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PPAR γ agonism attenuates cocaine cue reactivity

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Abstract

Cocaine use disorder is a chronic relapsing condition characterized by compulsive drug seeking and taking even after prolonged abstinence periods. Subsequent exposure to drug-associated cues can promote intense craving and lead to relapse in abstinent humans and rodent models. The responsiveness to these cocaine-related cues, or “cue reactivity”, can trigger relapse and cocaine-seeking behaviors; cue reactivity is measurable in cocaine-dependent humans as well as rodent models. Cue reactivity is thought to be predictive of cocaine craving and relapse.

Here we report that PPAR γ agonism during abstinence from cocaine self-administration reduced previously active lever pressing in Sprague Dawley rats during cue-reactivity tests, while administration of the PPAR γ antagonist, GW9662, reversed this effect. PPAR γ agonism also normalized nuclear ERK activity in the medial prefrontal cortex and hippocampus which was reversed with GW9662. Our results support the utility of PPAR γ agonism as a relapse prevention strategy to maintain abstinence in the presence of cocaine-associated cues.

INTRODUCTION

Cocaine use disorder is associated with emotional and cognitive impairments accompanied by dysfunctional behaviors that engender susceptibility to relapse (Goldstein and Volkow, 2002). Even after long durations of abstinence, relapse to cocaine-seeking and -taking can be triggered by exposure to drug-associated contexts and stimuli (e.g., paraphernalia) which

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evoked conditioned physiological and subjective effects as well as appetitive approach behaviors (“cue reactivity”) via activated limbic-cortico-striatal circuits (*For review, see* Weiss et al., 2001). Recent studies suggest that cue reactivity escalates during abstinence and predicts relapse in cocaine-dependent subjects (Bedi et al., 2011; Moeller et al., 2013a; Moeller et al., 2013b; Wang et al., 2013). In rodent models, cue reactivity (assessed by lever presses reinforced by the discrete cocaine-paired cue complex) escalates across months of forced abstinence (FA) from self-administration (SA) (Ghitza et al., 2003; Grimm et al., 2001; Neisewander et al., 2000). This escalation of cue reactivity evolves within the framework of long-term neuroadaptations in which extracellular signal-regulated kinase (ERK) signaling in limbic-cortico-striatal circuits plays an important role (Koya et al., 2009; Lu et al., 2005; Lu et al., 2007; McGinty et al., 2010; McGinty et al., 2014; Shaham and Hope, 2005; Sun et al., 2013; Whitfield et al., 2011). ERK is a key mediator of the cell cycle, cell maturation and differentiation; in the CNS, it mediates several forms of plasticity, learning and memory (Sweatt, 2004).

We have recently discovered that activated, phosphorylated ERK (pERK) and the nuclear receptor and transcription factor peroxisome proliferator activated receptor gamma (PPAR γ) directly interact in a dynamic multiprotein complex that facilitates ERK-dependent memory consolidation within the hippocampus (HIP) (Jahrling et al., 2014). The compelling interrelationship between pERK and PPAR γ engages induction of PPAR γ - and ERK-dependent transcriptomes and a proteome that alleviated synaptic and network abnormalities (Denner et al., 2012; Jahrling et al., 2014; Nenov et al., 2014; Rodriguez-Rivera et al., 2011). These findings prompted the current hypothesis that PPAR γ modulates ERK to attenuate cue reactivity during late FA from cocaine SA (Anastasio et al., 2014a; Anastasio et al., 2014b; Nielsen et al., 2012; Swinford-Jackson et al., 2016).

The work herein shows that PPAR γ agonism with the FDA-approved drug pioglitazone (PIO) significantly attenuated both behavioral sensitization to cocaine and cue reactivity in late FA without affecting general locomotor activity or motivation for the natural reward sucrose; this outcome was accompanied by normalization of pERK levels in the medial prefrontal cortex (mPFC), HIP, amygdala (AMG) and nucleus accumbens (NAc), important nodes in the limbic-cortico-striatal circuit which mediate cocaine-related behaviors (Everitt and Wolf, 2002; Koob and Le Moal, 2001; Koob and Nestler, 1997). The selective PPAR γ antagonist GW9662 reversed both the behavioral and molecular effects of PPAR γ agonism. Co-immunoprecipitated pERK and PPAR γ and their association in mPFC and HIP were reciprocally modulated with PPAR γ agonism and antagonism. These findings indicate that, in an analogous manner to memory consolidation processes in HIP (Jahrling et al., 2014), PPAR γ agonism resolves dysregulated pERK to ameliorate cue reactivity in late FA. Thus, a PPAR γ agonist may be a useful therapeutic approach to counteract cue reactivity and extend abstinence.

MATERIALS AND METHODS

Animals

Experimentally-naïve, male Sprague-Dawley rats (n=143; Harlan, Inc.) weighing approximately 225g (7–8 weeks of age) upon arrival, were allowed to acclimate for 5–7 days

in a colony room with controlled temperature (21–23°C) and humidity (45–50%) on a 12 hr light dark cycle (on between 0700–1900 hr). Rats were housed two per cage with food and water *ad libitum*. All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council, 2011) and with the approval of the Institutional Animal Care and Use Committee at the University of Texas Medical Branch (UTMB).

Drugs and Treatments

(–)-Cocaine (National Institutes on Drug Abuse) was dissolved in sterile 0.9% NaCl. The PPAR γ agonist PIO (Actos®) was obtained from the UTMB pharmacy. Via contract with a certified provider (Bio-Serve), PIO was milled into standard rodent chow at a dosage of 50 mg/kg, as previously described (Denner et al., 2012; Jahrling et al., 2014; Nenov et al., 2014; Rodriguez-Rivera et al., 2011). Food was weighed and replenished daily. Rats' weight and food intake was monitored throughout the experiments in which treatment ensued and PIO had no effect on body weight or food intake (Supplementary Figure 1). Pair-housed rats received the same treatment. While we cannot determine individual dosage or individual food intake, both rats had access *ad libitum* and, again, no difference between treatment groups (pair-housed cages) was noted. Sucrose pellets were purchased from BioServ (45 mg/pellet). GW9662 (Sigma) was dissolved in 100% DMSO.

Behavioral Sensitization to Cocaine

Up to this point, PPAR γ agonism has been explored as affecting alcohol intake (Blednov et al., 2015; Ferguson et al., 2014; Stopponi et al., 2013), opioid consumption (de Guglielmo et al., 2015), morphine tolerance (de Guglielmo et al., 2014), and behavioral sensitization to methamphetamine (Maeda et al., 2007)() and cannabinoids (Enayatfard et al., 2013). Behavioral sensitization to psychostimulants is a noncontingent drug exposure paradigm designed to model the long-lasting behavioral and neurochemical adaptations that underlie enhanced locomotor behavior in response to repeated psychostimulant exposure. Here, we evaluated the effects of PIO on the development of cocaine sensitization. Four days prior to initiating the behavioral sensitization protocol, PIO treatment was started based on pilot studies showing that four days of PIO intake is required to achieve steady-state effects on CNS PPAR γ DNA binding activity (Supplementary Figure 2). Locomotor activity was monitored and quantified under low light conditions using a modified open field system (San Diego Instruments). Clear Plexiglas chambers (40×40×40 cm) were surrounded by a 4×4 photobeam matrix positioned 4 cm from the chamber floor. Vertical activity was also recorded using a row of photobeams positioned approximately 16 cm above the chamber floor. The control software counted total horizontal activity as well as vertical beam interruptions and stored the counts in 5-min bins; these data are reported as total ambulation (De La Garza and Cunningham, 2000; Lee et al., 1991; McCreary et al., 1999; Paris et al., 1991; Sell et al., 2002).

Rats were randomly assigned to untreated and PIO-treated groups yielding four experimental groups (n=6/group): 1) saline untreated, 2) saline PIO-treated, 3) cocaine untreated, 4) cocaine PIO-treated. Each rat was habituated daily for one hour in the locomotor chamber for four days prior to intraperitoneal (IP) injection of cocaine (15 mg/kg)

or saline (1 ml/kg). Immediately after cocaine injection, each rat was returned to the locomotor chamber for a one hour locomotor activity assessment. This process was repeated once a day for five days. All groups then underwent a seven-day withdrawal period during which time they remained in their home cages but were handled and weighed daily. After seven days of withdrawal all rats were exposed to the locomotor chamber for one hour prior to cocaine challenge (15 mg/kg, IP) and locomotor assessment.

Cocaine Self-Administration

Implantations of intravenous catheters with back mounts were performed under anesthesia with a cocktail containing xylazine (8.6 mg/kg), acepromazine (1.5 mg/kg) and ketamine (43 mg/kg) in bacteriostatic saline. The catheter was inserted into the right jugular vein and exited dorsally (Anastasio et al., 2014a; Anastasio et al., 2014b; Nielsen et al., 2012). Daily catheter flushes were performed to ensure catheter patency using a solution of 0.1 ml bacteriostatic saline and heparin sodium salt (10 U/ml; American Pharmaceutical Partners), streptokinase (0.67 mg/ml; Sigma) and ticarcillin disodium (66.67 mg/ml; Research Products International). Following catheter surgery, rats were allowed at least five days of recovery before initiation of SA training. In all experiments, a control group was included in which saline was delivered upon active lever pressing.

Cocaine SA took place in standard operant chambers housed within ventilated, sound-attenuating chambers (Med-Associates, Inc.). Each chamber was equipped with a pellet receptacle flanked by two retractable levers, a stimulus light above each response lever, and a houselight opposite the levers. Cocaine infusions were delivered via syringe attached to an infusion pump (Med-Associates) mounted outside the cubicle. The infusion pumps were connected to the catheters via liquid swivels (Instech, Plymouth Meeting, PA) and polyethylene tubing encased inside a metal spring leash (Plastics One, Roanoke, VA). Cocaine SA training consisted of 14 daily 180-min sessions during which rats were trained to lever press for cocaine infusions (0.75 mg/kg/0.1 mL infusion) (Anastasio et al., 2014a; Anastasio et al., 2014b; Nielsen et al., 2012). Schedule completions on the active lever resulted in delivery of a cocaine infusion over a 6-sec period paired simultaneously with illumination of the house and stimulus lights and activation of the infusion pump (discrete cue complex paired with cocaine delivery); inactive lever presses produced no scheduled consequences. Following cocaine delivery, the stimulus light and infusion pump were inactivated; the house light remained on for an additional 20 sec to indicate a timeout period during which lever presses had no scheduled consequences. Rats were trained on a fixed ratio (FR) 1 schedule of reinforcement and progressed to an FR5 schedule, with stability defined upon achieving seven infusions/hr on the FR5 with less than 10% variability in infusions for three consecutive days.

As a control for subsequent neurochemical studies, we included a “no cue reactivity test” group of rats which were trained to SA cocaine then split into two treatment groups, PIO or untreated, and subjected to 30 days FA then sacrificed in an identical manner as cohorts that underwent cocaine cue reactivity testing.

Sucrose Self-Administration

Sucrose SA training consisted of 14 daily 180-min sessions during which freely-fed rats were trained to lever press for 45 mg sucrose pellets (Bio-Serv) (Cunningham et al., 2011a). Experimental parameters were identical to those employed in cocaine SA training except that sucrose was substituted as the reinforcer.

Note: After either cocaine or sucrose SA acquisition and maintenance, rats were divided into treatment groups balanced for infusions and drug/natural reward intake during SA acquisition and maintenance then subjected to 30 days FA followed by cue reactivity tests, described in detail in the 'Self-Administration Research Design' section following.

Self-Administration Research Design

Experiment 1: Effect of PPAR γ agonism on cue reactivity—Using a 2 $\times$ 2 factorial design, two separate cohorts of rats were trained to either SA cocaine or saline then pseudo-randomly divided (balanced for lever pressing and drug intake) into four treatment groups to test the effect of PIO on cue reactivity following cocaine or saline SA: 1) Saline untreated (n=12), 2) saline PIO-treated (n=10), 3) cocaine untreated (n=20), 4) cocaine PIO-treated (n=22). PIO treatment began following the final session of cocaine SA and continued during the 30 days FA period. Food intake and body weight was monitored weekly during the 30 day treatment/FA period; no significant differences in either parameter were measured in any of the treatment groups (data not shown).

Following 30 days of FA, the four groups were subjected to cue reactivity testing by quantifying active and inactive lever presses during a 60 min session. Presses on the previously-active lever were reinforced by the discrete cue complex (stimulus light illuminated, pellet dispenser activated) on an FR1 schedule; presses on the inactive lever were recorded but produced no scheduled consequences.

Experiment 2: Effect of PPAR γ antagonism on PIO attenuation of cue reactivity—Following cocaine SA, rats were pseudo-randomly divided (balanced for lever pressing/drug intake) into four treatment groups using a 2 $\times$ 2 factorial design to test the effect of the specific and irreversible PPAR γ antagonist GW9662, on PIO attenuation of cue reactivity: 1) cocaine untreated (n=8), 2) cocaine untreated + GW9662 (n=10), 3) cocaine PIO-treated (n=9), 4) cocaine PIO-treated + GW9662 (n=9). PIO treatment began following the final session of cocaine SA and continued during the 30 days FA period.

Following 30 days of FA, the four groups were subjected to cue reactivity testing by quantifying active and inactive lever presses during a 60 min session. Treatment groups designated to receive GW9662 received an IP injection two hrs prior to cue reactivity testing based upon timing of previous studies (Denner et al., 2012; Jahrling et al., 2014). During the test, presses on the previously-active lever were reinforced by the discrete cue complex (stimulus light illuminated, pellet dispenser activated) on an FR1 schedule; presses on the inactive lever were recorded but produced no scheduled consequences.

Experiment 3: Effect of PPAR γ agonism on acquisition of sucrose SA and cue reactivity—Rats (n=28) were trained to SA sucrose pellets (45 mg/pellet, BioServ) as

above (Cunningham et al., 2011) followed by 30 days of FA and a 60 min cue reactivity test on day 44. Three treatment groups were included: 1) untreated (n=9), 2) PIO-treated during days 1–14 during acquisition of sucrose SA (n=9), 3) PIO-treated during days 15–44 of FA (n=10). Rats were allowed food and water *ad libidum*.

To assess cue reactivity, presses on the previously-active lever were reinforced by the discrete cue complex (stimulus light illuminated, pellet dispenser activated) on an FR1 schedule during the 60 min session; presses on the inactive lever were recorded but produced no scheduled consequences.

We also included a set of “no cue reactivity test” rats that were killed upon removal from their home cages on FA Day 30 without re-exposure to the operant chambers to control for the behavioral experience during the cue reactivity session for the *ex vivo* neurochemical studies.

Immunoblot

Immediately following cue testing, rats were anesthetized, decapitated, and brains immediately micro-dissected on ice for -80°C storage following flash freezing in liquid nitrogen. Following the procedure described by Heffner et al. (Heffner et al., 1980), we collected the following brain regions from coronal sections prepared using a rat brain slicing guide (Zivic Instruments): dorsomedial prefrontal cortex, amygdala, dorsal hippocampus, ventral tegmental area and the nucleus accumbens. After rinsing with ice cold PBS, We sliced first at optic chiasm, two in front (every other 2 mm slot), four behind (every other slot) to generate six 2-mm coronal sections sandwiched between seven blades. After arranging sections on filter paper soaked with ice cold PBS, chosen brain regions were collected.

We also included a set of “no cue reactivity test” rats that were killed immediately upon removal from their home cages on FA Day 30 without re-exposure to the operant chambers to control for the behavioral experience during the cue reactivity session for the *ex vivo* neurochemical studies. Thus, reported changes in protein expression or phosphorylation state represent cue-induced alterations in PPAR γ and ERK. Nuclear extracts were prepared according to manufacturer’s instructions (Activ Motif) followed by BCA protein assay (Thermo Scientific, #23225,) then stored frozen at -80°C .

As a control for subsequent *ex vivo* neurochemical studies a set of “no cue reactivity test” rats were analyzed to control for the behavioral experience during the cue reactivity session. These rats were trained to SA cocaine then were killed immediately upon removal from their home cages on FA Day 30 without re-exposure to the operant chambers.

Nuclear samples (20 μg) were thawed on ice, resolved by SDS-PAGE (7.5% Bio-Rad Mini-PROTEAN TGX) and electro-blotted onto PVDF membranes (Immobilon, Millipore). To quantitatively compare between immunoblot film bands, a crude whole-brain lysate (20 μg /well) prepared from Sprague-Dawley rat brains was included in triplicate on each gel as an internal standard (further described below). After 15 min in TBS-T (50 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween-20) blocking buffer supplemented with 2% ECL Advance

Blocking agent (GE Healthcare), membranes were incubated overnight at 4°C with the antibody PPAR γ (1:500; Millipore, 07-466), and for one hr with the following antibodies: ERK (1:1000; Cell Signaling, 9102), and pERK (1:1000; Cell Signaling, 9101). Lamin-A/C (1:1000; Santa Cruz, SC-20681) was used as a loading control for nuclear fractions. All antibodies were diluted in TBS-Tween blocking buffer with 2% ECL Advance Blocking agent (GE Healthcare). After five washings with TBS-Tween, the blots were incubated for 1 hr at room temperature with anti-mouse IgG conjugated with horseradish peroxidase (1:50,000; GE Healthcare, NA931V) or anti-rabbit IgG conjugated with horseradish peroxidase (1:50,000; GE Healthcare, NA934V), in TBS-Tween containing 2% ECL Advance Blocking agent. After five washings with TBS-Tween, protein bands were detected by chemiluminescence via Advance ECL (GE Healthcare) detection system in accordance with the manufacturer's instructions. Films exposed in the linear range for the antibody combinations employed were developed with a Kodak imager.

As previously published by us (Jahrling et al., 2014), film exposures of immunoblot membranes were scanned at 300 dpi, and numeric band density and background values were acquired using ImageJ software [National Institutes of Health (NIH)]. The numeric values for lamin A/C in the loading control from each of the three identical rat brain homogenate internal standards was averaged, and all samples run on the gel were normalized to the loading control average density value. Lamin A/C was chosen as a normalization value so that density values for the loading control would remain in the linear range of the samples we were investigating. A standard loading control such as actin would have generated a signal too intense to accurately quantify the PPAR γ and pERK. Thus, this method allows for more precise comparison between different gels and across different experiments. In all instances, the total ERK signal was unchanged and was not reported. Immunoblot bands shown represent the mean of each experimental group and were cropped from contiguous lanes from a single immunoblot probed sequentially for PPAR γ , pERK, and lamin A/C then stripped and probed for total ERK. All samples were run at least three times and averaged prior to calculating group means and SEMs; film exposure times between experimental repeats were controlled with a stopwatch yet films in the linear range for the primary and secondary antibody combination (determined empirically) was the overall objective. Thus, the histograms in Figures 4, 5, and 6 represent the group population mean of the total number of animals for each SA/FA/cue reactivity experiment normalized to lamin A/C of a rat brain crude homogenate. We analyzed a cohort of "no cue reactivity test" rats that were killed immediately upon removal from their home cages on FA Day 30 without re-exposure to the operant chambers to compare our sham and

Immunoprecipitation

PPAR γ and ERK protein complexes were immunoprecipitated according to Jahrling et al. (2014). Briefly, tissue extract buffer (25 μ M HEPES, 0.1 M Triton X-100, 1 M Glycerol, and ddH₂O supplemented with 0.02 M Sigma protease inhibitor cocktail (P8340), 0.02 M NaF, and 0.02 M sodium orthovanadate) was combined with nuclear extract prepared from 600 μ g wet weight starting material. 600 μ g input was determined as optimal by performing input-output curves as demonstrated in Jahrling et al. (2014); 0 μ g input served as negative control and immunoblot of the unbound fraction determined the IP efficiency ~50%. A 500 μ l

reaction volume was put into a 1.5 ml Eppendorf tube followed by PPAR γ polyclonal antibody (10 μ l, Millipore 07-466) then incubated overnight on a rotating rocker at 4° C. The following day, Protein A sepharose beads 20 μ l (Cell Signaling) were added and samples placed on a rotating platform at 4°C for an additional 3 hr. Samples were centrifuged at 3,000 $\times$ g for one minute and supernatant carefully removed and discarded. Pelleted beads were washed by adding 500 μ L of extract buffer and placed on a rotating rocker in 4° C for 20 min; washes were repeated four times. After the final centrifugation the supernatant was removed without removing pelleted beads and 3 $\times$ SDS PAGE sample buffer was added. Samples were heated to 95–100 °C for 5 min then allowed to cool to room temperature. Samples were then centrifuged (14,000 $\times$ g) for 1 min and supernatant was loaded onto a 7.5% SDS-PAGE gel (Biorad), electrophoresed, then transferred onto PVDF membranes (Immobilon, Millipore). After 15 min in TBS-T (50 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween-20) blocking buffer supplemented with 2% ECL Advance Blocking agent (GE Healthcare), membranes were incubated overnight at 4°C with the following antibodies: PPAR γ -HRP (1:1000; Novus Biologicals, NBP2-22106H), then pERK-HRP (1:1000; Cell Signal, 8544s) then quantified as described previously to a standard control loaded in triplicate (Jahrling et al., 2014).

Statistical Analyses

Statistical power analyses (SAS for Windows, Version 6.12, SAS Institute) on previous cocaine SA data (Anastasio et al., 2014b; Cunningham et al., 2013; Cunningham et al., 2011b; Nic Dhonnchadha et al., 2009; Nielsen et al., 2012) indicated that at least n=8 rats/group is required to achieve 95% confidence against type 1 error at p=0.05. Therefore, all SA experiments were carried forth with a minimum group size of n=8 rats per group.

Data are reported as mean $\pm$ SEM. Two-factor (treatment $\times$ drug) ANOVA was employed to determine group differences (treatment [e.g., PPAR γ] $\times$ drug [e.g., cocaine]) as well as treatment $\times$ drug interaction, followed by Tukey to detect pairwise differences. Where appropriate, repeated measures two-way ANOVA was used to analyze whether treatment or drug influenced the primary outcome measure as well as whether an interaction occurred (e.g., treatment $\times$ drug). The experimenter was blinded to the group allocation (e.g., PIO treatment) throughout the duration of the study. Statistics are reported in the figure legends and additional tables.

RESULTS

PPAR γ agonism blocks development of behavioral sensitization to cocaine

A previous study showed that PPAR γ agonism with PIO or rosiglitazone during the withdrawal period attenuated methamphetamine induced locomotor sensitization (Maeda et al., 2007). We therefore tested the hypothesis that PIO would prevent the development of cocaine-induced locomotor sensitization. In contrast to Maeda et al. (2007), we began PIO treatment four days prior to the experiment. Cocaine (15 mg/kg) evoked hyperactivity relative to saline treated rats. PIO-treated rats exhibited stable cocaine-evoked hyperactivity across the five days of cocaine delivery (Figure 1A) as well as following a challenge

injection delivered after seven days of withdrawal (Figure 1B). Thus, PIO tempered behavioral sensitization to cocaine.

PPAR γ agonism with pioglitazone attenuated cue reactivity following prolonged abstinence from cocaine self-administration

The first cohort of rats (Figure 2A) acquired cocaine SA to stability (Figure 2B) and then were assigned to PIO or untreated groups to balance for active lever presses during cocaine SA. Immediately following the last SA session, treatment groups were placed on either PIO treatment (50 mg of PIO/kg chow) or untreated. PIO delivered in rat chow activates brain PPAR γ without altering food intake, body weight or locomotor activity (Figure 1; Supplementary Figure 1), consistent with our previous work (Rodriguez-Rivera et al., 2011). Following 30 days of FA, cue reactivity was assessed. As expected, saline-SA groups exhibited low pressing for the previously active lever that was unaffected by PIO treatment (Figure 2C). Animals trained to SA cocaine and left untreated during FA displayed high levels of lever pressing for cocaine-associated cues. PIO-treated rats exhibited significantly fewer previously active lever presses during the cue reactivity test compared to the untreated cocaine SA group. Finally, inactive lever presses (Figure 2C, *inset*) and latency to lever press was unaffected by any of our treatments (*data not shown*).

PPAR γ agonism had no effect on sucrose intake or cue reactivity

Since we are ultimately interested in neural mechanisms specific to re-exposure to cocaine context and cues following chronic cocaine SA and FA, we included saline SA above as a control for un-reinforced lever pressing. We next tested if SA of a “natural” reinforcer (e.g., sucrose pellets) is affected by PPAR γ agonism with PIO. Rats were trained to SA sucrose pellets under the same contingency schedule as cocaine (refer to SA Methods; Figure 3A). We found that PIO treatment, during acquisition (Days 1–14) did not alter sucrose intake (Figure 3B). PIO treatment during 30 days of FA (Days 14–44) did not affect cue reactivity in rats trained to SA sucrose (Figure 3C) and inactive lever pressing was unaltered across groups (Figure 3C, *inset*). Since PPAR γ agonism prevented behavioral sensitization to cocaine yet had no effect on acquisition of sucrose SA or cue reactivity to this natural reward, we conclude that, under the present experimental conditions, PPAR γ agonism exerted a selective influence on limbic-corticostriatal circuit function dysregulated by the psychostimulant cocaine.

PPAR γ agonism counteracted reductions in nuclear PPAR γ and pERK following cue reactivity test

Nuclear extracts of brain regions involved in cue reactivity circuitry were subjected to immunoblot (IB) to measure PPAR γ and active ERK (pERK) in individual rats (Anastasio et al., 2014a; Anastasio et al., 2014b) using established methods (Jahrling et al., 2014). While ERK1 has been shown to mediate cocaine-induced alterations in the striatum (Ferguson et al., 2006; Mazzucchelli et al., 2002), we measured the more abundant ERK2 isoform in the brain regions studied. ERK2 has been shown to dominate in the types of behavior we are measuring (Fijal et al., 2015; Girault et al., 2007; Velazquez et al., 2013). Furthermore, ERK1 phosphorylation changes tracked with ERK2 (Supplementary Figure 3).

When exposed to the cocaine SA chamber and allowed to lever press for cocaine-associated cues, the cocaine untreated group, which displayed the highest levels of cue reactivity (Figure 2C, 2D), exhibited attenuated pERK and PPAR γ levels in PFC, HIP, and AMG compared to all other groups (Figure 4A, B, C). The NAc of cocaine untreated SA rats exhibited decreased nuclear PPAR γ but no change in pERK; however, nuclear pERK in cocaine PIO-treated rats was *decreased* relative to all other groups (Figure 4D). In all instances, the total ERK protein signal in nuclear extracts was unchanged. Importantly, “no cue reactivity test” rats taught to SA cocaine with or without PIO treatment during 30 days of FA, exhibited similar levels of PPAR γ , ERK1/2, and pERK1/2 in all brain regions interrogated (Supplementary Figure 4). Thus, lever pressing for cocaine-associated cues induced a rapid reduction of nuclear pERK and PPAR γ in PFC, HIP, and AMG that was counteracted by PPAR γ agonism during FA. Furthermore, comparable samples from the brains of rats trained to SA sucrose (Figure 3) exhibited no alterations in nuclear ERK, pERK or PPAR γ (Supplementary Figure 5), suggesting that the observed changes in the brains of rats trained to SA cocaine are due to chronic cocaine SA and FA rather than effects of lever pressing during the cue reactivity test. Taken together, one hypothesis is that PPAR γ agonism counteracts loss of ‘top down’ control over cocaine cue reactivity, possibly by preventing ERK dysregulation in the PFC, HIP, AMG and NAc, during prolonged abstinence.

PPAR γ antagonism reversed effects of PIO treatment on cue reactivity and nuclear PPAR γ and pERK in PFC and HIP, but not AMG or NAc

To demonstrate the necessity of PPAR γ in suppression of cue reactivity, we employed the specific and irreversible antagonist, GW9662, as previously described (Denner et al., 2012; Jahrling et al., 2014). In the present experiment, we injected GW9662 or vehicle (IP) two hours prior to the cue reactivity test. As before, PIO-treated rats trained to cocaine SA demonstrated suppressed cue reactivity compared to cocaine untreated rats (Figure 5A). Cocaine untreated rats injected with either vehicle or GW9662 showed equivalent cue reactivity as did PIO-treated rats injected with GW9662, suggesting that acute GW9662 reversed the effect of PIO treatment; GW9662 alone had no effect on lever presses for cocaine-associated cues and inactive lever pressing was unchanged across groups (Figure 5A, *inset*). As a control, we tested whether IP GW9662 (1 mg/kg) affected locomotor activity and found that total ambulations were unaltered by IP injection of GW9662 (Supplementary Figure 6).

We next investigated the status of PPAR γ and ERK in PFC, HIP, AMG, and NAc with IB to find that PPAR γ antagonism, that blocks PIO-mediated suppression of cue reactivity, reversed the effects of PIO on nuclear PPAR γ and pERK in PFC and HIP (Figures 5B, 5C), but not in the AMG or NAc (Supplementary Figure 7). This finding indicates that PPAR γ agonism influences cue reactivity in the PFC and/or HIP whereas PPAR activity AMG and NAc does not appear to mediate lever pressing behavior to cocaine-associated cues.

pERK:PPAR γ protein complexes are increased by exposure to cocaine-associated cues in PIO-treated rats; PPAR γ antagonism reversed PIO effects

Based on our previous work showing that nuclear PPAR γ -pERK protein complexes facilitate ERK-dependent memory consolidation (Jahrling et al., 2014), we co-immunoprecipitated (IPed) nuclear PPAR γ -pERK complexes and found that PPAR γ -pERK complexes track with immunoblot (IB) results in PFC and HIP and that the irreversible PPAR γ antagonist GW9662 reversed both PIO-mediated suppression of cue reactivity and alterations in PPAR γ and pERK within the PFC and HIP (Figure 6A, 6B). Lastly, while IB of AMG and NAc nuclear extracts revealed cocaine and PIO-mediated changes, PPAR γ antagonism failed to reverse these effects and we observed no effect of GW9662 on PPAR γ -pERK complexes (Supplementary Figure 7).

DISCUSSION

Drug-induced neuroadaptations within the limbic corticostriatal system are postulated to underlie cocaine craving and relapse (Everitt and Wolf, 2002; Koob and Le Moal, 2001; Koob and Nestler, 1997; Marchant et al., 2015). Clinical studies in cocaine-dependent subjects reveal reduced PFC cerebral blood flow and metabolic activity, as well as loss of grey and white matter integrity that accompany enhanced reactivity to cocaine-associated cues, impaired decision-making, and impulsivity (Dias et al., 2015; Fein et al., 2002; Franklin et al., 2002; Lane et al., 2010; Ma et al., 2009; Ma et al., 2015; Ma et al., 2014a; Moeller et al., 2005; Moeller et al., 2007; Parvaz et al., 2015; Volkow et al., 1992; Volkow et al., 1988). Rodent models for cocaine SA and FA show these same deficits both structurally (Ma et al., 2014b; Narayana et al., 2014) and behaviorally (Anastasio et al., 2014a; Anastasio et al., 2014b; Fijal et al., 2015; Pickens et al., 2011; Swinford-Jackson et al., 2016) and thus are an ideal model to study the biochemical mechanisms that underlie cue reactivity and risk for relapse in cocaine-dependent humans.

We used cue reactivity to test the efficacy of PPAR γ agonism during FA in reducing previously active lever pressing when rats are subsequently re-exposed to the context and cues previously associated with cocaine SA since cocaine cue reactivity predicts relapse and treatment success in cocaine dependent subjects (Carter and Tiffany, 1999; Rohsenow et al., 1990). PPAR γ agonism has previously been used in models of behavioral sensitization to methamphetamine (Maeda et al., 2007), opioid consumption (de Guglielmo et al., 2015), and alcohol drinking and relapse (Stopponi et al., 2013; Stopponi et al., 2011). These studies found a main effect of PPAR γ agonism on consumption, with a specific effect on stress-induced relapse to alcohol rather than cue-induced relapse. To date, the utility of PPAR γ agonism in cocaine relapse had not been investigated. We found that treatment with the potent and selective PPAR γ agonist, PIO, administered throughout the cocaine FA period, significantly attenuated active lever pressing upon re-exposure to drug cues. Furthermore, delivery of the specific and irreversible antagonist, GW9662, prior to the cue reactivity test reversed the effects of PIO treatment on previously active lever pressing. Given the extensive differences between experimental design, animal strain, and drug substance utilized in the previous reports, it is difficult to predict if PIO treatment would affect cocaine SA, as is the case for alcohol and opioids (de Guglielmo et al., 2015). However, our data thus far support

PPAR γ as a viable therapeutic target to attenuate drug-seeking behavior to help maintain abstinence.

In support of this, PIO treatment prevented pERK reductions in nuclear fractions from PFC, HIP, and AMG (no change in total ERK). Within nuclear fractions of the NAc, re-exposure to cocaine cues and context resulted in reduced pERK whereas in untreated rats trained to SA cocaine, nuclear pERK remains unaltered (compared to saline controls) in the NAc. These results indicate that *re-exposure* to cocaine-associated context and cues drives the loss of nuclear ERK activity in PFC, HIP, and AMG whereas PIO treatment during cocaine FA protects from this loss. We make these assertions because rats that were not re-exposed to cocaine-associated cues, with or without PIO treatment, exhibited no alterations in PPAR γ , ERK or ERK phosphorylation.

It is important to note, however, that while GW9662 PPAR γ antagonism reversed the behavioral effect of PIO on cocaine cue reactivity, GW9662 reversed the biochemical effects of PIO *only* in the PFC and HIP. Thus, PPAR γ regulation of ERK and ERK-dependent behaviors is limited to the PFC and HIP and further suggests that the HIP also performs an important role in cocaine-seeking behavior in addition to the PFC. For example, in tests targeting context-dependent cue reactivity, previous work established overlapping neural substrates within the PFC, HIP, and AMG (Fuchs et al., 2005). The HIP is clearly an important element of this circuit contributing to neuroadaptations resulting from repeated exposure to a cocaine-paired context in the absence of discrete cocaine cues (Ito et al., 2008; Tropea et al., 2008). Thus, we put forth the hypothesis that HIP ERK and PPAR γ are mechanistically involved in cocaine-seeking behavior triggered by cocaine-associated cues in addition to the well-established PFC ERK system (reviewed in (McGinty et al., 2014). Future studies testing the effect of GW9662 delivery to HIP versus PFC on previously active lever pressing to cocaine-paired cues in PIO-treated rats may reveal important mechanisms on cocaine-seeking behavior. Furthermore, our strategy to begin PIO treatment early in the withdrawal phase is supported by several previous studies utilizing strategies to activate ERK in PFC (e.g., BDNF; Berglind et al., 2007; Berglind et al., 2009; Sun et al., 2013; Whitfield et al., 2011) and likely contributed to treatment efficacy and may have implications for treatment strategies in humans trying to maintain abstinence.

On another note, the relatively rapid (~2 hr pretreatment) reversal of PIO effects on cue reactivity with GW9662 antagonism and co-IP of these two proteins suggests that PPAR γ dynamically regulates ERK activity. This concept is supported by our studies on HIP-dependent memory consolidation (Denner et al., 2012; Jahrling et al., 2014) in which we found that PPAR γ agonism enhanced ERK-dependent cognitive performance via an increase in PPAR γ -pERK protein complexes during memory consolidation and ‘acute’ GW9662 PPAR γ antagonism both prevented memory consolidation and PPAR γ -pERK protein complex increase (Jahrling et al., 2014). We found analogous effects on PPAR γ -pERK protein complexes induced by the cue reactivity test in PFC and HIP. Taken together, these data support our fundamental hypothesis that, in behaviors and cognitive tasks in which ERK is pivotal, PPAR γ may serve as an intermediary to resurrect proper ERK function.

Finally, a series of supportive experiments provided additional evidence that PPAR γ agonism is a valid therapeutic approach for protection against maladaptive plasticity resulting from chronic cocaine versus a natural reward, e.g., sucrose. In addition, we found that PPAR γ agonism blocked the development of behavioral sensitization whilst having no effect on the acquisition of sucrose SA or cue reactivity similar to previous work (Stopponi et al., 2011). The fact that our biochemical studies tracked with the behavioral outcomes on sucrose SA and previously active lever pressing, in that sucrose cue reactivity tests induced no differences in nuclear PPAR γ or pERK, is consistent with our overall postulate that PPAR γ agonism alters the behavioral and biochemical landscape in which ERK is dysregulated and an essential mediator of said behaviors (Denner et al., 2012; Jahrling et al., 2014).

In summary, these studies support our hypothesis that PPAR γ agonism attenuates cocaine-seeking behavior via pERK-dependent mechanisms within limbic-corticostratial brain regions that underlie drug dependence. Thus, PPAR γ represents a viable therapeutic target for the prevention of relapse to cocaine abuse; as PIO is a FDA approved ligand, ongoing studies include a small placebo-controlled clinical trial in which cocaine-dependent subjects are treated with PIO and cocaine cue reactivity and cocaine-positive urine is monitored (NIH clinicaltrials.gov identifier: NCT02774343). We expect that the novel role that PPAR γ agonism plays in reigning in dysregulated ERK function will have applicability to cognitive aging, aging-related neurodegenerative disease, as well as addiction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. PPAR γ agonism blocks the development and expression of behavioral sensitization to cocaine

Untreated or PIO-treated rats were injected (IP) with saline (sham) or cocaine over 5 days followed by locomotor activity assessment (A). Repeated measures two-way ANOVA detected a significant effect of time [**** $p < 0.0001$; $F(4,76) = 6.26$] and treatment [$p < 0.0001$; $F(3,19) = 287$] as well as an interaction [$p < 0.0001$; $F(12,76) = 10.2$] on cocaine-evoked locomotor activity. (B) Following seven days abstinence, all rats received a cocaine challenge injection prior to assessment of locomotor activity; two-way ANOVA detected a significant effect of PIO treatment [***** $p < 0.0001$; $F(3,15) = 18.2$]. Data is expressed as mean $\pm$ SEM.

Figure 2. PPAR γ agonism attenuated cue reactivity following prolonged abstinence from cocaine self-administration

(A) Experimental timeline for 14 days cocaine self-administration (SA) followed by 30 days forced abstinence (FA), and cue reactivity test on day 44. (B) Rats were trained to self-administer cocaine during daily 3hr sessions. Cocaine was initially delivered on a fixed ratio-1 (FR1) schedule of reinforcement until meeting criterion, then FR5 schedule was introduced. (C) After 14 days of cocaine SA and 30 days of FA, rats were subject to a 60 min cue reactivity test. Two way ANOVA and Tukey multiple comparison tests showed that PPAR γ agonism significantly decreased active lever pressing in the cocaine PIO-treated group compared to the cocaine untreated group. $F(1,58)=18.39$ $^{**}p=0.004$. A main effect of cocaine was noted as well $F(1,58)=267.0$ $^{****}p<0.0001$. Arrow: inactive lever pressing was unaffected by either drug or treatment. (D) A separate cohort of rats were subjected to cue reactivity tests on day 44, after 30 days of FA, under three conditions: within the context alone, followed by our standard cue reactivity test, then in response to IP cocaine (15 mg/kg, IP). (E) PIO treatment attenuated active lever pressing in the context (*left*), in the presence of cocaine cues (*center*), and cocaine primed cocaine seeking (*right*). $^{*}p<0.05$, $^{**}p<0.01$, Student's two-sided t-test. Data is reported as mean $\pm$ SEM.

Figure 3. PPAR γ agonism had no effect on sucrose intake or cue reactivity

(A) Rats were trained to SA sucrose pellets with and without PIO treatment during acquisition and FA. (B) While lever pressing changed with days of training, PIO treatment did not affect acquisition; one-way repeated measures ANOVA. $F(13,351)=7.05$, $p<0.0001$; $F(2,27)=1.39$, $p=2.27$; $F(26,351)=0.64$, $p=0.92$ for training day, treatment, and interaction, respectively. (C) Neither PIO treatment during acquisition nor forced abstinence affected lever pressing to sucrose-associated cues after 30 days FA. One-way ANOVA $F(2,25)=0.48$, $p=0.62$. Arrow: inactive lever pressing was unaffected by either drug or treatment. Data expressed as mean $\pm$ SEM.

Figure 4. PPAR γ agonism counteracted cue reactivity reductions in nuclear PPAR γ and pERK2
Immediately following the cue reactivity test (Figure 1C), rats were euthanized and brain regions of interest sub-dissected and processed for immunoblot analysis. Exposure to cues associated with cocaine SA led to rapid changes in nuclear PPAR γ (*left*) and phosphorylated, therefore activated, ERK2 (pERK, *right*) in (A) medial prefrontal cortex (PFC); (B) hippocampus (HIP); (C) amygdala (AMG); (D) nucleus accumbens (NAC). PPAR γ agonism (PIO) prevented these changes in PFC, HIP, and AMG. In the NAC, PPAR γ agonism and exposure to cues associated with cocaine SA led to rapid nuclear PPAR γ

protein increase and nuclear pERK reduction. No significant changes were found in total nuclear ERK in any of the brain regions tested. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Table I lists two-way ANOVA and Tukey results. Data expressed as mean $\pm$ SEM.

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Figure 5. PPAR γ antagonism reversed effects of PIO treatment on cocaine seeking behavior and blocked PIO-mediated cue reactivity effects on nuclear PPAR γ and pERK2 in PFC and HIP

Two hours prior to the cue reactivity test rats were injected with either 1 mg/kg GW9662 or vehicle (DMSO). (A) PIO treatment suppressed cocaine seeking behavior as previously (black bar), whereas GW9662 reversed PIO effects on lever pressing to cues associated with cocaine (light grey bar). Two way ANOVA $F(1,30)=9.35$ $p=0.005$. ** $p<0.01$ compared to all other groups, Tukey multiple comparisons. No significant effect of drug or treatment on inactive lever pressing, *inset*. Immunoblot on brain regions collected immediately after the cue reactivity test revealed that GW9662 reversed PIO-mediated changes in nuclear PPAR γ (*left*) and pERK2 (*right*) in (B) medial prefrontal cortex (PFC) and (C) hippocampus (HIP).

No significant changes were found in AMG or NAc and total nuclear ERK2 in any of the brain regions tested was unaltered. ** $p < 0.01$; ns, not significant. Table II lists two-way ANOVA and Tukey results. Data expressed as mean $\pm$ SEM.

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Figure 6. pERK:PPAR γ protein complexes are increased by exposure to cues related to cocaine self-administration in PIO-treated rats; PPAR γ antagonism reversed effects of PIO treatment Immunoprecipitation of PPAR from (A) PFC and (B) HIP demonstrates that PIO treatment promotes the formation of pERK-PPAR γ complexes following exposure to cues related to cocaine SA (white bars) that is blocked by GW9662. Two way ANOVA $F(1,31)=11.3$ and 9.94 , $p=0.002$ and 0.007 for PFC and HIP, respectively. $*p<0.05$ compared to all other groups, Tukey pairwise analysis. Data expressed as mean $\pm$ SEM.

Table I
Two-way ANOVA and Tukey multiple comparisons test results for data depicted in Figure 4

Abbreviations: PFC, medial prefrontal cortex; HIP, hippocampus; AMG, amygdala; NAc, nucleus accumbens.

	PPAR γ		pERK	
	ANOVA F(1,32)	Tukey p value	ANOVA F(1,32)	Tukey p value
PFC	5.25	0.02	12.8	0.002
HIP	24.9	0.002	5.86	0.03
AMG	17.5	0.0002	3.55	0.03
NAc	38.7	0.006	54.1	0.007

Table II
Two-way ANOVA and Tukey multiple comparisons test results for data depicted in Figure 5

Abbreviations: PFC, medial prefrontal cortex; HIP, hippocampus; AMG, amygdala; NAc, nucleus accumbens.

	PPAR γ		pERK	
	ANOVA F(1,28)	Tukey p value	ANOVA F(1,28)	Tukey p value
PFC	11.8	0.002	12.8	0.002
HIP	9.44	0.007	6.16	0.02
AMG	0.566	0.5	0.411	0.5
NAc	3.38	0.1	0.762	0.4