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Chronic Administration of the Methylxanthine Propentofylline Impairs Reinstatement to Cocaine by a GLT-1-Dependent Mechanism

Kathryn J Reissner*[,1], Robyn M Brown[1,2], Sade Spencer[1], Phuong K Tran[1], Charles A Thomas[1] and Peter W Kalivas[1]

[1]Department of Neurosciences, Medical University of South Carolina, Charleston, SC, USA; [2]Florey Institute of Neuroscience and Mental Health, University of Melbourne, Parkville, Australia

In recent years, interactions between neurons and glia have been evaluated as mediators of neuropsychiatric diseases, including drug addiction. In particular, compounds that increase expression of the astroglial glutamate transporter GLT-1 (*N*-acetylcysteine and ceftriaxone) can decrease measures of drug seeking. However, it is unknown whether the compounds that influence broad measures of glial physiology can influence behavioral measures of drug relapse, nor is it clear whether the upregulated GLT-1 is functionally important for suppressing of drug seeking. To address these questions, we sought to determine whether the glial modulator and neuroprotective agent propentofylline (PPF) modifies drug seeking in rats using a reinstatement model of cocaine relapse. We found that 7 days of chronic (but not acute) administration of PPF significantly decreased both cue- and cocaine-induced reinstatement of cocaine seeking. We next determined whether the effect of systemic PPF on reinstatement depended upon its ability to restore expression of GLT-1 in the nucleus accumbens. PPF restored the cocaine-induced decrease in GLT-1 in the accumbens core; then, using an antisense strategy against glutamate transporter GLT-1, we found that restored transporter expression was necessary for PPF to inhibit cue-primed cocaine seeking. These findings indicate that modulating glial physiology with atypical xanthine derivatives like PPF is a potential avenue for developing new medications for cocaine abuse, and support the hypothesis that neuron–glial interactions contribute to mechanisms of psychostimulant addiction, particularly via expression and function of astroglial glutamate transporters.
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INTRODUCTION

Important functional roles have been increasingly ascribed to the glial cells in both health and disease (Aguzzi *et al*, 2013; Barres, 2008). Beyond sustaining normal nervous system function, in many instances of injury or disease, the activation of both microglia and astrocytes is a fundamentally important process (Liu *et al*, 2011). Activation of astrocytes refers to a morphological and functional responsiveness to nervous system trauma including ischemia, disease, or injury, and is typically associated with increased expression and release of proinflammatory cytokines, and increased expression of cytoskeletal proteins including glial fibrillary acidic protein and vimentin (Pekny and Nilsson, 2005; Sofroniew, 2009). Reactive astrocytes are also frequently associated with decreased expression and function of glutamate transporters including GLT-1/EAAT2 and GLAST/EAAT1, which can exacerbate excitotoxicity (Binns *et al*, 2005; Cata *et al*, 2006; Pekny and Nilsson, 2005; Sung *et al*, 2003; Sweitzer *et al*, 2001; Tawfik *et al*, 2008).

Exposure to drugs of abuse leads to activation of both astrocytes and microglia. For example, noncontingent administration of cocaine upregulates glial fibrillary acidic protein and vimentin 3 weeks after drug cessation (Bowers and Kalivas, 2003). Likewise, noncontingent administration of methamphetamine and opiates also increase measures of glial activation, including cell morphology and expression of inflammatory markers (Beitner-Johnson *et al*, 1993; Reichel *et al*, 2012; Schwarz *et al*, 2011; Watkins *et al*, 2009). Moreover, GLT-1 and GLAST are chronically downregulated following cocaine self-administration and withdrawal (Fischer-Smith *et al*, 2012; Knackstedt *et al*, 2010; Reissner *et al*, 2011), and chronic administration of ceftriaxone or *N*-acetylcysteine, compounds which upregulate GLT-1, reduce reinstated drug seeking in animals trained to self-administer cocaine (Sari *et al*, 2009; Knackstedt *et al*, 2010; Moussawi *et al*, 2011).

Propentofylline (PPF) is an atypical methylxanthine derivative characterized as an adenosine uptake and phosphodiesterase inhibitor (Sweitzer and De Leo, 2011), that also

*Correspondence: Dr KJ Reissner, Department of Psychology, UNC-Chapel Hill, CB 3270, Chapel Hill, NC 27599, USA, Tel: +1 919 843 6885, Fax: +1 919 962 2537, E-mail: reissner@unc.edu
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reverses markers for reactive gliosis following nerve injury, including reduced expression of GLT-1 (Tawfik *et al*, 2008). Methylxanthines are a broad class of drugs that include caffeine, theophylline, and theobromine. FDA-approved methylxanthines include pentoxifylline for improved circulation in intermittent claudication (Muir, 2009) and theophylline and aminophylline as bronchodilators for chronic obstructive pulmonary disease and asthma (Dzierba and Jelic, 2009; Tilley, 2011). Further, systemic treatment with glial modulator PPF can block conditioned place preference to both methamphetamine and morphine (Narita *et al*, 2006). We hypothesized that the capacity of PPF to restore GLT-1 might confer capacity for this class of drugs to be medications for cocaine relapse. To evaluate PPF as a potential anti-relapse treatment for cocaine addiction, we trained rats to self-administer cocaine and determined if reinstated cocaine seeking initiated by conditioned cues was reduced by PPF pretreatment. Daily, but not acute, PPF reduced reinstatement to cocaine, and using a vivo-morpholinos oligomer antisense (AS) strategy to inhibit translation of GLT-1, we determined that the ameliorative effect of PPF relies on its ability to raise levels of GLT-1 in the nucleus accumbens (NAc).

MATERIALS AND METHODS

Reagents

Vivo-morpholinos were custom synthesized and purchased from Gene Tools, LLC. PPF was purchased from Sigma (P9689) and prepared in saline. Sequences for vivo-morpholinos were as described (Reissner *et al*, 2012). Animals were purchased from Charles River, and cocaine was provided by NIDA.

Animal Care and Surgical Techniques

All animal treatment was in accordance with National Institutes of Health guidelines for laboratory animal care, and all protocols were approved by the Medical University of South Carolina Institutional Animal Care and Use Committee. Male Sprague–Dawley rats ($\sim$350 g) were individually housed on a 12 h reverse light cycle and were food restricted (20 g chow per day). Catheters were surgically implanted into the right jugular vein (0.02 ID, 0.047 OD, Bio-sil). Prophylactic antibiotic (Timentin 10 mg/0.1 ml, i.v.) was administered during surgery and 3 days postoperatively. Catheters were flushed daily with heparin until the end of self-administration. Cannulae (26 gauge) were implanted into the NAc using the following coordinates: +1.5 A/P, +1.8 M/L, −5.5 D/V (Paxinos and Watson, 2005).

Behavioral Analysis

Prior to the onset of self-administration, animals received one 15 h food training session, to facilitate acquisition of the operant task. Self-administration was performed on an FR1 reinforcement schedule (2 h per day) during which an active lever press resulted in a 0.2 mg infusion (0.05 ml) of cocaine paired with light and tone (5 s each). All sessions were performed at the same time each day. Following a minimum of 10 days of ten infusions or more, animals entered extinction training, during which time lever presses no longer resulted in infusion or cue presentation. Extinction active lever presses are shown for the average of the last 2 days of extinction. In all cases, PPF or saline was administered i.p. 30 min prior to extinction training or reinstatement test. Cue-primed reinstatement tests were for 2 h and measured lever pressing in response to availability of contingent cues; in the case of cocaine-primed reinstatement, animals were given a 10 mg/kg i.p. injection of cocaine immediately before the onset of reinstatement testing in the absence of conditioned cues. Locomotor testing was performed in a photocell apparatus (Omnitech Electronics, Columbus, OH) 30 min after the last treatment (saline or PPF), and activity was recorded in 10 min increments for a total of 120 min. Activity was quantified as total distance traveled. PPF was prepared in saline, and doses were selected based on systemic doses used in studies on allodynia (Sweitzer *et al*, 2001; Tawfik *et al*, 2008).

Sucrose self-administration was performed identically to cocaine administration on an FR1 schedule of reinforcement, except instead of a drug infusion animals received a 45 mg sucrose pellet (Test Diet, Richmond, IN).

Microinjections and Histology

Control and AS vivo-morpholinos against GLT-1 (30 pmol per injection) were microinjected 2 mm below the cannula base using 33-gauge microinjectors at 0.5 μl/min for a total volume of 1 μl per hemisphere. Microinjectors were then left in place for 1 min before removal, to allow diffusion. Microinjections were made once per day for 3 days prior to treatment with PPF or saline, such that reinstatement testing would be performed 7 days after the last microinjection, based on a protocol previously optimized for suppression of GLT-1 (Reissner *et al*, 2012). The AS sequence was designed around the translation start site; vivo-morpholino AS sequence: 5′-TGTTGGCACCCTCGG TTGATGCCAT-3′. For control, a reverse sequence of the same bases was used. A BLAST search of the *Rattus norvegicus* genome revealed that neither sequence should recognize any nonspecific gene. Upon completion of behavioral analysis, animals were sedated with pentobarbital (200 mg/kg, i.p.) and perfused with saline. Brains were postfixed in 10% formalin, sliced 100 μm thick, and stained using Cresyl Violet.

For validation of GLT-1 knockdown, control and AS sequence microinjections were performed in contralateral hemispheres of the same animal. Seven days after the last injection, sham microinjections were performed approximately 30 min prior to tissue harvest, for visual identification of the microinjection site. Tissue surrounding the microinjection site was taken no more than 1 mm from the site of injection in any dimension.

Western Blotting

For analysis of GLT-1 protein expression, a crude membrane subfraction was prepared from fresh tissue (Knackstedt *et al*, 2010). The final pellet was resuspended in RIPA buffer supplemented with 1.0% SDS and Halt protease/phosphatase inhibitors and EDTA (Thermo Scientific, 1 : 100). Protein

content was determined by the BCA method (Thermo Scientific) and 10 μg were separated on Bio-Rad BisTris gels and transferred to PVDF membranes, then probed using anti-GLT-1 (abcam, ab41621, 1 : 1000) and anti-Calnexin (Enzo ADI-SPA-860, 1 : 1000). Prior to electrophoresis, samples were heated for 30 min at 50°C.

Statistical Analyses

All data presented are mean and standard error about the mean. For all experiments, reinstatement criteria for control animals receiving saline i.p. was set at a minimum of 12 active lever presses ($n = 5$ did not reach criteria). A Student's *t*-test was used to compare reinstated lever presses in the saline and PPF groups. For acute PPF, a one-way ANOVA was used since there were three treatment groups. A three-way ANOVA was used in the case of animals receiving GLT-1 vivo-morpholinos. The three factors for the ANOVA were drug treatment (PPF *vs* saline), morpholino treatment (AS *vs* control) and day (extinction *vs* reinstatement). Where appropriate, this was followed by two-way ANOVA split by corresponding factor with a Bonferroni correction. For analysis of GLT-1 knockdown in naïve animals, a paired *t*-test was used for within animal comparison. In the case of western blotting, significant difference was determined using a one-way ANOVA followed by Neuman–Keuls direct comparisons between groups (Figure 3) or a paired Student's *t*-test (Figure 4b). All tests were considered significant at $p < 0.05$.

RESULTS

Daily PPF Pretreatment Inhibits Reinstatement to Cocaine

Animals were administered either a single injection of PPF prior to reinstatement (Figure 1b), or daily just prior to the last six extinction sessions, as well as prior to the reinstatement session (Figure 1d). There was no effect by either dose of acute PPF pretreatment on the number of cue-induced active lever presses during the reinstatement session ($F_{(1,15)} = 0.4372$). In contrast, for animals receiving chronic saline *vs* 10 mg/kg PPF, there was a significant reduction in the PPF compared with the saline group after either cue- or cocaine-induced reinstatement (cue $t_{(17)} = 2.494$, $p = 0.028$; cocaine $t_{(8)} = 3.238$, $p = 0.012$), (Figure 1d).

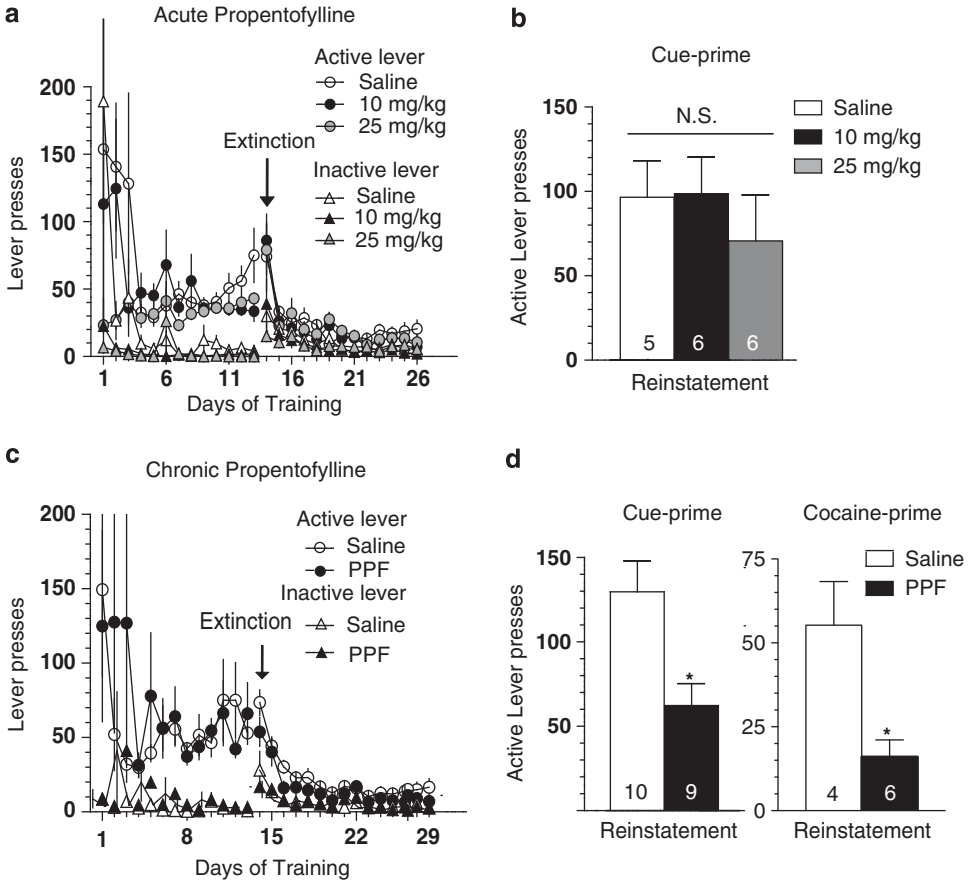

Figure 1 Chronic, but not acute, treatment with PPF impairs cocaine reinstatement. Top, lever presses during self-administration and extinction (a) and reinstatement (b) for acute treatment groups. (b) A repeated measure two-way ANOVA revealed significant reinstatement, but no difference in reinstatement between treatment groups receiving acute saline, or PPF 10 or 25 mg/kg. Bottom, lever presses during self-administration and extinction (c) and reinstatement (d) for chronic treatment groups. In contrast to acute PPF, chronic administration of 10 mg/kg PPF significantly impaired cue- and cocaine-primed reinstatement compared with saline treatment. Self-administration and extinction profiles were not significantly different for animals receiving chronic saline or PPF prior to cocaine-primed reinstatement (data not shown). *$p < 0.05$.

In order to determine whether the effect of chronic PPF administration on reinstatement was due to nonselective motor inhibition or memory for the drug-paired cues, locomotor and sucrose reinstatement experiments were performed (Figure 2a). For this purpose, animals were trained in self-administration and extinction exactly as described for Figure 1. However, following chronic PPF treatment, instead of reinstatement, animals were placed into a novel open field for 2 h. No difference was observed in the locomotor activity for animals receiving either saline or PPF (Figure 2b). In order to determine whether PPF-induced inhibition of reinstatement was enduring, these same animals were then allowed to re-enter extinction training without further PPF administration. In this case, there was no difference in reinstatement between the groups, indicating that the effect was reversible and required ongoing daily administration (Figure 2c).

A separate group of animals was trained to self-administer sucrose using a protocol of self-administration and extinction similar to the cocaine experiment. Rats receiving chronic PPF or saline showed equivalent levels of cue-induced reinstatement of sucrose seeking, indicating the effect on reinstatement to cocaine seeking was not a generalized effect on motivation nor extended to pursuit of non-drug reward (Figure 2).

Modulation of GLT-1 by PPF is required for the Behavioral Therapeutic Effect

PPF has been shown to manifest a variety of cellular effects, including restoration of GLT-1 suppression (Sweitzer *et al*, 2001). Because expression of GLT-1 is reduced following cocaine self-administration and extinction, or incubation of cocaine craving (Fischer-Smith *et al*, 2012; Knackstedt *et al*, 2010), and because reduced GLT-1 expression has been postulated to be an important mediator of drug seeking (Kalivas, 2009; Knackstedt *et al*, 2010), we sought to determine whether PPF might restore the cocaine-dependent decrease in GLT-1 in the NAc. Animals were trained to self-administer cocaine, or received yoked infusions of saline, and were then injected with either daily saline or PPF (10 mg/kg, i.p.). Following self-administration and extinction, animals were killed for preparation of a membrane subfraction from NAc core or the dorsomedial prefrontal cortex, including prelimbic and anterior cingulate regions. We observed a significant cocaine-dependent decrease in GLT-1 expression in the NAc, and a non-significant trend toward a decrease in the PFC (core: ($F_{(2,24)} = 3.833$, $p = 0.037$); PFC: ($F_{(2,24)} = 0.872$, $p = 0.432$), Figure 3). Moreover, a chronic regimen of PPF fully restored expression of GLT-1 in the NAc. These results suggest that restored expression of GLT-1 in the NAc might

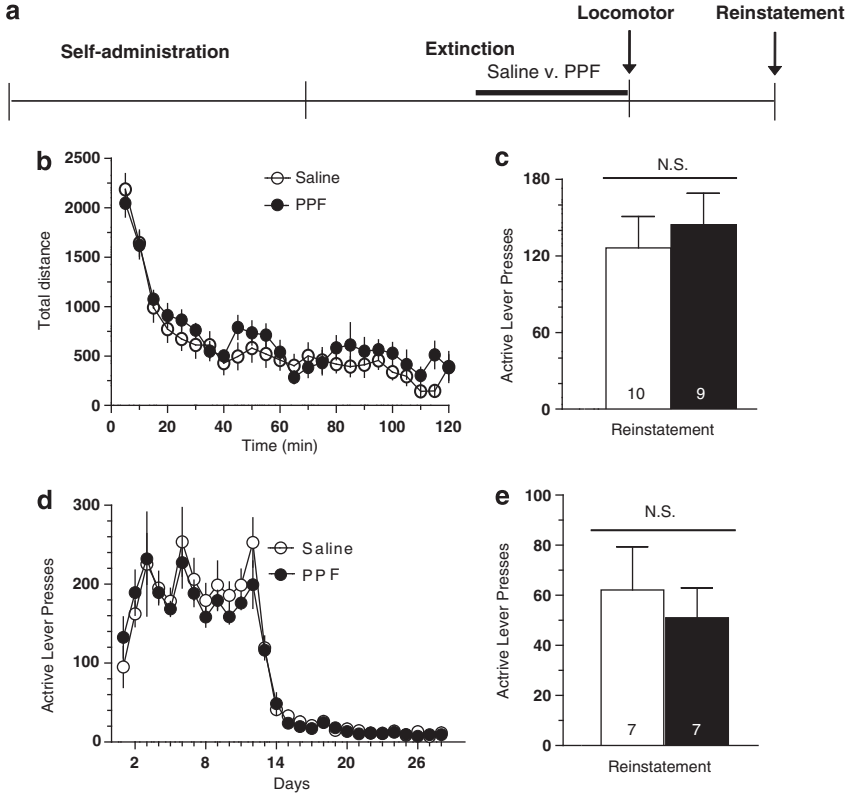

Figure 2 Chronic treatment with PPF does not have an enduring effect on drug seeking, nor does it affect locomotor activity or reinstatement for sucrose. (a) Experimental design for panels b and c. Animals were trained in self-administration and extinction, and treated with chronic PPF (10 mg/kg, ip) or saline, as described in Figure 1. (b) Instead of reinstatement, locomotor testing was performed on day 14 of extinction in an open field. (c) These animals were then allowed to continue an additional 6 days of extinction training without treatment, followed by a final cue-primed reinstatement test. (d,e) A separate cohort of animals were trained to self-administer sucrose pellets in a protocol identical to cocaine self-administration, followed by extinction and daily PPF or saline treatment. Cue-primed sucrose reinstatement was performed identically as for cocaine reinstatement. No significant difference was observed between sucrose-administering animals that received saline or PPF (10 mg/kg).

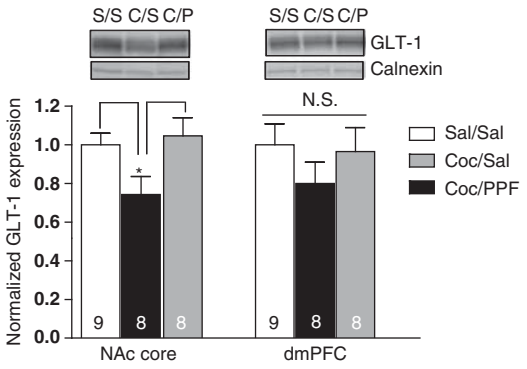

Figure 3 PPF restores the cocaine-dependent decrease in GLT-1. Animals were trained on cocaine (Coc) self-administration and extinction as in other experiments. Saline (Sal) animals received noncontingent infusions of saline dependent on behavior from cocaine-administering animals. Chronic treatment with PPF (10 mg/kg, ip, for 7 days) or saline was performed exactly as in Figure 1, except accumbens tissue was obtained instead of a reinstatement test, 24 h after the last extinction test. GLT-1 levels in a membrane-enriched subfraction were assessed by western blot and normalized to a Calnexin loading control. GLT-1 in cocaine-administering animals was then normalized to saline controls. $^{*}p < 0.05$.

contribute to PPF-induced antagonism of reinstated cocaine seeking.

To test this hypothesis, control or AS vivo-morpholinos were microinjected into the NAc core, using a protocol previously demonstrated to reduce GLT-1 expression by ~40% for 7–14 days after the last AS microinjection (Reissner *et al*, 2012), combined with chronic saline or PPF administration. Analysis by three-way ANOVA revealed a significant interaction between vivo-morpholino treatment (control *vs* AS), drug treatment (PPF *vs* saline) and day (extinction *vs* reinstatement) ($F_{(1,32)} = 4.498$, $p = 0.042$). Thus, comparison between groups was performed by a two-way ANOVA split by corresponding factor with a Bonferroni correction. Analyses revealed a main effect of reinstatement compared to extinction in both AS and control morpholino groups that had been treated with saline ($F_{(1,16)} = 15.126$, $p = 0.001$); in contrast, an interaction was observed in PPF-treated groups ($F_{(1,8)} = 18.007$, $p = 0.003$). Although the group treated with GLT-1 AS plus PPF reinstated ($p = 0.003$), the PPF-treated control vivo-morpholino group did not reinstate ($p \geqslant 0.05$) and a significant difference was found between this group and the PPF-treated AS group (Figure 4b, far right). Moreover, reinstatement in the AS/PPF group was no different from reinstatement in animals that received saline i.p. and were microinjected with either control or AS vivo-morpholinos. Thus, restored expression of GLT-1 is required for PPF to suppress cue-primed reinstatement.

In order to validate control of GLT-1 protein expression, AS or control vivo-morpholinos against GLT-1 were microinjected in an independent, naive group of animals. In this case, control *vs* AS vivo-morpholinos were microinjected into contralateral hemispheres in an identical manner as performed for behavioral analysis. Seven days after the last microinjection, tissue surrounding the microinjected site was harvested for subcellular fractionation and western blotting (Figure 4c). Compared with control vivo-morpholino microinjection, AS against GLT-1 results in ~30% suppression, similarly as reported previously ($t_{(5)} = 3.45$, $p = 0.018$), (Reissner *et al*, 2012).

DISCUSSION

Restoration of GLT-1 is a Critical Mechanism for Control of Reinstatement for Cocaine

These data show that daily, but not acute, systemic administration of the glial modulator PPF can impair cue-primed reinstatement to cocaine. The impairment of cocaine reinstatement was not due to a sedative or generalized effect by PPF on motivation, as the same protocol that blocked reinstatement did not affect locomotor activity or reinstated sucrose seeking. However, the effect of PPF depended on its ability to partially restore expression of the glutamate transporter GLT-1 within the NAc. Several studies have demonstrated that administration of compounds *N*-acetylcysteine and ceftriaxone inhibit cocaine sensitization, self-administration, or reinstatement, and also increase GLT-1 expression (Knackstedt *et al*, 2010; Sari *et al*, 2009; Sondheimer and Knackstedt, 2011; Ward *et al*, 2011). However, no studies have directly shown that this increase is requisite for these behavioral effects. Results presented here indicate that when PPF is administered systemically, AS-mediated suppression of GLT-1 specifically within the NAc core, a nucleus known to be critically important in cue-primed reinstatement to multiple drugs (Feltenstein and See, 2008; Kalivas and McFarland, 2003), is sufficient to prevent PPF reductions in cue-reinstated cocaine seeking.

GLT-1 is predominantly localized near synapses to rapidly eliminate synaptically released glutamate and minimize access to the extrasynaptic space (Cholet *et al*, 2002; Minelli *et al*, 2001; Shigeri *et al*, 2004). Reinstatement to cocaine, heroin, alcohol, and nicotine is associated with potentiated release of glutamate within the NAc (Gass *et al*, 2011; Gipson *et al*, 2013; LaLumiere and Kalivas, 2008; Lutgen *et al*, 2012; McFarland *et al*, 2003). Given that all of these drugs reduce GLT-1 in the NAc, the reduced uptake of synaptically released glutamate is a likely mediator of the increase in extracellular glutamate associated with reinstatement. Accordingly, restoring astrocyte-mediated clearance of synaptic glutamate in the NAc is a hypothesized mechanism whereby PPF inhibits cocaine seeking in the present study.

Although our data indicate a necessity to restore GLT-1 expression for the therapeutic effect of PPF, contributions by other known effects of PPF cannot be conclusively ruled out. For example, PPF is a phosphodiesterase inhibitor, and phosphodiesterase inhibition reduces cocaine sensitization and reinforcement (Liddie *et al*, 2012; Meskini *et al*, 1994; Schroeder *et al*, 2012; Zhong *et al*, 2012). In addition, PPF can act as an agonist of A_1 or antagonist of A_2 adenosine receptors in a concentration-dependent manner (Borgland *et al*, 1998), and adenosine receptors have been implicated in cocaine-mediated behaviors (Brown *et al*, 2012; O'Neill *et al*, 2012; Soria *et al*, 2006; Tozzi *et al*, 2012).

Finally, the fact that the therapeutic effect of PPF requires daily administration and depends on GLT-1 expression suggests that it may be working through a transcriptionally dependent mechanism. However, the effect of daily PPF did

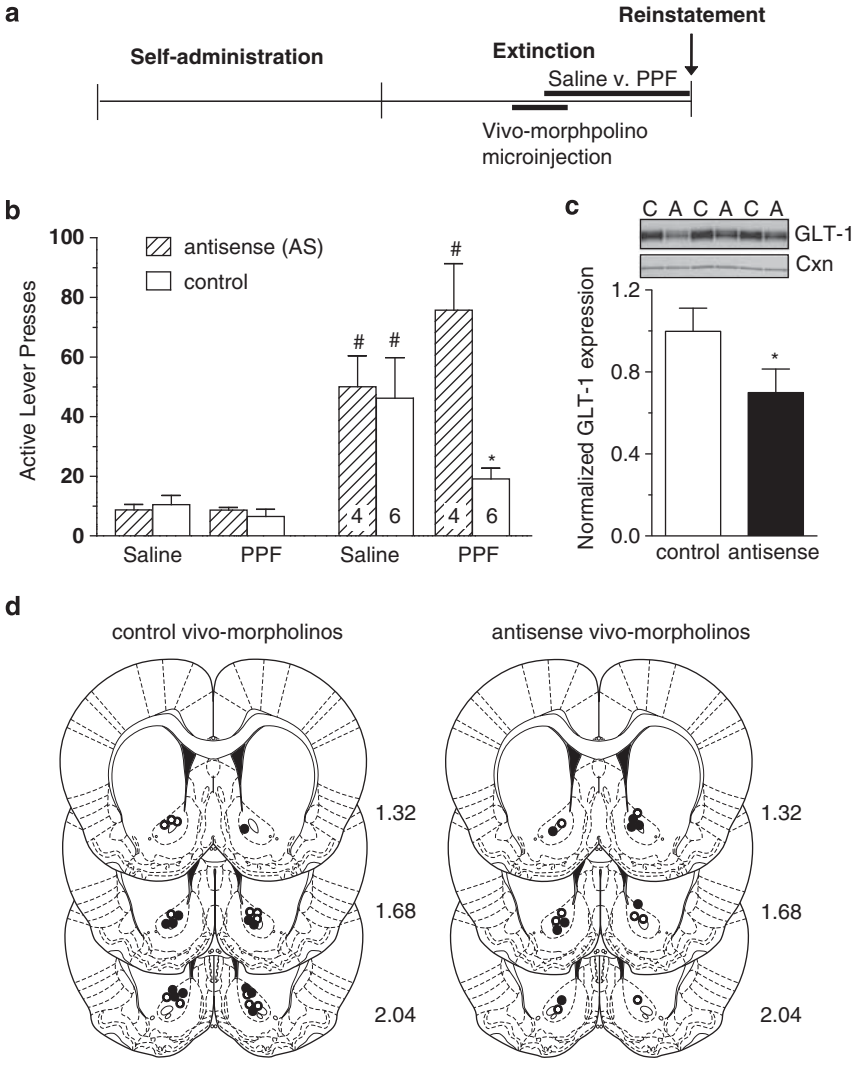

Figure 4 Restored expression of GLT-1 is required for the PPF-dependent decrease in reinstatement. (a) Experimental design. Vivo-morpholino microinjections were performed on 3 sequential days, and saline or PPF administration began on the third day of vivo-morpholino microinjection. Animals then received chronic treatment with saline or PPF (10 mg/kg, i.p.). (b) Active lever presses during cue-primed reinstatement to cocaine. ($^{\#}p<0.001$). (c), In a separate group of animals, efficacy of antisense to GLT-1 was determined using western blot ($n=6$ per group). C, control sequence; A, antisense. (d) Placement of microinjections for use in behavioral analysis (b) was determined by Cresyl violet staining. Open circles indicate saline treatment, closed circles indicate PPF treatment. $^{*}p<0.05$.

not endure after discontinuing daily administration. PPF has a relatively short half-life ($\sim$1 h) (Kwon *et al*, 1998; Kwon and Ryu, 2000) and is taken three times daily in most human studies. Thus, it is possible that a more frequent or longer dosing regimen would be required to observe an enduring effect.

PPF as a Medication in Cocaine Addiction and other Neuropsychiatric Disorders

Numerous human studies testing effects of PPF on cognition have demonstrated efficacy in age-related dementia, Alzheimer's disease, and vascular dementia (Frampton *et al*, 2003; Kittner *et al*, 1997; Marcusson *et al*, 1997; Mielke *et al*, 1998; Saletu *et al*, 1990). However, phase II and III clinical trials for use in Alzheimer's Disease and vascular dementia were discontinued due to variable results (for review, see Sweitzer and De Leo, (2011)). Use of the related compound pentoxifylline resulted in a non-significant trend toward a decrease in cocaine abuse in addicted individuals; however, in the case of this outpatient study, no measures of medication compliance were obtained (Ciraulo *et al*, 2005; Cooper *et al*, 2012). Although these studies collectively indicate a complex profile of clinical utility, they also demonstrate safety and tolerability of this class of molecules. Data presented here have important implications regarding the role of glial activation in the behavioral and cellular adaptations induced by chronic drug abuse, particularly with respect to GLT-1. Future studies will be important to more fully elucidate the mechanism(s) by which responsiveness of glial cells to drug exposure contributes to the behavioral pathologies, and to clarify the relationship between GLT-1 expression, astroglial function, and drug reward. For example, how do adaptations of glial cells in response to drug abuse affect neuronal signaling and synaptic strength? Also, can targeting glial cells directly restore these measures? A more thorough understanding of these fundamental mechanisms of

cocaine-induced glial pathology will help identify new therapeutic mechanisms and inform on developing compounds to target these mechanisms.

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The authors declare no conflict of interest.

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