracellular glutamate for approximately 90 min after injection (**Fig. 6b**)[11]. When animals were pretreated with *N*-acetylcysteine, the basal levels of extracellular glutamate increased, and the cocaine priming injection caused no further increase in glutamate. *N*-acetylcysteine also blocked the reinstatement of drug seeking (**Fig. 6c**).

As cocaine-primed reinstatement is biphasic with respect to cocaine dose[26], it is possible that the cysteine pro-drugs increased the reinstating efficacy of cocaine, thereby reducing active lever presses. However, both cysteine pro-drugs blocked reinstatement after priming with a lower dose of cocaine (5 mg/kg, i.p.), indicating that the blockade did not arise from the cysteine pro-drugs potentiating the effects of cocaine (**Fig. 7a**). The blockade of reinstatement was selective for cocaine-primed reinstatement since food-primed reinstatement of lever pressing was not altered by pretreatment with *N*-acetylcysteine (**Fig. 7b**). These data also verify that *N*-acetylcysteine did not nonspecifically disrupt the operant behavior. *N*-acetylcysteine also had no effect on basal or cocaine-induced locomotor activity (data not shown). Finally, the rate of cocaine self-administration in animals who achieved criterion for acquiring cocaine self-administration (see Methods) was not altered by a 4-h pretreatment with either *N*-acetylcysteine or OTC (**Fig. 7c**), indicating that the cysteine pro-drugs did not alter the ability of the animal to regulate cocaine intake. In no experiment (**Figs. 6** and **7**) did *N*-acetylcysteine or OTC

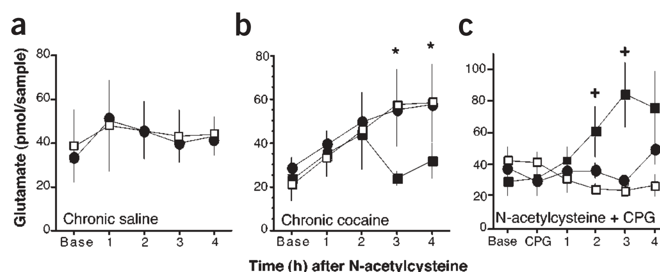

Figure 4 Three weeks after discontinuing from repeated cocaine (× 7 days), systemic administration of the cysteine pro-drug *N*-acetylcysteine elevated extracellular glutamate by increasing cystine/glutamate exchange. (**a**,**b**) After repeated saline treatment (**a**), *N*-acetylcysteine did not alter extracellular glutamate. Data are shown as mean ± s.e.m. per 60-min sample, $n = 5$–6 in each group (■ 6 mg/kg, s.c.; □ 60 mg/kg, s.c.; ● 600 mg/kg, s.c.). (**b**) After repeated cocaine treatment, *N*-acetylcysteine produced a dose-dependent increase in extracellular glutamate that was significant at the two highest doses of *N*-acetylcysteine. A two-way ANOVA with repeated measures over time revealed a significant effect of time ($F_{4,60} = 5.38$, $P < 0.001$; $n = 5$–7; symbols as in **a**). (**c**) The elevation in glutamate produced by *N*-acetylcysteine in cocaine-withdrawn subjects was blocked by infusion of the cystine/glutamate exchange antagonist CPG (50 μM) through the dialysis probe beginning 1 h before systemic injection of *N*-acetylcystine (■ *N*-acetylcysteine, 60 mg/kg, s.c.; □ CPG, 50 μM; ● *N*-acetylcysteine plus CPG). A two-way ANOVA with repeated measures over time revealed a significant interaction between treatment and time ($F_{10,75} = 2.17$; $P = 0.029$; $n = 4$–7). $^*P < 0.05$ compared to basal levels of glutamate. $^+P < 0.05$ compared to CPG + *N*-acetylcysteine treatment.

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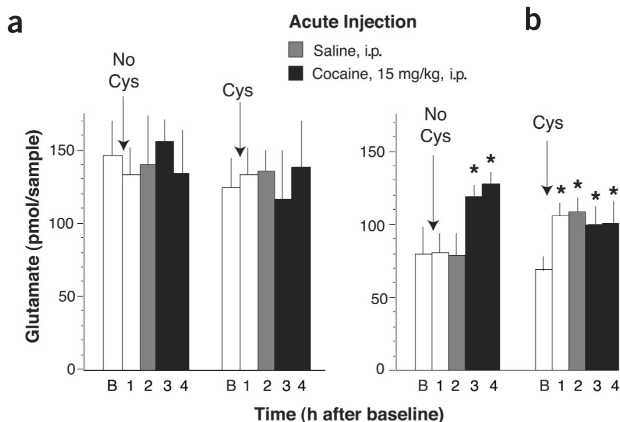

Figure 5 Infusion of cystine into the nucleus accumbens prevented the increase in extracellular glutamate by an acute cocaine injection given at 3 weeks withdrawal from repeated cocaine. Rats were treated for 7 d with experimenter-administered saline (**a**, $n = 13$) or cocaine (**b**, $n = 14$). After collecting baseline (B) samples of extracellular glutamate, 30 nM cystine was added to the dialysis buffer in half of the subjects and continued for the next 4 h. One hour later, all rats were injected with saline (1 ml/kg, i.p.). One hour after saline administration, all subjects were injected with cocaine (15 mg/kg, i.p.) and glutamate levels were measured for two more hours. Data are presented as mean ± s.e.m. pmol glutamate/60 min. A two-way ANOVA revealed a significant interaction between treatment and time ($F_{8,96} = 2.53$, $P < 0.05$).

affect pressing on the inactive lever ($P > 0.05$; one- or two-way ANOVA depending on experiment).

DISCUSSION

Repeated cocaine treatment produces persistent neuroadaptations in the concentration of extracellular glutamate in the nucleus accumbens[27,28], including a reduction in basal extracellular levels of glutamate[9,10]. We found that the reduction in extracellular glutamate was associated with reduced affinity of the cystine/glutamate exchanger, and that restoration of cystine/glutamate exchange in rats trained to self-administer cocaine prevented cocaine-primed reinstatement. These results indicate that the reduction in nonvesicular glutamate release from cystine/glutamate exchange after withdrawal from cocaine increases relapse susceptibility, and that ameliorating this reduction may be a viable therapeutic strategy in treating cocaine addiction.

Cocaine withdrawal reduces cystine/glutamate exchange

Basal extracellular glutamate is derived primarily from nonvesicular release[13] via the exchange of extracellular cystine for intracellular glutamate[12]. Withdrawal from repeated cocaine administration reduces basal extracellular glutamate levels selectively in the nucleus accumbens. Although the amount of xCT mRNA was not altered, three experiments indicate that withdrawal from repeated cocaine produces a reduction in cystine/glutamate exchange. First, the K_m of [^{35}S]cystine uptake through the cystine-glutamate exchanger was increased following repeated cocaine. Second, the activity of cystine-glutamate exchangers is regulated by the concentration of extracellular cystine[19], and increasing cystine in the nucleus accumbens restored the level of extracellular glutamate in cocaine-pretreated subjects. Third, blockade of cystine-glutamate exchangers in control subjects mimicked the effect of repeated cocaine administration.

The mechanism underlying the down-regulated activity of cystine/glutamate exchange in cocaine-withdrawn rats is unclear. However, certain explanations can be excluded and others proposed. As described above, there was no cocaine-induced change in the level mRNA encoding xCT in the nucleus accumbens. Unfortunately, no antibody is currently available to evaluate if the level of translated xCT protein is also unaltered by withdrawal from cocaine. There was no difference in the *in vivo* levels of extracellular cystine, and the level of extracellular glutamate and cystine was controlled in the *in vitro* measurement of the *K*m of [^{35}S]cystine uptake through the exchanger. Thus, the elevated *K*m in cocaine-withdrawn subjects may reflect differences in the concentration of the intracellular substrates for the exchanger, including cystine or glutamate. Although neither of these values have been neurochemically quantified in cocaine-withdrawn subjects, there is anatomical evidence that the intracellular concentration of glutamate in the nucleus accumbens is reduced after withdrawal from repeated cocaine administration[29]. Increasing *in vivo* extracellular cystine levels did not elevate extracellular glutamate in control subjects. The effectiveness of cystine in cocaine-withdrawn subjects and the lack of effect in controls points to possible neuroadaptations in cystine transporters in addition to the cystine/glutamate exchanger. Cystine is also transported into cells by high-capacity, low-affinity carriers, including XAG[30] and γ-glutamyl transpeptidase[31]. These other carriers, which were pharmacologically blocked in the [^{35}S]cystine uptake assay (see Methods), may compensate for the reduced activity of the exchanger and thereby maintain relatively normal *in vivo* basal levels of extracellular cystine after cocaine withdrawal. A possible mechanism whereby cocaine withdrawal could regulate cystine-glutamate exchange is via phosphorylation of the exchanger. Although little work has been done in this area, two studies have demonstrated that the activity of cystine-glutamate exchangers is regulated by cAMP-dependent protein kinase[12,32], and both studies found evidence that PKA stimulated activity of the exchanger.

Restoring Cys/Glu exchange prevents reinstatement

The reduced levels of extracellular glutamate in cocaine-pretreated animals were restored by increasing cystine/glutamate exchange,

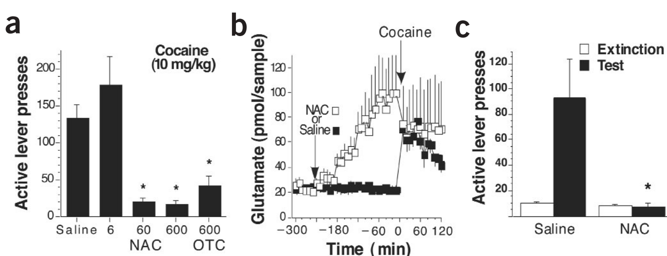

Figure 6 Cocaine-primed reinstatement of lever pressing and the increase in extracellular glutamate was blocked by pretreatment with *N*-acetylcysteine or (–)-2-oxothiazolidine-4-carboxylic acid (OTC). (**a**) *N*-acetylcysteine or OTC was administered 4 h before priming reinstatement of lever pressing with cocaine (10 mg/kg, i.p.). A one-way ANOVA revealed a significant effect of drug treatment ($F_{4,43} = 11.34$; $P < 0.001$; $n = 7$–16). (**b**,**c**) Simultaneous microdialysis and cocaine-primed reinstatement. *N*-acetylcysteine (60 mg/kg, s.c.) or saline was given 4 h prior to a cocaine prime. *N*-acetylcysteine elevated extracellular glutamate (**b**) and prevented cocaine-primed reinstatement (**c**). A two-way ANOVA with repeated measures over time revealed a significant interaction between treatment (*N*-acetylcysteine vs. saline) and time ($F_{4,60} = 5.38$; $P < 0.001$; $n = 5$ in each group). A one-way ANOVA revealed that *N*-acetylcysteine pretreatment blocked cocaine-primed reinstatement ($F_{1,9} = 7.34$; $P = 0.027$).

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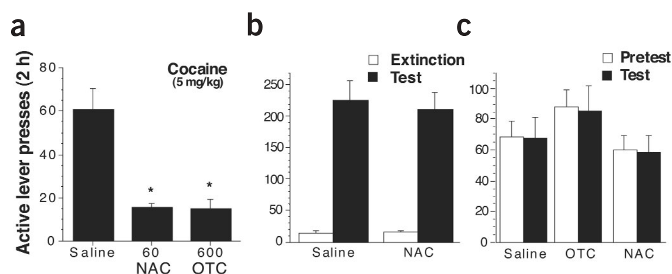

Figure 7 Neither *N*-acetylcysteine nor OTC blocked food-primed reinstatement or cocaine reward, and were still effective against a lower cocaine-priming dose. (**a**) Animals were primed with a lower dose of cocaine (5 mg/kg, i.p.) and reinstatement was blocked by pretreatment with *N*-acetylcysteine or OTC given 4 h before the priming injection ($F_{2,17}$ = 5.38; P < 0.001; n = 5–7). (**b**) A one-way ANOVA revealed no effect by *N*-acetylcysteine on food-primed reinstatement of lever pressing. (**c**) A two-way ANOVA revealed no effect by *N*-acetylcysteine or OTC on pressing for delivery of cocaine (0.25 mg/infusion) in animals trained to criterion for stable lever pressing. Pretest refers to day 11 of training, and the test with cysteine pro-drug was conducted on day 12. *P < 0.05 compared with saline.

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either by supplying the extracellular substrate cystine directly into the nucleus accumbens, or by systemic administration of the cysteine pro-drug *N*-acetylcysteine. The elevation in glutamate required cystine/glutamate exchange, as blocking the exchanger with intra-accumbens perfusion of CPG inhibited the *N*-acetylcysteine-induced rise in glutamate (**Fig. 4c**). Interestingly, neither treatment altered extracellular glutamate in the nucleus accumbens of control subjects. The lack of effect in controls may indicate that the exchanger is already functioning near maximum *in vivo*, which is consistent with cystine uptake through the exchanger being a rate-limiting step in glutathione synthesis[16,33]. Importantly, these data support an effect of *N*-acetylcysteine on cystine/glutamate exchange that is selective for the potentially pathogenic situation where extracellular glutamate has been reduced by repeated cocaine, and is without measurable consequence in control subjects.

To evaluate whether the reduction in glutamate after withdrawal from repeated cocaine may indeed be pathogenic in cocaine addiction, we used the reinstatement animal model of cocaine-primed relapse. Restoring levels of extracellular glutamate by systemically administering the cysteine pro-drugs *N*-acetylcysteine or OTC abolished cocaine-primed reinstatement. This effect did not occur in a reinstatement experiment for a natural reward (food), indicating that the cysteine pro-drugs may be selectively ameliorating a pathology produced by cocaine. Also, pretreatment with neither cysteine pro-drug altered cocaine self-administration, supporting a selective effect of the cysteine pro-drugs on a reinstatement model of relapse. The role of cysteine pro-drugs to elevate extracellular glutamate and to selectively inhibit cocaine-primed reinstatement is consistent with the emerging understanding that reinstatement and relapse to cocaine-taking behavior involve corticofugal glutamatergic projections[1,4,11].

It is paradoxical that a cocaine-priming injection elevates extracellular glutamate in cocaine-experienced animals to cause reinstatement, while elevating glutamate with cysteine pro-drugs prevents cocaine-primed reinstatement. One explanation for this apparent paradox is that the elevation by cysteine pro-drugs in subjects withdrawn from repeated cocaine occurs more than 3 h after *N*-acetylcysteine injection. This is in contrast to the elevation in glutamate that occurred within the first 10 min after a cocaine priming injection. Moreover, the increase in glutamate by a cocaine priming injection arises from increased synaptic release of glutamate in the projection from the prefrontal cortex to the nucleus accumbens[11], whereas the increase by *N*-acetylcysteine depends on non-synaptic release via cystine/glutamate exchange (**Fig. 4c**). Thus, the rapid rise in extracellular glutamate by cocaine is probably associated with excitatory postsynaptic signaling[34]. In contrast, the *N*-acetylcysteine-induced rise in extrasynaptic glutamate likely preferentially modulates extrasynaptic glutamate receptors, such as presynaptic group-II mGluRs[12] that are located, in part, outside the synaptic cleft and inhibit synaptic glutamate release[35,36]. The likelihood that glutamate derived from cystine/glutamate exchange will preferentially affect presynaptic mGluRs is supported by a study showing a relatively weak effect of cystine on postsynaptic excitatory potentials except at micromolar concentrations of cystine[19]. Nonetheless, *in vivo* it is possible that extrasynaptic glutamate from the exchanger may influence postsynaptic glutamate receptors. Studies in nucleus accumbens tissue slices or in anesthetized animals indicate that withdrawal from repeated cocaine reduces the efficacy of ionotropic glutamate receptor stimulation to produce excitatory postsynaptic currents or to induce depolarization block, respectively[37,38]. If the rise in extracellular glutamate induced by the cysteine pro-drug increased tone on the postsynaptic excitatory receptors, this may restore postsynaptic glutamate signaling and decrease relapse susceptibility. Along these lines, it has recently been shown that increasing GluR1 or GluR2 synthesis in the nucleus accumbens during extinction training in cocaine self-administering rats inhibits stress-primed reinstatement[39]. Although the precise roles for pre- and post-synaptic glutamate receptors in the *N*-acetylcysteine inhibition of cocaine-primed reinstatement are unclear, both mechanisms may reduce the signal-to-noise ratio of phasic glutamate release in the synapse, and thereby decrease the effectiveness of a cocaine injection to signal reinstatement behavior.

This study identifies the cystine/glutamate exchanger as a novel pharmacotherapeutic target for treating cocaine addiction. Withdrawal from cocaine caused a reduction of extracellular glutamate in the nucleus accumbens that arose from compromised cystine/glutamate exchange. This neuroadaptation seems to be involved in cocaine addiction since normalizing extracellular glutamate by stimulating cystine/glutamate exchange prevented cocaine-primed reinstatement.

METHODS

Animals and surgeries. Male Sprague Dawley rats (Harlan) weighing 275–300 g were individually housed in a temperature-controlled colony room with a 12-h light/dark cycle (lights on at 7 a.m.) with food and water available *ad libitum*. The housing conditions and care of the rats was in accordance with the Animal Welfare Act, and all procedures were approved by the Medical University of South Carolina's Institutional Animal Care and Use Committee. Rats were anesthetized with ketamine HCl (87.5 mg/kg Ketaset, Fort Dodge Animal Health) and xylazine (5 mg/kg Rompum, Bayer). Bilateral guide cannulae (20 gauge, 14 mm, Plastics One) were implanted above the prefrontal cortex (+3.0 mm anterior and ±1.0 mm mediolateral to bregma, and –1.0 mm ventral from the surface of the skull), striatum (+0.5 mm anterior and ±3.4 mm mediolateral to bregma, and –4.0 mm ventral from the surface of the skull) or nucleus accumbens (+ 1.6mm anterior and ±2.5 mm mediolateral to bregma, and –4.7 mm from the surface of the skull at a 6° angle from vertical) to allow the active region of the microdialysis probe, which begins 2 mm beyond the ventral tip of the guide cannulae, to be placed into the region of interest[40]. Following surgery, rats were given at least 5 d to recover before testing began.

In the reinstatement experiments, subjects were handled daily for 1 week after arrival and before surgery. Animals were given *ad libdum* access to food (Purina Rat Chow) until 7 d after surgery, when they received a 20-g daily ration of food for the remainder of the experiment. This regimen aided in the

acquisition of the lever press response, while allowing them to gain weight throughout the course of the experiment. Rats were anesthetized (see above) and implanted with intravenous catheters as described previously[4], and in some experiments microdialysis guide cannulas were aimed at the core of the nucleus accumbens (see above). The implanted catheter was flushed daily with both heparinized saline (0.2 ml of 100 IU) to maintain catheter patency and cefazolin antibiotic (0.2 ml of 0.1g/ml) to protect against infection.

Compounds. Cocaine was a gift from the National Institute of Drug Abuse and was dissolved in 0.9% saline. (S)-4-carboxyphenylglycine (CPG; Tocris Cookson) was dissolved in 1 equivalent NaOH and then diluted to appropriate concentration using dialysis buffer. Stock solutions of glutamate and cystine (Sigma) were dissolved in dialysis buffer and 1 equivalent HCl, respectively, and diluted using dialysis buffer. [^{35}S]cystine (Amersham) was dissolved in 0.05 M HCl and diluted in Krebs-Ringer phosphate buffer (KRP; 118 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5.0 mM hepes and 10mM glucose, pH 7.4). *N*-acetylcysteine and (–)-2-oxothiazolidine-4-carboxylic acid (OTC) were dissolved in sterile saline and water, respectively, and administered by subcutaneous injection.

Self-administration and extinction procedures. Behavioral training began 7 d after surgery. All training and testing was conducted in operant chambers fitted with two retractable levers (ENV-008, Med Associates). Initially, subjects were trained in a single 15-h training session to press a lever on an FR-1 schedule of reinforcement for one 45-mg food pellet (Noyes). The next day, the reinforcer was switched to a cocaine-HCl solution (0.25 mg/kg). Presses on the active lever now resulted in an intravenous infusion of cocaine (over 4 s), whereas presses on the inactive lever had no programmed consequences. Cocaine reinforcement was delivered on a modified FR-1 schedule such that each infusion of cocaine was followed by illumination of a stimulus over the lever and a 20-s timeout when active lever presses were counted but did not result in reinforcer delivery. After 20 s, the stimulus light was extinguished, and the first lever press again resulted in cocaine delivery. Daily training sessions lasted 2 h or until a subject earned 200 cocaine infusions, whichever came first. Subjects remained in cocaine self-administration training until they met an acquisition criterion that required the average presses on the active lever over three consecutive training days to vary by less than 10%. This criterion was achieved by 10–20 d of training in all cocaine self-administration experiments.

Once subjects met the acquisition criterion, extinction procedures were instituted where pressing on the active lever resulted in infusions of saline (0.9% saline over 4 s) instead of cocaine. Subjects remained in behavioral extinction until responding on the active lever averaged 10% or less of active lever pressing for three consecutive days. In all animals used for data analysis, this criterion was achieved between 12 and 20 d after beginning extinction training.

Reinstatement testing. A total of 43 subjects were tested for the reinstatement of drug-seeking behavior following a challenge injection of cocaine (10 mg/kg i.p. in a volume of 1 ml/kg bodyweight) or saline (0.9% in a volume of 1 ml/kg bodyweight). Four hours before systemic cocaine or saline injection, some subjects received injections of *N*-acetylcysteine, OTC or saline vehicle. During reinstatement testing, active lever presses were counted but resulted in saline, not cocaine, delivery. When microdialysis was conducted during reinstatement testing, 10 min microdialysis samples were collected for 6 h before beginning the behavioral trial and throughout the 2-h test session. Subjects were then disconnected and returned to their home cages.

Food reinstatement. A total of 11 food reinstatement subjects were trained in parallel to cocaine self-administering rats. They were trained to lever-press on an FR-1 schedule of reinforcement (each reinforcement consisting of a single 45-mg Noyes food pellet) in daily 2-h sessions. Once stable responding was achieved, the schedule of reinforcement was increased to FR-2 and then to FR-5. Subjects remained in maintenance until lever press responding stabilized at less than 10% variation across three consecutive days (ranging from 12 to 15 d of training). Subjects then entered behavioral extinction, where lever press responding no longer resulted in food delivery. Once lever presses fell to less than 10% of maintenance levels across three consecutive days (requiring a range of 10–12 d), rats were tested for their propensity to reinstate responding for noncontingent food delivery. Subjects received two pellets immediately upon the initiation, and an additional ten pellets for the first 20 min of the reinstatement session (one pellet at 2-min intervals). Lever presses never resulted in food delivery.

Investigator-administered cocaine. The cocaine procedure used has been previously shown to produce behavioral and neurochemical sensitization[9]. Briefly, rats received seven daily injections of saline (1 ml/kg i.p.) or cocaine (15 mg/kg i.p. on days 1 and 7; 30 mg/kg i.p. on days 2–6) and were then withdrawn for 3–4 weeks before further experimentation.

***In vivo* microdialysis.** Microdialysis probes were constructed as previously described[9], except that both the inlet and outlet tubing consisted of fused silica. The night before the experiment, the probes were inserted through the guide cannulae into the nucleus accumbens. In the reinstatement study, subjects were housed overnight in the operant chamber following insertion of the dialysis probe. The next day, dialysis buffer was advanced through the microdialysis probes, and 2 h later, 20 or 10 min baseline samples were collected. Additional samples were then collected with the addition of various drugs to the dialysis buffer or following systemic injection of *N*-acetylcysteine or saline. The no-net flux microdialysis technique involved adding varying concentrations of the analyte of interest (*i.e.*, glutamate or cystine) into the microdialysis buffer. The net flux of the analyte diffusing into or out of the probe was determined by subtracting the concentration of the analyte added to the buffer from the concentration of the analyte in the samples. A plot of the analyte flux at each concentration added to the buffer yields an estimate of basal levels ($y = 0$; or the point at which there is no-net flux of analyte into or out of the probe) and elimination (slope, or clearance of the analyte from the probe).

Glutamate and cystine quantification. The concentration of glutamate[9,11] and cystine[41] in dialysis samples was determined using HPLC coupled to fluorescence detection as described elsewhere. Concentrations of glutamate and cystine were quantified by comparing peak heights from samples and external standards.

RT-PCR. Total RNA was extracted from frozen rat brain tissues and treated with DNase I. Single-strand cDNAs were synthesized from 1 μg of total RNA using Superscript II reverse transcriptase and random primers (Promega). Rat xCT mRNA-specific primers were designed according to the highly conserved sequences between human- and mouse-xCT mRNA. To control for variations in sampling and processing between samples, rat 28S ribosomal RNA was co-amplified as a multiplex PCR reaction. PCR primer sequences were as follows: xCT (399 bp), 5′-ACCTTTTGCAAGCTCACAGCAA-3′ and 5′-AGCAGGAGAGGGCAACAAAGAT-3′; 28S ribosomal RNA (208 bp), 5′-TGACTCTCTTAAGGTAGCCAAA-3′ and 5′-CCCACTTATTCTACACCTCTCA-3′. 80% of 28S RNA primers were phosphorylated at their 3′ end so that the efficiency of control cDNA amplification could be the same as that of target cDNA amplification when they are co-amplified exponentially in the same tube. A 2.5-μl aliquot of cDNA was used as a template for each PCR reaction. The PCR amplification condition was 94 °C for 50 s, 56 °C for 50 s and 72 °C for 1 min for a total of 25 cycles. PCR products were separated in 1.5% agarose gel and subsequently stained with ethidium bromide. Band intensities were quantified using NIH Image (v. 1.62). The ratio between the initial amount of xCT mRNA and that of 28S ribosomal RNA was calculated for each sample.

[^{35}S]cystine uptake. Rats were decapitated and the nucleus accumbens was rapidly dissected and cut into 1-mm coronal slices, and then into 350 × 350 micron slices using a McIlwain tissue chopper. The slices were washed 5× for 10 min at 37 °C in oxygenated Krebs-Ringer phosphate buffer (KRP; 118 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5.0 mM HEPES and 10mM glucose, pH 7.4), and incubated at 37 °C in oxygenated KRP containing 0.1–30.0 μM [^{35}S]cystine for 2–15 min to determine the rate of uptake (*i.e.* slope) across a range of concentrations. To isolate cystine uptake to cystine-glutamate exchange, the X_{AG} inhibitor aspartate (1 mM) and the γ-glutamyl transpeptidase inhibitor acivicin (1 mM) were added to the incubation buffer. Incubation was terminated by rapidly washing the tissue 3× using ice-cold KRP. Slices were then solubilized using 1% SDS and

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the level of radioactivity was determined using a liquid scintillation counter. Radioactivity counts from known concentrations of [^{35}S]cystine were used to determine the concentration of [^{35}S]cystine in tissue slices. Protein content in the slices was measured using the Bio-Rad DC protein assay (Bio-Rad). [^{35}S]cystine uptake in the presence of unlabeled 1 mM cystine was used to identify nonspecific labeling and was subtracted from all data. Kinetics of cystine/glutamate exchange were determined using a Lineweaver-Burke plot in which 1/slope of uptake at each concentration is plotted against 1/[^{35}S-cystine].

Histology and statistical analyses. Rats included in the microdialysis studies were given an overdose of pentobarbital, and the brains were fixed by intracardiac infusion of 0.9% saline followed by 1% formalin solution. Brains were then removed and stored in 1 % formalin for at least 1 week before sectioning. The tissue was then blocked and coronal sections (100 μM) were cut and stained with cresyl violet to verify probe placements.

The SPSS statistics package was used to perform the statistical analyses. Data from microdialysis experiments involving drug infusions were analyzed using two-way analysis of variance (ANOVA) with cocaine history as a between-subject factor and drug concentration as a repeated factor. Significant main effects and interactions were further evaluated using Fisher LSD *post hoc* analyses. Data from no-net flux microdialysis experiments, kinetic estimates of [^{35}S]cystine uptake, and xCT mRNA and protein levels were analyzed using two-tailed Student *t*-tests with cocaine history as the between-subject factor.

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COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

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