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PPARγ agonism attenuates cocaine seeking behavior via association with ERK MAPK

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PPARγ agonism attenuates cocaine seeking behavior via association with ERK MAPK

By

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Dedication

This work is dedicated to my parents Sandra Bracken Miller and Stephen Massey and the wonderful Christina van Lier.

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I have relied heavily on my friends and family during my time as a graduate student. I would first like to thank all of the friends I have made at UTMB for providing me with comfort and someone to lean on during difficult times. I would also like to give a special thank you to my parents Sandra Bracken Miller and Stephen Massey for being there when I needed them the most and for helping me stay the course.

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Abstract

The nucleus accumbens integrates information from a number of cortical and limbic structures such as the prefrontal cortex and hippocampus and alterations of this neurocircuitry during drug taking and abstinence is thought to underlie relapse behaviors. ERK, a protein vital for learning and memory, is altered by cocaine use and abstinence. Our lab demonstrated PPARγ agonism rescued cognitive impairment in an animal model of Alzheimer's disease and that this rescue involved ERK. Given the role of altered learning and memory circuitry in addiction, and our recent evidence that PPARγ is involved in restoring hippocampal cognitive deficits through ERK, the current study tested the hypothesis that the prevention of drug-induced alterations of ERK in

brain regions critical for the integration of drug cues and context, which lead to increased cocaine seeking, could be attenuated by targeting PPARγ. We found PPARγ agonism attenuated cocaine cue reactivity in Sprague Dawley rats while administration of a selective PPARγ antagonist, GW 9662, reversed the behavioral effects of PPARγ agonism. We determined PPARγ agonism rescued dysregulated ERK activity in the medial prefrontal cortex, hippocampus, amygdala, and nucleus accumbens and GW 9662 prevented this rescue in the medial prefrontal cortex and hippocampus. PPARγ agonism during forced abstinence prevented a decrease in a nuclear pERK/PPARγ protein complex after a cocaine cue reactivity test in the hippocampus and medial prefrontal cortex demonstrating a direct molecular mechanism for PPARγ agonism action on pERK dependent plasticity. Our results demonstrate that PPARγ agonism attenuated cocaine seeking through a pERK dependent mechanism which prevented dysregulation of basic reward and memory circuitry that is known to become altered with drug taking, abstinence, and cue reactivity.

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List of Abbreviations

ACC Anterior Cingulate Cortex

Amg Amygdala

BBB Blood Brain Barrier

CPP	Cocaine conditioned place preference
ERK	Extra Cellular Signal Related Kinase
FA	Forced Abstinence
FR	Fixed ratio
HIP	Hippocampus
ICV	Intracerebroventricular
IP	Intraperitoneal
OFC	Orbitofrontal Cortex
pERK	phosphorylated Extra Cellular Signal Related Kinase
PFC	Prefrontal Cortex
PIO	Pioglitazone
PP2A	Protein Phosphatase 2 A
PPARγ	Perioxisome Proliferator Activated Receptor Gamma
PPRE	Perioxisome proliferator response element
RSG	Rosiglitazone
RXR	Retinoid X Receptor
SA	Self-Administration
STEP	Striatal-enriched protein tyrosine phosphatase
TZD	Thiazolidinedione
VTA	Ventral Tegmental Area

Chapter 1: Introduction to addiction

Psychostimulant abuse and dependence (addiction) is a chronic, relapsing brain disorder. However, only 8.5% of those who needed treatment for drug abuse and addiction received care; cost and inaccessibility were cited as primary barriers (SAMHSA, 2009). In order to bridge this gap, cheaper and more effective therapeutics must be developed, particularly for relapse during abstinence.

In the last century, new discoveries in addiction science have begun to put to rest misconceptions concerning the nature of addiction. Addiction is now recognized as a disordered integration of cognitive and motivational aspects of reward-directed behavior involving higher order limbic cortico-striatal structures (Kalivas and Volkow, 2005). These brain circuits involving the hippocampus (HIP), prefrontal cortex (PFC), nucleus accumbens (NAc), orbitofrontal cortex (OFC), and amygdala (Amg) are predominantly sensitive to plasticity incurred due to repeated pairing of environmental stimuli (e.g., drug paraphernalia, *etc*.) with exposure to abused drugs. These associations between environmental cues and drug-taking are essential drug-associated memories that can trigger conditioned emotional responses in addicts and “craving” (desire for drug).

These responses are often cited as strong motivating factors to explain relapse to drug-seeking and drug-taking during abstinence and it is repeated relapse that serves as a major determinant of the chronicity and severity of stimulant addiction (Volkow et al., 2009).

Addiction produces profound changes in brain function and plasticity that can be long lasting and is thought to lead to the high rate of recidivism seen in addicts. In the next sections we will discuss in detail a few of the brain regions known to be affected in addiction including those of the frontal cortices and limbic brain regions. Together these brain regions are all part of the mesocortical limbic circuit.

The frontal cortex includes several distinct regions known to have unique properties in addiction such as the PFC, anterior cingulate cortex (ACC) and the OFC to name a few. The frontal cortices are thought to regulate the motivational salience of reward and correlate with the intensity of responding for said reward (Jentsch *et al*., 1999). The ACC and OFC are highly involved with reward predictability and their recruitment to excitatory stimuli such as drug reward is highly dependent on the predictability of the drug reward itself (Berns, *et al.,* 2001). Positron emission tomography (PET) studies have shown that giving a cocaine addict small doses of methylphenidate will increase dopaminergic output in the OFC compared to placebo (Volkow *et al*., 2005). In addition to this increase in dopaminergic activity one can also see an increase in metabolic activity within the ACC and OFC and this increase in metabolic activity positively correlates with an addicts self-report of drug craving, (Volkow *et al*., 1999; Wexler *et al.,* 2001). It has also been noted that ACC and OFC are inhibited in addicts when using experimental paradigms that involve decision

making (Kaufman *et al.,* 2003). It should be noted that this increase in metabolic activity is only seen during drug craving and that addicts as well as animal subjects tend to have a decrease in PFC activity when measured at basal levels compared to control subjects (Jentsch *et al*., 1999; Goldstein *et al.,* 2002). Studies have implicated hypoactivity in the PFC of human cocaine addicts (Franklin *et al.* 2002; Matochik *et al.,* 2003). In fact withdrawal from repeated administration of psychostimulants has been shown to cause dymorphisms in the dendritic tree of pyramidal cells, the main dopaminergic output neurons for the PFC (Robinson *et al.,* 2001). Thus a weakening of PFC output to other brain regions is noted with prolonged FA from psychostimulants. The PFC contains significant interconnections with the limbic system including the Amg and HIP. This implies that drug addiction has a profound effect on the basis of emotional and cued learning as well as higher order executive functions.

The limbic brain regions underlie basic emotional learning and reward driven motivational behaviors. Limbic brain regions include areas such as the Amg, NAc, HIP, and ventral tegmental area (VTA). The Amg is highly involved in emotional learning and has been shown to be heavily involved in fear motivated behavior (Kluver and Bucy, 1939). The Amg is a critical brain region for establishing neutral stimuli as predictors of motivationally relevant events such as access to drug reward (Everitt *et al.,* 2003). However PET scan studies among human addicts have failed to show a correlation between self-reports of drug craving and Amg activity (Breiter *et al.,* 1997; Kilts *et al.,* 2004). The Amg is highly connected to the NAc and PFC via glutamatergic interconnections as well as dopamine efferents (Cardinal *et al.,* 2002). The Amg consists primarily of three distinct regions, the basolateral Amg, the central nucleus of

the Amg, and the extended Amg. The extended Amg is a cluster of interconnected neurons that together with the stria terminalis and central Amg form an outlet for environmental stressors (Shalev *et al.,* 2002). The extended Amg has also been shown to be involved in stress-induced reinstatement of drug seeking behavior whereas the basolateral Amg is not (Shaham *et al.,* 2000). Instead, the basolateral Amg has been shown to be involved in cue-induced as well as context-driven reinstatement of drug seeking behavior (Meil WM and See RE, 1997; Fuchs *et al*., 2005). The basolateral Amg and PFC are connected via glutamatergic efferents and have been shown to influence complex behaviors (Cardinal *et al.*, 2002). The functional integration of the basolateral Amg and frontal cortex regions such as the PFC has been demonstrated in healthy subjects via PET scan when exposed to cues meant to elicit a motivational behavior (Morris *et al.*, 2001; Rilling *et al.*, 2002). These studies demonstrate the role for the Amg in the establishment of learned associations between drug reward and previously neutral environmental cues.

The NAc has been shown to be involved in reward motivated behavior (Olds and Milner, 1954). The NAc serves as a convergence point for excitatory afferents from several brain regions including the HIP and mPFC. These brain regions are thought to send distinct information to the NAc during drug reward situations (MacAskill *et al.,* 2012; Papp et al., 2012). Specifically the HIP is involved in establishing contextual cues associated with prior drug taking increasing the reinforcing properties of cocaine and the mPFC is thought to send action-outcome information to the NAc (Robbins *et al*., 1996; Fuchs *et al.,* 2006; Berridge *et al.,* 2013). This neurocircuitry of the mPFC, HIP, and NAc is vital for key features of addiction such as drug craving and relapse following the

presentation of drug-associated cues after prolonged withdrawal periods (Everitt *et al.,* 2005; Kalivas *et al.,* 2005). The NAc contains two sub-regions which are morphologically and functionally distinct known as the shell and core, (Kelley *et al*., 2004). The shell has dopaminergic input from the VTA and is thought to underlie the establishment of the associations between motivational events and the expression of learned behaviors (Di Ciano *et al.,* 2000; Kelley *et al*., 2004). After learning the brain regions involved in the mesocortical limbic circuit and their functional connectivity to one another we will now discuss how these brain regions are involved in the multistage disease known as addiction.

Addiction is a multi-stage disease in which casual use of a drug escalates, and withdrawal leads to a negative emotional state which leads to drug craving and ultimately addiction (Koob and Volkow, 2010). During these three stages of addiction several brain regions are activated and lead to a decrease in natural reward stimuli as well as an increase in craving for the abused drug. Cocaine blocks the re-uptake of serotonin, norepinephrine, and dopamine (Koe, 1976). Prolonged dopamine release leads to changes in synaptic plasticity of the mesolimbic dopamine system (Wolf *et al*., 2002). Dopaminergic cell bodies in the VTA have been shown to project to the NAc, PFC, and Amg (Ungerstedt *et al.,* 1971; Oades *et al.,* 1987; Fallon *et al.,* 1988). Serotonin has been shown to modulate the cortico-limbic dopamine system and is involved in cocaine-induced hyperlocomotion (Filip *et al.,* 2004). In addition to dopaminergic projections from limbic to cortical brain regions, glutamatergic afferents project from the PFC and HIP to the NAc, VTA, and Amg (Tzschentke*.,* 2001). The Amg has also been shown to send glutamatergic input into the NAc (Kalivas, 2004).

These plasticity changes cause a shift that leads to increased habitual drug taking and continual relapse to drug in spite of negative consequences (Everitt and Wolf, 2002; Hyman *et al*., 2006). This continual relapsing of drug use even after long periods of abstinence defines drug addiction and may happen long after subjects have experienced withdrawal symptoms (Langleben *et al*., 2008).

Many brain regions are involved in the acute rewarding effects of cocaine before it transitions into habitual use. For example it has been demonstrated that lesions of the mesocortical limbic dopamine system blunt the rewarding effects of cocaine (McGregor and Roberts, 1993). Of the brain regions involved, the NAc and Amg have been found to be of increasing importance in the transition from casual to habitual drug use. Lesions of the NAc and basolateral Amg have been found to block the acquisition of cocaine self-administration (Whitelaw *et al*., 1996). The NAc receives projections from higher cortical areas such as the HIP, PFC, and Amg. Thus the NAc is an important relay for the conversion of limbic action (Amg and HIP) executive function (PFC), and declarative memory (HIP). Human imaging studies have shown that the rewarding effects of cocaine taking are closely associated with the NAc (Volkow *et al*., 1996). It has also been noted that behavioral sensitization (a measure of increased psychostimulant motor excitation in animals) has led to continued potentiation of excitatory NAc synapses (Kourrich *et al*., 2007).

The withdrawal phase of cocaine addiction involves an overall decrease in D2 dopamine receptors in several brain regions and is associated with fatigue and depression in humans (Barr and Phillips, 1999). Acute withdrawal has been shown to cause a decrease in mesolimbic dopamine in nearly all animal studies (Rossetti *et al*.,

1992; Weiss *et al*., 1996). In animal models withdrawal symptoms include decreased motor excitation and decreased motivation to seek natural (nondrug) rewards (Pulvirenti and Koob, 1993). These decreases in mesolimbic dopamine may underlie human addicts self-reports of amotivation and anhedonia during withdrawal (Volkow *et al*., 1997; Martinez, 2004).

Another common feature of the withdrawal stage of addiction is an increase in anxiety like behavior. Brain imaging studies in humans have found an overall decrease in endogenous brain opioids during cocaine withdrawal which is thought to underlie at least in part the irritability and anxiolytic behavior in humans (Zubieta *et al*., 1996). It has been shown that cocaine abstinent rats have increased anxiolytic behavior as measured by the elevated plus maze and that this behavior is associated with increased Amg activity (Koob *et al*., 2008).

The craving stage of addiction has been difficult to clinically define in humans and does not necessarily correlate with likelihood of relapse (Tiffany *et al*., 2000). Regardless, the craving stage of cocaine addiction, defined as the incubation of cocaine craving in animal models, (Grimm *et al.,* 2001) certainly remains the best clinical target for the prevention of cocaine relapse in humans. During the craving stage of addiction it is noted that neural connections between higher cortical structures such as the PFC and limbic structures (Amg, NAc) tend to weaken. This has led to the hypothesis that increased drug craving is associated with a loss of executive function or control. Brain lesion studies have shown that the PFC is vital for proper emotion-related decision making (Clark *et al*., 2008). Additionally, it has been shown that cue-induced cocaine reinstatement in animal studies involves the basolateral Amg (Everitt and Wolf, 2002)

and that this involvement of the basolateral Amg may include a feed forward projection to the PFC (Weiss *et al*., 2001). Human imaging studies have also demonstrated that the PFC and basolateral Amg are vital for cue and drug induced craving (Franklin *et al.,* 2007).

Addictive drugs have been shown to disrupt neurogenesis in the HIP (Canales *et al*., 2007). The HIP is also involved in the craving stage of addiction by the recognition of contextually related cues that have been previously associated with drug taking. Both the HIP, associating drug taking context, and the basolateral Amg, associating drug cue, send glutamatergic projections to the NAc core which is thought to lead to the increased reinforcing properties of cocaine as well as other psychostimulants. In fact, human imaging studies have shown that cue-induced craving activates both the Amg and HIP (Volkow *et al.,* 2004). Damage to the HIP has been shown to affect cocaine self-administration in rats (Caine *et al.,* 2001). Human cocaine addicts often score poorly on behavioral tasks that involve spatial and verbal recognition; behavioral tasks known to be mediated by the HIP. Human cocaine addicts also tend to perform poorly on tasks involving attention and cognitive flexibility, tasks known to involve the PFC and OFC. Taken together, poor outcomes on the described cognitive behavior tasks have been shown to positively correlate with relapse and demonstrate a fundamental loss of executive function (Aharonovich *et al.,* 2006; Bolla *et al.,* 2003). With these studies in mind, it has been postulated that preventing a loss of executive function in addicts may help maintain abstinence from psychostimulants and thus prevent drug relapse. It has been demonstrated that over time, environmental contexts and discrete stimuli often become associated with cocaine use leading to conditioned responses (cocaine cue

reactivity) that have been shown to predict relapse and treatment success (Rohsenow *el al.,* 1990; Carter and Tiffany 1999; janes *et al.,* 2010). Therefore utilizing a model in which rats self-administer cocaine then undergo a forced abstinence period when pharmacotherapies could be applied would be clinically relevant. Using a cocaine cue reactivity model we hypothesized that PPARy agonism administered during forced abstinence would attenaute cocaine cue reactivity. The results presented within this dissertation demonstrate that PPARγ agonism significantly attenuated cocaine cue reactivity following a forced abstinence period and that PPARγ represents a novel therapeutic target for cocaine addiction.

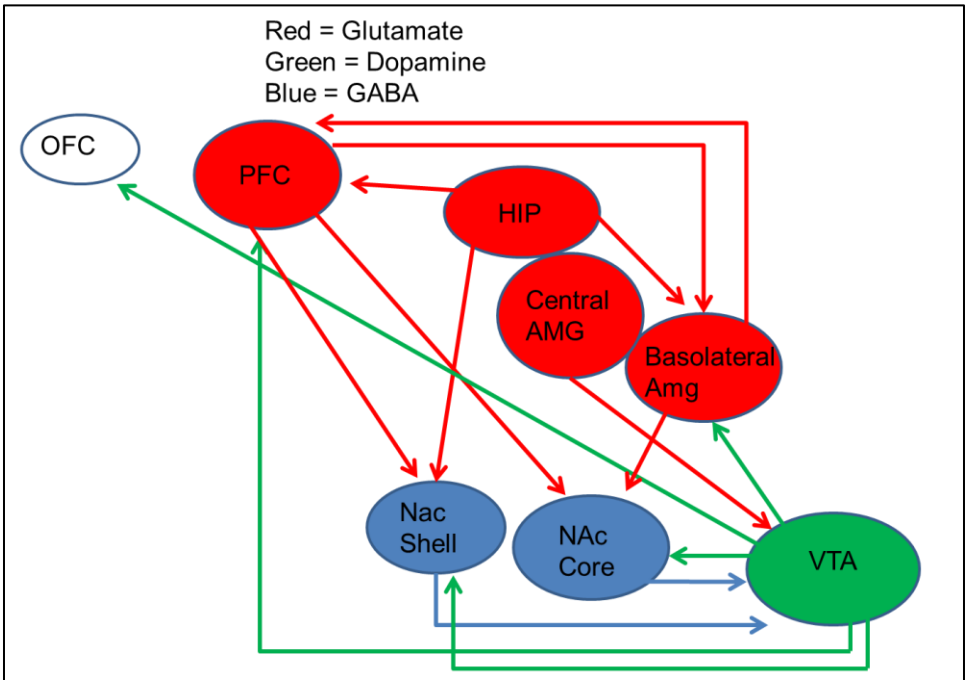

Figure 1.1 Schematic representation of the mesocortical limbic circuit. The frontal cortices (OFC, PFC) and limbic brain regions (HIP, Amg, NAc, and VTA) are highly interconnected and form the mesocortical limbic circuit. These circuit is known to be "hijacked" duirng addiction leading to increased drug seeking and relapse during forced abstinence.

Chapter 2: Oral delivery of Thiazolidinediones increased CNS PPARγ-DNA binding with no peripheral side effects

Introduction

PPARγ is a ligand-activated transcription factor belonging to the nuclear hormone receptor superfamily. There are three members of the PPAR receptor family including α, β and γ; each of which is differentially expressed in the periphery (Desvergne and Wahll, 1999). PPARα is expressed in adipocytes, heart, and kidney whereas PPARβ is primarily expressed in the placenta and large intestine. However all three isoforms are expressed to varying degrees in the CNS by both neurons and glia. All three of these nuclear hormone super receptors heterodimerize with the PPAR binding partner Retinoid X Receptor (RXR) in order to bind to specific target genes. In the CNS, RXR is expressed by neurons and glia (Schrage *et al.,* 2006). Interestingly upon CNS insult RXR subcellular location switches primarily from the cytoplasm to the nucleus which indicates transcriptional activation of RXR heterodimers during times of cellular stress/injury (Schrage *et al.,* 2006).

PPARγ is a highly conserved protein with a 99% similarity between human and mouse isoforms (Fajas *et al.,* 1997). The human genome of PPARγ can give rise to four different mRNAs of PPARγ; however, due to differential splicing only two isoforms PPARy1 and PPARy2 are expressed.

PPARγ is known to act as a master gene of insulin receptor sensitivity as well as play a large role in adipogenesis and lipid uptake (Lehrke and Lazar, 2005). PPARγ is also expressed abundantly in the immune system specifically within macrophages and lymphocytes as well as the vascular system (smooth muscle cells). In these systems, as well as the CNS, PPARγ is thought to have an anti-inflammatory role (Daynes and Jones, 2002; Kaundal *et al.,* 2011). In fact activation of both PPARγ and PPARα has been shown to attenuate the inflammatory response of astrocytes that were previously treated with amyloid beta (Benito *et al.,* 2012).

Like all members of the nuclear hormone receptor superfamily PPARγ is composed of four structural domains: A/B, C, D, and E/F (Fajas, *et al.,* 1997). The A/B domain has a ligand binding independent AF-1 domain. The C domain is one of three PPARγ domains necessary for PPARγ heterodimerization with RXR (Chandra *et al.,* 2008). The C region also contains two cysteine rich zinc fingers responsible for the DNA binding of PPARγ to peroxisome proliferator response elements (PPREs) found in the promoter region of target genes of PPARγ transcription factor activity. PPREs are a hexanucleotide repeat (AGGTCA) which bind PPARγ and RXR to PPARγ target genes. Both PPARγ and RXR bind to a single PPRE resulting in a polar arrangement of their prospective binding domains in which PPARγ is upstream of RXR (Chandra *et al.,* 2008). The D region of PPARγ is thought to affect DNA binding specificity and also

contains a nuclear localization sequence (Zielenlak, *et al.,* 2008). The E/F region of PPARγ contains the ligand binding domain (AF2) upon which synthetic PPARγ agonists known as the Thiazolidinediones (TZD's) act. This region is also thought to enhance PPARγ-DNA binding by interacting with the DNA binding domain of both PPARγ and RXR thus stabilizing the heterodimer (Chandra *et al.,* 2008).

Endogenous ligands for PPARγ activation include the eicosanoids and poly-unsatured fatty acids (Camp and Tafuri 1997). Synthetic compounds known as the TZD's bind PPARγ with a very high affinity, (Lehman *et al.,* 1995). These compounds include rosiglitazone (Avandia), pioglitazone (Actos), and troglitazone (Rezulin). TZD's serve as highly selective PPARγ agonists and are used clinically for the treatment of type 2 diabetes.

Pioglitazone (PIO) and rosiglitazone (RSG) represent the most commonly used PPARγ agonists and GW 9662 is a specific and irreversible antagonist of PPARγ. Of the TZD's, both PIO and RSG have been shown to be efficacious for the treatment of hyperinsulinemia, hyperlipidemia and hyperglycemia that underlie inflammatory and metabolic symptoms of type 2 diabetes. PIO and RSG are bioavailable, and readily cross the blood brain barrier (Strum *et al.,* 2007). Using these drugs, numerous laboratories are exploring the potential of PPAR receptor activation as a method to treat various disease states of the CNS including neurodegeneration, demyelination, and inflammation. Although RSG and PIO are FDA approved they are known clinically known to have side effects such as edema, or weight gain. Therefore validation that the dosage of TZD's chosen for these experiments would not induce any noticeable side effects was required. Validation that orally delivered TZD's when laced into standard

rodent chow could penetrate the blood brain barrier (BBB) thus eliminating the need for repeated intracerebroventricular (ICV) injection was also required. It was hypothesized that the PPARγ agonists RSG and PIO, when laced into standard rodent chow at 30mg/kg, would be a sufficient dose to penetrate the BBB of rats' and induce activation of PPARγ, It was also hypothesized that RSG and PIO laced feed would not effect rats' weight or feed consumption.

Materials and Methods

Assessment of orally delivered RSG.

Rats were divided into two groups and fed either standard rodent chow (untreated) or chow laced with 30mg/kg RSG as described previously (Rodriguez-Rivera *et al.,* 2011; Denner *et al.,* 2012; Jahrling *et al.,* 2014). Rats were fed these perspective diets for a period of one, two, four, six, or eight days and were then sacked and whole brain homogenate prepared for the PPARγ-DNA binding assay.

DNA binding assay

PPARγ DNA binding was performed using the Trans AM® ELISA kit (Active Motif). Approximately 8 µg of nuclear extract was added to each well of a 96-well plate in which a DNA oligonucleotide representing the peroxisome proliferator response element (PPRE) had been immobilized. After 1hr of incubation wells were washed using the provided buffer and then incubated with a PPARγ antibody provided with the assay (dilution 1:1,000) for 1hr at room temperature. After three washes wells were

incubated for 1hr with diluted horseradish developing solution. After a 10 min incubation period, the reaction was stopped by the addition of 100 µL stop solution (provided in the kit), and absorbance (450nm) was read on a FlexStation microplate reader to determine PPARγ-DNA binding.

Assessment of consumption rates and weight gain with PIO laced feed

PIO was milled into standard rodent chow at a dosage of 30 mg/kg (Bio-Serv) as previously described (Rodriguez-Rivera *et al.,* 2011; Denner *et al.,* 2012; Jahrling *et al.,* 2014). Rats were divided equally into groups fed either standard rodent chow (control) or standard rodent chow supplemented with 30mg/kg PIO for a period of 30 days. Rat food intake and body weights were monitored daily during the 30 day period.

Results

An orally delivered Thiazolidinedione increased CNS PPARγ-DNA binding in the rat brain

To determine if orally delivered RSG effects CNS PPARγ activity, rats were provided either RSG laced feed or control diet (Sham). RSG treatment was administered by supplementation of rat chow with 30 mg RSG per kg standard rat chow and provided *ad libidum* as previously described (Rodriguez-Rivera *et al.,* 2011; Denner *et al.,* 2012; Jahrling *et al.,* 2014). Rats were sacrificed at pre-designated time points of 1, 2, 4, 6, and 8 days. Brains were removed, homogenated, and nuclear extracts prepared at the designated time points. An ELISA-based DNA binding assay was used to determine PPARγ-DNA binding to a PPRE oligonucleotide (Active Motiff). PPARγ-transfected COS-7 cells (positive control) and blank wells that received no nuclear

extract served as controls. **Figure 2.1** demonstrates that orally-delivered RSG maximizes PPARγ-DNA binding by 4 days in the rat brain two sided t test (P≤0.05). This indicates that PPARγ agonists such as RSG penetrate the BBB and exert an effect on PPARγ in this case increased PPARγ-DNA binding.

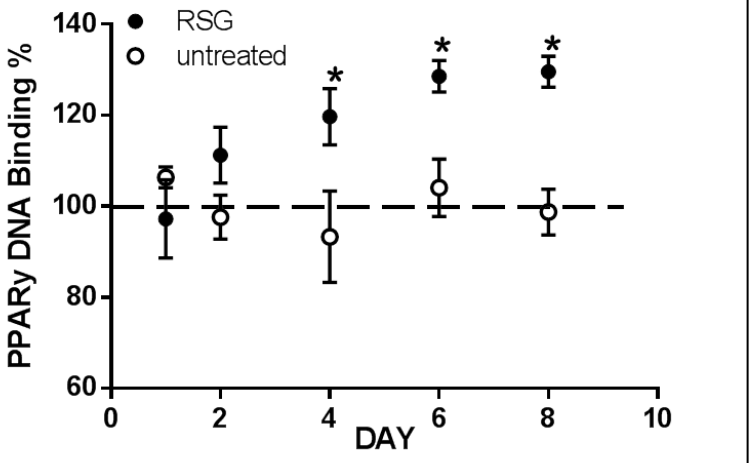

Figure 2.1 Orally delivered RSG increased CNS PPARγ DNA binding activity. Sprague-Dawley rats were fed regular rodent chow or chow supplemented with 30mg RSG per kg chow. PPARγ DNA binding is significantly increased in rat brain by four days treatment. * Indicates p< 0.05 two sided t test.

Orally delivered Pioglitazone did not cause an increase in rat weight

Pioglitazone was laced into rodent feed to simulate a human oral dose (PIO at 30mg/kg of feed) Treatment with PIO started immediately after cocaine self-administration and continued for a period of thirty days. During PIO treatment rats were weighed and handled daily. After 30 days rats eating Pioglitazone laced feed did not weigh significantly more than control two way ANOVA $F(3,24)=.8833$, $P=.4637$ **Fig 2.2**. This indicates that feed laced with PIO at 30mg/kg did not induce significant weight gain

relative to standard rat chow. Validation that rat chow laced with PIO was not causing a disruption in consumption behavior was then measured.

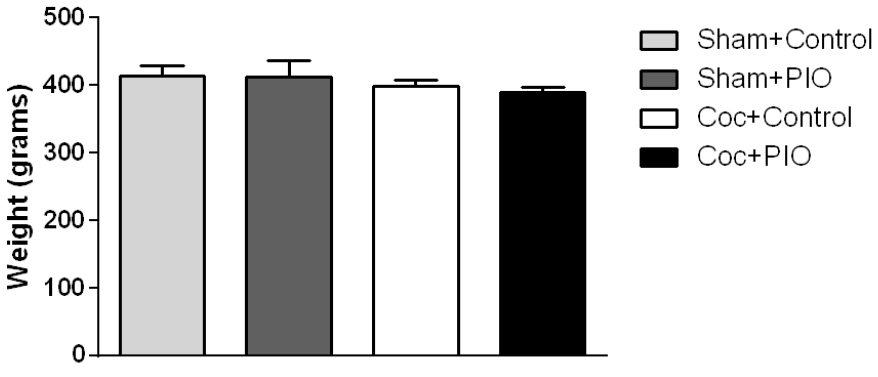

Figure 2.2 Pioglitazone did not increase rat weight. PIO treatment (30mg/kg in feed) was given to rats for a period of thirty days. During PIO treatment rats were weighed and handled daily. Rats consuming PIO laced feed were not significantly heavier than those eating control diet two way ANOVA (F (3,24) =.8833, P=.4637).

Pioglitazone addition to standard rat chow did not effect feed consumption in rats

Pioglitazone was laced into rodent feed to simulate a human oral dose (PIO at 30mg/kg of feed) Treatment with PIO started immediately after cocaine self-administration and continued for a period of thirty days. During PIO treatment feed was weighed daily to determine consumption rates. Repeated Measures One Way ANOVA reveled that PIO did not affect feed consumption F (3, 116) =3649, P=.7785 **Fig 2.3**. This indicates that Feed laced with PIO and 30mg/kg did not effect consumption rates and rats given PIO were therefore not food deprived.

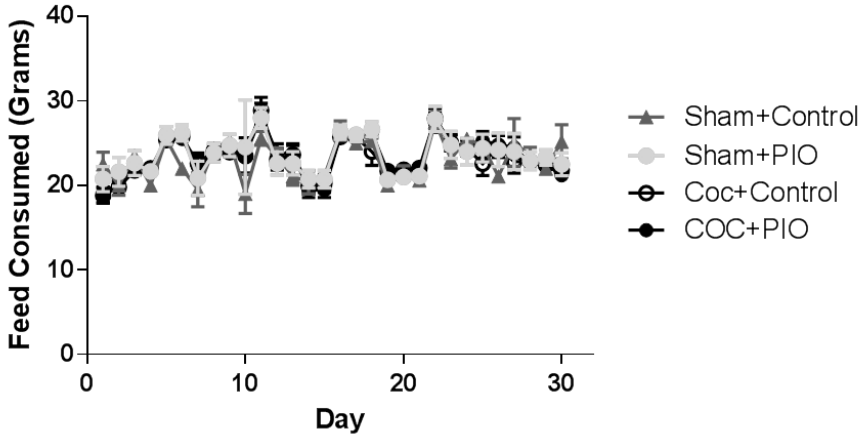

Figure 2.3 Pioglitazone did not effect consumption. PIO treatment (30mg/kg in feed) was given to rats for a period of thirty days. During PIO treatment rat feed was weighed daily to determine consumption. Repeated Measures One Way ANOVA reveled that PIO did not affect feed consumption $F(3,116)=.3649$, $P=.7785$.

Discussion

Here we show that standard rodent chow, when laced with PPARγ agonists PIO and RSG, penetrated the BBB and caused an increase in CNS PPARγ-DNA binding as measured by ELISSA, (One way ANOVA $p \leq 0.05$) (**Fig 2.1)**. These results are in agreement with previous findings showing that the TZD's can penetrate the blood brain barrier (Strum, 2007).

A clinical side effect of TZD's is a general increase in weight or edema. Knowing this we wanted to determine if our dosage of PIO would cause an increase in rat weight when administered for a period of thirty days. We also wanted to know if PIO addition to rodent chow would result in a decrease in feed consumption, thus affecting our rats general health via food deprivation. Thus PPARγ agonism when introduced into the

feed has no effect on basic consumption rates or overall weight of our rats at the dosage of 30mg/kg of standard rodent chow.

To determine the approximate dosage of PIO for human studies, we can calculate an approximation based on our rats average daily consumption of PIO laced feed (approximately twenty-three grams) utilizing the body surface area calculation (BSACU). Taking into account that rats consume an average of 0.69mg of PIO (30mg/kg PIO feed multiplied by 0.023kg feed daily = 0.69mg) daily we can determine that the human equivalent dosage would be approximately 64mg of PIO per day. Typical human dosage of PIO is between 30 or 45mg daily, meaning our model system is very close to the typical dosage for a 70kg human.

Knowing that PPARγ agonism, when laced in feed, penetrated the BBB and that PPARγ agonism does not affect overall consumption or health of the rats as measured by weight; It was decided that PPARγ agonists laced into the feed was an acceptable mode of drug delivery for the continuation of these studies.

Chapter 3: PPARγ agonism blocked the expression of cocaine-induced locomotor sensitization

Introduction

Having established that PPARγ agonist laced feed targeted CNS PPARγ, we needed to determine if PPARγ agonism had any effect on maladaptive behaviors that are known to be associated with cocaine. Sensitization is a non-associative learning process in which repeated administration of a stimulus results in an amplified response over time. All psychostimulants cause an increase in locomotion over time which is evident even after a withdrawal period. This phenomenon is known as locomotor sensitization. Locomotor sensitization is thought to at least in part involve associative learning. For example, studies have shown the effect of increased locomotor activity with psychostimulants to be increased when paired to a dedicated test chamber rather than a novel test environment (Badiani *et al.,* 1995). Several studies have shown this context dependency to be nearly complete suggesting that repeated administration of a drug in a dedicated test environment may be necessary to induce locomotor sensitization suggesting overlapping neural networks between locomotor sensitization

and learned reward (Anagnostaras and Robinson, 1996; Tirelli and Terry, 1998). A recent study has demonstrated that the expression of locomotor sensitization to methamphetamine was blocked by daily repeated ICV injection of the PPARγ agonists rosiglitazone (RSG) and pioglitazone (PIO) during a 7-day withdrawal period. The behavioral effects of PPARγ agonist administration was blocked by PPARγ antagonism with GW-9662 (Maeda *et al.* 2007). Since locomotor sensitization is a maladaptive behavior of psychostimulant abuse, and since previous studies have demonstrated that PPARγ agonism with PIO or RSG could block the expression of methamphetamine induced locomotor sensitization, we decided to test the hypothesis that PPARγ agonism with TZD's would prevent the expression of cocaine induced locomotor sensitization.

Materials and Methods

Locomotor activity

Apparatus

Locomotor activity was monitored and quantified under low light conditions using a modified open field system (San Diego Instruments, CA, USA). Clear Plexiglas chambers (40x40x40cm) were surrounded by a 4x4 photobeam matrix positioned 4cm from the floor of the chamber. Consecutive beam breaks within the central 16x16cm of the box were recorded as central ambulation. Peripheral beam breaks in the surrounding perimeter were recorded as peripheral ambulations. Central and Peripheral beam breaks were summed up to give a measure of total ambulation. Vertical activity was also recorded using a row of 16 photobeams positioned approximately 16cm above the chamber. Beam breaks in these beams were recorded as vertical activity.

Procedure

Rats were divided into RSG-treatment or control diet (Sham-treatment) groups. RSG treatment was started four days prior to locomotor activity assessment and continued throughout the entirety of the experiment. Each group was habituated for one hour daily in the locomotor chamber prior to IP injection of cocaine (15mg/kg) or saline (1ml/kg) yielding four experimental groups (RSG+Cocaine, RSG+Saline, Sham+Cocaine, Sham+Saline). After IP injection, rats were quickly returned to locomotor chambers for a period of one hour where locomotor activity was assessed. This process was repeated once a day for a period of five days. All groups then underwent a withdrawal period of 7 days in which they remained in their home cages but were handled and weighed daily. After seven days of withdrawal all groups were habituated to locomotor chambers for one hour prior to a cocaine challenge of 15mg/kg that was administered to all groups. This same procedure was repeated with the PPARγ agonist PIO.

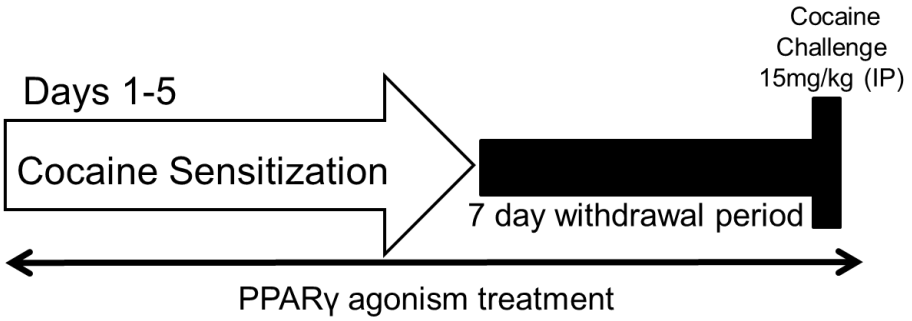

Figure 3.1 Timeline for cocaine-induced locomotor sensitization. Rats were divided into PPARγ agonist treatment or control diet (Sham-treatment) groups. PPARγ agonism was started four days prior to locomotor activity assessment and continued throughout the entirety of the experiment. On days 1-5 IP injections of cocaine (15mg/kg) or saline (1ml/kg) yielded four experimental groups (RSG+Cocaine, RSG+Saline, Sham+Cocaine, Sham+Saline). All groups underwent a 7 day withdrawal period in which they remained in their home cages but were handled and weighed daily. After 7 days of withdrawal all groups were administered a challenge dose of cocaine 15mg/kg (IP).

Results

PPARγ agonism attenuated the expression of locomotor activity

Before beginning cocaine self-administration experiments, we wanted to see if PPARγ agonism had any effect on maladaptive behaviors that are known to be associated with cocaine. Therefore we decided to perform a locomotor sensitization experiment. All psychostimulants cause an increase in locomotion over time with repeated injection which is evident even after a short withdrawal period. This phenomenon is known as locomotor sensitization. Rats were divided into RSG-treatment or control diet (Sham-treatment) groups. RSG treatment was started four days prior to locomotor activity assessment and continued throughout the entirety of the experiment. Each group was habituated for one hour daily in the locomotor chamber prior to IP injection of cocaine (15mg/kg) or saline (1ml/kg) yielding four experimental groups (RSG+Cocaine, RSG+Saline, Control+Cocaine, Control+Saline). After IP injection, rats were quickly returned to locomotor chambers for a period of one hour where locomotor activity was assessed. This process was repeated once a day for a period of five days (**Fig. 3a**). All groups subsequently underwent a withdrawal period of 7 days in which they remained in their home cages but were handled and weighed daily. After seven days of withdrawal all groups were habituated to locomotor chambers for one hour prior to a cocaine challenge of 15mg/kg, administered to all groups. Upon cocaine challenge the RSG+Cocaine group had similar locomotor activity compared to control groups (Control+Saline and RSG+Saline) that had never received cocaine. Furthermore, tukey multiple comparison analysis revealed that the Control+Cocaine rats exhibited significantly greater locomotor activity compared to all remaining groups (one-

way ANOVA F (8, 16) =4.34 P=0.001) (**Fig. 3.2c**). These results implicate that PPARγ agonism blocked the expression of cocaine locomotor sensitization indicating that PPARγ agonism prevented an increase in locomotor activity associated with psychostimulants. It should be noted that rats administered both cocaine and RSG had an increased locomotor activity level compared to rats administered saline (Two way ANOVA F (8, 16) =2.03 P=0.031) (**Fig 3.2a**). These data indicate that rats treated with RSG experienced the acute locomotor effects of cocaine administration without developing locomotor sensitization. PIO, another potent selective PPARγ agonist, was used in a separate locomotor sensitization experiment and found to be just as efficacious (one-way ANOVA; F (8, 16) =64.5, P=0.002) (**Fig 3.2b, d**). Therefore subsequent studies were conducted with PIO.

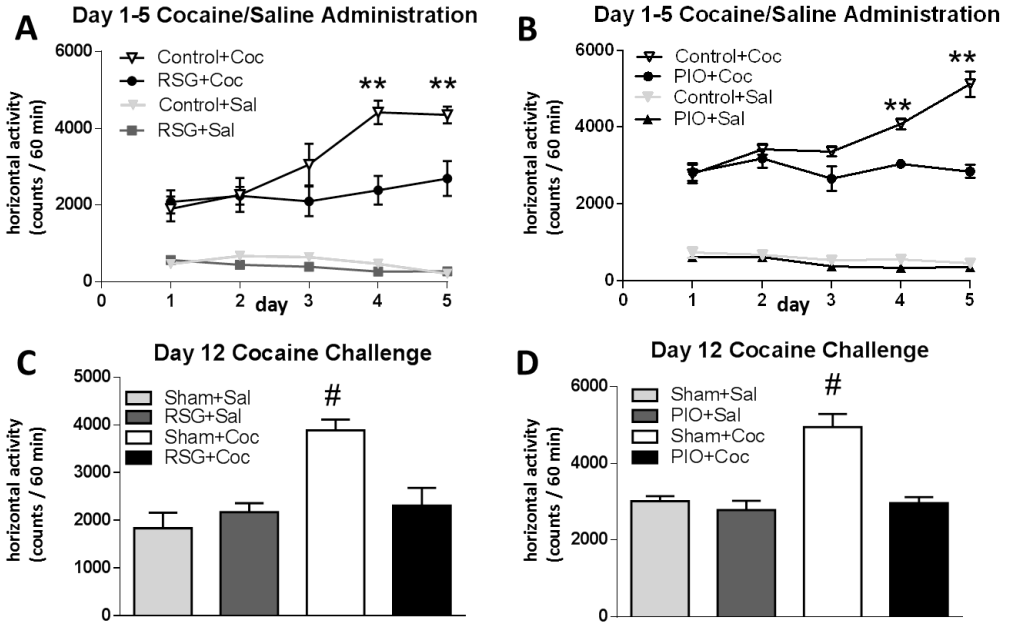

Figure 3.2 PPARγ agonism blocked the expression of cocaine-induced locomotor sensitization. Rats were divided into PPARγ agonist treated (RSG/PIO) or untreated groups. Treatment was started four days prior to locomotor activity assessment and continued throughout the entirety of the experiment. **A, B,** on days 1-5 rats received either a saline or cocaine injection followed by 1hr locomotor activity assessment. ** indicates difference from day 1; two-way repeated measures ANOVA and Bonferonni post hoc analysis $F(4,12)=.84$ $P<0.001$ and $F(4,12)=.6311$, $P<0.001$ for RSG and PIO respectively. On days 6-11, rats went through a withdrawal period. **C, D,** on day 12, all rats received a cocaine challenge injection and locomotor activity assessment. #Indicates difference between untreated cocaine injected and all other groups based upon one-way ANOVA followed by Tukey multiple comparison analysis $F(8,16)=4.34$, $P=0.001$ and $F(8,16)=64.5$, $P=0.002$ for RSG and PIO, respectively.

Discussion

Before beginning cocaine self-administration, we first wanted to see if PPARγ agonism had any effect on maladaptive behaviors that are known to be associated with cocaine. Locomotor sensitization is a behavioral model of psychostimulant induced neuroplasticity in the brain elicited by repeated administration of said psychostimulant. The neuronal network that underlies behavioral sensitization is distributed among limbo-cortical striatal structures and is thought to underlie motivational behavior. We noted that both RSG and PIO blocked the expression of locomotor sensitization and that PPARγ agonism when administered throughout the entirety of the study prevented the

expression of locomotor sensitization (**Fig 3.1 a, c**). This data is similar to other studies which demonstrated that the expression of locomotor sensitization to methamphetamine was alleviated by injection of PPARγ agonists PIO and RSG over the withdrawal period of locomotor sensitization (Maeda *et al.* 2007). Interestingly, the authors in this study did not observe any changes in hyperlocomotion when PPARγ agonists were administered concurrently with repeated methamphetamine injection during locomotor sensitization. This difference in our data could be reflected by the author's choice of animal model (mouse) or their method of TZD delivery (ICV). Our method of drug delivery has been shown to induce a steady state of PPARγ activation as measured by CNS PPARγ-DNA binding. This steady state is unlikely with once daily ICV injections. With the knowledge that PPARγ agonism laced into rodent chow could prevent the expression of locomotor sensitization to cocaine, the next logical step was to utilize cocaine self-administration, a more clinically relevant model of cocaine addiction (Panlilio *et al.,* 2007).

Chapter 4: PPARγ agonism attenuated cocaine seeking behavior via an ERK-MAPK/PPARγ protein complex

Introduction

The rodent model of self-administration (SA) is a form of operant conditioning and to date is one of the most clinically relevant behavioral models of human cocaine addiction. Here, cocaine is administered via an intravenous catheter that has been surgically implanted. Intravenous catheterization is commonly used since it greatly increases the bioavailability of the drug and has rapid onset. In regards to clinical relevance, humans suffering from addiction often begin to use intravenous drugs for similar reasons. Therefore, this route of administration is thought to increase the face validity of the rat self-administration paradigm (Panlilio *et al.,* 2007). In our model, i.v. cocaine was delivered once rats pressed an active lever that also illuminated a discrete cue light. This light was used to help stimulate associative drug taking memories similar to that of a human drug abuser with drug paraphernalia. Over time, environmental contexts and discrete stimuli (the cue light) become associated with cocaine use leading to conditioned responses (cocaine cue reactivity) that have been shown to predict

relapse and treatment success (Rohsenow *el al.,* 1990; Carter and Tiffany 1999; janes *et al.,* 2010).

Our model utilizes a fixed ratio (FR) schedule of drug reinforcement. The fixed ratio model requires a predefined number of operant responses (active lever presses) to dispense a single cocaine dosage. Male Sprague-Dawley rats began a reinforcement schedule of one (FR1) and move to a reinforcement schedule of 5 (FR5), requiring 1 and 5 active lever presses, respectively, to dispense a predetermined amount of cocaine. After fourteen days of SA, rats were returned to their home cages for a period of forced abstinence (FA). During this FA period rats were weighed and handled daily to check for any signs of stress or illness but were not taken to the operant SA chambers. In the FA model, rats acquire cocaine self-administration and are then immediately removed from the self-administration situation (operant chamber). This preserves the integrity of the drug-taking behavior with drug-associated cues as rats are not exposed to either in the absence of drug. Increased cocaine seeking following FA relies on the association of drug-associated cues and drug-taking behavior to be intact as comparison studies between cocaine FA and extinction training consistently show that cocaine extinction training attenuates, whereas FA promotes cocaine seeking (Neisewander *et al.,* 2000; Zavala *et al.,* 2007). The FA model has a greater clinical relevance to human populations as opposed to extinction/reinstatement since treatment seeking addicts do not undergo extinction training (Ling, *et al.,* 2006). This means that addicts which may come across drug-associated cues (such as drug paraphernalia) have their prior drug conditioning history and learned association with drug-cues and drug-taking behaviors completely intact. This is vital when considering that addicts

often report increased craving for cocaine during abstinence upon re-exposure to cocaine related cues (Ehrman *et al.,* 1992; Robbins *et al.,* 1997). For these reasons we decided to use a FA model for all cocaine SA studies presented within this dissertation.

After thirty days FA, rats were reintroduced to their original operant SA chambers and allowed to freely lever press for cocaine associated cues. Pressing the previously active cocaine lever triggered the cue light which was previously paired with cocaine delivery causing an increase in active lever pressing compared to rats which had no cocaine experience. This is known as cocaine cue reactivity. Drug cue reactivity is the attentional bias of an animal or human towards drug-associated cues which can be measured by physiological effects (heart rate) or an increase in appetitive approach behaviors (lever pressing for drug-associated cues) (Carter and Tiffany, 1999; Field and Cox, 2008; Buccafusco *et al.,* 2009). Clinical relevancy of cue reactivity has been demonstrated as individuals with a higher magnitude of cue reactivity are known to be at a greater risk for developing addiction and/or subsequent relapse (Mahler *et al.,* 2010; Hendershot *et al.,* 2011). Cue reactivity has also been shown to be an accurate predictor for relapse as well as treatment success in treatment seeking addicts (Rohsenow *et al.,* 1990; Janes *et al.,* 2010). Therefore, we decided to implement a rat model of cocaine cue reactivity for all cocaine SA studies presented within this dissertation. Numerous proteins have been shown to be dysregulated following chronic cocaine SA and FA, of particular interest to the studies presented within this dissertation are the Extracellular Signal Related Kinases, due to their definitive role in plasticity and learning and memory.

Extracellular Signal Related Kinases (ERK1 and ERK2) are 44 and 42kd respectively. ERK is expressed robustly in mature neurons and the ERK MAPK signaling cascade is critical in early nervous system development and differentiation. As stated previously, ERK protein is vital for the long term synaptic changes seen in learning and memory. Associative cue conditioning and various forms of spatial learning are all subject to disruption by inhibiting ERK activation (Sweatt *et al.,* 2003). ERK protein has also been demonstrated to be vital for the plasticity associated with increased drug taking in both locomotor sensitization and the incubation of cocaine craving associated with prolonged cocaine abstinence (Lu *et al.,* 2006; Li *et al.,* 2008). ERK activity in these paradigms is often dynamic and fast. For example, a cocaine conditioned place preference paradigm saw a marked increase in ERK activity in as little as five minutes (Valjent *et al.*, 2004).

ERK1 and ERK2 are highly conserved proteins across all mammalian species. ERK 1 knockout mice are functionally healthy and appear to have no deficits except for a marked decrease in CD4+ and CD8+ T cells indicating a role for ERK1 in thymocyte maturation (Pages *et. al.,* 1999). It has been noted that ERK2 knockout mice are embryonic lethal due to severe deficits in placental vasculature (Yao *et al.,* 2003; Hatano, *et al*., 2003). However through a series of gene ablation studies, it has been demonstrated that it is the gene dosage of ERK which is vital for survival, meaning mice with a single copy of ERK1 and ERK2 or two ERK2 alleles are functionally normal (Lefloch, *et al.,* 2008). Since ERK2 is more abundantly expressed, in most cells, it was concluded that the gene dosage of ERK was what was necessary for survival.

ERK protein is activated via phosphorylation by a number of different receptors albeit traditionally by tyrosine kinase receptors. Here Ras protein, when activated by the autophosphorylated tyrosine kinase receptor dimer, leads to activation of the Raf protein (Schaeffer and Weber, 1999). The Raf kinases only phosphorylate MEK 1 and MEK2 which in turn specifically activate only ERK1 and ERK2 (Ray *et al.,* 1988). To activate ERK1/2 both tyrosine and threonine resides must be phosphorylated.

A number of phosphatases have been known to deactivate ERK1/2. Protein phosphatase 2 A (PP2A) and striatal-enriched protein tyrosine phosphatase (STEP) are the best characterized. PP2A has been shown to induce a rapid deactivation of ERK 1/2 in vitro (Alessi et al., 1995). However PP2A has also been implicated in activating the ERK MAPK pathway via its association with Raf-1 (Abraham *et al.,* 2000). STEP is another potent phosphatase of ERK1/2 and is abundantly expressed in the mesocortical limbic circuit (Boulanger *et al.,* 1995). STEP has been shown to dephosphorylate ERK and prevent its nuclear translocation (Zuniga *et al.,* 1999; Nika *et al.,* 2004). In neuronal cultures, STEP has been shown to reduce NMDA mediated long term potentiation as well as reduce NMDA-induced pERK expression (Pelkey et al., 2002). Phosphorylation of STEP has been shown to deactivate this protein phosphatase (Nika *et al.,* 2004).

It has been noted that ERK protein is an important mediator of neuronal activation, in particular with drugs of abuse. ERK protein has been shown to be involved in all three stages of addiction (acquisition, abstinence, and relapse). In terms of cocaine taking/acquisition of drug abuse, it has been shown that ERK protein is activated with noncontingent, repeated cocaine administration (Miller and Marshall, 2005) as well as with a single administration of cocaine (Fumagelli *et al*., 2009). This

activation of ERK protein has also been shown to be brain region specific. A single injection of cocaine does not activate ERK in the ventral tegmental area (VTA) whereas repeated cocaine injections do (Berhow *et al.,* 1996; Valjent *et al.,* 2000). Cocaine SA has been shown to cause a dysregulation in pERK in both the medial prefrontal cortex (mPFC) and nucleus accumbens (NAc) immediately after cocaine administration cessation and during early withdrawal (Edwards, *et al.,* 2007; Whitfield, *et al*., 2011).

Several studies have found ERK to be necessary for cocaine conditioned place preference (CPP). In this procedure, animals are given an injection of cocaine (unconditioned stimulus) in one of two connected chambers (deemed drug paired and non-paired context) during a training phase. Over time the animal will begin to associate the drug paired context with the rewarding effects of cocaine and therefore spend more time in this area during the testing phase when no cocaine is given. Previous studies have reported that injections of SL327 (a MEK inhibitor and thus a blockade of ERK activation) before the daily training phase of CPP prevented the expression of CPP on test day (Valjent *et al*., 2000). It is important to note that when SL327 was injected alone it did not produce a place aversion leading the authors of this study to conclude that it was the lack of ERK activity that blocked the expression of CPP (Valjent *et al.,* 2000).

ERK protein has also been shown to be necessary for the consolidation and reactivation of cocaine taking memories and produces epigenetic changes during morphine withdrawal (Ciccarelli, *et al*., 2013). ERK activation in the basolateral Amygdala (Amg), but not the NAc is necessary for the reactivation and reconsolidation of cocaine related contextual memories (Wells, *et al.,* 2013). Another study

demonstrated that ERK antagonism in the NAc after daily amphetamine CPP attenuated CPP expression on testing day, presumably due to ERK's role in CPP memory consolidation (Gerdjikov *et al.,* 2004).

Studies have also shown that ERK is involved in the neuroadaptations that take place after withdrawal from chronic cocaine SA. Studies have noted a decrease in ERK activity during abstinence in the mPFC and NAc (Berglind *et al.,* 2007; Whitfield *et al.,* 2011). Increased ERK activity in these brain regions attenuated cocaine seeking indicating that decreased ERK activity in these brain regions is necessary for neuroadaptations that lead to increased cocaine seeking. In a follow up to these studies, it was noted that chronic cocaine SA followed by a short withdrawal period leads to an elevation of activated STEP but not PP2A suggesting that STEP is responsible for the deactivation of ERK seen in previous studies (Sun, *et al.,* 2013).

Other studies have noted a deactivation of STEP which was concurrent with ERK activation following acute cocaine or methamphetamine administration (Valjent *et al.,* 2005). The phosphatase PP2A has also been implicated in restraint stress-induced hyperlocomotion in cocaine sensitized mice (Maeda *et al.,* 2006).

ERK activity has been shown to be necessary for all aspects of drug abuse. Animal studies have shown that dysregulation of this protein in multiple brain regions is vital for the transition of casual drug use into addiction and subsequently that ERK activation in brain regions, such as the central Amg, are vital for memory retrieval of cues previously associated with cocaine. Previous studies have suggested that ERK2 acts as a gateway for dopaminergic-mediated rewarding information and glutamatergic context-mediated information of drug-induced plasticity to converge (Girault *et al.,*

2007). For these reasons pharmacotherapies that rescue or prevent dysregulated ERK activity in the mesocortical limbic system may be critical for the treatment of human addiction.

Through a series of studies, our lab has revealed a compelling interrelationship between pERK and the nuclear receptor and transcription factor, PPARγ, in hippocampus-dependent learning and memory under conditions of dysregulated ERK activity. We demonstrated that PPARγ agonism enhanced cognition in a rodent model of Alzheimer's disease via the induction of both PPARγ- and ERK-dependent transcriptomes and a proteome that alleviated synaptic and network abnormalities (Rodriguez-Rivera *et al.,* 2011; Denner *et al.,* 2012; Jahrling *et al.,* 2014; Nenov *et al.,* 2014). Furthermore, we discovered that pERK and PPARγ participate in a dynamic multiprotein complex to facilitate hippocampus (HIP) memory consolidation. Knowing that there is significant overlap between PPAR and ERK response genes, and that PPARγ agonism rescues cognitive impairment in an animal model of Alzheimer's and that this cognitive rescue involves pERK, we hypothesized that drug induced alterations in ERK, which can lead to increased cocaine seeking, could be attenuated by targeting PPARγ and that PPARγ and pERK are in a multiprotein complex thus providing a direct molecular mechanism for PPARγ action on pERK.

Materials and Methods

Self-administration

Surgery

Rats were anesthetized (i.m.) with a cocktail containing xylazine (8.6 mg/kg), acepromazine (1.5 mg/kg), and ketamine (43mg/kg) in bacteriostatic saline. The

catheter was inserted into the right jugular vein and exited dorsally (Cunningham *et al.,* 2011; Cunningham *et al.,* 2013; Anastasio *et al.,* 2014a; Anastasio *et al.,* 2014b). Daily catheter flushes were performed after each cocaine self-administration session to ensure catheter patency using a solution of 0.1ml bacteriostatic saline and heparin sodium salt (10U/ml.; American Pharmaceutical Partners, East Schaumburg, IL, USA), streptokinase (0.67mg/ml; Sigma Chemical) and ticarcillin disodium (66.67mg/ml; Research Products International, Mt. Prospect, IL, USA). Rats were allowed at least 5 days of recovery before beginning cocaine SA procedure

Operant conditioning chambers

Standard operant conditioning chambers (Med-Associates, Inc., St. Albans, VT, USA) housed in ventilated sound attenuating cubicles with fans (Med-Associates, Inc.) were utilized for cocaine self-administration studies. Each chamber was equipped with two response levers, a stimulus light located above each response lever, and a house light opposite the levers. Cocaine infusions were delivered by a syringe attached to an infusion pump (Med-Associates Inc.) mounted inside the cubicle. The infusion pumps were connected to liquid swivels (Instech, Plymouth Meeting, PA, USA) that were attached to the catheters via polyethylene tubing encased inside a metal spring leash (Plastics One).

Self-Administration procedure

Self-administration training consisted of fourteen daily 3hr sessions during which rats were trained to press the active lever on a fixed ratio (FR) schedule of reinforcement to obtain cocaine (0.75 mg/kg/0.1 mL, i.v.). Rats were not food restricted or food trained prior to commencement of SA and no priming infusions were given

(Cunningham *et al.,* 2011; Cunningham *et al.,* 2013; Anastasio *et al.,* 2014a; Anastasio *et al.,* 2014b). Scheduled completions on the active lever resulted in the simultaneous activation of the cue light, followed by activation of the infusion pump. The infusions were delivered over a 6 sec period, after which the cue light and pump were inactivated simultaneously; no infusions were delivered in the sham control groups. In all groups, the light-stimulus duration was 7 sec and a 20 sec timeout period followed each reinforcer. The house light remained illuminated for the 20 sec timeout period, during which lever presses had no scheduled consequences. Responses on the inactive lever had no scheduled consequences. Rats were initially trained on a FR1 schedule of reinforcement, until meeting a criterion of 7 reinforcers/hr. for 3 consecutive days with < 10% variation in the number of infusions received. After rats meet this acquisition criterion, and demonstrate stable responding on the FR1 schedule, an FR5 schedule of reinforcement was introduced.

Forced abstinence and cue reactivity

After self-administration training (14 days), rats underwent forced abstinence during which they remained in their home cages, but were weighed, handled, and cannula checked daily. Rats were divided into four treatment groups: 1) Cocaine+PIO, 2) Cocaine+Control, 3) Sham+Control, 4) Sham+PIO and matched for their mean drug intake during training to assess cue reactivity at forced abstinence day 30 (FA30). Pioglitazone maleate (Actos GlaxoSmithKilne) was pulverized and mixed with standard rodent chow (Bio-Serv, Frenchtown, NJ, USA) at 30mg/kg. Rats were fed control chow (Control) or PIO laced chow (PIO) during the forced abstinence period. Daily food consumption was recorded and each animal ate an average of 23g of chow daily giving

an average daily PIO dose of 0.69mg. Consumption did not differ between PIO and control chow.

At day 30 of the study, the animals were reintroduced to the SA chambers and assessed for cue reactivity. During this 1hr test session, responses on the previously active lever are reinforced by presentation of the conditioned-paired stimulus complex (cue light, drug infusion pump motor) using an FR1 schedule. Responses on the previously active and inactive levers were recorded throughout the 1hr session and the former is used as a measure of cue reactivity. Food was available ad-libidum throughout the course of the experimental sessions. A small contingent of rats (N=4 per group) underwent 14 days of self-administration followed by 30 days of forced abstinence but were not subject to a cue test on day 30. These rats are referred to as no cue no context since they failed to undergo cue reactivity testing but did experience cocaine self-administration and forced abstinence (Anastasio *et al.,* 2014a). The purpose of these rats was to determine if PIO prevented rapid, transient protein changes induced by cue reactivity and the incubation of cocaine craving.

Contextual cocaine seeking

After self-administration training and 30 days forced abstinence rats were placed back into their original operant chambers (the previously paired drug taking context) and allowed to freely lever press without conditioned paired stimuli (cue light or drug infusion pump) for a period of ten minutes (Anastasio *et al.,* 2014a). This period of time was deemed contextual cocaine seeking. After ten minutes a non-contingent cue light would flash indicating the beginning of 60 minutes of cocaine seeking in which the paired

stimulus complex would be present on an FR1 schedule. This period of time was identical to our prior cocaine cue reactivity test.

Cocaine primed cocaine seeking

After contextual and cued drug seeking, rats were briefly taken out of their operant chambers and injected IP with 15mg/kg cocaine. Rats were then placed back into their original operant chamber and allowed to freely lever press for a period of 60 minutes. The paired stimulus complex was not present at this time and lever presses were recorded but had no scheduled consequences.

Sucrose self-administration

Procedure

Self-administration training of sucrose consisted of fourteen daily 3hr sessions during which rats were trained to press the active lever on a fixed ratio (FR) schedule of reinforcement to obtain one 45mg dustless precision pellet of sucrose (BioServ) (Cunningham *et al.,* 2011). Rats were not food restricted or food trained prior to commencement of SA and no priming pellets were given. Experimental parameters were identical to cocaine self-administration except that sucrose was substituted as the reinforcer. Scheduled completions on the active lever resulted in the simultaneous activation of the cue light, followed by activation of the pellet dispenser. In all groups, the light-stimulus duration was 7 sec and a 20 sec timeout period followed each reinforcer. The house light remained illuminated for the 20 sec timeout period, during which lever presses had no scheduled consequences. Responses on the inactive lever had no scheduled consequences. Rats were initially trained on a FR1 schedule of

reinforcement, until meeting a criterion of 7 reinforcers/hr for 3 consecutive days with < 10% variation in the number of infusions or pellets received. After rats meet this acquisition criterion, and demonstrate stable responding on the FR1 schedule, an FR5 schedule of reinforcement was introduced.

Forced abstinence and cue reactivity

After self-administration training (14 days), rats underwent forced abstinence during which they remained in their home cages, but were weighed and handled daily. Rats were divided into three treatment groups: 1) Sucrose+PIO during Acquisition, 2) Sucrose+Control, 3) Sucrose+PIO during abstinence and matched for their mean sucrose pellet intake during training to assess cue reactivity at forced abstinence day 30 (FA30). Pioglitazone maleate (Actos GlaxoSmithKilne) was pulverized and mixed with standard rodent chow (Bio-Serv, Frenchtown, NJ, USA) at 30mg/kg. Rats were fed control chow (Control) or PIO laced chow (PIO) during the forced abstinence (FA) period. A small contingent of rats were given PIO laced chow during sucrose SA training to determine of PIO had any effects on the acquisition of operant conditioning behavior. Daily food consumption was recorded and each animal ate an average of 23g of chow daily giving an average daily PIO dose of 0.69mg. Consumption did not differ between PIO and control chow.

At day 30 of the study, the animals were reintroduced to the SA chambers and assessed for cue reactivity. During this 1 hr test session, responses on the previously active lever are reinforced by presentation of the conditioned-paired stimulus complex (cue light, sound of pellet dispenser) using an FR1 schedule. Responses on the

previously active and inactive levers were recorded throughout the 1 hr session and the former is used as a measure of cue reactivity. Food was available ad-libidum throughout the course of the experimental sessions.

Protein fractionation

Immediately following the cue test on FA30, rats were anesthetized with chloral hydrate solution (400mg/kg), decapitated, and brain regions (HIP, Amg, PFC, NAc) microdissected immediately on a cool tray (4^{o}C) (Heffner et al. 1980). Samples were flash frozen in liquid nitrogen and stored at -80^{o}C for subsequent protein extraction. Protein fractionation by differential centrifugation from brain tissues was performed to assess the cellular and regional localization of PPARγ.

Nuclear extraction

The nuclei of individual rats' HIP, NAc, mPFC, and Amg were extracted with the Active Motif nuclear extraction kit (Active Motif) following manufacturer's instructions. Brain regions were weighed then transferred to a pre-chilled Dounce homogenizer and homogenized in 1X hypotonic buffer supplemented with DTT (1mM) as well as detergent (0.1%, Nonidet P-40). The total homogenates were incubated on ice for 15 min and then centrifuged for 10 min (850 x g at 4ºC). The pellet was resuspendend in 500µL 1X hypotonic buffer then incubated on ice for 15 min. Thirty µL of the provided detergent (10% NP-40) was added to the suspension and vortexed for 10 sec. This suspension was then centrifuged for 30 sec (14,000 x g at 4ºC). The supernatant (cytoplasmic fraction) was collected into a pre-chilled microcentrifuge tube and stored at -80ºC. Immediately after centrifugation, the nuclei containing pellet was resuspended in

50µL of the Active Motif Lysis Buffer and vortexed for 10 sec. The nuclear suspension was incubated on ice for 30 min on a rocking platform (150 rpm). Samples were vortexed for 30 sec then centrifuged for 10 min (14,000 x g at 4ºC). The supernatant (nuclear fraction) was collected and aliquots were stored at --80ºC for later use.

Quantitative western Blot

Nuclear samples (20µg) were electrophoresed by 7.5% SDS-PAGE and blotted onto PVDF membranes (Immobilon, Millipore). After 15 min in TBS-Tween (50mM Tris pH 7.5, 150mM 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 PPARγ (1:500; Millipore, 07-466), and for one hour with the following antibodies: ERK (1:1000; Cell Signaling, 9102), and phospho-ERK (1:1000; Cell Signaling, 9101), Pan-Cadherin (1:1000; Abcam, ab6528), lamin-B (1:1000; Santa Cruz, SC-20681), diluted in TBS-Tween blocking buffer with 2% ECL Advance Blocking agent. After five washings with TBS-Tween, the blots were incubated for 1 h 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 Detection system in accordance with the manufacturer's instructions (GE Healthcare) and film exposures. Films were developed with a Kodak imager (Kodak). Densitometric quantification was performed using ImageJ (NIH).

Quantitative immunoprecipitation

Approximately 500µl of extract buffer (consisting of 25µM HEPES, 0.1M Triton X-100, 1M Glycerol, and Water) was combined with 600 µg of tissue sample in a 1.5ml Eppendorf tube and supplemented with 0.02M sigma protease inhibitor cocktail (P8340), 0.02M NaF, and 0.02M sodium orthovanadate. PPARγ anti-body 10µl (Millipore) was added and samples incubated overnight on a rotating rocker at 4^{o}C. Protein A sepharose beads 20µl (Cell Signaling) are then added and samples placed on a rotating platform at 4^{o}C for an additional 3 hours. Samples were centrifuged at 3,000 X g for a period of one minute and supernatant carefully removed and discarded. Pelleted beads were washed by adding 500µL of extract buffer and placed on a rotating rocker in 4^{o}C for a period of twenty minutes. This wash cycle was repeated four times. After the final centrifugation as much supernatant was removed without removing pelleted beads and 3x sample buffer was added. Samples were heated to 95-100 ^{o}C for a period of five minutes then allowed to cool to room temperature. Samples were then centrifuged (14,000 x g) for one minute and supernatant was loaded onto a 7.5% SDS-PAGE gel (Biorad) and then transferred onto PVDF membranes (Immobilon, Millipore). After 15 min in TBS-T (50mM Tris pH 7.5, 150mM 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: PPARγ-HRP (1:1000; Novus Biologicals, NBP2-22106H), and pERK-HRP (1:1000; Cell Signal, 8544s) then quantified as described previously to a standard loading control of sham mPFC (Jahrling, Hernandez et al. 2014).

Results

Rats acquired cocaine self-administration

Rats were trained to press the active lever on a FR1 schedule of reinforcement to obtain cocaine (0.75 mg/kg/0.1 mL, i.v.). This FR1 schedule continued until rats meet a criterion of at least 7 reinforcers/hr for 3 consecutive days. After this criterion was met, an FR5 schedule was then introduced in which every five active lever presses resulted in an infusion of cocaine (**Fig 4.1**). SA training lasted for a period of fourteen days and rats that failed to meet an FR5 criterion were excluded from the study.

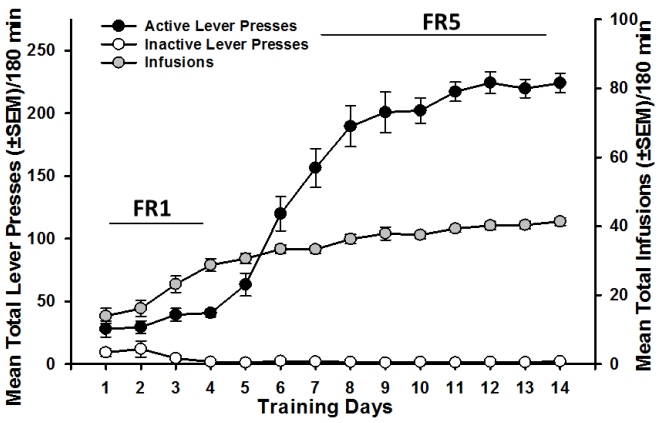

Figure 4.1 Rats acquired cocaine self-administration. Rats were trained to press the active lever to obtain cocaine (0.75 mg/kg in 0.1 mL, i.v.) during 3hr sessions. Rats were trained on a fixed ratio 1 (FR1) schedule of reinforcement until meeting a criterion of at least 7 reinforcers/hr for 3 consecutive days then a FR5 schedule was introduced. Rats that did not meet these criteria were excluded from these studies.

Nuclear PPARγ and pERK protein are increased in rats that underwent one day of forced abstinence from cocaine but not thirty

In order to determine if PPARγ and pERK are dysregulated following chronic SA, followed by FA, HIP and PFC nuclear samples were collected immediately after FA day

one or FA day thirty and immunoblots probed for PPARγ, pERK, and ERK were quantified. Nuclear PPARγ protein expression was significantly increased in rats that had undergone one day FA but not thirty in both the HIP and mPFC (**Fig 4.2 a, b**). One-way ANOVA followed by tukey multiple comparison analysis determined significance between FA D1 Cocaine and all other groups. $F(3, 10) = 50.32$, $P=0.001$ and $F(3, 10) = 3.85$, $P=0.04$ for HIP and PFC, respectively. We also noted an increase in nuclear pERK protein expression following one day of FA but not thirty in both the HIP and mPFC (**Fig 4.2 c, d**). One-way ANOVA followed by Tukey post hoc analysis determined significance between FA D1 Cocaine and all other groups. $F(3, 10) = 4.45$, $P=0.02$ and $F(3, 10) = 13.85$, $P=0.003$ for HIP and PFC, respectively. Sham groups did not display any alteration in PPARγ or ERK in either the nuclear or cytosolic fractions. This indicates that PPARγ as well as pERK are altered following chronic cocaine SA and FA. Learning this we decided to apply PIO treatment during prolonged FA with the hypothesis that PIO treatment during FA would attenuate cocaine cue reactivity.

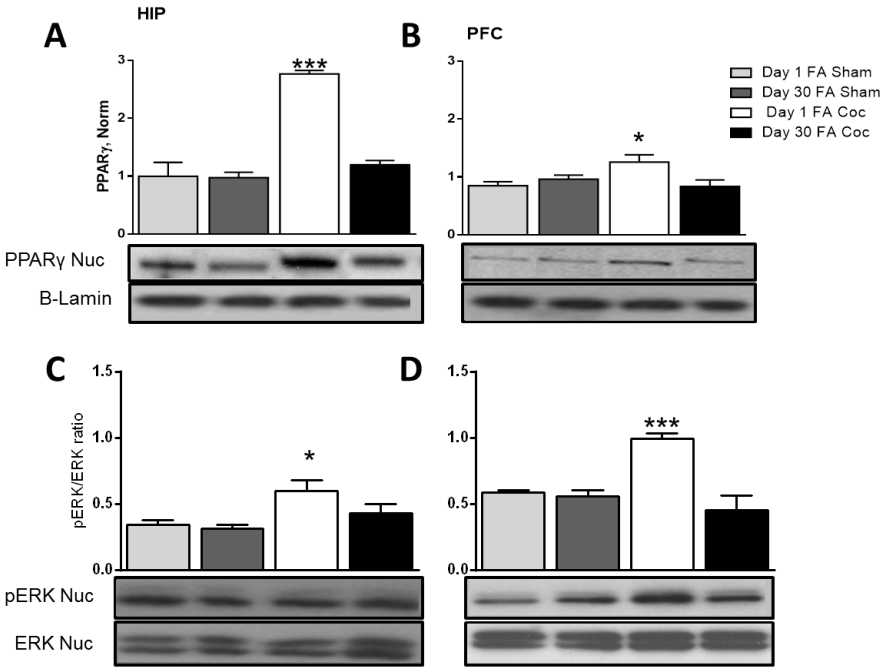

Figure 4.2 Nuclear PPARγ and pERK protein are increased in rats that underwent one day of FA from cocaine but not thirty (A) PPARγ is elevated in the HIP following 1 day FA but not 30. One-way ANOVA followed by Tukey multiple comparison analysis determined significance between FA D1 Coc and all other groups $F(3,10)=50.32$, $P=0.001$.(**B**) PPARγ is elevated in the PFC following one day FA but not 30. One-way ANOVA followed by Tukey multiple comparison analysis determined significance between FA D1 Coc and all other groups $F(3,10)=3.85$, $P=0.04$. (**C**) pERK is elevated in the HIP following 1 day FA but not 30. One-way ANOVA followed by Tukey multiple comparison test determined significance between FA D1 Coc and all other groups $F(3,10)=4.45$, $P=0.02$ (**D**) pERK is elevated in the PFC following 1 day FA but not 30. One-way ANOVA followed by Tukey multiple comparison test determined significance between FA D1 Coc and all other groups $F(3,10)=13.85$, $P=0.003$.

PPARγ agonism attenuated cocaine cue reactivity

Male Sprague-Dawley rats underwent fourteen days of cocaine self-administration (SA) where they received cocaine or no infusions (Sham) followed by 30 days FA. During the SA paradigm, pressing an active lever results in 0.75mg/kg/0.1ml infusion of cocaine as well as activation of a cue light whereas pressing the inactive lever results in no consequences for the rat. These rats were divided into two groups control diet (control) and feed laced with the PPARγ agonist PIO at 30mg/kg chow respectively. Groups were given these treatments during the FA period and underwent

a cue reactivity test on day 30 of FA. The cue reactivity test exposes rats to cues previously associated with cocaine SA which can trigger cocaine seeking by acting as conditioned stimuli, even during abstinence, (Buccafusco *et al.*, 2009). Environmental surroundings (the operant chamber) serve as contextual cues whereas the cue light serves as a discrete cocaine cue. It is important to note that our model does not have cocaine on board during these cue tests and is therefore not cue-induced reinstatement but rather a measure of cocaine seeking when exposed to cues previously paired with cocaine. This way we can quantify cocaine seeking by the number of previously active lever presses. I found that PPARγ agonism significantly attenuated cocaine seeking as measured by a decrease in previously active lever presses. Two way ANOVA revealed a main effect of both cocaine ($F(1, 58) = 267.0$ $P < 0.0001$) and PIO treatment ($F(1, 58) = 38.93$ $P=0.0212$) with a significant interaction ($F(1, 58) = 14.11$, $P =0.0041$) (**Fig 4.4**). No difference in inactive lever pressing between sham groups or the Coc+PIO group was found ($F(1, 58) = 18.39$ $P =0.1694$) (**Fig 4.5**). These results indicate that PPARγ agonism significantly attenuated cocaine cue reactivity.

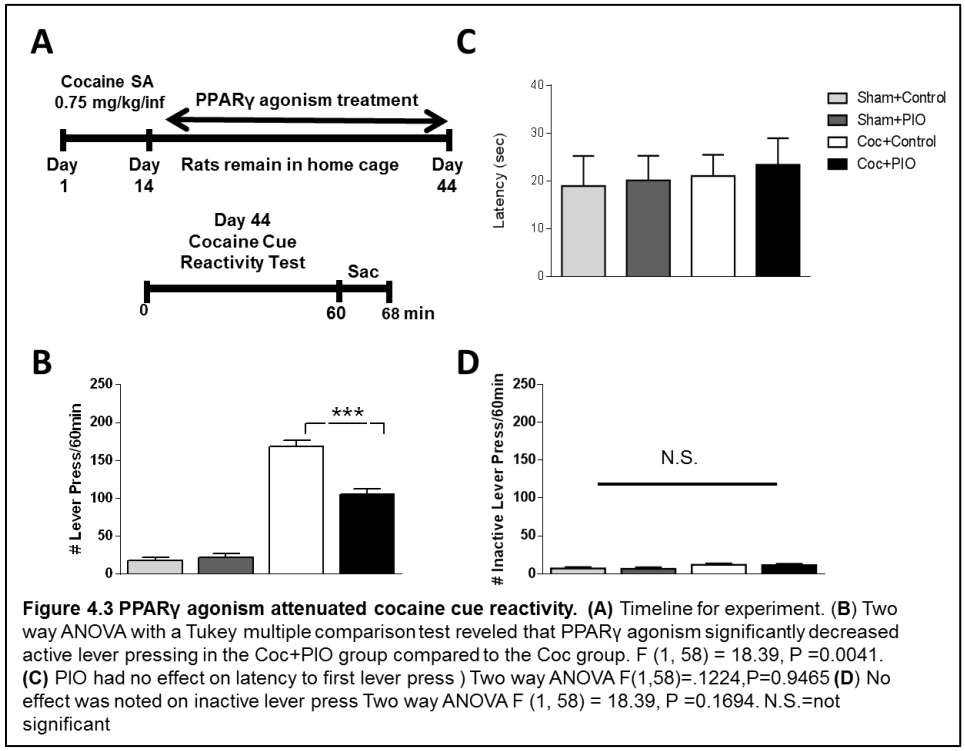

Figure 4.3 PPARγ agonism attenuated cocaine cue reactivity. (A) Timeline for experiment. (**B**) Two way ANOVA with a Tukey multiple comparison test reveled that PPARγ agonism significantly decreased active lever pressing in the Coc+PIO group compared to the Coc group. F (1, 58) = 18.39, P =0.0041. **(C)** PIO had no effect on latency to first lever press) Two way ANOVA F(1,58)=.1224,P=0.9465 **(D**) No effect was noted on inactive lever press Two way ANOVA F (1, 58) = 18.39, P =0.1694. N.S.=not significant

PPARγ agonism prevented attenuation in nuclear PPARγ and pERK protein expression in the amygdala following cocaine cue reactivity.

PPARγ agonism prevented attenuation of nuclear PPARγ and pERK protein expression in the Amg Two way ANOVA F (1, 32) = 17.47, P= 0.0002) for PPARγ F (1, 32) = 3.550, P = 0.0241 for pERK1 F (1, 32) = 3.551, P= 0.0487 for pERK2 respectively (**Fig 4.5**). Tukey multiple comparison tests showed that the Coc+control group was significantly different from all other groups. This indicates that PIO treatment is rescuing dysregulated pERK in the Amg following FA treatment.

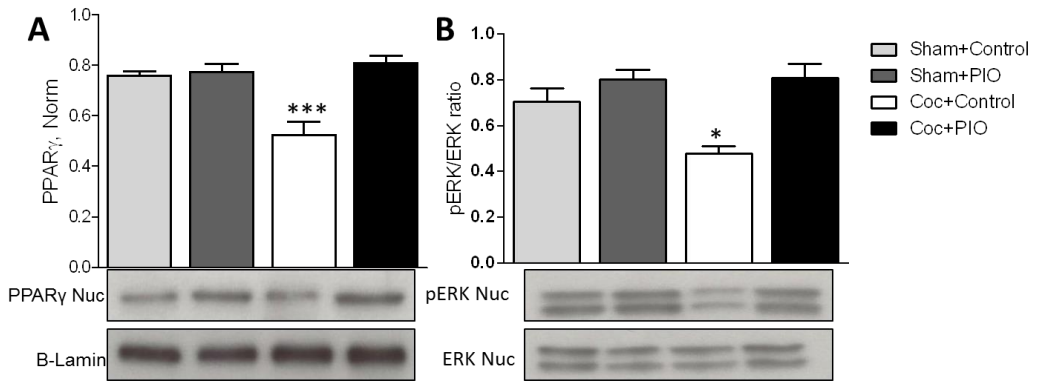

Figure 4.4 PPARγ agonism prevented attenuation of nuclear PPARγ and pERK protein expression in the Amg following cocaine cue reactivity. After the cocaine cue reactivity test rats were immediately sacked and brain regions sub-dissected. (**A**) PPARγ agonism prevented attenuation of nuclear PPARγ protein expression in the Amg. Two way ANOVA F (1, 32) = 17.47, P= 0.0002 *** indicates significant difference between Coc+Control and all other groups as determined by a tukey multiple comparison test. (**B**) PPARγ agonism prevented an attenuation of pERK protein expression. Two way ANOVA F (1, 32) = 3.550, P = 0.0241 for pERK1 F (1, 32) = 3.551, P= 0.0487 for pERK2 respectively. * indicates a significant difference between Coc+Control and all other groups as determined by a tukey multiple comparison test.

PPARγ agonism prevented attenuation in nuclear PPARγ and pERK protein expression in the medial prefrontal cortex following cocaine cue reactivity.

PPARγ agonism prevented attenuation of nuclear PPARγ and pERK protein expression in the mPFC Two way ANOVA (F (1, 33) =5.247, P=0.0218 for PPARγ and F (1, 32) =12.75 P=0.0011 for pERK1 and F (1, 32) =12.75, P=0.002, respectively) (**Fig 4.5**). Tukey multiple comparison tests showed that the Coc+control group was significantly different from all other groups. This indicates that PIO treatment is rescuing dysregulated pERK in the mPFC following FA treatment.

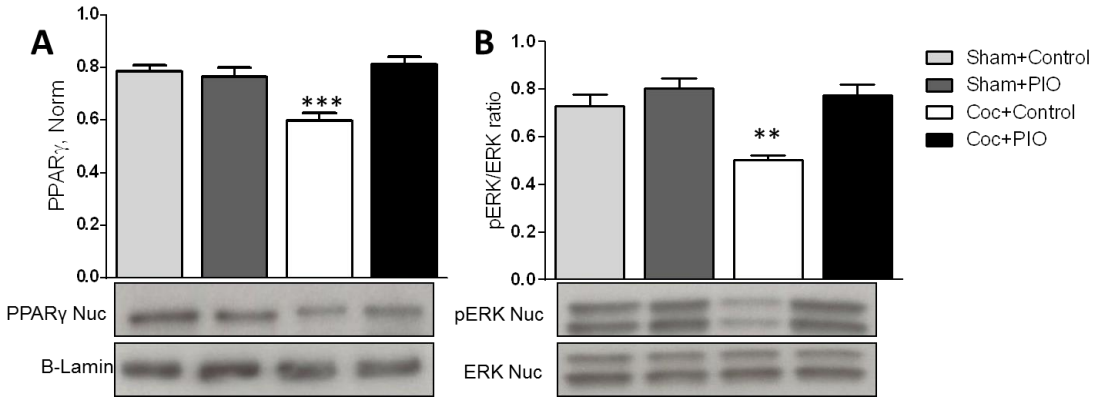

Figure 4.5 PPARγ agonism prevented attenuation of nuclear PPARγ and pERK protein expression in the mPFC following cocaine cue reactivity. After the cocaine cue reactivity test rats were immediately sacked and brain regions sub-dissected. (**A**) PPARγ agonism prevented attenuation of nuclear PPARγ protein expression in the mPFC Two way ANOVA F (1, 33) = 5.247, P = 0.0218 * indicates significant difference between Coc+Control and all other groups as determined by a tukey multiple compariosn test (**B**) PPARγ agonism prevented an attenuation of pERK protein expression in the mPFC Two way ANOVA F (1, 32) = .12.75, P = 0.0011 for pERK 1 F (1, 32) = 12.75, P = 0.002 for pERK2 respectively. ** indicates a significant difference between Coc+Control and all other groups as determined by tukey multiple comparison test.

PPARγ agonism prevented attenuation in nuclear PPARγ and pERK protein expression in the hippocampus following cocaine cue reactivity.

PPARγ agonism prevented attenuation of nuclear PPARγ and pERK protein expression in the HIP Two way ANOVA F(1,32)=24.92 P=0.0023 for PPARγ and F(1,32)=5.837 P=0.0216 for pERK1 F(1,32)=5.857, P=0.0289 respectively (**Fig 4.6**). Tukey multiple comparison tests showed that the Coc+control group was significantly different from all other groups. This indicates that PIO treatment is rescuing dysregulated pERK in the HIP following FA treatment.

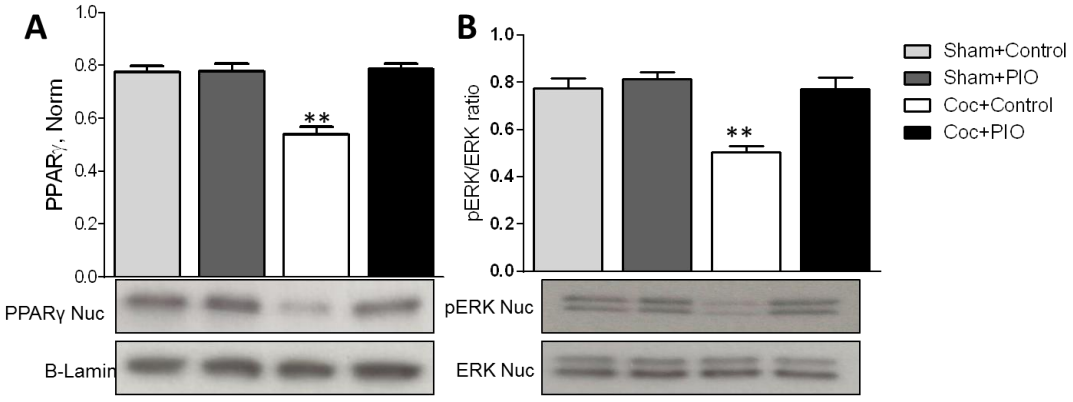

Figure 4.6 PPARγ agonism prevented attenuation of nuclear PPARγ and pERK protein expression in the HIP following cocaine cue reactivity. After a cocaine cue reactivity test rats were immediately sacked and brain regions sub-dissected. (**A**) PPARγ agonism prevented attenuation of nuclear PPARγ protein expression in the HIP Two way ANOVA F (1, 32) = 24.92, P = 0.0023 ** indicates significant difference between Coc+Control and all other groups. (**B**) PPARγ agonism prevented an attenuation of pERK protein expression in the HIP Two way ANOVA F (1, 32) = 5.837, P = 0.0216 for pERK1 F (1, 32) = 5.857, P = 0.0289 for pERK2 respectively. ** indicates a significant difference between Coc+Control and all other groups.

PPARγ agonism increased nuclear PPARγ and attenuated pERK protein in the nucleus accumbens following cocaine cue reactivity.

In contrast to the PFC, Amg, and HIP, PPARγ agonism increased nuclear PPARγ protein expression in the NAc. Two-way ANOVA analysis revealed a significant interaction effect between cocaine and PIO (F (1, 32) =38.68, P=0.0061 for PPARγ (**Fig 4.7a**). Two way ANOVA reveled that PPARγ agonism decreased pERK protein expression in the NAc F (1, 32) =54.10, P=0.0071) for pERK1 F (1, 32) =54.10, P=0.0067 for pERK2 respectively (**Fig4.7b**). Tukey multiple comparison tests showed that the Coc+PIO group was significantly different from all other groups. This indicates that PIO treatment is attenuating pERK protein expression in the NAc during FA. We

then wanted to determine if the changes we note in PPARγ and pERK protein expression occur during FA or the cocaine cue reactivity test.

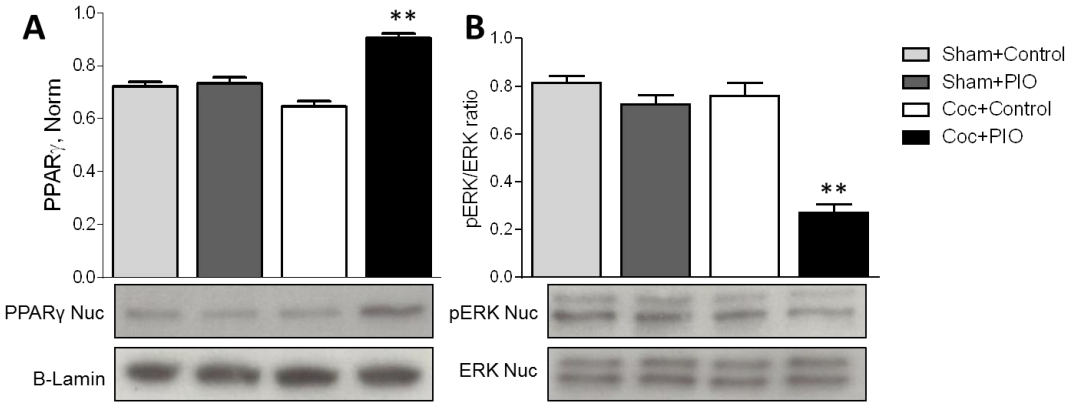

Figure 4.7 PPARγ agonism increased nuclear PPARγ and attenuated nuclear pERK protein expression in the NAc following cocaine cue reactivity. After the cocaine cue reactivity test rats were immediately sacked and brain regions sub-dissected. (**A**) PPARγ agonism increased nuclear PPARγ protein expression in the NAc Two way ANOVA F (1, 32) = 38.68, P = 0.0061 ** indicates significant difference between Coc+PIO and all other groups as demonstrated with a tukey multiple comparison test. (**B**) PPARγ agonism decreased pERK protein expression in the NAc Two way ANOVA F (1, 32) = 54.10, P = 0.0071 for pERK1 F (1, 32) = 54.10, P = 0.0067 for pERK2 respectively. ** indicates a significant difference between Coc+PIO and all other groups as determined by a tukey multiple comparison test.

PPARγ agonism during forced abstinence prevented pERK attenuation in the medial prefrontal cortex and hippocampus and attenuated pERK in the nucleus accumbens during a cocaine cue reactivity test

To determine if the changes we note in PPARγ and pERK protein expression occur during FA or the cocaine cue reactivity test a small contingent of rats underwent cocaine SA training and FA but were immediately sacrificed prior to a cocaine cue reactivity test on day thirty of FA. We noted attenuation of nuclear PPARγ and pERK protein expression in the mPFC and HIP of the Coc+control group after a cue reactivity test (**Fig. 4.8 a-d**). Two way ANOVA revealed a main effect of cue reactivity as rats that self-administered cocaine and underwent FA but did not undergo the cue reactivity test failed to demonstrate a change in either PPARγ or pERK (F (1, 33) = 8.236, P=0.0157)

for PPARγ and (F (1, 32) =12.05, P= 0.032) for pERK2 in the mPFC and F(1,32)=24.92 P=0.0213 for PPARγ and F(1,32)=5.837 P=0.0216 for pERK2 in the HIP. Two way ANOVA also demonstrated an interaction between PIO treatment and cue reactivity F(1,33)=5.247, P=0.0218) for PPARγ (F(1,32)=12.75, P= 0.002) for pERK2 in the mPFC and F(1,32)=24.92 P=0.0023 for PPARγ and (F(1,32)=5.837 P=0.0289) for pERK2 in the HIP. This indicated that the cue reactivity test is responsible for the attenuation of nuclear PPARγ and pERK protein expression in the Coc+control group and PIO treatment during FA is preventing this attenuation. In contrast to the PFC and HIP, the NAc of rats that underwent a cue reactivity test show an attenuation of pERK protein expression when compared to Coc+Control rats and a significant increase in PPARγ protein expression for the Coc+PIO group (**Fig 4.8 e, f**). Two-way ANOVA analysis revealed a significant interaction effect between cue reactivity and PIO (F (1, 33) =37.40, P=0.031 for PPARγ and F (1, 33) =48.10, P=0.0091) for pERK1 F (1, 33) =44.10, P=0.0072 for pERK2 respectively. These results indicated that PPARγ agonism during FA is responsible for the prevention of dysregulated ERK activity in a cue reactivity test.

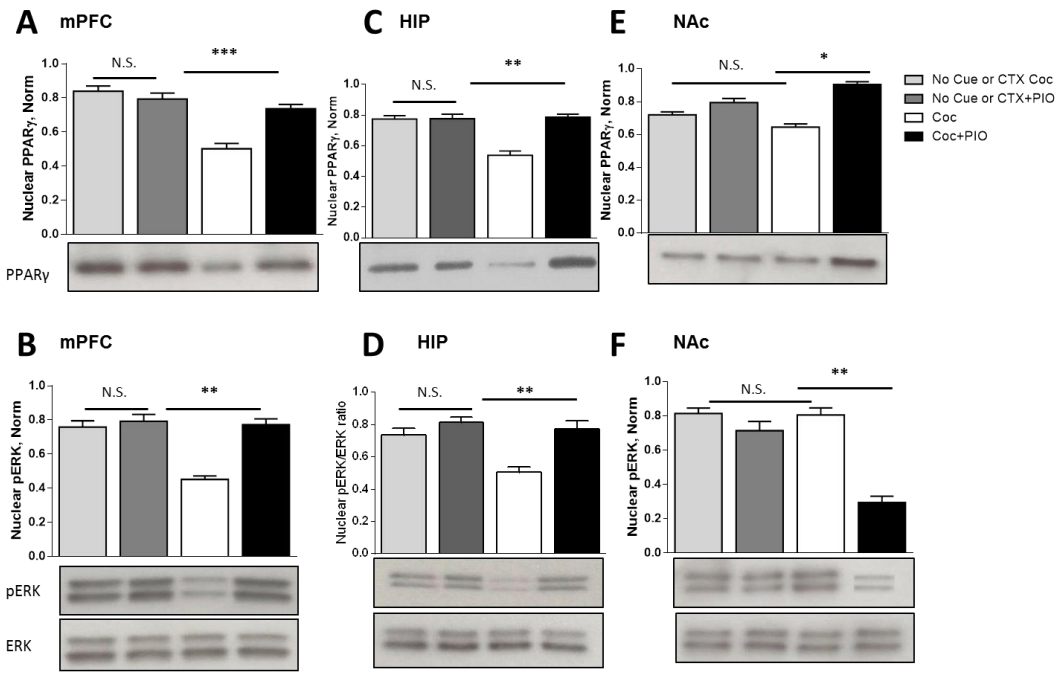

Figure 4.8 PPARγ agonism during FA prevented pERK attenuation in the mPFC and HIP and attenuated pERK in the NAc during a cocaine cue reactivity test After a cocaine cue reactivity test rats were immediately sacked and brain regions sub-dissected. A small contingent of rats (deemed no cue no context) underwent 14 days SA and 30 days FA but did not undergo a cue reactivity test. No cue no context rats were compared to rats that had undergone the cue reactivity test to determine if PPARγ agonism had an effect during cue reactivity. **(A)** PIO given during FA prevented nuclear PPARγ protein attenuation during a cocaine cue reactivity test in the mPFC Two way ANOVA ($F(1,33)=5.247$, $P= 0.0218$) *** indicates significant difference between Coc+Control and all other groups. **(B)** PIO given during FA prevented attenuation of nuclear pERK protein in the mPFC Two way ANOVA ($F(1,32)=12.75$, $P= 0.002$) for pERK2 ** indicates significant difference between Coc+Control and all other groups. **(C)** PIO given during FA prevented nuclear PPARγ protein attenuation during a cocaine cue reactivity test in the HIP Two way ANOVA $F(1, 32) = 24.92$, $P = 0.0023$ ** indicates significant difference between Coc+Control and all other groups. **(D)** PIO given during FA prevented attenuation of nuclear pERK protein in the HIP Two way ANOVA ($F(1, 32) = 5.857$, $P = 0.0289$) for pERK2. ** indicates a significant difference between Coc+Control and all other groups. **(E)** PIO given during FA increased nuclear PPARγ protein in the NAc during a cocaine cue reactivity test (two way ANOVA (($F(1,33)=37.40$, $P=0.031$) ** indicates significant difference between Coc+PIO and all other groups. **(F)** PIO given during FA attenuated pERK in the NAc during a cocaine cue reactivity test (two way ANOVA $F(1,33)=44.10$, $P=0.0072$ for pERK2) ** indicates significant difference between Coc+PIO and all other groups. N.S.=not significant.

pERK protein expression in the Coc+PIO group correlated with the total number of active lever presses in a cocaine cue reactivity test.

Correlational analysis shows a negative correlation between pERK protein expression and number of previously active lever presses in a cocaine cue reactivity test in the Coc+PIO group. This was observed for both the mPFC ($P=0.0147$, $R= 0.5737$) and HIP ($P=0.0365$, $R=0.3989$) (**Fig 4.9 a, b**). In contrast to the HIP and PFC, the NAc shows a positive correlation with pERK protein expression and number of

previously active lever presses in a cocaine cue reactivity test in the Coc+PIO group (P= 0.023, R=0.5605) (**Fig 4.9, c**). These data indicate that upregulation of pERK protein expression in both the HIP and mPFC and a down regulation of pERK protein expression in the NAc is driving the attenuation of cocaine cue reactivity seen in the Coc+PIO group.

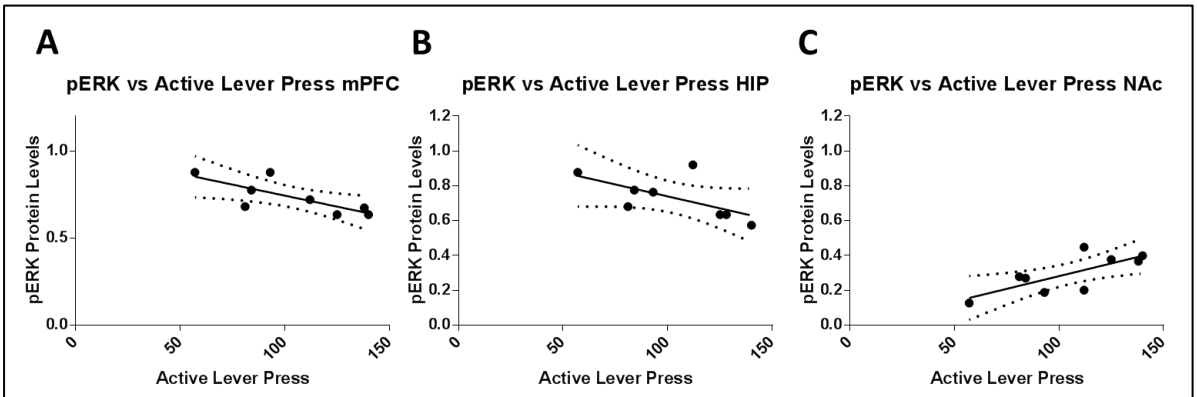

Figure 4.9 pERK protein expression in the Coc+PIO group correlated with active lever press during cocaine cue reactivity. Correlational analysis showing a negative correlation between pERK protein expression and number of previously active lever presses in a cocaine cue reactivity test in the Coc+PIO group for (**A**) mPFC (P=0.0147, R= 0.5737) and (**B**) HIP (P=0.0365, R=0.3989). (**C**) In contrast to the HIP and PFC, the NAc shows a positive correlation with pERK protein expression and number of previously active lever presses in a cocaine cue reactivity test in the Coc+PIO group (P= 0.023, R=0.5605).

PPARγ agonism decreased cocaine context, cue, and cocaine primed seeking

Since biochemical evidence that PPARγ agonism was affecting brain regions known to be involved in context-induced cocaine seeking such as the HIP and cocaine primed cocaine seeking such as the NAc, we wanted to determine if PPARγ agonism administered during an FA period would attenuate cocaine context-induced as well as cocaine primed lever pressing. Therefore a cue train in which we could measure the effect of PPARγ agonism administration during FA on contextual, cued, and cocaine primed cocaine seeking was performed in lieu of a cocaine cue reactivity test (**Fig 4.10 a**). PPARγ agonism attenuated context-induced cocaine seeking compared to the

Coc+Control group (two-tailed t test P=0.0334) (**Fig 4.10 b**). PPARγ agonism also significantly attenuated cocaine cue reactivity (two tailed t test P=0.005) but had no effect on inactive lever pressing during a cue reactivity test (two-tailed t test P=0.5876) (**Fig 4.10 c, d**). After the cue reactivity test rats were administered 15mg/kg of cocaine IP prior to being placed into their original operant chambers and were allowed to freely lever press for a period of one hour. PPARγ agonism significantly attenuated cocaine primed cocaine seeking (unpaired t test P=0.0073) (**Fig. 4.10 e**). This indicates that PPARγ agonism is effective at attenuating context, cue and cocaine primed cocaine seeking. Since PPARγ agonism had successfully attenuated cocaine seeking in three separate cocaine seeking paradigms, and since PPARγ agonism was affecting multiple brain regions involved in motivation and reward such as the NAc, we wanted to determine if PIO treatment had any effect on natural reward seeking.

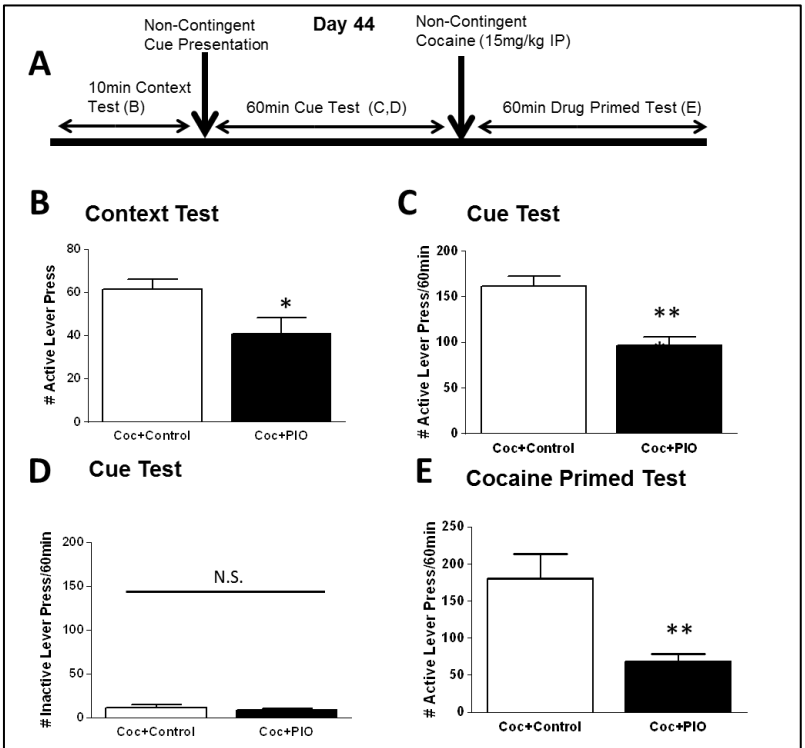

Figure 4.9 PPARγ agonism decreased cocaine context, cue, and cocaine primed seeking. **(A)** Timeline for experiment. **(B)** PIO given during FA attenuated context-induced cocaine seeking (two sided t-test P=0.0334). **(C)** PIO given during FA attenuated cocaine cue reactivity (two sided t-test, P=0.005). **(D)** No effect was observed on inactive lever press during the cocaine cue reactivity test (two-sided t-test P=0.5876) **(E)** PIO given during FA attenuated cocaine primed cocaine seeking test (two sided t-test P=0.0073). N.S.=not significant

Pioglitazone had no effect on sucrose self-administration acquisition or cue reactivity

Thirty male Sprague-Dawley rats underwent fourteen days of sucrose SA where they received sucrose pellets followed by 30 days of FA. During the SA paradigm, pressing an active lever resulted in delivery of one 45mg sucrose pellet as well as activation of a cue light whereas pressing the inactive lever resulted in no consequences for the rat. Rats were divided into three groups 1) Sucrose+PIO during Acquisition (Sucrose PIO Acq), 2) Sucrose+Control, 3) Sucrose+PIO during FA (Sucrose PIO FA). This way we could determine if PIO administration would effect

sucrose acquisition or sucrose cue reactivity (**Fig 4.11 a**). Repeated measures one way ANOVA revealed that PIO had no effect on the number of pellets received F (13, 26) =83.53, P=0.2660) during the acquisition of sucrose SA (**Fig 4.11 c**). All groups then underwent a cue reactivity test on day 30 of FA.

PPARγ agonism had no effect on sucrose cue reactivity as measured by previously active lever presses Two way ANOVA (F=0.7438, P=0.4855) or inactive lever presses (F=0.6901, P=0.5108) (**Fig 4.11 b, d**). After demonstrating that PPARγ agonism had no behavioral effect on sucrose acquisition or sucrose cue reactivity, we wanted to determine if PPARγ agonism had any effect on PPARγ or pERK protein expression following a sucrose cue reactivity task.

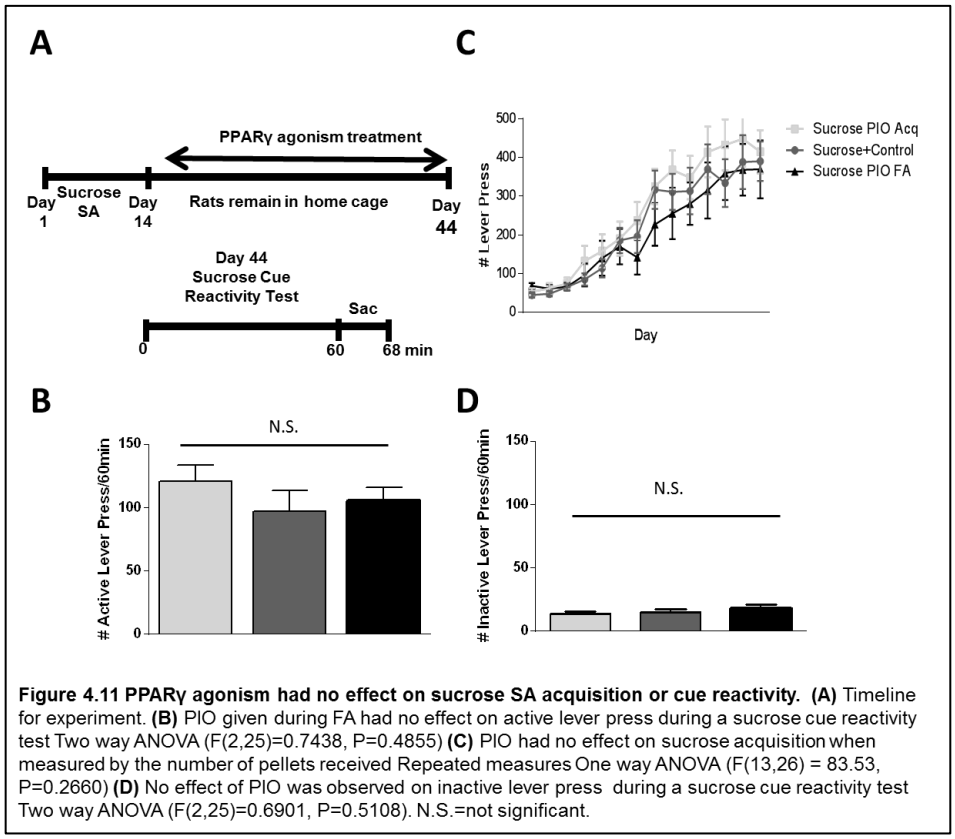

Figure 4.11 PPARγ agonism had no effect on sucrose SA acquisition or cue reactivity. (A) Timeline for experiment. **(B)** PIO given during FA had no effect on active lever press during a sucrose cue reactivity test Two way ANOVA (F(2,25)=0.7438, P=0.4855) **(C)** PIO had no effect on sucrose acquisition when measured by the number of pellets received Repeated measures One way ANOVA (F(13,26) = 83.53, P=0.2660) **(D)** No effect of PIO was observed on inactive lever press during a sucrose cue reactivity test Two way ANOVA (F(2,25)=0.6901, P=0.5108). N.S.=not significant.

Pioglitazone had no effect on PPARγ or pERK protein expression in the medial prefrontal cortex following sucrose cue reactivity test.

Nuclear PPARγ as well as pERK protein expression was determined among the three sucrose administration groups (Sucrose+PIO during Acquisition, Sucrose+Control, and Sucrose+PIO during FA respectively) in mPFC following a sucrose cue reactivity test. Two way ANOVA showed that neither PPARγ nor pERK protein expression was significantly altered among groups F (1, 36) =0.5976, P=0.5607 for PPARγ F (1, 36) =0.3176 P=0.7319 for pERK) (**Fig 4.12**). This indicated that PPARγ agonism had no effect on PPARγ or pERK protein expression in the mPFC following a sucrose cue reactivity test.

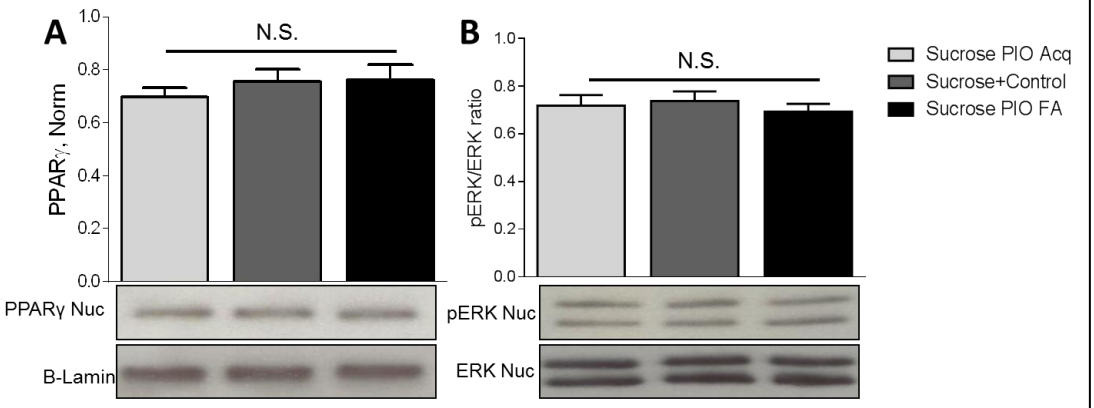

Figure 4.12 PIO had no effect on PPARγ or pERK protein expression in the mPFC following sucrose cue reactivity. Rats were divided into 3 groups (Sucrose+PIO during Acquisition, Sucrose, and Sucrose+PIO during FA respectively) and underwent Sucrose SA followed by a cue reactivity test after 30 days FA. Two way ANOVA shows that neither PPARγ nor pERK proteins were significantly altered F(1,36)=0.5976, P=0.5607 for PPARγ F(1,36)=0.3176, P=0.7319 for pERK by PIO. This indicates that PPARγ agonism has no effect on PPARγ or pERK protein levels in the mPFC and that PIO does not effect natural reward. N.S.=not significant

Pioglitazone had no effect on PPARγ or pERK protein expression in the hippocampus following sucrose cue reactivity test.

Nuclear PPARγ and pERK protein expression was determined in the HIP following a sucrose cue reactivity test. Two way ANOVA showed that neither PPARγ nor pERK protein expression was significantly altered among groups F (1, 36) =0.4751, P=0.6294 for PPARγ F (1, 36) =0.4571, P=0.6195 for pERK) (**Fig. 4.13**). This indicated that PPARγ agonism had no effect on PPARγ or pERK protein expression in the HIP following a sucrose cue reactivity test.

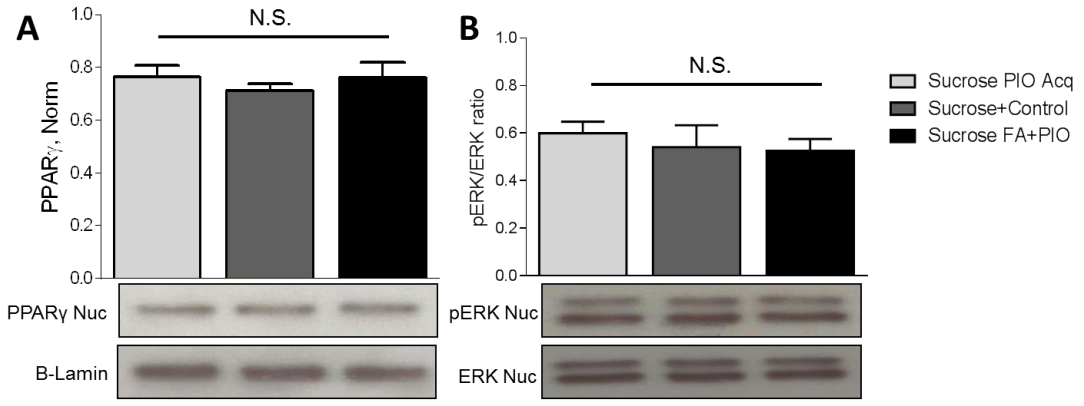

Figure 4.13 PIO had no effect on PPARγ or pERK protein expression in the HIP following sucrose cue reactivity. Rats were divided into 3 groups (Sucrose+PIO during Acquisition, Sucrose, and Sucrose+PIO during FA respectively) and underwent Sucrose SA followed by a cue reactivity test after 30 days FA. Two way ANOVA shows that neither PPARγ nor pERK proteins were significantly altered F(1,36)=0.4751, P=0.6294 for PPARγ F(1,36)=0.4571 P=0.6195 for pERK by PIO . This indicates that PPARγ agonism has no effect on PPARγ or pERK levels in the HIP and that PIO does not affect natural reward. N.S.=not significant

Pioglitazone had no effect on PPARγ or pERK protein levels in the nucleus accumbens following sucrose cue reactivity test.

Nuclear PPARγ and pERK protein expression among the three sucrose administration groups (Sucrose+PIO during Acquisition, Sucrose+Control, and Sucrose+PIO during FA respectively) was determined in the NAc following a sucrose cue reactivity test (**Fig 4.14**). Two way ANOVA shows that neither PPARγ nor pERK protein expression was significantly altered among groups F (1, 36) =0.5976, P=0.5607

for PPARγ F (1, 36) =0.3176, P=0.7319 for pERK). This indicated that PPARγ agonism had no effect on PPARγ or pERK protein expression in the NAc following a sucrose cue reactivity test.

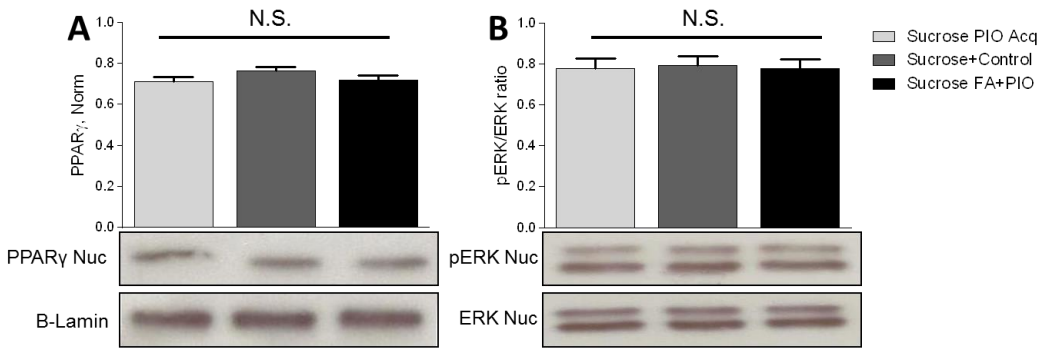

Figure 4.14 PIO had no effect on PPARγ or pERK protein expression in the NAc following sucrose cue reactivity. Rats were divided into 3 groups (Sucrose+PIO during Acquisition, Sucrose, and Sucrose+PIO during FA respectively) and underwent Sucrose SA followed by a cue reactivity test after 30 days FA. Two way ANOVA shows that neither PPARγ nor pERK proteins were significantly altered F(1,36)=0.5976, P=0.5607 for PPARγ F(1,36)=0.3176, P=0.7319 for pERK by PIO. This indicates that PPARγ agonism has no effect on PPARγ or pERK levels in the NAc and that PIO does not effect natural reward. N.S.=not significant

Pioglitazone had no effect on PPARγ or pERK protein levels in the amygdala following sucrose cue reactivity test.

PPARγ as well as pERK protein levels among the three sucrose administration groups (Sucrose+PIO during Acquisition, Sucrose+Control, and Sucrose+PIO during FA respectively) were determined in the nuclear fraction of the Amg following a sucrose cue reactivity test. Two way ANOVA shows that neither PPARγ nor pERK proteins were significantly altered F(1,36)=0.5976, P=0.5607 for PPARγ F(1,36)=0.3176, P=0.7319 for pERK This indicated that PPARγ agonism had no effect on PPARγ or pERK in the Amg following a sucrose cue reactivity test (**Fig 4.15**). After it was demonstrated that PPARγ agonism, when administered during FA, attenuates cocaine cue reactivity and has no effect on sucrose cue reactivity, we wanted to demonstrate that prior cocaine cue reactivity results were PPARγ dependent. Therefore, we decided to apply a potent and

selective PPARγ antagonist, GW 9662, prior to cocaine cue reactivity. If GW 9662 could reverse the behavioral effects of PPARγ agonism, then we could demonstrate that the attenuation of cocaine cue reactivity with PIO was indeed PPARγ dependent.

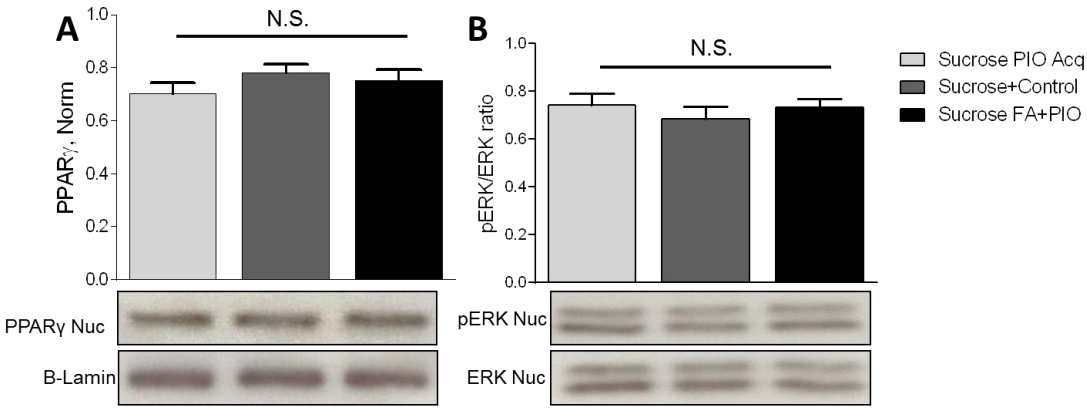

Figure 4.15 PIO had no effect on PPARγ or pERK protein expression in the Amg following sucrose cue reactivity. Rats were divided into 3 groups (Sucrose+PIO during Acquisition, Sucrose, and Sucrose+PIO during FA respectively) and underwent Sucrose SA followed by a cue reactivity test after 30 days FA. Two way ANOVA shows that neither PPARγ nor pERK proteins were significantly altered $F(1,36)=0.5976$, $P=0.5607$ for PPARγ $F(1,36)=0.3176$ $P=0.7319$ for pERK. This indicates that PPARγ agonism has no effect on PPARγ or pERK levels in the Amg and that these changes in protein are not a consequence of the operant behavior (lever pressing). N.S.=not significant

GW-9662 did not affect locomotor activity in rats

Previous studies in our lab have used an ICV method of delivery (Rodriguez-Rivera et al., 2011; Jahrling et al., 2014). For these studies we wanted to administer GW 9662 IP. However, since PPARγ is located in the periphery of the body as well as the CNS we wanted to determine if GW 9662 IP administration would lead to increased lethargy. To test the hypothesis that GW-9662 does not cause lethargy when injected

IP; rats were divided into three groups: control (10% DMSO), 0.5mg/kg GW, or 1.0mg/kg GW, and then subjected to a locomotor activity test. Rats were allowed to habituate to a locomotor activity room for a period of one hour before GW-9662, a

potent PPARγ antagonist, was injected IP into rats at a dosage of either 0.5mg/kg, or 1mg/kg. Rats were allowed to roam for 2 hours to ensure that GW-9662 had crossed the blood brain barrier. Rats were then subject to a locomotor activity test where activity was measured in a beam break box for a period of 1 hour. One way ANOVA demonstrates that GW 9662 had no significant effect on locomotor activity compared to a vehicle control (10% DMSO) as measured by total horizontal activity $F(2,27)=0.3346$ $P=0.7186$, or rearing $F(2,27)=0.2760$ $P=0.7609$ (**Fig 4.16**). After it was determined that a single dose of GW 9662 administered IP would not decrease basal locomotor activity compared to control, we decided to administer this PPARγ antagonist prior to a cocaine cue reactivity test. It was hypothesized that GW 9962 administration prior to a cocaine cue reactivity test would reverse the behavioral effects of PPARγ agonism and demonstrate that our prior results showing an attenuation of cocaine cue reactivity following PIO administration during FA was indeed PPARγ dependent.

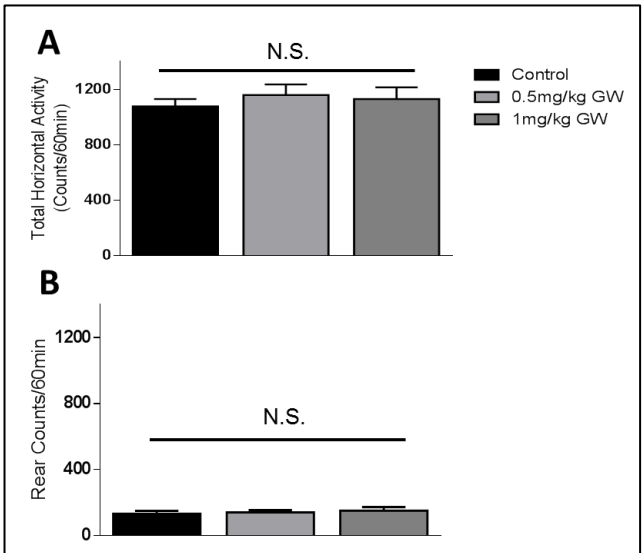

Figure 4.16 GW-9662 did not effect locomotor activity. GW-9662 was injected IP into rats at a dosage of either 0.5mg/kg, or 1mg/kg. Rats were then allowed to habituate to the locomotor activity room for a period of one hour and then subject to a locomotor activity test. (A) GW-9662 had no significant effect on total horizontal activity compared to a vehicle control (10% DMSO). One-way ANOVA $F(2,27)=0.3346$, $P=0.7186$. (B) GW-9662 had no significant effect on total horizontal activity compared to a vehicle control (10% DMSO). One-way ANOVA $F(2,27)=0.2760$, $P=0.7609$ N.S.= not significant

PPARγ antagonism with GW 9662 restored cocaine cue reactivity

In order to demonstrate that PPARγ agonism attenuated cocaine cue reactivity, we decided to administer a selective PPARγ antagonist, GW 9662, prior to cue test. Male Sprague Dawley rats underwent fourteen days SA followed by thirty days FA. Rats were split into four groups: Control+DMSO, Control+GW, PIO+DMSO, PIO+GW. During abstinence rats received either feed laced with PIO at 30mg/kg or control as described previously. Two hours before cue test rats were taken out of their home cages and given a dose of either GW 9662, (1mg/kg), or vehicle (10% DMSO) IP. Rats then underwent a standard cocaine cue reactivity test as described previously (**Fig 4.17 a)**. PPARγ antagonism significantly restored cocaine cue reactivity (two way ANOVA $F(1, 30) =9.350$, $P=0.0047$) (**Fig. 4.17 b**). We also noted a main effect of PIO ($F(1, 30) =21.39$, $P=0.004$) and GW 9662 ($F(1, 30) =9.710$, $P=0.0034$). Neither PIO nor GW-

9662 had a significant effect on latency or inactive lever pressing during the cocaine cue reactivity test (F (1, 30) =0.6140, P=0.5946 for latency (F (1, 30) =0.1180, P=0.7336) for inactive lever press (**Fig. 4.17 c, d**). This data indicated that PPARγ antagonism reversed the behavioral effects of PPARγ agonist treatment and our previous results showing an attenuation of cocaine cue reactivity with PPARγ agonist treatment during FA are PPARγ dependent. We then wanted to determine what effect, if any, GW 9662 treatment had on PPARγ and pERK protein levels in the nuclear fraction of the HIP, mPFC, Amg, and NAc immediately following a cocaine cue reactivity test.

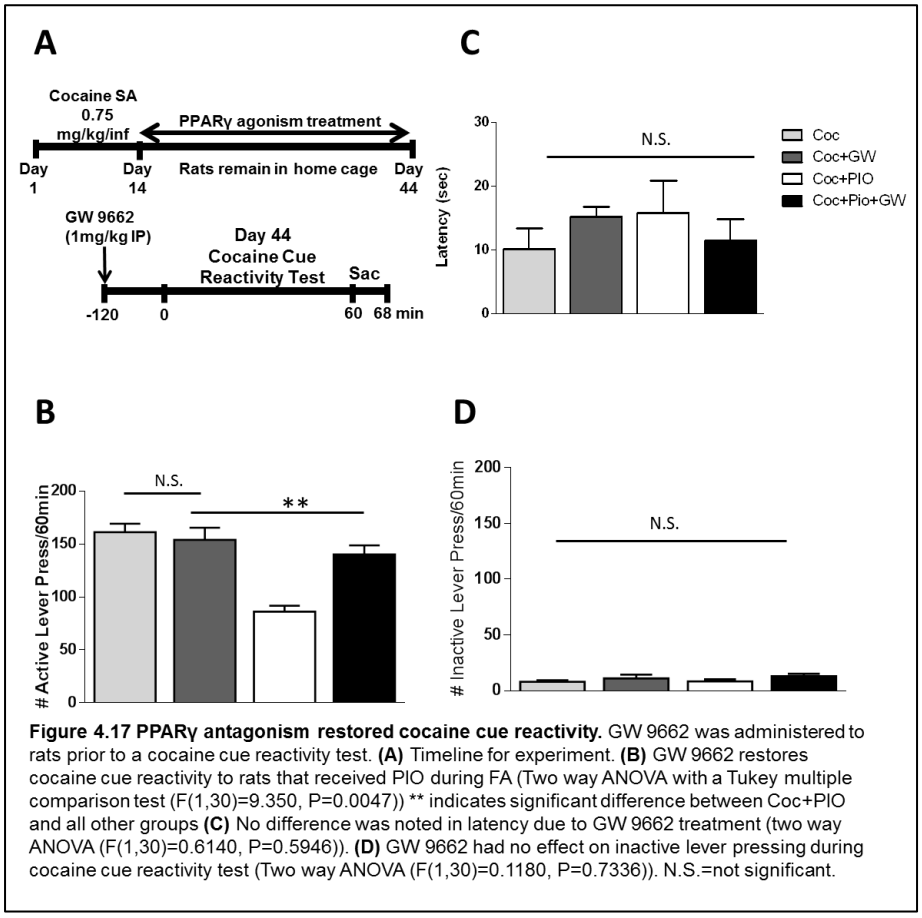

Figure 4.17 PPARγ antagonism restored cocaine cue reactivity. GW 9662 was administered to rats prior to a cocaine cue reactivity test. **(A)** Timeline for experiment. **(B)** GW 9662 restores cocaine cue reactivity to rats that received PIO during FA (Two way ANOVA with a Tukey multiple comparison test (F(1,30)=9.350, P=0.0047)) ** indicates significant difference between Coc+PIO and all other groups **(C)** No difference was noted in latency due to GW 9662 treatment (two way ANOVA (F(1,30)=0.6140, P=0.5946)). **(D)** GW 9662 had no effect on inactive lever pressing during cocaine cue reactivity test (Two way ANOVA (F(1,30)=0.1180, P=0.7336)). N.S.=not significant.

GW 9662 attenuated nuclear PPARγ and pERK protein expression in the medial prefrontal cortex

GW 9662 attenuated both nuclear PPARγ and pERK protein expression in the mPFC following a cocaine cue reactivity test (**Fig. 4.18).** Two way ANOVA revealed a main effect of PIO treatment in preventing nuclear PPARγ and pERK attenuation F(1,28)=11.78, P=48.69 for PPARγ (F(1,28)=11.05, P=0.021) for pERK 1 and (F(1,28)=12.05, P= 0.032) for pERK2. We also noted a statistically significant interaction effect of PPARγ agonism with PIO and GW 9662 treatment for the attenuation of nuclear PPARγ and pERK protein expression in the mPFC (Two way ANOVA F(1,28)=11.78, P=0.0019) for PPARγ (F(1,28)=0.12.75, P= 0.0011) for pERK 1 and (F(1,28)=12.75, P= 0.002) for pERK2 Tukey multiple comparison test revealed that significance was driven by the Coc+PIO group in comparison to all other groups. These results indicated that PPARγ antagonism attenuates nuclear PPARγ and pERK protein expression in the mPFC.

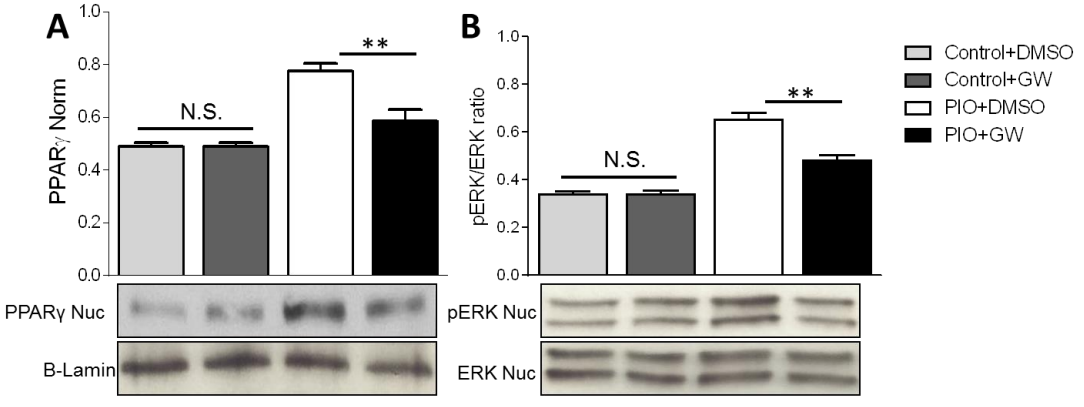

Figure 4.18 GW 9662 attenuated nuclear PPARγ and pERK protein expression in the mPFC. Rats were given either 10% DMSO or GW 9662 (1mg/kg) prior to cue test. After cue test rats were immediately sacked and brain regions sub-dissected. (**A**) GW 9662 attenuates nuclear PPARγ protein expression Two way ANOVA F(1,28)=11.78, P=0.0019). (**B**) GW 9662 attenuates nuclear pERK protein expression Two way ANOVA F(1,28)=12.75, P= 0.0011) for pERK 1 and (F(1,28)=12.75, P= 0.002) for pERK2. N.S.=not significant

GW 9662 had no effect on nuclear PPARγ or pERK protein expression in the nucleus accumbens

In contrast to the mPFC, GW 9662 showed no significant effect on nuclear PPARγ or pERK protein expression in the NAc (**Fig 4.19**). Two way ANOVA revealed a main effect of PIO treatment on nuclear PPARγ and nuclear pERK ($F(1,28)=60.81$, $P=0.0019$) for PPARγ ($F(1,28)=47.38$, $P= 0.0001$) for pERK 1 and ($F(1,28)=101.4$, $P= 0.001$) for pERK2. However GW 9662 administration exhibited no significant effect on either nuclear PPARγ or pERK protein expression in the NAc ($F(1,28)=3.384$, $P=0.0864$) for PPARγ ($F(1,28)=1.760$, $P= 0.1954$) for pERK 1 and ($F(1,28)=.7621$, $P= 0.3901$) for pERK2. Since GW 9662 did not have an effect on the NAc, but we see a restoration of cocaine cue reactivity following GW 9662 administration, this indicates that the NAc may not be the primary brain region responsible for the restoration of cocaine cue reactivity in these studies.

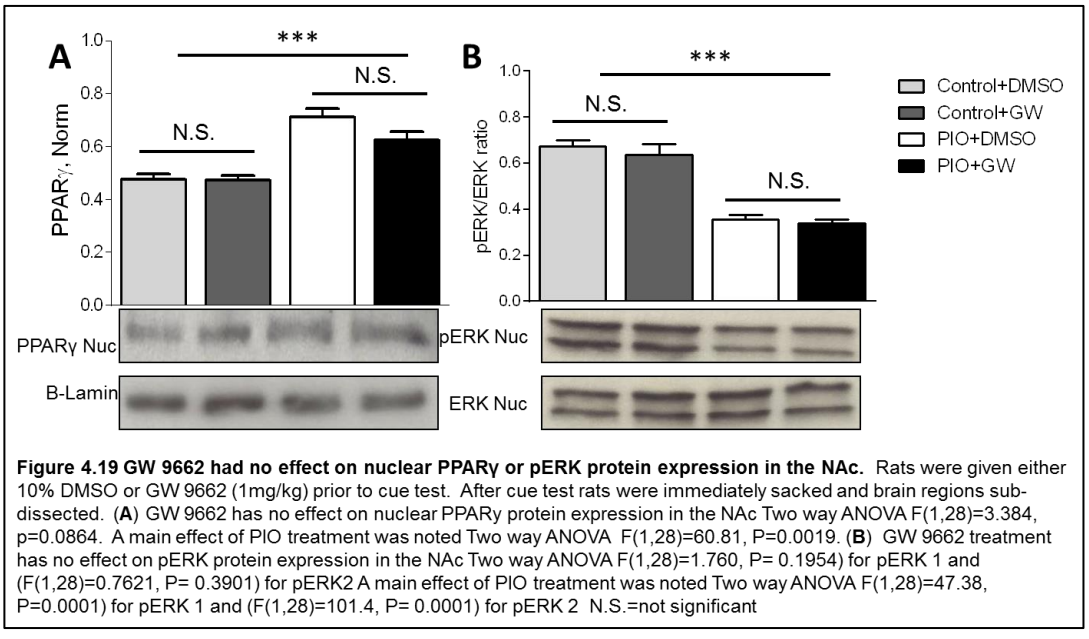

Figure 4.19 GW 9662 had no effect on nuclear PPARγ or pERK protein expression in the NAc. Rats were given either 10% DMSO or GW 9662 (1mg/kg) prior to cue test. After cue test rats were immediately sacked and brain regions sub-dissected. (**A**) GW 9662 has no effect on nuclear PPARγ protein expression in the NAc Two way ANOVA $F(1,28)=3.384$, $p=0.0864$. A main effect of PIO treatment was noted Two way ANOVA $F(1,28)=60.81$, $P=0.0019$. (**B**) GW 9662 treatment has no effect on pERK protein expression in the NAc Two way ANOVA $F(1,28)=1.760$, $P= 0.1954$) for pERK 1 and ($F(1,28)=0.7621$, $P= 0.3901$) for pERK2 A main effect of PIO treatment was noted Two way ANOVA $F(1,28)=47.38$, $P=0.0001$) for pERK 1 and ($F(1,28)=101.4$, $P= 0.0001$) for pERK 2 N.S.=not significant

GW 9662 had no effect on nuclear PPARγ or pERK protein expression in the amygdala

GW 9662 exhibits no significant effect on nuclear PPARγ or pERK protein expression in the Amg (**Fig 4.20**). Two way ANOVA reveals a main effect of PIO treatment on nuclear PPARγ and nuclear pERK (F(1,28)=50.86, P=0.001) for PPARγ (F(1,28)=42.97, P=0.0001) for pERK 1 and (F(1,28)=43.34, P= 0.0001) for pERK2. However GW 9662 administration exhibited no significant effect on either nuclear PPARγ or pERK protein expression in the Amg (F(1,28)=0.5657, P=0.4582) for PPARγ (F(1,28)=2.118, P= 0.1585) for pERK 1 and (F(1,28)=0.4109, P= 0.5306) for pERK2. Since GW 9662 does not have an effect on the Amg, but we see a restoration of cocaine seeking behavior following GW 9662 administration, this indicates that the Amg, like the NAc, may not be the primary brain region responsible for the restoration of cocaine cue reactivity in these studies.

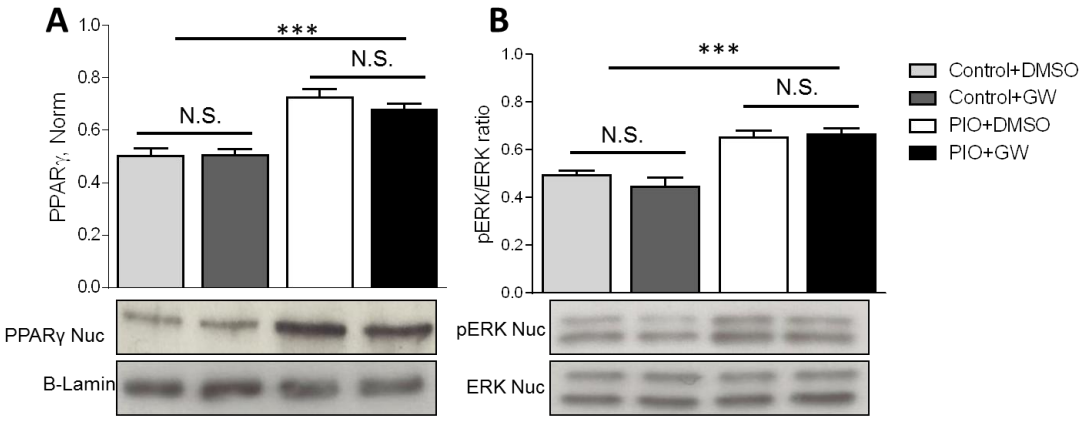

Figure 4.20 GW 9662 had no effect on nuclear PPARγ or pERK protein expression in the Amg. Rats were given either 10% DMSO or GW 9662 (1mg/kg) prior to cue test. After cue test rats were immediately sacked and brain regions sub-dissected. (**A**) GW 9662 treatment had no effect on nuclear PPARγ protein expression in the Amg Two way ANOVA F(1,28)=0.5657, P=0.4582. A main effect of PIO was noted F(1,28)=50.86, P=0.001. (**B**) GW 9662 has no effect on nuclear pERK protein expression in the Amg Two way ANOVA F(1,28)=2.118, P= 0.1585) for pERK 1 and (F(1,28)=0.4109, P= 0.5306) for pERK2. A main effect of PIO treatment was noted Two way ANOVA F(1,28)=42.97, P=0.0001) for pERK 1 and (F(1,28)=43.34, P= 0.0001) for pERK2. N.S.=not significant

GW 9662 attenuated nuclear PPARγ and pERK protein expression in the hippocampus

GW 9662 attenuated both nuclear PPARγ and pERK protein expression in the HIP following a cocaine cue reactivity test (**Fig. 4.21**). Two way ANOVA revealed a main effect of PIO treatment in preventing nuclear PPARγ and pERK attenuation in the HIP following a cocaine cue reactivity test, $F(1,28)=9.846$, $P=.0052$ for PPARγ ($F(1,28)=22.60$, $P=0.001$) for pERK 1 and ($F(1,28)=23.46$, $P=0.001$) for pERK2. We also note a statistically significant interaction effect of PPARγ agonism with PIO and GW 9662 treatment for the attenuation of nuclear PPARγ and pERK protein expression in the HIP, Two way ANOVA ($F(1,28)=9.443$, $P=0.0069$) for PPARγ ($F(1,28)=7.120$, $P= 0.0211$) for pERK 1 and ($F(1,28)=6.160$, $P= 0.0221$) for pERK2. Tukey multiple comparison test revealed that significance was driven by the Coc+PIO group in comparison to all other groups. These results indicated that PPARγ antagonism attenuates nuclear PPARγ and pERK protein expression in the HIP.

Since GW 9662 attenuated nuclear PPARγ and pERK protein expression in both the HIP and mPFC, we wanted to determine if PPARγ and pERK are in a multiprotein complex. Recent studies have discovered that pERK and PPARγ participate in a dynamic multiprotein complex and that targeting this complex with PPARγ agonism can facilitate HIP memory consolidation in a mouse model of Alzheimer's disease (Jahrling et al., 2014). Knowing that previous studies have demonstrated a pERK/PPARγ complex, and the current results demonstrating that PPARγ agonism with PIO during FA can alter pERK in multiple brain regions, we hypothesized that PPARγ and pERK are in a multiprotein complex and that this complex would be altered following GW 9662 administration and cocaine cue reactivity.

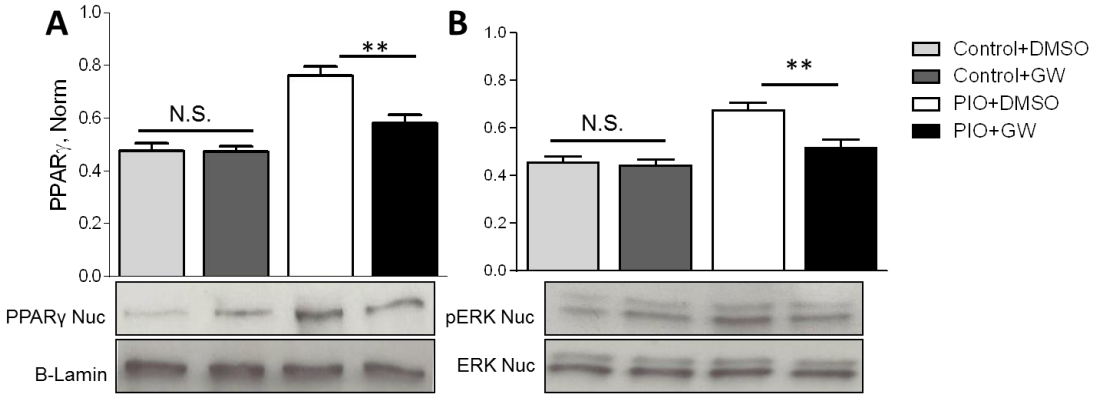

Figure 4.21 GW 9662 attenuated nuclear PPARγ and pERK protein expression in the HIP. Rats were given either 10% DMSO or GW 9662 (1mg/kg) prior to cue test. After cue test rats were immediately sacked and brain regions sub-dissected. (**A**) GW 9662 attenuated nuclear PPARγ protein expression in the HIP Two way ANOVA F(1,28)=9.443, P=0.0069). (**B**) GW 9662 treatment attenuated pERK protein expression in the HIP Two way ANOVA F(1,28)=7.120, P=0.0211) for pERK 1 and (F(1,28)=6.160, P=0.0221) for pERK2. N.S.=not significant

Nuclear PPARγ and pERK protein associate in vivo in multiprotein complexes

Nuclear mPFC samples from sham rats were washed with total PPARγ antibody and pulled down using protein A agarose beads. PPARγ and pERK can be found in the same protein complex within the nuclear fraction of mPFC (**Fig. 4.22**). Plotted are the ninety-five percent confidence bands demonstrating that PPARγ and pERK protein are detected well within the linear range between 300 to 1200 micrograms of nuclear protein sample (P=0.013, R=.9012) for PPARγ and (P=.0287, R=.8396) for pERK2 respectively. This data demonstrate that PPARγ and pERK are in a multiprotein complex and provide a direct molecular mechanism of action for the effects of PPARγ agonism on pERK. We then wanted to determine if pERK/PPARγ protein complexes were altered following GW 9662 administration and cocaine cue reactivity.

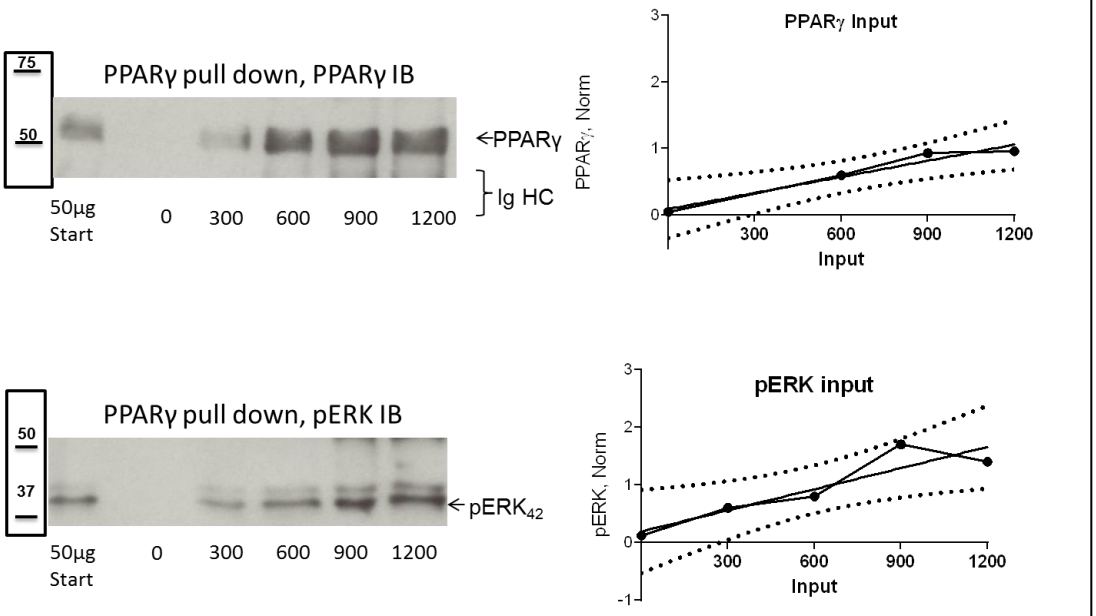

Figure 4.22 Nuclear PPARγ and pERK protein associate in vivo in multiprotein complexes. Nuclear mPFC samples from sham rats were washed with total PPARγ antibody and pulled down using protein A agarose beads. PPARγ and pERK can be found in the same protein complex within the nuclear fraction of mPFC. Plotted are the ninety-five percent confidence bands demonstrating that PPARγ and pERK protein are detected well within the linear range between 300 to 1200 micrograms of nuclear protein sample (P=0.013, R=.9012) for PPARγ and (P=.0287, R=.8396) for pERK2 respectively.

GW 9662 decreased the pERK/PPARγ protein complex in the medial prefrontal cortex and hippocampus but not the nucleus accumbens.

Utilizing the PPARy pull down assay, we show that PPARy and pERK are in a protein complex and that this complex is decreased with cocaine cue reactivity in both the HIP and mPFC (**Fig 4.23).** Specificity of this interaction was demonstrated when we note a decrease in the pERK/PPARγ complex following GW 9662 treatment (two way ANOVA F (1, 30) =11.32, P=0.0021 for mPFC and F (1, 30) =9.937, P=0.007 for HIP. Tukey multiple comparison test showed that the PIO+DMSO group had a significantly greater pERK/PPARγ complex ratio in both the mPFC F (1, 30) =4.453, P=0.0430 and HIP F (1, 30) =7.056, P=0.010 (**Fig 4.23 a, b**). We also note that GW 9662 has no

effect on the NAc pERK/PPARγ complex (two way ANOVA F (1, 28) = 0.3392, P = 0.5650). However we do note a main effect of PIO treatment on the NAc F (1, 28) = 17.07, P=0.0312 **(Fig 4.23, c).** These results demonstrated that PPARγ and pERK exist in a protein complex and pERK-PPARγ association in mPFC and HIP was attenuated by GW9662 antagonism during reversal of the behavioral effect of PPARγ agonism on cocaine cue reactivity thus giving us a molecular mechanism upon which to attribute PPARγ agonism on pERK-dependent plasticity resulting from chronic cocaine SA, abstinence, and cocaine cue reactivity. These findings support our hypothesis that PPARγ agonism attenuates cocaine seeking behavior via a pERK-dependent mechanism.

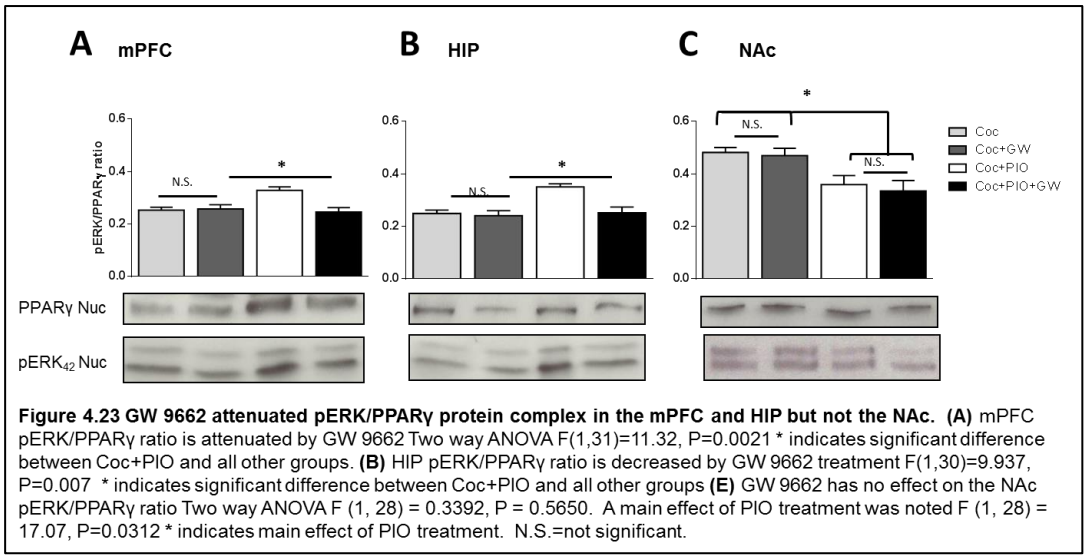

Figure 4.23 GW 9662 attenuated pERK/PPARγ protein complex in the mPFC and HIP but not the NAc. (A) mPFC pERK/PPARγ ratio is attenuated by GW 9662 Two way ANOVA F(1,31)=11.32, P=0.0021 * indicates significant difference between Coc+PIO and all other groups. **(B)** HIP pERK/PPARγ ratio is decreased by GW 9662 treatment F(1,30)=9.937, P=0.007 * indicates significant difference between Coc+PIO and all other groups **(E)** GW 9662 has no effect on the NAc pERK/PPARγ ratio Two way ANOVA F (1, 28) = 0.3392, P = 0.5650. A main effect of PIO treatment was noted F (1, 28) = 17.07, P=0.0312 * indicates main effect of PIO treatment. N.S.=not significant.

Discussion

After determining that PPARγ agonism could block the expression of locomotor sensitization we wanted to see if PPARγ or ERK proteins were altered in rats that had undergone cocaine SA and FA. Recent studies from our lab have demonstrated a significant overlap between peroxisome proliferator response element-containing PPAR target genes and ERK-regulated, cAMP response element-containing target genes (Denner, Rodriguez-Rivera *et al.* 2012). Given that there is a significant overlap between PPAR and ERK response genes, and our labs' previous data which demonstrated PPARγ agonism rescued cognitive impairment in a rodent model of Alzheimer's disease by ERK (Rodriguez-Rivera, Denner *et al.* 2011). We hypothesized that drug induced alterations in ERK, which lead to increased cocaine seeking during abstinence, could be attenuated by targeting PPARγ. Rats underwent 14 days SA and were immediately sacked following one or 30 days FA. We noted that both nuclear PPARγ and pERK are elevated in the HIP and mPFC after one but not thirty days FA (**Fig 4.2**). These data indicated that cocaine SA does disrupt both nuclear PPARγ and pERK protein expression. Other labs have shown an elevation of pERK immediately following SA indicating its key role in drug induced plasticity (Berglind *et al.*, 2007). Based on this data we decided to test the hypothesis that PPARγ agonism, when administered during an FA period, could attenuate cocaine cue reactivity.

The cue reactivity test exposes rats to cues previously associated with cocaine SA which can trigger cocaine seeking by acting as conditioned stimuli, even after an abstinence period, (Buccafusco *et al.*, 2009). PPARγ agonism during FA significantly

attenuated cocaine cue reactivity (**Fig 4.3**). It is important to note PIO treatment did not effect latency indicating that PIO treatment during FA did not effect basal locomotor activity (**Fig 4.3**). We then wanted to determine if PPARγ agonism had an effect on nuclear PPARγ or pERK.

Here we report that PPARγ agonism prevents the attenuation of nuclear pERK following a cocaine cue reactivity test (**Fig 4.4-4.8**). We noted that treatment with PIO prevents attenuation of nuclear pERK in the HIP, mPFC, and Amg of rats that underwent cocaine SA followed by FA and a cue reactivity test. Rats that underwent cocaine SA but did not undergo a cue reactivity test had nuclear pERK and PPARγ levels similar to sham rats. This indicates that the changes we note in nuclear pERK and PPARγ protein expression following a cocaine cue reactivity test are due to the re-exposure of cocaine related context and cues following a prolonged FA period. We also note that nuclear pERK protein expression negatively correlates with the number of previously active lever presses during a cocaine cue reactivity test in the COC+PIO group (**Fig 4.9 a, b**). Interestingly, pERK protein expression is positively correlated with the number of previously active lever presses during a cocaine cue reactivity test (**Fig 4.9 c**). These data indicate that upregulation of pERK protein expression in both the HIP and mPFC and a down regulation of pERK protein expression in the NAc is driving the attenuation of cocaine cue reactivity seen in the COC+PIO group. These correlational analyses justified further study of the link between pERK protein expression in these brain regions and attenuation of cocaine cue reactivity.

Once it was established that PPARγ agonism with PIO decreased cocaine cue reactivity we wanted to determine if PPARγ agonism had the same effect with cocaine

context-induced cocaine seeking as well as cocaine primed cocaine seeking. The timeline for this experiment can be seen in **Fig 4.10**. For cocaine context-induced cocaine seeking, rats were placed back into their original operant chamber and allowed to freely lever press in the absence of a discrete cocaine cues (cue light, drug infusion pump activation). PPARγ agonism attenuated cocaine context-induced cocaine seeking (**Fig. 4.10 b**). It is important to note that neither group was significantly different in terms of inactive lever pressing indicating that PIO did not affect operant behavior. PPARγ agonism also significantly attenuated cocaine primed cocaine seeking (**Fig 4.10 e**). This indicated that PPARγ agonism may attenuate reward seeking. To determine if PPARγ agonism would attenuate all reward seeking rats were subject to sucrose SA followed by a sucrose cue reactivity test. This experiment was carried out in the exact same manner as our cocaine cue reactivity test except rats received one 45mg sucrose pellet per active lever press instead of 0.75mg/kg/inf cocaine. Rats were divided into three groups 1) Sucrose+Control 2) Sucrose + PIO Acq, and 3) Sucrose + PIO FA. By including a group of rats that received PIO treatment only during the acquisition period of sucrose SA we could determine if PIO had any effect on sucrose training acquisition. PIO given during acquisition did not significantly alter the acquisition of sucrose administration (**Fig 4.11 c**). Giving PIO treatment to a separate group of rats during the FA phase effectively mirrored our cocaine cue reactivity experiment in order to determine if PPARγ agonism would attenuate sucrose cue reactivity. Here we show that PPARγ agonism had no effect on either active or inactive lever pressing during a sucrose cue reactivity test (**Fig 4.11 b, d**). Biochemistry results confirmed this showing that nuclear pERK and PPARγ protein expression was not significantly altered in the

mPFC, Hip, Amg, or NAc immediately following sucrose cue reactivity testing (**Fig 4.12-4.15)**.

PPARγ agonism significantly attenuated three separate tests for cocaine seeking behavior (cued, context, and cocaine primed). These results are unique to drug reward since PPARγ agonism had no effect on either sucrose acquisition or sucrose cue reactivity. We now wanted to determine if the behavioral effects of PIO administration were PPARγ dependent.

Thiazolidinediones have been known to have several "off target effects" that is actions which do not involve PPARγ directly. These actions include the inhibition of Acyl CoA synthetase and the induction of the proapoptotic gene proline oxidase (Pandhare *et al.,* 2006; Zhang *et al.,* 2006; Askari *et al.,* 2007). To test if the behavioral results we observed were due to a direct result of PPARγ agonism, we decided to use a potent and selective PPARγ antagonist GW-9662. We hypothesized that GW-9662 would restore cocaine cue reactivity when administered to PIO treated rats. We found that GW 9662 administration prior a cocaine cue reactivity test reverses the behavioral effects of PPARγ agonism. We also show that neither GW-9662 nor PIO had any significant effect on inactive lever pressing or latency indicating that neither GW 9662 nor PIO had any effect on basal locomotor activity (**Fig 4.17**). Since GW-9662 treatment restored cocaine cue reactivity we can infer that the attenuation of cocaine cue reactivity with PIO treatment is a direct result of PPARγ.

Utilizing a PPARy pull down assay, we also show that PPARy and pERK are in a protein complex in vivo and that this complex is attenuated with cocaine cue reactivity in

both the HIP and mPFC. Specificity of this interaction was demonstrated when we note a decrease in the pERK/PPARγ complex following GW 9662 treatment in both the HIP and mPFC (**Fig 4.23 a, b**). We also note that GW 9662 has no effect on the NAc pERK/PPARγ complex but we do note a main effect of PIO treatment (**Fig 4.23 c**). This suggests that PIO treatment during FA is preventing attenuation of a pERK/PPARγ protein complex during a cocaine cue reactivity test in the mPFC and HIP but not the NAc. This data provides a direct molecular mechanism upon which to attribute PPARγ agonism effect on pERK protein expression.

The PFC is implicated in planning complex cognitive behaviors as well as decision making and moderating correct social behavior, (Yang *et al.,* 2009). Addicts have a decrease in prefrontal cortical activity when measured at basal levels compared to control subjects (Goldstein and Volkow 2002; Matochik *et al.,* 2003). In fact withdrawal from repeated administration of psychostimulants has been shown to cause dymorphisms in the dendritic tree of pyramidal cells, the main dopaminergic output neurons for the mPFC, thus weakening PFC output to other brain regions (Robinson *et al.,* 2001). In agreement with our results, previous studies have also seen a cognitive rescue when ERK functionality is restored in the mPFC following psychostimulant administration, (Gonzalez *et al.,* 2014). It should be noted that previous work has demonstrated partially overlapping neural substrates which mediate both cued and context cocaine reinstatement behavior, of particular note was the mPFC, HIP, and Amg, (Fuchs *et al,* 2005). In these studies it was noted that inactivation of the dorsomedial PFC or dorsal HIP abolished cocaine context-induced reinstatement. Our rats underwent a period of FA in which they did not extinguish cocaine seeking behavior

unlike the previous study (Fuchs *et al,* 2005). This may contribute to our different findings since previous work has shown that extinction behavior can alter the function of brain regions when comparing them to an abstinence model, (Fuchs *et al,* 2006; LaLumiere *et al,* 2010). Likewise the Amg, in particular the basolateral amygdala (BLA) has been shown to be vital for cue-induced reinstatement and that inactivation of this brain region leads to an abolition of cue-induced cocaine reinstatement (Fuchs *et al,* 2002). However it should be noted that in our present studies we did not sub-dissect the Amg and that the different sub-regions of this brain area have been known to have alternate effects on cocaine seeking behavior, (Fuchs *et al,* 2002; Kalivas and Volkow, 2005).

The HIP is responsible for contextual relevance and is known to send glutamatergic afferents to medium spiny neurons of the NAc, (Papp *et al,* 2012). Some of the strongest inputs to the NAc arise from the HIP (Britt *et al,* 2012). In fact chronic, non-contingent cocaine injections have been shown to increase the AMPA/NMDA ratio of glutamatergic afferents from the ventral HIP to the NAc, (Britt *et al,* 2012).

Proper functionality of the NAc core depends on glutamatergic afferents from the PFC which are known to become disrupted during addiction, (Di Ciano, Cardinal *et al*. 2001). Administration of glutamate antagonists into the core have been shown to decrease both cue and drug induced reinstatement (Cornish and Kalivas, 2001; Park *et al.,* 2002). Recent studies have demonstrated that proper functionality of glutamatergic afferents from the HIP and mPFC are necessary for proper NAc regulation and that restoration of both these afferents was necessary to abolish cocaine cue reactivity (Pascoli *et al,* 2014). Therefore our studies showing a prevention of nuclear pERK

attenuation with PPARγ agonist treatment in both the mPFC and HIP being vital for a reduction of cocaine cue reactivity is comparable to current literature in the addiction field.

Chapter 5: Summary and future directions

Summary

We began our studies with a number of control experiments. We successfully demonstrated that PIO laced feed causes a significant increase in PPARγ-DNA binding following four days of consumption (**Fig 2.1**). We also note that pioglitazone (PIO) laced feed does not affect overall feed consumption in comparison to control feed nor does it cause any significant fluctuations in rat weight (**Fig 2.2-2.3**). These data indicated that our method of drug delivery was both adequate for PPARγ agonism and would not produce any basic side effects such as weight gain.

We utilized the locomotor sensitization paradigm to determine if PPARγ agonism could prevent or reverse at least one maladaptive behavior of cocaine such as hyperlocomotion. We found that PPARγ agonism prevents the induction as well as expression of locomotor sensitization (**Fig 3.2**).

Previous studies have found that PPARγ agonism prevented the expression but not the induction of locomotor sensitization (Maeda *et al,* 2007). However, these

studies had several differences with ours including choice of psychostimulant, (methamphetamine versus cocaine) and animal model, (mice versus rats) which may delineate the differential results. Based on our positive results with locomotor sensitization, we then decided to move forward with our studies utilizing the self-administration (SA) procedure, a paradigm that has more face validity with human addiction and relapse, (Panillo *et al,* 2007).

Using the SA paradigm we show that PPARγ agonism administered during forced abstinence (FA) attenuates cocaine context-induced, cue-induced, and cocaine primed cocaine seeking (**Fig 4.9**). We also note that both nuclear PPARγ and pERK protein expression are disrupted following a chronic SA and FA (**Fig 4.2**). Knowing that PPARγ and ERK are dysregulated following chronic cocaine administration, and given previous results from our lab which demonstrated that a cognitive rescue of Alzheimer's with PPARγ agonism involved ERK (Rodriguez-Rivera *et al*, 2011; Denner *et al,* 2012; Jahrling *et al,* 2014) We hypothesized that cocaine induced alterations in ERK, which lead to increased cocaine cue reactivity, could be attenuated by targeting PPARγ.

We found that PPARγ agonism prevents dysregulated nuclear pERK in several distinct brain regions (**Fig 4.4-4.8**) Furthermore, this dysregulation of pERK occurs upon re-exposure to context and cues that had been previously associated with cocaine since rats that did not undergo a cue reactivity test on day 30 of FA did not exhibit altered nuclear PPARγ or pERK protein expression (**Fig 4.8**). We also note that pERK protein expression negatively correlates with the number of previously active lever presses during a cocaine cue reactivity test in the Coc+PIO group (**Fig 4.9 a, b**). Interestingly, pERK protein expression is positively correlated with the number of previously active

lever presses during a cocaine cue reactivity test (**Fig 4.9 c**). These data indicate that upregulation of pERK protein expression in both the hippocampus (HIP) and medial prefrontal cortex (mPFC) and a down regulation of pERK protein expression in the nucleus accumbens (NAc) is driving the attenuation of cocaine cue reactivity seen in the Coc+PIO group. These correlational analyses justified further study of the link between pERK protein expression in these brain regions and attenuation of cocaine cue reactivity. These results also indicated that the attenuation of cocaine cue reactivity of our Coc+PIO group was due to a prevention of nuclear pERK attenuation in the HIP and mPFC. This is important to consider given numerous studies have shown addicts to have hypoactive cortical activity (Goldstein and Volkow 2002; Matochik *et al.,* 2003). This means that PIO treatment during FA is preventing an attenuation of cortical activity.

PPARγ agonism had no effect on sucrose cue reactivity indicating that PPARγ agonism does not affect natural reward. Biochemistry results show that nuclear pERK and PPARγ protein expression was not significantly altered in the mPFC, amygdala (Amg), HIP, or NAc following sucrose cue reactivity (**Fig 4.12-4.15)**.

Biochemical analysis revealed that GW 9662 attenuated nuclear PPARγ and pERK protein expression in the mPFC and HIP but not the Amg or NAc (**Fig 4.18-4.21**). To investigate the selective effects of GW 9662, we utilized a PPARγ pull-down assay which demonstrated that PPARγ and pERK are in the same protein complex (**Fig 4.22**). Treatment with GW 9662 prior to a cocaine cue reactivity test can attenuate this pERK/PPARγ complex in the HIP and mPFC but not the NAc (**Fig 4.23**). These data provided a direct mechanism for the effect of PPARγ agonism on pERK protein expression and suggests that preventing attenuation of pERK protein expression in the

HIP and mPFC is vital for the attenuation of cocaine cue reactivity. Given our labs previous work demonstrating a functional relationship with ERK and PPARγ in Alzheimer's, and our recent findings that PPARγ agonism prevents ERK dysregulation following cocaine cue reactivity, we propose that manipulation of PPARγ may be a novel regulator for ERK protein function.

Since drug addiction is a multi-modal dysfunction of brain reward circuitry it is not surprising that we find changes in multiple brain regions. It has been shown that increased mPFC pERK during early withdrawal leads to a decrease in cocaine seeking, (Whitfield *et al,* 2011). In a follow up to these studies, it was noted that chronic cocaine SA followed by a short withdrawal period leads to an elevation of activated striatal-enriched protein tyrosine phosphatase (STEP) but not protein phosphatase 2 A (PP2A) suggesting that STEP is responsible for the deactivation of ERK seen in previous studies (Sun, *et al.,* 2013). This is important since our studies demonstrate that pERK and PPARγ are in a multiprotein complex in vivo. It is possible that STEP is also involved in this complex allowing for rapid transition of active to inactive ERK. Previous studies have shown PP2A, another ERK phosphatase, to be in a multiprotein complex with biogenic amine transporters such as the serotonin transporter. The authors of this study suggest that this association demonstrates a dynamic stability between phosphatases, and amine transporter phosphorylation and sequestration (Bauman *et al.,* 2000). Therefore a multiprotein complex with PPARγ, pERK, and a known phosphatase of ERK such as PP2A or STEP is plausible. Still another possibility is that ERK association with PPARγ is preventing ERK dephosphorylation and allowing pERK translocation to the nucleus. In the studies presented within this dissertation we note

that pERK is elevated in the nuclear fraction of the HIP and mPFC in our Coc+PIO group immediately following a cocaine cue reactivity test. Other studies have noted that STEP dephosphorylation of ERK prevents ERK translocation to the nucleus (Zuniga *et al.,* 1999). The association of pERK with PPARγ may be preventing a deactivation of ERK via STEP, an ERK phosphatase known to be elevated in the mPFC following early withdrawal from cocaine SA (Sun *et al.,* 2013).

The data presented within this dissertation demonstrate that nuclear pERK protein expression is dysregulated in multiple brain regions immediately following a cocaine cue reactivity test and rescue of this dysregulated pERK via PPARγ agonism significantly attenuated cocaine cue reactivity. This attenuation in cocaine cue reactivity with PPARγ agonism was caused by a prevention of attenuated nuclear pERK protein expression in the mPFC and HIP. Application of a selective PPARγ antagonist demonstrated that the attenuation of cocaine cue reactivity was PPARγ dependent. Interestingly the attenuation of cocaine cue reactivity with PPARγ agonism appears to be due in large part to a prevention of attenuated nuclear pERK in the mPFC and HIP, two brain regions that are known to influence proper functionality of the NAc. Recent literature in the field of addiction has demonstrated that preventing a dysregulation of glutamatergic afferents from both the mPFC and HIP to the NAc is vital for the attenuation of cocaine cue reactivity after prolonged FA (Pascoli *et al.,* 2014). It is also important to mention that previous studies have suggested that ERK2 may act as a "gate" for the alterations in dopaminergic and glutamatergic signaling necessary for drug use to transition into addiction (Girault *et al.,* 2007). Given these findings, and the work presented within this dissertation demonstrating that prevention of nuclear pERK

attenuation in the mPFC and HIP is vital for attenuation of cocaine cue reactivity, we propose that PPARγ agonism's prevention of nuclear pERK attenuation in both the mPFC and HIP prevents disruption of pERK dependent plasticity thus maintaining proper top down signaling onto limbic structures such as the NAc thereby attenuating cocaine cue reactivity (**Fig 5.1).** Taken together, these studies demonstrate that PPARγ agonism may be a novel therapeutic target to prevent cocaine relapse in humans.

Future studies may include targeting specific brain regions with PPARγ agonism to determine which brain region, if any, is more vital for cocaine cue reactivity suppression. Since we utilized FDA approved ligands in this study, future directions may also include clinical trials in which PIO is administered to treatment seeking cocaine addicts.

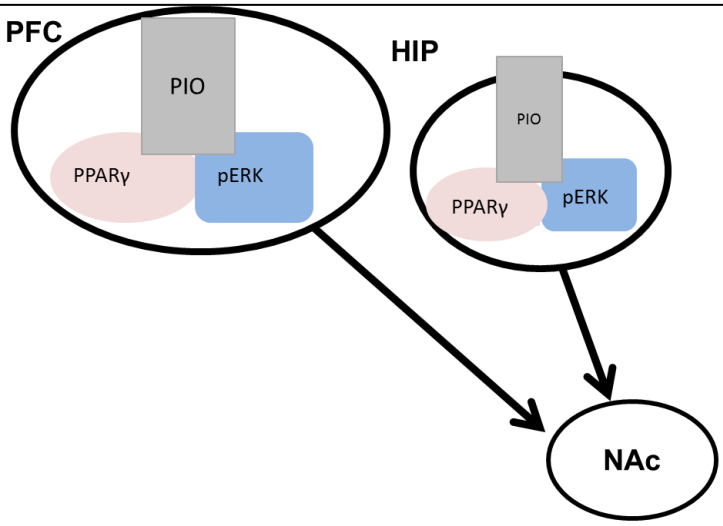

Figure 5.1 PIO prevents nuclear pERK attenuation in the mPFC and HIP.
Schematic representation of the proposed mechanism of action of PPARγ agonism. PPARγ agonist administered during FA prevent nuclear pERK attenuation in the mPFC and HIP thereby preventing pERK dependent plasticity changes observed following cocaine cue reactivity. Thus PPARγ agonism allows the mPFC and HIP to send proper top down function onto limbic brain regions such as the NAc.

Future directions

Although a recent study has demonstrated the importance of both HIP and mPFC afferents on the NAc during a cocaine cue reactivity test following FA (Pascoli *et al,* 2014), one region may be more vital for the suppression of cocaine cue reactivity with PPARγ agonism. In order to determine if either the HIP or mPFC are more vital for the suppression of cocaine cue reactivity, an experiment using targeted microinjections of GW 9662 could be performed. In this experiment rats would be trained to self-administer cocaine and undergo a period of FA with or without PIO treatment as performed previously. Just prior to cue test rats would then undergo targeted microinjections of either GW 9662 or vehicle into either the HIP or mPFC. If GW 9662 injection causes a greater restoration of cocaine seeking in either the HIP or mPFC then we can deduce that the afferents from this brain region are more vital for proper NAc function following a prolonged FA period from cocaine. Although this finding would help determine the specific brain region(s) necessary for suppression of cocaine seeking, it is highly likely that both the HIP and mPFC are necessary for the significant reduction in cocaine seeking since previous studies have demonstrated that restoration of either HIP or mPFC alone was not sufficient to attenuate cocaine cue reactivity following prolonged FA (Pascoli *et al.,* 2014). This dissertation has also shown evidence that upregulation of pERK protein expression in both the HIP and mPFC was necessary to attenuate cocaine cue reactivity (**Fig 4.9**).

Since we have successfully demonstrated that PPARγ agonism decreases cocaine seeking in at least three distinct behavioral paradigms, and since TZD's are FDA approved, the next logical step would be to establish human clinical trials. FDA

approved substances are permitted to move straight into phase two clinical trials with prove in scientific literature of their efficacious effects on a different disease model. PIO could be administered daily to treatment seeking cocaine dependent individuals. The trial would be double blind placebo controlled (with sucrose pills serving as a control) in order to eliminate bias. To demonstrate that these subjects are taking PIO and that it has little to no harmful effect on their basic metabolism, weekly blood draws would be taken to test glucose levels and liver enzymes. Urine drug screens for cocaine could also be used and tested for on a weekly basis. To determine if PIO treatment was effective, neuropsychological tests such as the cocaine Stroop or the computerized visual analog scale for craving could be used. The cocaine Stroop task is a derivative of the Stroop task which demonstrates interference in reaction time, (Stroop, 1935). In this task, words associated with cocaine such as “drug” or “dealer” are used and a subject’s reaction time or lingering on said word are measured in order to determine an attentional bias towards drug cues in comparison to neutral words (Hester *et al.,* 2006). In order to ensure that the control subjects are receiving some form of therapy to alleviate their drug addiction, cognitive behavioral therapy would be applied to all groups. Cognitive behavioral therapy (CBT) is a systematic approach taken in order to resolve subjects’ emotional dysregulations or maladaptive behaviors. This form of therapy is a blend between traditional cognitive therapy as well as behavior therapy and is often deemed “action oriented,” meaning therapists are seeking to help a subject control or maintain a certain behavioral and cognitive response, (Schacter *et al.,* 2010). This type of treatment could be used to try to prevent the maladaptive behaviors associated with cocaine abstinence and subsequent relapse. It is important to note that

according to the BCSA rats in the preceding studies took a human equivalent dose of around 60mg of Pioglitazone daily while the current FDA guidelines only allow a maximal human dose of 45mg daily. While these respective doses are very close one may not see optimal effects in a human clinical trial due to these restrictions in dosage.

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VITA

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