COCAINE-MEDIATED DISRUPTION OF RETINOID X RECEPTOR-γ SIGNALING: THE ROLE OF TNF-α

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ABSTRACT

Cocaine abuse poses a substantial health and economic burden for which no effective treatment currently exists. Exposure to cocaine results in altered signaling in a number of central nervous system (CNS) pathways. Previous studies have primarily focused on neurotransmitter systems, such as the dopaminergic and glutamatergic systems, as well as on drug-induced neuroplasticity within the mesolimbic system, which is believed to contribute to reward, addiction, and relapse following withdrawal. Furthermore, cocaine exerts a number of effects on gene regulation that contribute to many pathological conditions commonly afflicting users such as mood disturbances, psychotic symptoms, and long-term cognitive dysfunction. While some mechanisms by which cocaine regulates gene expression have been well-characterized, a large gap in our understanding regarding its downstream actions still exists and must be elucidated in order to develop effective treatment strategies.

One pathway we have discovered to be disrupted in an animal model of chronic cocaine abuse is the retinoid X receptor (RXR) signaling pathway. Retinoid X receptors serve as obligate heterodimer partners for a number of nuclear receptor transcription factors, including the thyroid hormone receptor (TR), retinoic acid receptor, vitamin D receptor, and peroxisome proliferator activated receptor. Heterodimeric complexes bind to specific recognition sequences in or around the promoter of target genes to activate, or in some cases, repress, transcriptional activity. Therefore, alterations in the levels and function of RXRs can potentially disrupt numerous signaling cascades. In this context, we observed a significant down-regulation in mRNA and protein levels of RXR-γ, an isoform predominantly expressed in the CNS that is involved in dopaminergic signaling, in brains of cocaine-administered mice. Additionally, we observed significantly decreased levels of the neuroplasticity protein, neurogranin, which is regulated transcriptionally by TR/RXR heterodimers.

Mechanisms underlying regulation of RXR levels in cells of the CNS are vastly unexplored. Studies in other organ systems, including liver and cardiac systems, demonstrate pro-inflammatory cytokines and cellular stress pathways exert repressive effects on RXR signaling, although these studies solely investigated regulation of the RXR-α isoform. Recently, studies have highlighted the role of the immune system during chronic drug abuse, and demonstrate that significant amounts of proinflammatory factors are produced in the brains of chronic cocaine abusers. Therefore, we hypothesized that cocaine-mediated induction of inflammatory cytokines, such as tumor necrosis factor (TNF)-α may contribute to decreased RXR-γ expression within the CNS. Utilizing *in vitro* neuronal systems, we have demonstrated that cocaine exposure induces neuronal expression of TNF-α and that this contributes to decreased levels of RXR-γ, as inhibition of TNF-α or its downstream effector c-Jun-NH2-terminal kinase (JNK) prevents cocaine-mediated reductions in RXR-γ protein levels. Furthermore, treatment of neurons with TNF-α alone mimics the effects on RXR-γ levels observed in cocaine-treated cells. Additionally, we show that proteasome-dependent protein degradation likely plays a role, as inhibition of the 26 S proteasome with Bortezomib during cocaine or TNF-α exposure blocks the down-regulation of RXR-γ levels. Degradation of RXR-γ in response to cocaine and TNF-α may involve nuclear export, as our results show an increased level of RXR-γ in the cytoplasmic compartment shortly after treatment, and inhibiting nuclear export during treatment with Leptomycin B prevents decreases in whole cell protein levels of RXR-γ.

In addition to the effects of chronic cocaine abuse on neurons, other CNS cell types such as oligodendrocytes may be negatively impacted by exposure to cocaine. Imaging studies and post-mortem microarray data from human cocaine abuse patients reveal loss of myelin and down-regulated expression of myelin-related genes in the nucleus accumbens and frontal cortex. Altered myelin integrity likely contributes to cognitive deficits that present in many chronic cocaine abuse patients and may also exacerbate damage to neurons. However, limited investigation has been performed to evaluate the effects of cocaine on oligodendrocyte health and function. We have employed an *in vivo* murine model of chronic cocaine administration to evaluate the impact of cocaine on white matter protein levels. Our data reveal that cocaine induces a significant decrease in white matter protein levels, even following an extended period of withdrawal, in the nucleus accumbens. One potential mechanism for cocaine-mediated white matter damage involves perturbations of glutamate homeostasis, as glutamatergic signaling can induce excitotoxicity in CNS cells, including oligodendrocytes. In this context, we found that administration of the β-lactam antibiotic, ceftriaxone, during cocaine withdrawal ameliorates loss of white matter proteins. Ceftriaxone has previously been shown to upregulate expression and activity of the glial glutamate transporter GLT-1, lending support to the theory that cocaine-mediated myelin loss may be due, in part, to disruption of glutamatergic signaling. Ceftriaxone treatment also decreased expression of cleaved caspase-3, a pro-apoptotic signaling molecule activated during excitotoxic cell death, in cocaine-administered mice.

Taken together, our studies characterize two novel consequences of cocaine exposure: (1) decreased neuronal RXR-γ expression and down-regulation of RXR-target genes, such as neurogranin, and (2) loss of myelin proteins in the nucleus accumbens which can be attenuated by administration of ceftriaxone. These findings yield insight into mechanisms underlying cocaine-mediated CNS cell death, and highlight potential treatment avenues for restoring brain health. Additionally, as inflammatory processes were identified as key mediators in some of these observations, our findings likely extend to a number of neurodegenerative diseases which are characterized by a neuroinflammatory component.

I dedicate this work to

former colleague and friend, Dr. Gladys Corley, who reminded me daily that the hardest battles are often those most worth fighting.

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3-NT	3-nitrotyrosine
4-HNE	4-hydroxy-2-nonenal
6-OHDA	6-hydroxy-dopamine
AC	Adenylyl cyclase
AD	Alzheimer's disease
AF-1	Activation function domain-1
AF-2	Activation function domain-2
AIDS	Aquired immune deficiency syndrome
ALS	Amyotrophic lateral sclerosis
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
APL	Acute promyelocytic leukemia
ATRA	All-*trans* retinoic acid
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
Bort	Bortezomib
cAMP	Cyclic adenosine monophosphate
Ceft	Ceftriaxone
CM	Conditioned Medium
CNAC	Comprehensive NeuroAIDS Center
CRABP	Cellular retinoic acid binding protein
CRBP	Cellular retinol binding protein
CREB	cAMP response element binding protein

CNS	Central nervous system
D2	Deiodinase-2 enzyme
D3	Deiodinase-3 enzyme
DAPI	4',6-diamidino-2-phenylindole
DARPP-32	Dopamine and cyclic AMP-regulated phosphoprotein molecular weight 32
DAT	Dopamine transporter
DBD	DNA binding domain
DHA	Docosahexaenoic acid
DM20	PLP alternate isoform
DR	Dopamine receptor
DTI	Diffusion tensor imaging
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
FA	Fractional anisotropy
GAP-43	Growth-associated protein-43
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLT-1	Excitatory amino acid transporter 2 (EAAT2)
HAD	HIV-associated dementia
HD	Huntington's disease
HIV	Human immunodeficiency virus
HIV-E	HIV-encephalitis
HPT	Hyptothalamic-pituitary-thyroid
IDE	Insulin-degrading enzyme
IIR	Innate immune response

IL	Interleukin
IP	Intraperitoneal or Immunoprecipitation
JNK	c-Jun-NH2-terminal kinase
LBD	Ligand binding domain
LMB	Leptomycin B
LXR	Liver X receptor
MAG	Myelin-associated glycoprotein
MBP	Myelin basic protein
MDA	Malondialdehyde
MKK	Map kinase kinase
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
N2a	Neuro-2a
NA	Nucleus accumbens
NCOR	Nuclear receptor co-repressor
Ng	Neurogranin
NGF-1B	Nerve growth factor-1B
NMDA	N-methyl-D-aspartate
OPC	Oligodendrocyte progenitor cell
PD	Parkinson’s disease
PFC	Pre-frontal cortex
PKC	Protein kinase C
PLP	Proteolipid protein
PPAR	Peroxisome proliferator activated receptor

PSD-95	Post-synaptic density-95
PTU	6-n-propylthiouracil
qRT-PCR	Quantitative real-time-polymerase chain reaction
RA	Retinoic acid
RALDH	Retinaldehyde dehydrogenase
RAR	Retinoic acid receptor
RARE	Retinoic acid responsive element
RBP	Retinol binding protein
RDH	Retinol dehydrogenase
ROS	Reactive oxygen species
RXR	Retinoid X receptor
SCH	Subclinical hypothyroidism
SMRT	Silencing mediator of retinoid and and thyroid hormone receptors
SNP	Single nucleotide polymorphism
T3	Triiodothryonine
T4	Thyroxine
TNF-α	Tumor necrosis factor-α
TR	Thyroid hormone receptor
TRE	Thyroid hormone responsive element
TSH	Thyrotropin
VAD	Vitamin A deficiency
VDR	Vitamin D receptor
VTA	Ventral tegmental area
WM	White matter

CHAPTER 1

INTRODUCTION

Effects of Acute and Chronic Cocaine Exposure on CNS Signaling

Cocaine remains among the most widely abused illicit drugs worldwide and in the United States, as a 2013 study reported an estimated 4.6% of Americans between the ages of 18 and 25 had used cocaine within the previous year [1]. Cocaine addiction represents a significant health and economic burden for which no effective treatment currently exists. All addictive drugs of abuse stimulate dopamine release from the ventral tegmental area (VTA), which projects to a number of mesocorticolimbic structures including the prefrontal cortex (PFC), nucleus accumbens (NA), amygdala, and hippocampus [2-4] (Figure 1A). The acute effects of cocaine are mediated primarily by its ability to bind to the dopamine transporter (DAT) and inhibit the reuptake of synaptic dopamine for recycling and/or degradation, leaving excess dopamine in the synaptic cleft for sustained stimulation of dopamine receptors on nearby neurons [5-7] (Figure 1B). Additionally, cocaine binds to norepinephrine and serotonin transporters to similarly prevent reuptake of these neurotransmitters, albeit with much lower affinity than that for DAT [8]. A primary role for the mesolimbic dopaminergic system in the acute reinforcing effects of cocaine has been well-established, and heightened stimulation of dopamine receptors leads to user euphoria and contributes to the drug's addictive properties [2, 9].

The effects of dopamine are mediated by two families of dopamine receptors – the dopamine 1 receptor (D1R) family (includes D1R and D5R) and the dopamine 2

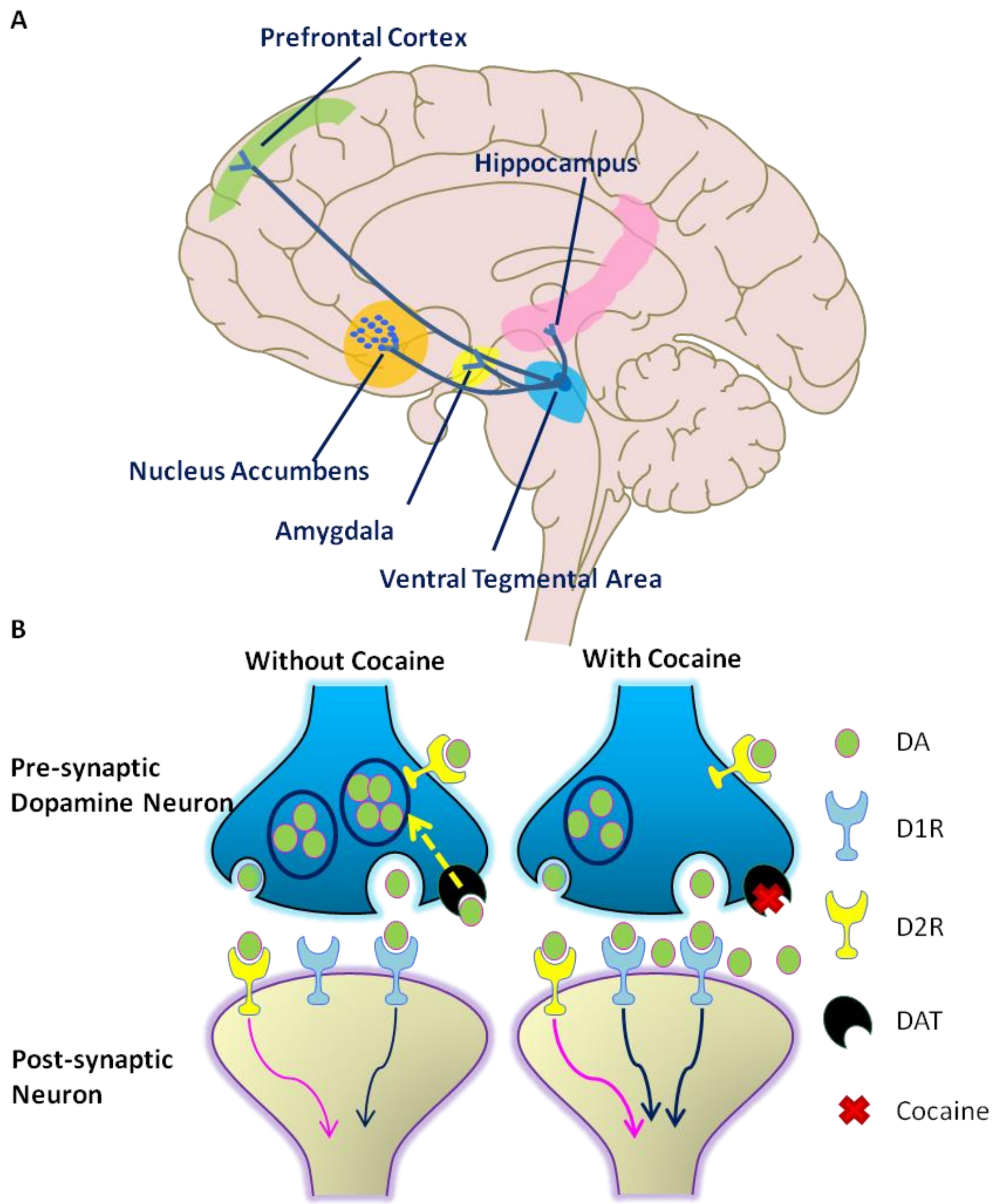


Figure 1. (A) Schematic of dopaminergic signaling within the mesocorticolimbic system. The ventral tegmental area (VTA) is activated by drugs of abuse, and sends dopaminergic projections to the amygdala, nucleus accumbens (NA), prefrontal cortex, and hippocampus. A large release of dopamine (DA) in the NA contributes to the immediate rewarding effects of cocaine. (B) Cocaine binds to the dopamine transporter (DAT) to inhibit reuptake of synaptic DA. In the absence of cocaine, following DA release, synaptic DA can stimulate post-synaptic dopamine receptors (D1R and D2Rs), be taken up by pre-synaptic D2 autoreceptors, or bind to DATs for reuptake and recycling (yellow, dashed arrow). In the presence of cocaine, the reuptake by DAT is blocked, allowing excess DA to remain in the cleft and continuously stimulate post-synaptic DA receptors, leading to amplification of DA signaling.

receptor (D2R) family (includes D2R, D3R, and D4R). Synaptic dopamine that accumulates in response to cocaine exposure primarily stimulates post-synaptic D1Rs, and both pre- and post-synaptic D2Rs. The D1R is a 7-transmembrane spanning, G protein-coupled receptor whose activation results in stimulatory signaling cascades and up-regulation of a number of regulatory molecules including adenylyl cyclase (AC), cAMP response element binding protein (CREB), and dopamine and cyclic AMP-regulated phosphoprotein molecular weight 32 (DARPP-32) [10, 11]. Dopamine 2 receptors are coupled to inhibitory G-protein pathways which can antagonize D1R signaling by directly inhibiting AC activity [12]. The immediate stimulant and rewarding effects of cocaine are thought to be primarily mediated through D1R signaling, however both receptor subtypes have been shown to contribute to reward processing, the development of addiction, and long-term behavioral changes [13-17]. Additionally, chronic exposure to cocaine is associated with a decrease in striatal D2R availability and reduced expression of D2R is correlated to increased predilection to addiction and relapse [14, 15]. Interestingly, formation of post-synaptic D1R/D2R heteromers has been observed in response to cocaine administration and these heterodimers are believed to play a role in a number of cocaine-mediated cellular events including increased intracellular calcium levels and protein kinase C (PKC) activation [18]. Both D1 and D2-type receptors are found abundantly in most areas of the reward pathway including the striatum, NA, and PFC, as well as within learning and memory centers such as the hippocampus, though D1Rs are typically found at a higher density than D2Rs within the mesolimbic dopaminergic pathway [19, 20].

Over the course of repeated cocaine administration and periods of withdrawal followed by relapse, additional neurotransmitter systems become critically important to the neuroadaptations associated with chronic drug abuse and to the formation of dependence. For instance, disruptions in glutamate homeostasis are now clearly implicated in the development of addiction and the propensity to relapse [21]. Additionally, a primary role for glutamatergic transmission in the development of behavioral sensitization in response to repeated administration of cocaine has been established, and targeting this system to restore basal signaling levels has led to decreased sensitization and diminished cocaine-seeking behavior in animal self-administration models [21-23]. Dopaminergic systems are also affected by chronic cocaine exposure, as decreased basal dopamine levels and reduced expression of D2R have been observed in the brains of human cocaine abusing patients and in animal models, even following extended periods of withdrawal [24-26]. Additionally, chronic exposure to cocaine is associated with enhanced neuroplasticity in a number of brain areas and can engage mechanisms normally involved in associative learning including induction of immediate early genes (IEGs), altered glutamate receptor subunit composition, epigenetic modification of chromatin structure, and synaptic rearrangements [27-30]. Such neuroplastic changes are believed to largely underlie the neurobiology of addiction and dependence. In this context, over the course of repeated cocaine administration, reward-predictive cues alone begin to enhance dopamine release even prior to the pharmacological effects of cocaine itself [6]. This response to environmental triggers confers extremely high abuse potential and contributes to the vulnerability to relapse in cocaine-withdrawn individuals.

Cocaine-Mediated Neuronal Dysfunction

Cocaine usage is associated with a number of pathological consequences including stroke, seizure, and brain hemorrhage; all of which can produce significant, widespread neuronal damage and even result in fatality [31]. While these consequences are readily observable and requiring of immediate attention, a number of additional brain abnormalities and dysfunctional signaling events take place less visibly over time in response to chronic drug use. Repeated or long-term exposure to cocaine affects the regulation of gene expression, which can lead to relatively stable and long-lasting changes in cells of the central nervous system (CNS). These changes are numerous and include alterations in dendrite morphology, dendritic branching, and neurotrophic support [4, 32, 33]. For instance, both acute and repeated cocaine administration have been shown to increase levels of brain-derived neurotrophic factor (BDNF) in the mesolimbic dopaminergic pathway [34, 35]. While BDNF is a critical factor for neuronal health and support in normal conditions, increased levels following cocaine exposure are believed to contribute to neuroplastic changes that underlie addiction and drug-craving during periods of withdrawal [4, 35]. Interestingly, individuals with intrinsically low levels of BDNF may exhibit increased vulnerability to the development of a drug addiction, and infusion of BDNF directly into the medial PFC or VTA can suppress cocaine-seeking behavior in withdrawn rodents [36, 37], while infusion directly into the NA appears to enhance cocaine self-administration [38].

Additional studies demonstrate that cocaine negatively regulates levels of other plasticity proteins, particularly within the hippocampus. For example, Yao *et al.* found that treatment of primary hippocampal neurons with cocaine resulted in dendritic beading and decreased levels of the plasticity-related protein Arc [39]. Studies performed in rat primary cortical cultures demonstrated decreased levels of the synaptic plasticity markers Neurogranin (Ng) and growth-associated protein-43 (GAP-43) in response to chronic stimulation of cAMP signaling by forskolin, which mimics D1R activation [40]. *In vivo*, significantly decreased hippocampal neurogenesis has been observed in adult rats exposed to chronic cocaine administration [41, 42]. Cocaine has also been shown to induce neurotoxicity in cultured neurons. For instance, studies by Dey and Snow revealed that *in vitro* treatment with a physiologically relevant dose of cocaine induced apoptosis in rodent primary locus coeruleus neurons [43]. Furthermore, studies performed in human neuronal progenitor cells demonstrate cocaine-induced oxidative damage leads to cell death [44].

Chronic cocaine abuse is associated with significant behavioral and cognitive abnormalities. Cognitive processes mediated by the PFC such as attention and executive decision-making appear to be particularly disrupted, and the severity of dysfunction correlates to duration of cocaine use [45, 46]. Imaging studies support these findings by showing decreased cerebral blood flow and metabolism in the PFC of cocaine-abusing individuals [47-50]. Chronic cocaine abuse patients also commonly exhibit learning and memory impairment, as well as some loss of motor function [51, 52]. Neuronal damage as a result of repeated cocaine exposure likely contributes to the neuropsychological impairments and behavioral alterations observed in abusing populations. Electrophysiological studies demonstrate changes in membrane potential of prefrontal cortical neurons indicative of defective cortical integration of synaptic inputs [53].

Overall, these data indicate that exposure to cocaine leads to cognitive impairment via neuronal damage and disruption of a number of signaling pathways, many of which have yet to be fully elucidated.

Cocaine-Mediated White Matter Damage

Few studies have investigated the effects of cocaine exposure on oligodendrocyte function and myelin integrity. However, data from imaging and post-mortem microarray studies indicate decreased white matter (WM) integrity and myelin gene expression in a number of brain regions in human cocaine abusing patients [54-57]. These studies suggest the PFC and NA are regions particularly vulnerable to myelin loss following chronic cocaine exposure [49, 54, 55]. Early imaging studies reported decreased frontal WM volume and MRI focal signal hyperintensities in WM of cocaine and heroin users, indicative of demyelination [56, 58, 59]. More recently, researchers have utilized diffusion tensor imaging (DTI) to evaluate WM integrity and brain connectivity in drug abuse patients. Evidence gathered from DTI studies reveals decreased fractional anisotropy (FA), a measure for the directionality of diffusion and indicator of myelin integrity, in the frontal WM of cocaine-dependent subjects [60-62]. Additionally, DTI of cocaine-treated rats demonstrated significantly decreased FA in the corpus callosum compared to saline-treated controls, which correlated to a 40% decrease in myelin basic protein (MBP) levels [63]. The mechanisms underlying cocaine-mediated myelin loss remain largely uninvestigated. Likewise, whether this loss is a direct effect of cocaine exposure or secondary to neuronal loss and/or dysfunction is currently unknown.

In spite of numerous reports of altered myelination in cocaine abuse patients and evidence that WM disruption correlates to cognitive dysfunction and behavioral abnormalities [64, 65], limited investigation of the effects of cocaine exposure on oligodendrocyte health and white matter composition in animal models has been described. Early *in vivo* studies evaluating the effects of cocaine on WM have focused on the developmental myelination period in offspring of cocaine-administered dams. For example, Wiggins *et al.* demonstrated delayed onset of myelination and decreased WM volume in rats exposed to cocaine *in utero* [66]. To our knowledge, except for the afore-mentioned DTI study by Narayana *et al.* [63], no studies to date have been published describing the effects of chronic cocaine exposure on WM levels and myelin integrity in adult animals.

Although only limited investigation of the impact of drugs of abuse on WM have been performed, studies demonstrate that perturbations in both dopamine and glutamate signaling significantly impact oligodendrocyte health and myelination capacity [67]. For example, dopamine induces caspase-3 activation and cell death in a dose-dependent manner in cultured oligodendrocyte progenitor cells (OPCs) [68, 69] and glutamate is known to be highly toxic to oligodendrocytes both *in vitro* and *in vivo* [70-72]. Oligodendrocyte processes and the myelin sheath itself appear particularly rich in *N*-methyl-d-aspartate (NMDA) glutamate receptors, which may mediate excitotoxic damage in response to elevated extracellular glutamate levels during cellular stress and/or injury [73]. Furthermore, treatment of glial cells with the D2/D3R agonist quinpirole results in an increased population of proliferating OPCs, but a decreased number of mature oligodendrocytes while treatment with the dopamine antagonist haloperidol significantly increases the number of mature, myelin-producing cells [74]. These findings suggest that dopamine may arrest OPC maturation at a progenitor-like stage, decreasing the number of mature oligodendrocytes available to myelinate axons. As cocaine is known to influence both dopaminergic and glutamatergic signaling within the CNS, cocaine-mediated alterations in neurotransmitter signaling could significantly impact oligodendroglial function.

In addition to neurotransmitter-related toxicity, oligodendrocytes are particularly vulnerable to lipid peroxidation events due to their high lipid-to-protein content and intrinsically low antioxidant defense [75]. Oligodendrocytes possess low levels of the anti-apoptotic protein Bcl2 and the antioxidant glutathione, but relatively high levels of the pro-apoptotic protein Bax, and of iron, which can catalyze the formation of hydroxyl radicals [75, 76]. These factors render oligodendrocytes highly susceptible to oxidative damage, leading to cell death and demyelination. Numerous neurodegenerative diseases including HIV-associated dementia (HAD), Alzheimer's disease (AD), and Parkinson's disease (PD) are associated with increased lipid peroxidation within the CNS [77-79]. Likewise, the demyelinating disease multiple sclerosis (MS) is characterized by increased oxidative stress and elevated levels of lipid peroxidation products, particularly within active lesions [75]. *In vivo* cocaine administration induces reactive oxygen species (ROS) production and lipid peroxidation in a number of organs including the liver and cardiac systems [80, 81]. However, few studies have evaluated cocaine-mediated lipid peroxidation in the CNS. Interestingly, the autoxidation of dopamine, such as that which occurs following drug-induced dopamine release, generates free oxygen radicals which can be toxic to myelinating cells of the CNS [82, 83]. Furthermore, exposure to oxidative stress has been shown to disrupt oligodendrocyte maturation and result in hypomyelination [83]. Potential modes of oligodendrocyte damage in response to drug abuse are depicted in Figure 2.

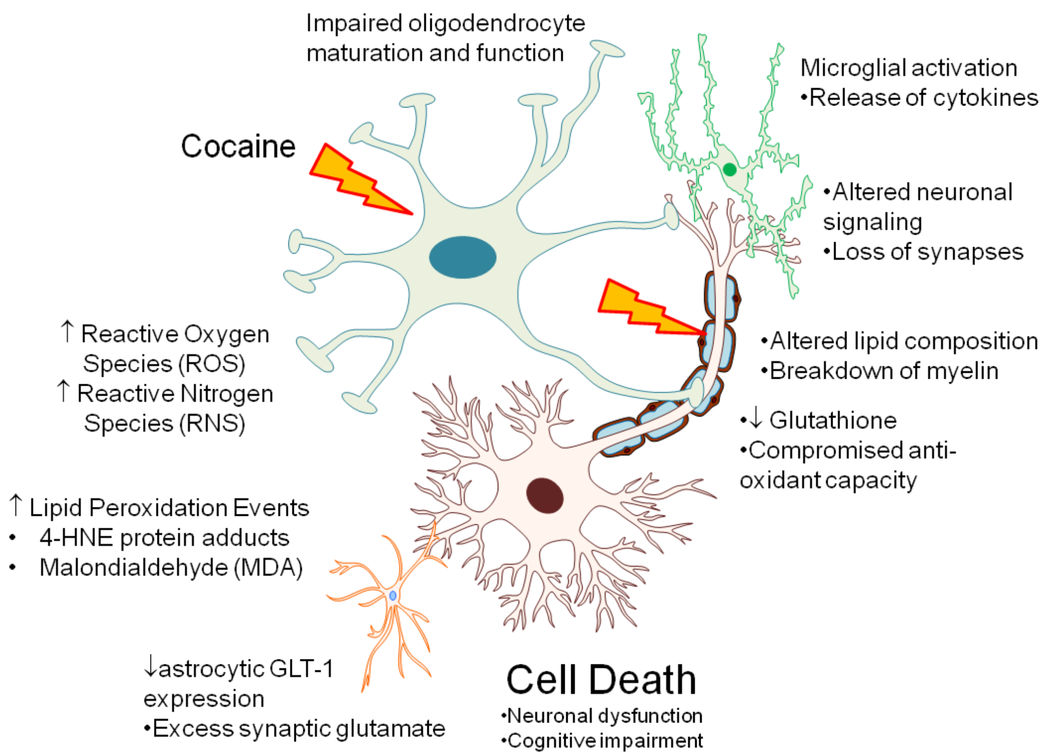


Figure 2. Mechanisms of cocaine-induced white matter loss
Schematic demonstrating potential impact of cocaine on oligodendrocyte health and subsequent myelination of axons and neuronal signaling. Cocaine induces generation of reactive oxygen and nitrogen species, and induces lipid peroxidation, producing toxic byproducts that deplete important cellular antioxidants such as glutathione. Cocaine induces a down-regulation in astrocytic excitatory amino acid transporter 2 (EAAT2; GLT-1) expression, leading to excess synaptic glutamate and excitotoxicity. Activation of microglia leads to release of pro-inflammatory cytokines which potentiate excititoxicity. These processes contribute to destruction of the myelin sheath, leading to subsequent neuronal dysfunction, loss of synapses, cell death, and ultimately cognitive impairment.

Damage to myelin can lead to impaired signal transduction and neuronal connectivity, ultimately resulting in neuronal dysfunction and potentially in axonal damage and neuronal loss. Previous studies demonstrate that chronic cocaine abuse is associated with a significant reduction in both WM and gray matter volume, particularly within the frontal cortex [61, 84]. Damage to this brain region, including loss of gray matter and loss or lesion of WM, is associated with impairments in executive decision making, attention, and impulse control, which may become critical factors in determining the propensity for relapse in cocaine-abstinent individuals [85, 86]. In fact, imaging studies indicate that the degree of myelin integrity negatively correlates to the probability of relapse in cocaine-abstinent individuals [87]. Therefore, the rationale for exploring cocaine-mediated WM loss and potential therapeutic interventions to preserve myelin integrity is compelling.

Cocaine and Neuroinflammation

Despite conflicting evidence regarding the direct neurotoxicity of cocaine, numerous *in vitro* and *in vivo* studies demonstrate that exposure to the drug depletes important cellular antioxidants such as glutathione, rendering cells more vulnerable to subsequent insults [88, 89]. Additionally, *in vitro* administration of physiologically relevant dosages of cocaine alone can induce apoptosis in primary neuronal cell culture and neuronal cell lines [43, 44, 90]. It is well-accepted that cocaine compromises the integrity of the blood-brain-barrier (BBB), allowing peripheral cytokines and chemokines to traffic into the CNS to further potentiate the inflammatory process [91-93]. For these reasons, among others, cocaine abuse represents a dangerous comorbidity for a number of pathological conditions, including HIV-1 infection, where immune cell trafficking into the CNS plays a significant role in disease progression. While the monocytes and macrophages of the periphery and the microglia of the CNS are primarily thought of in the context of inflammatory cytokine production and secretion, other cell types including astrocytes and neurons can produce and release certain inflammatory factors, such as TNF-α, as well [94, 95]. Early *in vivo* studies demonstrate TNF-α immunoreactivity in striatal neurons following surgical injury to the hippocampus and within a subset of hypothalamic neurons following systemic administration of lipopolysaccharide (LPS) [95, 96]. Additionally, it has been well-documented that activated astrocytes release a number of pro-inflammatory cytokines, including TNF-α [94, 97], and that astrocyte activation can occur in response to psychostimulant abuse [98]. Furthermore, TNF-α amplifies and prolongs the inflammatory response by inducing release of additional cytokines as well as other inflammatory mediators including ROS and nitric oxide [99].

In addition to peripheral cells trafficking to the CNS through BBB breakdown, recent studies highlight a localized inflammatory response to cocaine. Such studies have demonstrated that exposure to psychostimulant drugs including cocaine and methamphetamine induces the innate immune response (IIR) within the CNS, primarily through the activation of microglia [100]. Initiation of the IIR is associated with the release of the pro-inflammatory cytokines interleukin (IL)-1β, IL-6, and TNF-α, which can further activate the immune response and induce cell death signaling cascades [100-103]. Mechanisms by which exposure to psychostimulants activate the IIR and induce release of inflammatory cytokines are largely unexplored. One emerging hypothesis describes glutamatergic activation as a trigger for activation of microglia. It is known that cocaine exposure results in extensive glutamate release in multiple brain regions including the PFC and NA [100]. Additionally, altered glutamate homeostasis is highly associated with neurotoxicity and disruption of frontal cortical circuitry [49, 104]. TNF-α inhibits glial uptake of excess synaptic glutamate, contributing to excitotoxicity [105]. Therefore, cocaine-mediated increases in extracellular glutamate levels could be one contributing factor to the initiation of a neuroinflammatory response.

A number of *in vitro* studies demonstrate that neuronal TNF-α production is induced upon cocaine exposure. For instance, Dey and Snow reported that a physiologically relevant dose of cocaine induced cell death in primary rat locus coeruleus neurons in a TNF-α dependent manner [43]. Additionally, other studies have demonstrated induction of inflammatory gene expression in human brain endothelial cells [101], and oxidative stress leading to cell death in human neuronal progenitor cells [44, 103] following cocaine exposure. These findings could have far-reaching implications, as a growing body of evidence suggests innate immune gene expression drives addiction-like behavior and many neuropsychological comorbidities associated with chronic substance abuse.

Neuroinflammatory processes are considered integral to the neurodegeneration observed in a number of CNS diseases including, but not limited to, AD, PD, MS, and HIV-encephalitis (HIV-E) [79, 106-108]. Additionally, chronic neuroinflammation is associated with cognitive decline and psychiatric disorders such as depression and anxiety. Therefore, evidence gathered from studies examining cocaine-mediated neuroinflammation may yield relevance to a number of disease states beyond the scope of drug abuse.

Nuclear Receptor Signaling

The nuclear receptor superfamily is comprised of over 100 proteins that typically function as ligand-activated transcription factors [109]. There are multiple classes of nuclear receptors, but the two most widely studied are the class I steroid hormone receptors and the class II retinoid X receptor (RXR) heterodimers. Class I receptors bind steroids such as glucocorticoids, androgen, and estrogen in the cytosol, then translocate to the nucleus where homodimers bind DNA response elements on target genes [110]. For example, activation of the class II nuclear receptor peroxisome proliferator-activated receptor (PPAR)-α gene is regulated by glucocorticoids in this manner [111]. Class II comprises receptors for thyroid hormone, vitamin A-derived retinoic acid (RA), vitamin D3, fatty acids, and likely more ligands that have yet to be identified [112]. Class II receptors primarily reside within the nucleus and require heterodimerization with RXRs to bind DNA response elements and exert control over transcriptional regulation [109, 113] (Figure 3). Therefore, RXRs are an essential component to signaling cascades regulated by any of these ligands and their respective nuclear receptors.

When class II nuclear receptors are unliganded, they remain bound to their respective DNA response elements as part of the heterodimeric complex with RXRs. Additionally, co-repressors including nuclear receptor co-repressor 1 (NCOR) and silencing mediator of retinoid and thyroid hormone receptors (SMRT) are recruited to inactive heterodimers to form repressive complexes and maintain a state of transcriptional inhibition [114, 115] (Figure 3). Following activation by the appropriate ligand, the nuclear receptor complex undergoes a conformational change that results in dissociation of co-repressors and, in some cases, recruitment of co-activator proteins that

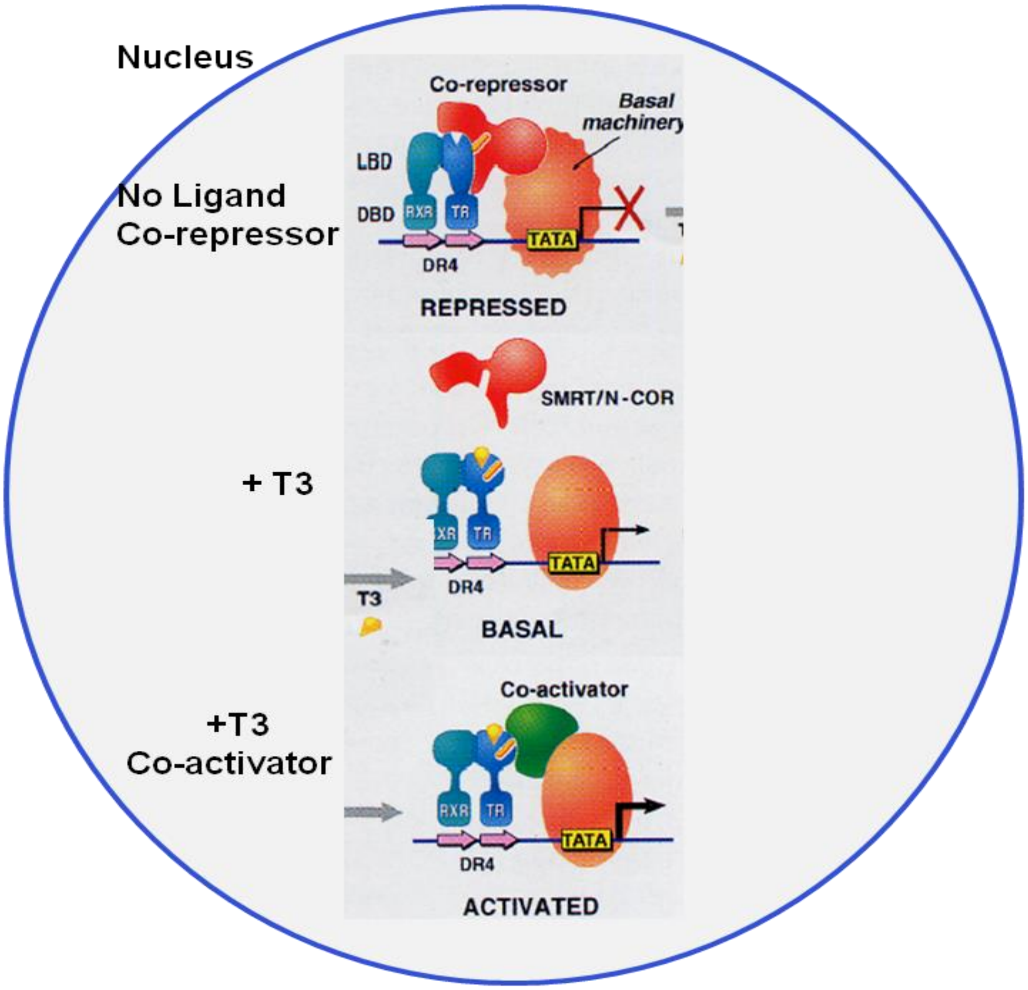


Figure 3. Nuclear TR/RXR heterodimers regulation of transcription
Schematic demonstrating de-repression of nuclear TR/RXR heterodimers by ligand (thyroid hormone; T3) binding, recruitment of co-activators, and activation of transcription of target genes. LBD, ligand binding domain; DBD, DNA binding domain; RXR, retinoid X receptor; TR, thyroid hormone receptor; DR4, direct repeat 4; SMRT, silencing mediator of retinoids and thryoid hormone; N-COR, nuclear receptor co-repressor (Adapted from [109]).

possess inherent histone acetyltransferase activity [114] (Figure 3). Following activation, mechanisms exist to terminate signaling of the nuclear receptor complex and/or induce degradation of the receptors to prevent over-activation [116]. In addition to their role as transcriptional activators, nuclear receptors can inhibit gene expression through trans-repression, which accounts for the powerful down-regulation of inflammatory gene expression by ligands of retinoic acid receptors (RARs), liver X receptors (LXRs), and PPARs [117].

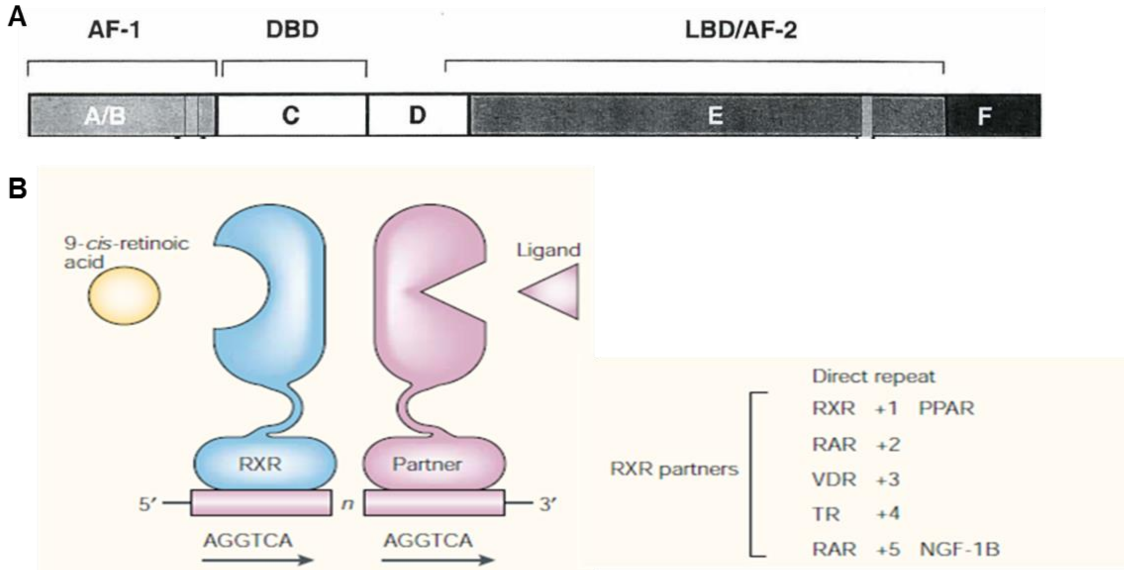


Figure 4. Structure and function of RXR heterodimers
(A) Organization and structure of RXRs includes an N-terminal ligand-independent activation domain "A/B", a DNA-binding domain "C", a hinge region "D", a ligand binding domain, and C-terminal ligand-dependent activation domain "E" (adapted from [190]). (B) Retinoid X receptors bind 9-*cis*-RA and heterodimerize with other liganded nuclear receptor partners including the RAR, VDR, TR, and RAR. A DNA direct repeat "*n*" lies between the two receptors when bound, and the number of nucleotides determines the response element. Additionally, RXRs can homodimerize, in which case $n = 1$. AF-1, activating function domain-1; DBD, DNA binding domain; LBD, ligand binding domain; AF-2, activating function domain-2; RXR, retinoid X receptor; RAR, retinoic acid receptor; VDR, vitamin D receptor; TR, thyroid hormone receptor; NGF-1B, nerve growth factor-1B (adapted from [115]).

Class II nuclear receptors are similar in structure and are composed of two activation function (AF) domains, one (AF-1) at the N-terminus is ligand-independent and the second (AF-2), at the C-terminus, is a ligand-dependent activation domain [113] (Figure 4A). Additionally, between the two activation domains, nuclear receptors contain a ligand binding domain (LBD) specific to their respective ligand, and a DNA binding domain (DBD) which recognizes specific nucleotide inverted repeats within the promoter region of target genes [115] (Figure 4, A and B). Among the RXR isoforms (α, β, and γ), the protein sequences are highly conserved except in the variable AF-1 domain [112, 118]. It is likely through this domain that differential expression and regulation among isoforms is controlled.

Regulation of Gene Expression by Retinoids

Retinoic acid signaling is thought to regulate over 500 genes directly or indirectly [119]. Retinoic acid is the biologically active form of vitamin A, which undergoes a series of oxidation steps to be converted to cellular RA. This process may be disrupted at several points. Retinoic acid is derived from dietary vitamin A and is first brought to cells of target organs via the bloodstream in the form of retinol and carried by retinol binding protein (RBP). Following transport into cells via plasma membrane receptors and transfer to cellular retinol binding protein (CRBP), retinol undergoes a number of enzymatic reactions before conversion to bio-active all-*trans* retinoic acid (ATRA). All-*trans* retinoic acid may then be transported to the nucleus by cellular retinoic acid binding protein (CRABP) to bind RARs, isomerized to 9-*cis* RA and transported to the nucleus where it can bind RXRs, stored in the form of retinyl esters, or it can be degraded into inactive metabolites by cytochrome P450 family enzymes, namely CYP26 [120]. This signaling cascade and subsequent activation of RXR-responsive genes such as the neuron-specific synaptic plasticity protein Ng are depicted in Figure 5.

In addition to binding 9-*cis* RA, RXRs have been shown to bind to certain long-chain fatty acids including docosahexaenoic acid (DHA) and arachidonic acid [121, 122].

Furthermore, DHA exerts numerous properties similar to those observed during RA administration, including anti-tumorigenic and neuroprotective effects [123-125]. Additionally, DHA is present at much higher concentrations in the CNS than 9-*cis* RA, whose *in vivo* bio-availability is subject of controversy, and therefore might mediate more RXR signaling events than initially anticipated [121, 126]. Regardless of ligand availability, RXRs are generally considered to be permissive partners for other nuclear receptors, meaning transcriptional activation of target genes does not require ligand-dependent activation of RXR in most cases so long as its heterodimer partner is bound by ligand and the co-repressor complex has been removed [127]. However, RXR activation likely enhances transcriptional regulation by heterodimeric complexes [128].

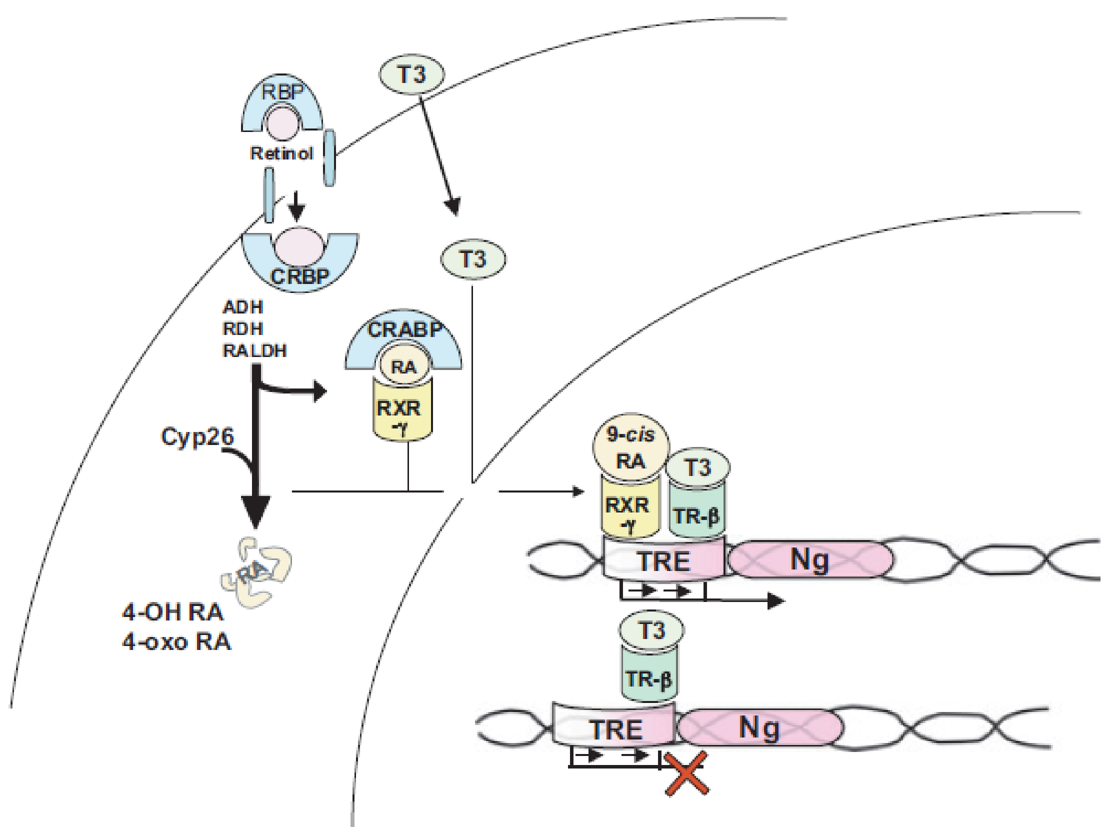


Figure 5. Thyroid Hormone and Retinoic Acid Regulate Transcription of Neurogranin through TR-β/RXR-γ Heterodimers
Retinol is transported through the bloodstream carried by RBP. Upon reaching target neurons, retinol is transported across the cell membrane and binds CRBP. Retinol then undergoes a series of dehydrogenase steps to be converted to bioactive forms including all-*trans* RA and its isomer 9-*cis* RA. At this point, RA can be degraded by CYP26 enzymes into inactive metabolites, or transported by CRABP to interact with nuclear receptors (RXR-γ in this illustration) in the cytoplasm or nucleus. Thyroid hormone is converted to bioactive T3 in astrocytes and subsequently trafficked to neurons where it can bind TR-β and heterodimerize with RXRs to form a transcriptional complex. Following heterodimer binding to TREs and ligand activation, transcriptional repression of target genes including Ng is released and transcription is initiated. Without T3 ligand or RXR, Ng expression is not induced (red 'X'). T3, triiodothryonine; RBP, retinol binding protein; CRBP, cellular retinol binding protein; CRABP, cellular retinoic acid binding protein; ADH, aldehyde dehydrogenase; RDH, retinol dehydrogenase; RALDH, retinaldehyde dehydrogenase; RA, retinoic acid; 9-*cis* RA, 9-*cis* retinoic acid; RXR-γ, retinoid X receptor-γ; TR-β, thyroid hormone nuclear receptor; TRE, thyroid hormone responsive element; Ng, neurogranin (from [238]).

Regulation of Neurogranin by Retinoid X Receptors

Neurogranin (designated RC3 in rodents) is a 78-amino-acid neuron-specific protein first identified by Watson *et al.* during subtraction hybridization studies [129]. Neurogranin is a post-synaptic, calmodulin-binding, PKC substrate that is enriched in dendrites of cortical, striatal, and hippocampal neurons and controlled at the transcriptional level by thyroid hormone receptor (TR)/RXR heterodimers [130, 131]. Neurogranin is highly conserved among species and plays a critical role in adult synaptic plasticity, as rodents with genetic deletion of the gene are viable but display severe spatial learning deficits as well as increased anxiogenic behaviors [132]. Neurogranin dysregulation is noted in a number of neurodegenerative diseases including AD and fronto-temporal dementia in which its levels are significantly decreased [133]. Furthermore, dendritic translocation of Ng mRNA has been shown to be disrupted in AD, particularly within the temporal cortex [133]. Additionally, single nucleotide polymorphisms (SNP) that are associated with the development of schizophrenia have been identified within the Ng gene [134]. Finally, Ng expression has been shown to decline over time, which is associated with the cognitive decline seen in normal ageing. In fact, administration of vitamin A or RA to aged rats increases Ng levels and subsequent neurocognitive function [135, 136].

Neurogranin is phosphorylated by PKC during LTP and function-blocking antibodies directed against Ng block LTP in rat hippocampal preparations [137]. The importance of Ng on cognitive function is highlighted in *in vivo* animal models of thyroid hormone deficiency. As the Ng gene is regulated by TR/RXR heterodimers, depletion of circulating thyroid hormone levels significantly decreases Ng levels in discrete brain regions including the striatum and hippocampus [138, 139]. Decreased Ng levels are accompanied by cognitive decline, which can be reversed by restoration of Ng levels through thyroid hormone administration [139]. Interestingly, decreased Ng is also observed in rodents in states of vitamin A deficiency (VAD). This is likely because rodents possess retinoic acid responsive elements (RAREs) within the Ng promoter in addition to TREs [138, 140]. Thus, it is debatable whether retinoid administration increases Ng levels through activation of the RARE or through activation of the RXR component of the TRE in thyroid hormone-depleted rodents. In humans, thyroid hormone deficiency during development leads to an irreversible form of mental retardation in humans termed cretinism [141], underscoring the importance of thyroid hormone in brain development and maintenance of neurocognitive function.

Although substance abuse is known to exert profound effects on synaptic plasticity within the striatocorticolimbic pathway, no previous studies have investigated the effects of drugs of abuse on Ng expression. However, one study evaluating the effects of cAMP activation in primary cortical neurons with forskolin, which mimics aspects of D1R activation, resulted in significantly reduced levels of Ng and of its pre-synaptic counterpart GAP-43 [40]. Furthermore, cocaine is known to induce an influx of calcium in many neuronal populations which could perturb normal Ng signaling, as the protein typically serves as a calcium sensor to regulate free calmodulin levels [131, 142]. Therefore, the interaction of chronic cocaine exposure and Ng expression and signaling seem worthy of investigation.

Retinoid Signaling and CNS Disease Pathogenesis

Retinoic acid signaling through RAR/RXR heterodimers is critical to a number of biological processes including cellular differentiation, embryonic development, vision, and suppression of tumor growth [143]. Retinoic acid displays powerful differentiating and anti-proliferative properties, thus can act as a teratogen to developing embryos that are exposed to higher than normal levels for their developmental phase [144]. These differentiation-inducing properties of RA have been harnessed clinically to combat a number of cancers, including squamous cell carcinomas, acute promyelocytic leukemia (APL), and some stages of neuroblastoma [145, 146]. Additionally, preliminary findings indicate retinoid therapy may be useful in treating liver, lung, breast, pancreatic, and colon carcinomas [147-150]. Decreased RA signaling and/or decreased levels of retinoic acid/retinoid receptors have been observed in number of cancers, including lung, liver, stomach, breast, and brain cancers [151-153]. Additionally, tumor cells display mechanisms to down-regulate RA signaling to evade differentiation and/or apoptosis, including upregulation of the RA-degrading enzyme CYP26A1 and epigenetic silencing of RARs and RXRs [154-156].

In addition to its implications in tumorigenesis and cancer progression, RA signaling has been shown to be disrupted in a number of human diseases. Additionally, restoration of retinoid signaling confers protection in many of these diseases. For instance, VAD in HIV+ patients is associated with a poorer prognosis and faster progression to AIDS, while RA inhibits HIV replication in monocytes/macrophages and could potentially provide antioxidative protection against viral proteins [157, 158]. Additionally, RA has been considered as a potential therapeutic in a number of autoimmune disorders including rheumatoid arthritis and inflammatory bowel disease due to its potent anti-inflammatory properties [159, 160]. Furthermore, in a mouse model of diabetic neuropathy, administration of RA increased serum and tissue levels of nerve growth factor, decreased neuropathy, and improved myelination of peripheral nerves [161]. However, until recently, RA signaling was viewed as critically important primarily during development and in maintenance of the visual system with little evaluation of RA signaling within the adult CNS.

Role of Retinoid Signaling in Neurodegenerative Disease

Over the past decade, the importance of RXR signaling in the maintenance of CNS health has started to emerge. Vitamin A/RA and ligands for other nuclear receptors such as the PPARs can exert anti-inflammatory and antioxidative properties. In this context, altered RA signaling has been described in a number of neurodegenerative diseases including amyotrophic lateral sclerosis (ALS), PD, AD, Huntington's disease (HD), and MS [162]. Additionally, targeting of RXRs and/or its nuclear receptor partners has proven to be a useful therapeutic strategy in animal models of a number of these diseases.

The importance of retinoid signaling for motoneuron health was demonstrated in a 2002 study by Corcoran *et al.*, which demonstrated motoneuron degeneration and increased astrogliosis in adult rats fed a retinoid-deficient diet [163]. These findings led to intense investigation of retinoid signaling in ALS patients, upon which microarray data revealed numerous altered genes related to the RA signaling pathway and decreased levels of RXR-γ in the spinal cords of human ALS patients [162, 164-166]. Retinoic acid has also been linked to dopaminergic neuron health and survival in a number of studies. Interestingly, dopaminergic neurons of the substantia nigra have been identified as a major source of RA synthesis in the adult brain [167]. Additionally, the D2R contains a functional RARE within its promoter [168], and inhibition of RA signaling results in decreased D2R expression and Parkinson's-like symptoms [169]. Furthermore, administration of 9-*cis* RA protects against 6-hydroxydopamine (6-OHDA)-mediated dopaminergic cell death and increases tyrosine hydroxylase immunoreactivity in both cultured rat ventromesencephalic neurons and in the striatum of adult rats [170].

Retinoid signaling now has clear implications in the pathogenesis of AD, even outside of its known effects on learning and memory. The insulin-degrading enzyme (IDE) is a key factor in the degradation of amyloid-β (Aβ) plaques and is decreased in AD. The IDE gene contains an RARE in its promoter and its expression can by induced by RA administration, clearly implicating RA as a potential therapeutic for AD [171]. Furthermore, rats fed a VAD diet developed forebrain Aβ deposits and a loss of choline acetyltransferase-expressing neurons [172]. Perhaps the most compelling evidence that retinoid signaling pathway disruption is critical to the pathogenesis of AD came from Cramer *et al.*, who demonstrated that administration of the FDA-approved RXR agonist bexarotene to APP/PSIΔE9 mice, a transgenic mouse model of AD, led to rapid Aβ clearance and reversal of cognitive deficits [173].

In addition to its potential role in neurodegenerative diseases, retinoid signaling has also been implicated in a number of psychiatric disorders. Interestingly, there is ongoing controversy regarding administration of 13-*cis* RA (Accutane) to acne patients and the development of clinical depression. Some studies describe an increased incidence of depression and suicide in Accutane patients [174], while others report no association or even a decrease in depressive symptoms [175, 176]. Animal studies describe suppressed hippocampal cell survival following administration 13-*cis* RA [177], while others provide evidence that RA is required for hippocampal neurogenesis in adult rats [178]. The discrepancy may be due to differences in the isoform (13-*cis* vs 9-*cis* RA or ATRA), as 13-*cis* RA requires isomerization to ATRA or 9-*cis* RA prior to being able to bind RARs or RXRs, and may exert off-target effects. Additionally, aberrant retinoid signaling has also been implicated in schizophrenia, as vitamin A deprivation during development in mice can lead to forebrain dysfunction and aberrant dopaminergic signaling resembling that seen in schizophrenic patients [179].

Investigation into retinoid therapies in the treatment of neurodegenerative diseases is still in its infancy, and identification of genetic and environmental factors that contribute to RA signaling dysfunction will likely exert profound effects on our understanding of mechanisms regulating retinoid signaling within the CNS.

Implications for RXR in Demyelinating Disease

The importance of RXR signaling has been identified not only in neurons, but in other CNS cell types, as well. Similarly to Ng, the oligodendrocyte specific gene myelin basic protein (MBP) contains a TRE within its promoter and is responsive to both thyroid hormone and the RXR ligand 9-*cis* RA [180]. Additionally, manipulation of RXR signaling and/or that of its nuclear receptor partners exerts neuroprotection in models of demyelinating diseases such as MS [181, 182]. Administration of 9-*cis* RA to experimental autoimmune encephalomyelitis (EAE) mice, a commonly used animal model of MS, at the onset of clinical disease reduced disease severity [183]. Furthermore, treatment was even more effective when administered in combination with a PPAR-γ agonist [183]. Ligands for both PPARs and LXRs have also been shown to exert protective and anti-inflammatory effects in EAE [184]. Furthermore, the RXR-γ isoform specifically was identified as a key mediator of oligodendrocyte maturation and remyelination capacity following WM lesion [182]. In spite of these findings, the role of nuclear receptor signaling in myelination and/or demyelinating disease remains relatively unexplored. Furthermore, whether nuclear receptor activation promotes protection in animal models of demyelinating disease primarily through repression of inflammatory processes or through activation of myelin-associated genes remains to be determined.

Effect of Inflammatory Mediators on RXR Expression and Function

Exposure to cocaine induces expression of inflammatory factors, including TNF-α, in both *in vitro* systems and *in vivo* [43, 91, 103, 185]. Induction of inflammatory signaling cascades results in activation of MAP kinase kinases (MKKs) MKK4 and MKK7, which respond to environmental stressors, and in turn activate c-Jun-NH(2)-terminal kinase (JNK) via phosphorylation of Thr183 and Tyr185 [186-188]. Phosphorylated JNK activates a number of pathways through its kinase activity, most notably the c-jun/AP-1 pathway which regulates transcription of a number of genes involved in cellular processes such as apoptosis, differentiation, cell cycle and inflammatory response [188, 189]. More recently, it has been demonstrated that JNK phosphorylates specific nuclear receptor transcription factors, which may impact their function, DNA-binding activity, and/or degradation [188, 190-193]. Among the nuclear receptors identified as substrates for phospho-JNK (P-JNK), RXR-α has been demonstrated to be phosphorylated at multiple residues in hepatocytes and COS-1 cells following treatment with pro-inflammatory cytokines such as IL-1β or activation of stress pathways through UV irradiation [190, 193]. Additionally, treatment with TNF-α or IL-1 decreases the levels of a number of nuclear receptors, including RXR-α, PPAR-α, and PPAR-γ in liver cells *in vitro* through an uncharacterized mechanism [194]. The IL-1β-induced reduction in liver RXR-α levels was demonstrated *in vivo*, as well [195]. Despite mounting evidence for a critical role of RXRs in maintaining neuronal health and neurocognitive function, very little literature exists on the regulation of these receptors in response to cellular stress within the CNS. Similarly, very little is known about the regulation of the alternative RXR isoforms, RXR-β and RXR-γ, which share high sequence homology to RXR-α, but differ in their N-terminal activation domains as well as in their tissue-specific expression patterns [116, 118].

Hypothesis and Proposed Aims of the Study

Despite the high prevalence of cocaine abuse and associated known health risks, no effective therapies currently exist to enhance rehabilitation and prevent relapse and long-term CNS damage in recovering users. Cocaine is known to disrupt a number of signaling pathways, due at least in part, to its regulation of specific neurotransmitter systems including the dopaminergic and glutamatergic systems. Additionally, previous studies by Langford *et al.* identified increased incidence of thyroid hormone abnormalities in a cohort of cocaine-abusing HIV+ individuals [196], raising the question of whether cocaine, HIV, or a combination of both mediates hypothalamic-pituitary-thyroid (HPT) axis dysfunction. Thyroid hormone serves as a transcriptional regulator of a number of genes within the CNS, including Ng, a neuron-specific PKC substrate required for adult neuroplasticity, as well as MBP, an oligodendrocyte-specific gene required for oligodendrocyte maturation and myelin production. Thus, disruptions in HPT signaling that ultimately lead to altered thyroid hormone levels could exert profound effects on transcriptional regulation of a number of genes across multiple CNS cell types.

Following cocaine exposure, neuroplastic adaptations are known to occur in both the striatum, a key site of action for drugs of abuse, as well as in brain regions receiving striatal projections such as the PFC and the hippocampus. Neurogranin is highly enriched in brain regions implicated during drug abuse, where it is known to play an integral role in the regulation of post-synaptic calcium signaling, and ultimately in synaptic plasticity in the adult brain. Therefore, we will evaluate the effects of cocaine on HPT signaling pathways, and will assess alterations in the pathway through analysis of downstream target genes, primarily Ng. Neurogranin is down-regulated in states of thyroid hormone deficiency, as well as in a number of neurocognitive diseases. We hypothesize that cocaine-mediated disruptions in HPT signaling and or TR/RXR nuclear receptor signaling will lead to alterations in Ng expression. Dysregulation of Ng expression may in turn contribute to cocaine-induced neuronal dysfunction and cognitive impairment.

To first investigate the effects of cocaine on brain levels of Ng, we will employ a murine model of chronic cocaine abuse. Following a repeated administration paradigm, brains will be harvested and assessed for regional expression of Ng. Additionally, to evaluate potential impact of cocaine on HPT function, we will assess levels of circulating thyroid hormone and of proteins involved in neuronal thyroid hormone processing. Additionally, as heterodimerization with RXR is required for TR to exert transcriptional regulation, we will evaluate levels of key factors involved in retinoid signaling cascades, including RXRs and enzymes involved in the metabolism of cellular RA.

Though neurons are a key target of cocaine's actions in the brain, other cell types including astrocytes and microglia are known to be impacted by chronic exposure to psychostimulant drugs. Therefore, to evaluate specific findings from our *in vivo* studies in a cell-specific context, we will utilize neuronal cell culture systems to further characterize changes in TR/RXR signaling and subsequent regulation of downstream target genes. Cocaine is known to induce immune-modulatory effects such as increasing production of pro-inflammatory cytokines in humans, animal models, and *in vitro*. As cellular stress cascades that activate kinases, particularly JNK, can impact the levels and function of nuclear receptors, we will assess whether any observed changes in TR/RXR signaling might be due to induction of cytokine expression and subsequent activation of JNK. Based on these results, we will employ pharmacological inhibitors of specific cytokines, and of the downstream cellular stress response to determine whether cocaine-induced alterations in TR/RXR signaling can be prevented and/or reversed.

Findings from these studies will characterize the effects of cocaine on TR/RXR signaling, which could prove to be a novel pathway disrupted by cocaine abuse not previously investigated in the extant literature. As TR/RXR signaling, and that of other RXR heterodimers, has recently been discovered to exert profound effects on CNS health and function both during development and in adulthood, our studies will highlight a potential mechanism for cocaine-mediated dysregulation of a large number of genes. For instance, over 500 genes have been identified as potential targets of retinoid signaling, including the neuronal plasticity-related proteins Ng and GAP-43, as well as MBP, the major protein constituent of the myelin sheath [119]. Additionally, as this pathway is implicated in numerous neurocognitive disruptions, it could be a clinically useful pathway to target in treatment of substance abuse and associated neurocognitive disorders.

Of the numerous harmful effects cocaine has on cells of the CNS, little attention has been paid to its impact on oligodendrocyte health and function. Yet, a number of clinical studies demonstrate significantly reduced WM in cocaine abusing patients. Furthermore, similarly to Ng, transcriptional activation of MBP can by induced by activation of TR/RXR heterodimers, and MBP levels are sensitive to bioavailable levels of both thyroid hormone and retinoids. Therefore, we have selected to evaluate cocaine-induced WM loss in the context of an *in vivo* murine model of chronic abuse. We hypothesize that chronic exposure to cocaine damages oligodendrocytes in specific brain regions such as the NA, which results in loss of WM proteins and demyelination. Additionally, MBP levels may be impacted by cocaine-mediated alterations in TR/RXR signaling. We will investigate levels of major myelin proteins such as MBP, proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), and myelin-associated glycoprotein (MAG) in the NA following a chronic cocaine abuse paradigm, and in the context of an extended period of withdrawal. Furthermore, we will investigate potential contributors to myelin loss, such as oligodendrocyte death and lipid peroxidation events in response to cocaine both in our *in vivo* model and in an oligodendrocyte progenitor cell culture system. Finally, we will evaluate whether cocaine-mediated effects on WM could be due, in part, to disruptions in glutamate homeostasis by administering the β-lactam antibiotic, Ceftriaxone, a known activator of the major glial transporter GLT-1, during cocaine administration or during withdrawal.

These studies will help define whether demyelination seen in cocaine-abusing human patients is a direct result of cocaine abuse, or a result of potential comorbid conditions and/or polydrug abuse that characterizes a number of patient populations. *In* *vivo* studies are required to determine direct effects of cocaine on oligodendrocytes and myelin integrity while limiting extraneous environmental and genetic variables. Our *in vitro* studies will address potential mechanisms of oligodendrocyte damage in response to drug. Furthermore, these aims will help characterize potential therapeutic targets for rehabilitation of cocaine abstinent individuals, as myelin integrity was a recently identified prognosticator of propensity to relapse.

Overall, our aims of investigating cocaine-mediated disruptions in nuclear receptor signaling, including effects on target neuroplasticity-related genes, and in myelin integrity highlight two previously unexplored areas of research. Furthermore, findings from these studies may ultimately aid the development of suitable therapeutics for the treatment of cocaine abuse and/or cocaine-associated neurocognitive impairments. As current therapies targeting systems more traditionally associated with substance abuse have not proven to be clinically useful, our studies may highlight potential treatment avenues that would not have previously been considered. Furthermore, as cocaine induces numerous cellular stress cascades, our findings will likely be applicable to cellular stress events in general, such as those which occur during neurodegenerative disease processes.

CHAPTER 2

MATERIALS AND METHODS

Animals

Ten-week old C57BL/6 male mice (Charles River Laboratories, Wilmington, MA) were utilized for all studies. All procedures were approved and conducted in accordance with Temple University IACUC guidelines. Mice were singly housed in an animal facility with constant airflow, controlled temperature (21–23^{o}C) on a 12-h light/dark cycle and supplied with food and water *ad libitum*.

In Vivo Drug Treatment

Cocaine hydrochloride (Sigma-Aldrich, St. Louis, MO) was dissolved in sterile saline and administered at 15 mg/kg by intraperitoneal (IP) injection. Mice receiving 6-*n*-propyl-2-thiouracyl-P (PTU) (Harlan Teklan, Madison, WI) were fed a low iodine diet supplemented with 0.15% PTU (hypothyroid) or 0.075% PTU (subclinical hypothyroid (SCH)). Ceftriaxone sodium (American Regent Inc., Shirley, NY) was dissolved in sterile water and administered (200 mg/kg, IP). Injection volumes were 1 ml/100 g body weight. Groups and treatment schedules are listed in Table 1.

Stereotypic Behavior Testing

Stereotypic behavioral testing was conducted on mice from selected groups (Table 1). Immediately following a challenge injection of saline or cocaine (15 mg/kg, IP) at the end of each cocaine administration paradigm, mice were observed for 1 min at

15, 30, 45, and 60 min. A '0' time reading served as the baseline measure taken 1 min

Table 1. Mouse Treatment Groups for *in Vivo* Experiments

Group (number of mice)	Treatment (n days)	Days of Withdrawal (Veh or Ceft)	Cocaine Challenge (Y/N)	Stereotypic Behavior Analyses (Y/N)
1. Control (n=6)	Veh (14 d)	2 d (Veh)	N	Y
2. Coc (n=6)	Coc (14 d)	0 d	N	N
3. Cocaine(n=6)	Coc (14 d)	2 d (Veh)	Y	Y
4. Hypo (n=6)	PTU + Veh (14 d)	2 d (Veh)	N	Y
5. SCH (n=6)	½ PTU + Veh (14 d)	2 d (Veh)	N	Y
6. Hypo+Coc (n=6)	PTU + Coc (14 d)	2 d (Veh)	Y	Y
7. SCH+Coc (n=6)	½ PTU + Coc (14 d)	2 d(Veh)	Y	Y
8. Control (n=10)	Veh (14 d)	30 d (Veh)	Y	Y
9. Ceft (n=9)	Ceft (17 d)	30 d (Ceft)	Y	Y
10. Coc (n=8)	Coc (14 d)	30 d (Veh)	Y	Y
11. Coc with Ceft (n=8)	Ceft (17 d)+Coc (14 d)	30 d (Veh)	Y	Y
12. Coc then Ceft (n=8)	Coc (14 d)	30 d (Ceft)	Y	Y
13. Control (n=4)	Veh (10 d)	0 d	N	N
14. Coc (n=4)	Coc (5 d)	0 d	N	N
15. Coc (n=4)	Coc (7 d)	0 d	N	N
16. Coc (n=4)	Coc (10 d)	0 d	N	N
17. Coc (n=4)	Coc (14 d)	0d	N	N

Coc, cocaine; Hypo, hypothyroid; SCH, subclinical hypothyroid; Ceft, ceftriaxone; Veh, vehicle; PTU, *n*-propyl-2-thiouracyl-P

before saline or cocaine injection. Stereotypic behavior was scored based on a modified rating scale as previously described [197-199]. The scoring scale consisted of the following rating system: (i) sleeping, inactive, (ii) alert, grooming, (iii) increased sniffing, (iv) intermittent rearing and sniffing, (v) increased locomotion, (vi) intense sniffing in one location, (vii) continuous sniffing and pivoting, (viii) continuous rearing and sniffing,

(ix) maintained rearing and sniffing, (x) splayed hind limbs. The scores for all time points were plotted and the stereotypic behavior was assessed by taking the area under the curve and by the average total activity at each time point.

Novel Object Recognition Task

To test the effect of cocaine and ceftriaxone treatments on working memory, a novel object recognition task was employed as previously described [200]. Briefly, mice were acclimated to a test box (same dimensions as their home cage with fresh bedding) for 15 minutes and then placed back into their home cages. Trial 1 consisted of object familiarization. Two identical objects were placed in mirror image fashion in the test box and mice were allowed to explore the objects for a total of 5 minutes before being placed back into their home cages. A one-hour inter-trial interval was selected, so Trial 2 was conducted 1 h after completion of Trial 1. Trial 2 consisted of removing one of the familiar objects and replacing it with a novel object. Mice were again allowed to explore freely for 5 min. The times spent exploring each object for both Trial 1 and Trial 2 were recorded by a blinded observer. To determine the degree of novel object recognition, the percentage of time spent exploring the novel object in Trial 2 out of total object exploration time was determined. Objects were randomly assigned and both orientation and which objects were selected to be familiar or novel were varied across subjects. All mice were tested individually and assigned to their own test cage. Bedding was not replaced or disturbed between trials.

Serum ELISA of Circulating Thyroid Hormone and Retinol Levels

Serum from mice receiving vehicle or 14 days of cocaine administration was obtained by ocular blood draw prior to euthanasia. Triiodothryonine (T3) and thyroxine (T4) levels were measured by enzyme-linked immunosorbent assay (ELISA) according to manufacturers' instructions (Calbiotech, Spring Valley, CA; and Alpha Diagnostic International, San Antonio, TX, respectively). Twenty-five microliters of each serum sample was measured in duplicate from each mouse and T3 and T4 levels were assessed by absorbance at 450 nm.

Serum from mice receiving vehicle or 7 days of cocaine administration was obtained by ocular blood draw prior to euthanasia. Retinol binding protein 4 (RBP4) levels were measured by ELISA according to manufacturers' instructions (Adipogen, San Diego, CA) as a measure of serum retinol levels [201]. Twenty-five microliters of each serum sample (diluted 1:10,000) was measured in duplicate from each mouse and RBP4 levels were assessed by absorbance at 450 nm. For all ELISA tests, samples and standards of known concentrations were assessed. Values were obtained and compared to the standard curve to extrapolate sample T3, T4, or RBP4 concentrations.

Tissue Harvest and Rodent Brain Dissection

Animals were heavily anesthetized with 5% isoflurane and decapitated. Brains were removed and placed into ice-cold phosphate-buffered saline (PBS). The hippocampus was dissected from each brain so that one hemisphere was used for protein analysis and the other for RNA analysis. Tissues for protein analysis were stored at -80^{o}C, and those for RNA analysis were placed in RNAlater© stabilization solution

(Qiagen, Valencia, CA, USA) and stored at -20^{o}C. For immunohistochemical analysis, whole brains from mice receiving cocaine and vehicle injections for 10 days were fixed in 10% buffered formalin for 24 h and processed for immunolabeling by standard paraffin-embedding and sectioning. Brains from control mice and those receiving cocaine for 7 days were similarly removed and the hippocampi dissected out and stored at -80^{o}C until malondialdehyde (MDA) content analysis was performed.

Western Blotting

Equal amounts of protein were loaded (20 μg per lane unless otherwise indicated) onto pre-cast midi-gels (4–12% Bis–Tris; Invitrogen, Carlsbad, CA), separated by electrophoresis and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were blocked in 5% non-fat milk or bovine serum albumin (BSA) in Tris-buffered saline, 0.1% Tween-20 (TBST) for 30 min before incubation with primary antibodies. Primary antibodies included: Ng (1:10,000; Upstate Biotechnology, Lake Placid, NY), D2 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), D3 (1:1,000; Novus, Littleton, CO), TR1β (1:1,000; Santa Cruz), Cyp26A1 (1:1,000; Abcam), RXR-γ (1:1,000; Abcam), phospho-JNK (1:1,000; Cell Signaling, Danvers, MA), total JNK (1:1,000; Cell Signaling), phospho-serine (1:500; Sigma-Aldrich), MBP (1:5,000; Millipore, Danvers, MA), MOG (1:1,000; Abcam), MAG (1:2,000; Santa Cruz), PLP/DM20 (1:2,000, Abcam), GLT1 (1:20,000; Millipore), cleaved caspase-3 (1:500; Millipore), 4-hydroxy-2-nonenal (4-HNE) (1:1,000; Abcam), Bax (1:2,000; Cell Signaling), ubiquitin (1:1,000; Invitrogen), and the loading controls tubulin (1:5,000; Sigma-Aldrich), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:4,000; Santa

Cruz), Histone H1 (1:750; Millipore), and Lamin A/C (1:1,000; Cell Signaling). Membranes were incubated for either 2 h at 23°C or overnight at 4°C, washed in 1× TBST, incubated with appropriate secondary anti-mouse or -rabbit antibodies (1:10,000; Thermo Scientific, Waltham, MA) for 1 h, and developed with ECL Prime (Amersham Biosciences, Piscataway, NJ). Band intensities were calculated using ImageJ software and normalized to the loading control [202].

RNA Extraction and qRT-PCR Analysis

Total RNA was extracted using the Qiagen RNeasy® kit according to manufacturers' instructions (Qiagen). Complementary DNA (cDNA) was generated using the iScript™ cDNA synthesis kit following the manufacturer's protocol (Bio-Rad). Generation of cDNA product was verified using primers for the housekeeping genes riboprotein (L13A) or GAPDH. The PCR reaction was performed using a 96-well thermocycler and a standard amplification program. PCR products were separated through 1% agarose gel and visualized under UV light. Once a quality PCR product was confirmed for all samples, quantitative real-time-polymerase chain reaction (qRT-PCR) analyses of Ng, TRβ1, RXR-γ, and TNF-α were performed. Riboprotein (L13A) and GAPDH were used as housekeeping genes for qRT-PCR normalization. All primer sequences utilized are listed in Table 2. Reaction mixtures for qRT-PCR consisted of LightCycler® 480 SYBR Green I Master Mix (Bio-Rad), forward and reverse primers, 2 μL of cDNA (diluted 1 : 10) and H_2O to bring the reaction volume up to 20 μL. Samples were placed in a 96-well plate and the qRT-PCR was performed on a Roche LightCycler® 480 (Roche, Indianapolis, IN). All reactions were performed in triplicate and amplification curves and C_p values were obtained for analysis. Results were normalized against riboprotein or GAPDH using the $\Delta\Delta C_t$ quantification method and expressed as mean ± SEM.

Table 2. Primer Sequences Utilized

Gene	Accession Number	Forward Primer	Reverse Primer
Mo. Ng	NM_022029.2	5-CACCCAGCATCGACAAA-3	5-CGCTCTTTATCTTCTTCCTC-3
Mo. TRβ1	NM_001113417.1	5-ATCAAGACAGTCACTGAGGC-3	5-GGGCATTCACAATGGGTGCTT-3
Mo. TRα1	NM_178060.3	5-GACTGACCTCCGCATGATCG-3	5-CCTGATCCTCAAAGACCTCC-3
Mo./Hu. RXR-γ	NM_009107	5-AGGCAGGTTTGCCAAGCTTCTG-3	5-GGAGTGTCTCCAATGAGCTTGA-3
Mo. Riboprotein	NM_009438	5-CCTGCTGCTCTCAAGGTTGTT-3	5-CGATAGTGCATCTTGGCCTTT-3
Hu. TNF-α	NM_000594.3	5′-CTGGGCAGGTCTACTTTGGG-3′	5′-CTGGAGGCCCCAGTTTGAAT-3′
Hu. GAPDH	NM_002046.4	5’-GTCTCCTCTGACTTCAACAGC-3’	5’-ACCACCCTGTTGCTGTAGCCAA-3’

Mo. mouse: Hu.

Immunofluorescence Labeling of Paraffin-Embedded Tissue

Coronal sections (4 μm thick) of formalin-fixed, paraffin-embedded tissues were placed on electromagnetically charged glass slides. Slides were deparaffinized in xylene and rehydrated through descending grades of ethanol up to water. After non-enzymatic antigen retrieval in 0.01 M sodium citrate buffer (pH 6.0) for 30 min at 97°C in a vacuum oven, slides were washed with 1× PBS and placed in blocking solution (5% normal goat or horse serum; Vector Laboratories, Burlingame, CA, USA) for 2 h. Primary antibodies consisted of Ng (1:200; Upstate), RXR-γ (1:100; Abcam), CRBPI (1:200; Santa Cruz), MBP (1:500; Millipore), neurofilament (1:200; Covance, Princeton, NJ), CC1 (1:50; Abcam), cleaved caspase-3 (1:50; Millipore), synaptophysin (1:200; Abcam), post-synaptic density-95 (PSD-95) (1:200; Abcam), 4-HNE (1:200; Abcam), 3-nitrotyrosine

(3-NT) (1:200; Abcam), Iba1 (1:200; Abcam), and SMI 32 (Covance) for hypo-phosphorylated NF. Sections were incubated with primary antibody overnight in a dark, humidified chamber at 23°C, rinsed 3× with PBS, and incubated with fluorescein isothiocyanate (FITC) (1:500; Vector Laboratories, Burlingame, CA) or Texas Red (1:500; Vector Laboratories)-conjugated secondary antibodies for 1 h at 23°C in the dark. Sections were again washed 3× with PBS, cover-slipped with an aqueous based mounting medium containing DAPI for nuclear labeling (Vectashield; Vector Laboratories), visualized with a Nikon ultraviolet inverted microscope, and processed with deconvolution software (Slidebook 4.0; Intelligent Imaging, Denver, CO, USA). Deconvolution was performed using SlideBook4 software, allowing acquisition of multiple 0.25 μm thick digital sections and 3-D reconstruction of the image.

Lipid Peroxidation Assay

The hippocampal malondialdehyde (MDA) content of mice receiving vehicle and cocaine injections for 7 days was assessed using a lipid peroxidation (MDA) assay kit according to manufacturers' instructions (Abcam). Briefly, 10 mg of hippocampal tissue from each brain was homogenized by mechanical dounce disruption on ice in 300 μL of MDA lysis buffer and centrifuged to remove insoluble material. Two-hundred microliters of supernatant from each sample was collected and added to 600 μL of thiobarbituric acid (TBA). Samples were heated for 60 min at 95^{o}C, cooled for 10 min on ice, and then analyzed for formation of MDA-TBA adducts colorimetrically at 532 nm. A standard curve was generated using serial dilutions of MDA standard, and the

MDA content in nmol/mg of tissue for each sample was calculated through interpolation of the standard curve.

Cell Culture

SH-SY5Y neuroblastoma cells (ATCC, Manassas, VA) were maintained in a 1:1 ratio of Dulbecco's Modified Eagle Medium: Nutrient Mixture F12 (DMEM/F12) (Invitrogen) containing 10% fetal bovine serum (FBS) (Thermo-Scientific). Twenty-four hours following initial plating, medium was replaced with neurobasal medium containing Glutamax and B27 supplement (Invitrogen) to induce a neuron-like phenotype [203, 204]. Neuro-2a (N2a) mouse neuroblastoma cells (ATCC®CCL-131™) were maintained in DMEM (Invitrogen) containing 10% FBS.

Human primary fetal neurons were provided by the Temple University Comprehensive NeuroAIDS Center (CNAC). Fetal brain tissue (gestational age, 16-18 weeks) was obtained from elective abortion procedures performed in full compliance with National Institutes of Health and Temple University ethical guidelines. The tissue was washed with cold Hanks balanced salt solution (HBSS) and meninges and blood vessels were removed. For neuronal isolation, tissue in HBSS was digested with papain (Sigma-Aldrich) for 30 min at 37°C. The tissue was further dissociated to obtain single-cell suspensions by repeated pipetting. Cells were plated at a density of approximately 1.8×10^6 cells/ 60mm dish coated with poly-D lysine in neurobasal media with B27 supplement, horse serum, and gentamicin (NM5). After approximately 2 h, neurons were re-fed with the same neurobasal media. Twenty-four hours later, cultures were re-fed with a complete change of neurobasal media as before except lacking horse serum (NM0). Four days later, one fourth of the media was removed and replaced with neurobasal media (NM0) supplemented with fluoro-deoxyuridine (FDU) and uridine. Following FDU treatment, neurons were maintained in neurobasal medium containing Glutamax and B27 supplement, with half-media changes every other day. Purity of cell type specific cultures was assessed by immunolabeling with anti-GFAP and -GLAST1 for astrocytes, -Iba-1 and –CD11b for microglia and -MAP2 or -neurofilament for neurons.

CG4 rat oligodendrocyte progenitor cells were cultured as previously described [205]. Briefly, CG4 cells were grown on poly-D-ornithine-coated dishes in DMEM containing 1% N1 (Invitrogen), 10 ng/ml biotin (Sigma-Aldrich), supplemented with 30% B104 conditioned medium (CM). B104 rat neuroblastoma cells (ATCC® CRL-1887™) were grown in DMEM containing 10% FBS and switched to DMEM containing 1% N1 and 10 ng/ml biotin to produce CM. Following 48-72 h growth in N1 medium, culture supernatant was collected and used as CM for the growth of CG4 cells. All cell culture media contained 1% penicillin/streptomycin (Invitrogen) or Gentamycin (Sigma-Aldrich) antibiotic.

Expression Vectors and Transfection of Neuro-2a Cells

For experiments requiring exogenous expression of RXR-γ, N2a cells were transfected with a myc-DDK-tagged human RXR-γ expression vector in a pCMV6 entry plasmid (Origene, Rockville, MD). Transfection was carried out using Fugene-mediated gene transfer according to manufacturers' instructions (Promega, Fitchburg, WI). Vector only (pCMV6)-transfected N2a cells served as controls. For luciferase assays, N2a cells were transfected with a human RXR-γ promoter *Renilla* luciferase reporter construct (Switchgear Genomics, Menlo Park, CA) or a random control construct (Switchgear Genomics). The random R01 construct contained 1 kb of non-conserved, non-genomic, and non-repetitive fragments from the human genome cloned upstream of the luciferase reporter in place of the RXR-γ promoter to serve as an optimal background control. Additionally, a human GAPDH promoter luciferase reporter construct (Switchgear Genomics) was utilized as a transfection efficiency control and to assess positive luciferase expression.

Site-Directed Mutagenesis

A QuickChange mutagenesis kit was used to introduce an amino acid substitution at predicted ubiquitination/sumoylation sites of RXR-γ according to the manufacturer's protocol (Agilent Technologies, Santa Clara, CA). Using mutagenic primers, the lysine residues at position 113 or 249 were replaced by arginines (K113R or K249R, respectively) in the RXR-γ-pCMV6 plasmid construct (Origene). The sense mutagenic primer sequence utilized for K113R was: 5'-tcagcagttcagaggacatcaggcccttaccag-3', and the antisense primer sequence was: 5'-ctggtaagggcctgatgtcctctgaactgctga-3'. Lysine 113 was predicted to be post-translationally modified by ubiquitination and/or sumoylation based on sequence homology to the experimentally verified sumoylation site in RXR-α (K108), as well as on post-translational modification prediction software sequence analysis [206, 207]. The sense mutagenic primer sequence utilized for K249R was: 5'-gaacttgctgttgaaccaaggacagaatcctatggtga-3', and the antisense primer was: 5'-tcaccataggattctgtccttggttcaacagcaagttc-3'. Lysine 249 was highly predicted to be ubiquitinated by post-translational modification software sequence analysis [206]. In brief, primers were incubated with plasmid and QuickChange kit components (Agilent), mutated DNA plasmid was transformed into ultra-competent cells, and purified by mini-prep (Qiagen). The mutations were confirmed by sequencing (Genewiz, South Plainfield, NJ).

In Vitro Drug Treatment

Cocaine hydrochloride (Sigma-Aldrich) was dissolved in sodium citrate buffer (pH 5.0) to increase stability [43]. One millimolar stock solution was prepared and subsequent dilutions were performed in cell culture medium. The physiologically relevant dose of 5 µM was utilized for all experiments [208]. Sodium citrate buffer was used as the control vehicle during cocaine treatments. Human recombinant TNF-α (Cell Signaling) was dissolved in PBS containing 5% FBS and added to cell culture medium at a final concentration of 10 to 100 ng/ml, depending on the cell type being treated. Tumor necrosis factor-α neutralizing antibody (TNF-α nAb) (Cell Signaling) was diluted in sterile water and added to cell culture medium at a concentration of 0.5 µg/ml. Cells were pre-treated with TNF-α nAb for 2 h prior to cocaine treatments. The JNK inhibitor

SP600125 (Cell Signaling) was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and added to cell culture medium at a concentration of 10 µM. Cells were pre-treated with SP600125 for 40 min prior to cocaine or TNF-α treatment. Leptomycin B (LMB) (Cell Signaling) was dissolved in ethanol and added to cell culture medium at a concentration of 1 nM. Cells were pre-treated with LMB for 1 h prior to cocaine or TNF-α treatment. Bortezomib (Bort) (Toronto Research Chemicals, Ontario, Canada) was dissolved in DMSO and added to cell culture medium at a final concentration of 100 pM. Cells were pre-treated with Bort for 16 h prior to cocaine or TNF-α treatment. Docosahexaenoic acid (Sigma-Alrich) was dissolved in DMSO and added to cell culture medium at a final concentration of 1.5 µM.

CG4 cells were plated on poly-D-ornithine-coated 6-well dishes in standard growth medium containing CM. Twenty-four hours following initial plating, the medium was replaced with fresh growth medium containing CM from drug-naïve B104 cells, or in the same conditioned medium from B104 cells that were exposed to cocaine for 48-72 h. CG4 cells were cultured an additional 24-48 h in control or cocaine CM before harvesting. In protection experiments, CG4 cells were treated with or without 1 µM 9-*cis*-RA (Tocris Biosciences, Bristol, United Kingdom) during cocaine treatment.

TNF-α ELISA of Cell Culture Supernatant

Cell culture supernatant from cocaine-treated SH-SY5Y cells and human primary neurons was analyzed by ELISA for levels of secreted TNF-α. Cells were treated with 5 µM cocaine and the cell culture medium was collected 30 min following treatment. Any non-adherent cells were removed by centrifugation and the remaining supernatants were analyzed using a human TNF-α ELISA kit according to manufacturers' instructions (BD Biosciences, San Jose, CA). One-hundred microliters of each sample (diluted 1:5 in sample diluent) was measured in duplicate and TNF-α levels were assessed by absorbance at 450 nm. Values were obtained and compared to the standard curve to extrapolate TNF-α concentration.

Cell Harvest and Protein Extraction

Following treatment, cells were washed in 1× PBS, then scraped and collected in ice-cold PBS. Cell suspension was centrifuged at 13,000 x g for 1 min and the supernatant was discarded. The cell pellet was then re-suspended in ice-cold HEPES lysis buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100) containing protease inhibitor cocktail (Calbiochem, San Diego, CA), vortexed, and incubated on ice for 10 min to complete cell lysis. Samples were then centrifuged at 13,000 x g for 10 min to separate insoluble material. The supernatant containing protein was collected and placed into clean, pre-chilled Eppendorf tubes and samples were stored at -80^{o}C until protein analysis was performed. For experiments requiring nuclear/cytoplasmic fractionation, cells were collected similarly but lysed and fractionated using the NE-PER nuclear and cytoplasmic extraction kit according to the manufacturer's protocol (Thermo-Scientific). Following fractionation, nuclear and cytoplasmic lysates were stored at -80^{o}C until analysis. Following protein extraction, western blot analyses were conducted as described above.

Immunoprecipitation

For immunoprecipitation (IP) experiments, cells were harvested and lysed in RIPA buffer (20 mM Tris (pH 8.0), 150 mM NaCl, 1% nonidet P-40 (NP-40), 2 mM EDTA, 10% glycerol 0.5% deoxycholic acid) containing protease inhibitor cocktail (1:100; Calbiochem), phosphatase inhibitor cocktail (1:100; Calbiochem), and 20 mM N-ethylmaleimide (Sigma) for 20 min on ice. Samples were centrifuged at 13,000 x g for 10 min to remove insoluble material, and supernatant containing protein was collected. One-hundred fifty μg of total protein was incubated with 1 μg/ml of IP antibody overnight at 4^{o}C with end-over-end rotation. After incubation, 50 μL of Protein-A or -G bead slurry (Abcam), depending on antibody host species, was added and samples were incubated with end-over-end rotation at 4^{o}C for 1 h. The beads with protein conjugates were washed 5 times with PBS and centrifuged to remove supernatant following each wash. After the final wash, protein was eluted from the beads by addition of 50 μL of 1X NuPAGE LDS sample buffer and reducing agent (Invitrogen) and boiling at 90^{o}C for 5 min. Samples were then centrifuged at 13,000 x g at 23^{o}C for 5 min and the supernatant was collected for western blotting, as described above.

Immunocytochemistry

Following the appropriate treatment, cells plated on chamber slides were washed once with 1× PBS and fixed with 4% formaldehyde for 15 min at 23^{o}C. Following fixation, slides were washed with 1× PBS and cells were permeabilized with 0.2% Triton X-100 for 15 min, washed again, and placed in blocking solution (5% normal goat or horse serum; Vector Laboratories) for 30 min. Slides were then incubated in primary antibody for 2 hr at 23^{o}C. Primary antibodies consisted of RXR-γ (1:200; Abcam), myc (1:200; Origene), and phospho-JNK (1:200; Cell Signaling). Following incubation with primary antibody, slides were rinsed 3× with PBS, and incubated with FITC (1:500; Vector Laboratories) or TRITC (1:500; Vector Laboratories)-conjugated secondary antibodies for 1 h at 23°C in the dark. Sections were again washed 3× with PBS, cover-slipped with an aqueous based mounting medium containing DAPI for nuclear labeling (Vectashield; Vector Laboratories), and visualized with Leica Advanced Widefield imaging system (Leica Microsystems; Buffalo Grove, IL).

Luciferase Assay

Neuro-2a cells transfected with the human RXR-γ promoter, GAPDH promoter, or a random 1 kb sequence (R01) driving a luminescent reporter gene (RenSP) were treated with TNF-α and assayed 24 h later according to the manufacturer's protocol (Swithgear Genomics). Briefly, cells were plated at a density of 10,000 cells per well of a 96-well cell culture plate and transfected with the appropriate promoter reporter constructs 24 h later. Following 36 h of transfection, cells were treated with 50 ng/ml of TNF-α for 24 h. Following treatment, the cell culture plate was frozen overnight at -80^{o}C and thawed the following day to increase cell lysis and enhance luciferase signal. Samples were incubated in Lightswitch assay reagent (Switchgear) for 30 min in the dark and read on a luminometer (Perkin Elmer, Waltham, MA). Relative luciferase units were obtained for each sample and normalized to the untreated average value. Data were compared to R01 and GAPDH promoter activity to assess background luminescence and ensure luciferase expression from control promoter constructs was not affected by treatment.

Statistical Analysis

All data were analyzed using student's t-test or one-way analysis of variance (ANOVA) with *post hoc* testing where appropriate using GraphPad Prizm® (GraphPad Software Inc., San Diego, CA). Results were expressed as mean ± SEM, $n \geq 3$. Values of $p \leq 0.05$ were considered statistically significant.

CHAPTER 3.

COCAINE EXPOSURE DISRUPTS RXR-γ SIGNALING IN THE CNS

Introduction

The hypothalamic-pituitary-thyroid (HPT) axis is comprised of a tightly regulated signaling cascade that ultimately controls the release of thyroid hormones triiodothryonine (T3) and thyroxine (T4) from the thyroid gland and subsequent regulation of thyroid hormone (TH)-responsive genes in the brain. Processing of these hormones in the CNS occurs via crosstalk between astrocytes and neurons, as deiodinase-2 (D2), the enzyme responsible for conversion of T4 into its more potent T3 form, is expressed in astrocytes [209, 210]. T3 is then transported to neurons where it can activate TH-responsive target genes or be inactivated by deiodinase-3 (D3) enzyme [209, 211]. Both D2 and D3 are sensitive to circulating levels of TH and serve as important regulatory elements.

Hypothyroidism, characterized by insufficient levels of circulating T3 and T4, contributes to cognitive impairments, decreased hippocampal neurogenesis, and even dementia [212-215]. Additionally, both overt and subclinical hypothyroidism (SCH) are implicated as leading risk factors for major depressive disorder [216, 217]; in itself a risk factor for the development of a substance abuse disorder [218, 219]. In this context, the association between cocaine abuse and HPT dysfunction represents a valid and under-represented field of research, as a number of cocaine abusers may suffer from underlying TH abnormalities. Additionally, TH deficits may be exacerbated with continued cocaine use/abuse, further contributing to cognitive dysfunction and hindering effective treatment. A small number of studies have been conducted to examine the effects of cocaine abuse on TH levels and HPT functioning, both in clinical settings and in experimental animal models. However, few conclusions have been reached and numerous discrepancies exist among the findings. For instance, Dhopesh *et al* reported that heavy cocaine abuse does not affect thyroid functioning in humans [220], and Budziszewska *et al* substantiated this evidence in rats [221]. However, other studies reported that cocaine abusers exhibit an impaired thyrotropin (TSH) response to thyroid releasing hormone [222, 223]. Furthermore, dopamine, the neurotransmitter through which cocaine exerts a majority of its CNS effects by inhibition of its reuptake, is a known inhibitor of TSH secretion from the pituitary, and dopamine agonists induce hypothyroidism in humans [224, 225]. Importantly, none of these studies examined the expression of TH responsive genes following cocaine abuse. Additional research is needed to increase understanding of the molecular interactions between cocaine abuse and HPT disruption.

Although conflicting evidence exists regarding the degree to which cocaine is directly neurotoxic, numerous studies have shown that chronic abuse can lead to cognitive dysfunction, mood disturbances, and vulnerability to the development of co-morbid psychiatric disorders [45, 46, 226]. Microarray studies from post-mortem human brain tissue from cocaine abusers have provided valuable insight into some of the transcriptional changes occurring in response to cocaine. Changes in genes related to the maintenance of the extracellular matrix, myelination, signal transduction, and synaptic plasticity have been consistently observed [54, 227, 228]. Similarly, microarray studies involving hypothyroid human patients and animals also show changes in neural plasticity [214, 229].

One gene that is directly regulated by TH levels and that is implicated in synaptic plasticity is Ng, also called RC3, which contains a TR/RXR response element within its first intron (Figure 5) [230]. It is well-documented that hypothyroidism results in decreased Ng expression, particularly in the cerebral cortex and hippocampus [231, 232]. Transcriptional activation of the Ng gene requires both TH-bound TR, as well as RXRs, which bind 9-*cis*-retinoic acid (9-*cis* RA), an isomer of all-trans retinoic acid, which is present in the brain [143, 167, 232-236]. Retinoic acid binds to CRABP in the cytoplasm, during which time it can either be degraded by the enzyme CYP26A1, a member of the cytochrome p450 family, or transported into the nucleus to bind RXRs. In rodents, RA is required for transcriptional activation of Ng, as decreased levels of Ng comparable to those observed in hypothyroidism are also evident during VAD [236]. It has not been fully determined whether RA signals through retinoic acid response elements (RAREs) or through the RXR component of TR/RXR heterodimers within the Ng promoter. Furthermore, the effects of VAD on Ng expression in humans have not been investigated.

In the present study, we assessed the effects of cocaine on HPT signaling in the CNS with particular focus on the expression of Ng in a murine model of chronic abuse. Cocaine was administered to both euthyroid and propylthiouracil (PTU)-induced hypothyroid mice to determine if cocaine abuse may exacerbate deficits observed in hypothyroidism. Consistent with previous studies, PTU treatment resulted in the depletion of serum T4 and decreased expression of Ng [139, 232]. While chronic cocaine treatment did not alter serum TH levels, it significantly decreased Ng mRNA and protein levels in euthyroid mice, leading to the hypothesis that regulation may be occurring at the level of the TR/RXR nuclear receptors. In support of this hypothesis, mRNA and protein levels of RXR-γ, an RXR isoform predominantly expressed in adult hypothalamus, striatum, pituitary, and hippocampus, were significantly decreased in the brains of cocaine-treated animals. Increased protein levels of CYP26A1 were also observed in the brains of mice exposed to cocaine, suggesting that induction of RA metabolizing enzymes following cocaine exposure could lead to a down-regulation in RA signaling.

Overall, our data demonstrate that cocaine increases CYP26A1 levels leading to dysregulation of RA signaling and RXR expression and function, potentially contributing to altered transcription of a number of RXR-responsive genes, including Ng. Similarly, we have found the pre-synaptic counterpart of Ng, growth-associated protein-43 (GAP-43) to be similarly down-regulated by chronic cocaine exposure. The GAP-43 promoter contains an RARE, requiring RA for its transcriptional activation. Thus, our observation that cocaine down-regulates hippocampal GAP-43 expression supports the hypothesis that cocaine leads to dysregulated RA signaling.

Few studies exist regarding the regulation of RXR levels, particularly within CNS cells. However, studies in non-CNS cell types demonstrate that RXR-α is negatively regulated by cellular stress events, such as UV irradiation or exposure to inflammatory cytokines including TNF-α and IL-1β [191, 193, 237]. In this study, we investigated the neuron-specific mechanism(s) underlying the cocaine-mediated decrease in RXR-γ levels in response to chronic cocaine exposure that we previously reported *in vivo* [238]. We hypothesized that cocaine-mediated induction of TNF-α leads to phosphorylation and subsequent nuclear export and proteasomal-dependent degradation of RXR-γ in a JNK-dependent manner. We found significantly decreased expression of RXR-γ in SH-SY5Y neuroblastoma cells and human primary fetal neurons in response to cocaine treatment, which could be attenuated by administration of a TNF-α function-blocking antibody or JNK inhibitor, as well as by proteasomal inhibition. Treatment with TNF-α alone mimicked the effects of cocaine, and degradation of RXR-γ appeared to be dependent upon its phosphorylation and nuclear export. Taken together, these studies indicate that neuronal RXR signaling is significantly disrupted in response to cocaine and/or inflammatory cytokines. These findings could have significant implications for addressing neuronal dysfunction in drug-abusing populations, and likely extend to a number of neurodegenerative disease states that are characterized by chronic neuroinflammation.

Results

Cocaine does not alter serum levels of thyroid hormones

Hypothyroidism and subclinical hypothyroidism were confirmed by measuring serum levels of T4 from weekly blood draws in all groups of mice [139, 239-241]. T4 levels in euthyroid control mice ranged from 3.3 to 4.98 µg/dL (mean = 4.05 µg/dL); whereas, levels of T4 in mice fed 0.15% PTU for 15 days ranged from 0 (undetectable) – 0.22 µg/dL (mean = 0.03 µg/dL) (Figure 6A). Mice fed 0.075% PTU-containing chow for 15 days had T4 levels ranging from 0.37 to 2.4 µg/dL (mean = 1.87 µg/dL). Mice given cocaine for 14 days had normal T4 levels ranging from 2.8 to 4.1 µg/dL (mean = 3.62 µg/dL). Cocaine administration in combination with half or full dose PTU had no significant effects on T4 levels compared with PTU alone (Figure 6A). These results suggest that during PTU-induced overt or SCH in mice, as measured by free T4 levels, concurrent cocaine administration does not alter free T4 levels. As expected and as previously reported, levels of T3 were not affected by PTU (data not shown) [211]. Additionally, cocaine had no effect on T3 levels (data not shown).

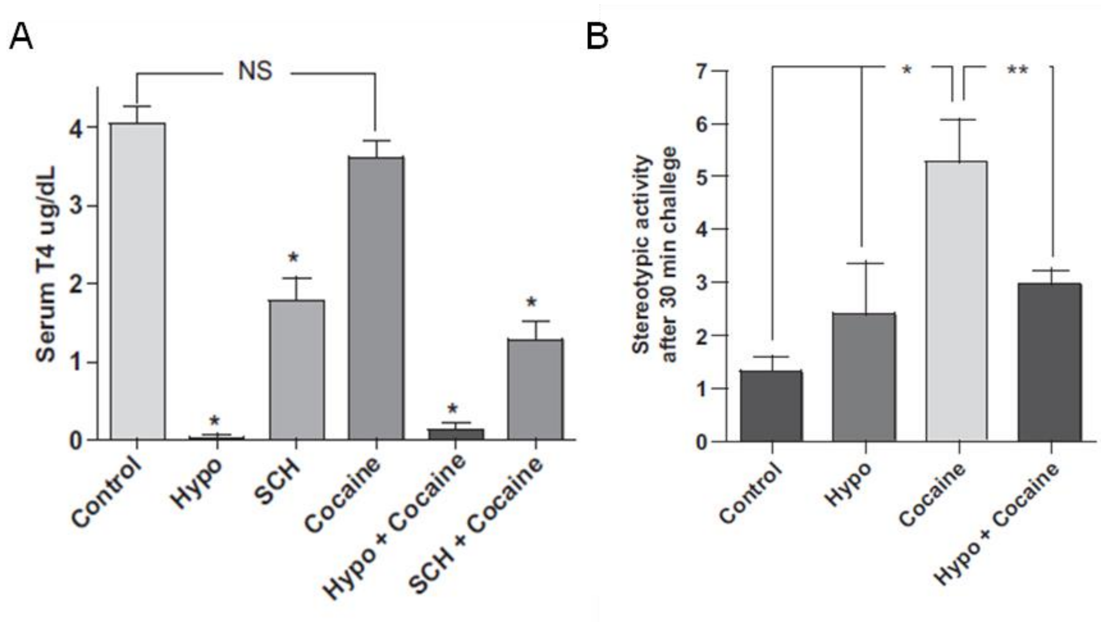


Figure 6. Chronic cocaine administration does not alter circulating thyroid hormone levels but stereotypic activity in response to cocaine challenge is diminished in hypothyroid mice

(A) Chronic cocaine administration had no effect on serum levels of free thyroxine (T4) in mice. Levels of T4 were significantly lower in propylthiouracyl (PTU)-fed mice with or without cocaine administration (hypothyroid and subclinical hypothyroid mice) compared to euthyroid control (*$p < 0.01$). (B) Hypothyroidism diminished stereotypic behavior in cocaine-administered mice. After 14 days of cocaine injections (15 mg/kg, IP, 1 x daily), mice were withdrawn from cocaine for 48 h followed by a challenge injection (15 mg/kg, IP). Stereotypic behavior was assessed and cocaine-treated mice displayed significantly greater activity at 30 min post-challenge than control mice (*$p < 0.01$). Stereotypic behavior in hypothyroid mice administered cocaine was significantly diminished compared to euthyroid cocaine-treated mice (**$p < 0.05$). Hypo, hypothyroid; SCH, subclinical hypothyroid; NS, not statistically significant. Data were analyzed by one-way ANOVA with Tukey's multiple comparisons *post hoc* test. $n \geq 6$ mice per group (from [238]).

Cocaine-induced stereotypic behaviors are diminished in hypothyroid mice

The effects of cocaine and hypothyroidism on stereotypic behavior were assessed in all groups of mice. As expected, cocaine-treated mice displayed significantly increased stereotypic behavior after receiving a challenge dose of cocaine following 48 h of withdrawal, indicative of sensitization (*$p < 0.01$) (Figure 6B). Stereotypic behavior in hypothyroid cocaine-treated mice was significantly decreased compared with euthyroid cocaine-treated mice (**$p < 0.05$) (Figure 6B). Stereotypic behavior in vehicle-treated hypothyroid (Figure 6B) and SCH (data not shown) mice was similar to behavior observed in euthyroid mice that did not receive cocaine. Likewise, behavior observed in SCH mice that received cocaine (data not shown) was similar to that of euthyroid mice that received cocaine injections. These results suggest that in the mouse model of chronic cocaine abuse, overt hypothyroidism diminishes typical stereotypic behavior associated with cocaine withdrawal and challenge. As cocaine alone did not alter T4 levels, but cocaine-induced stereotypic behavior was diminished in hypothyroid mice, we next addressed changes in the signaling that may be responsible, at least in part, for these observations.

Cocaine decreases posterior forebrain expression of neurogranin independently of changes in thyroid hormone signaling in vivo

While the thyroid gland produces both T3 and T4, the vast majority of TH produced is in the form of T4 that is subsequently converted to the more bioactive T3 form by D2 [241]. Our data from PTU-fed mice showed that less circulating T4 was available for conversion to T3 while serum levels of T3 were not changed (Figure 6A).

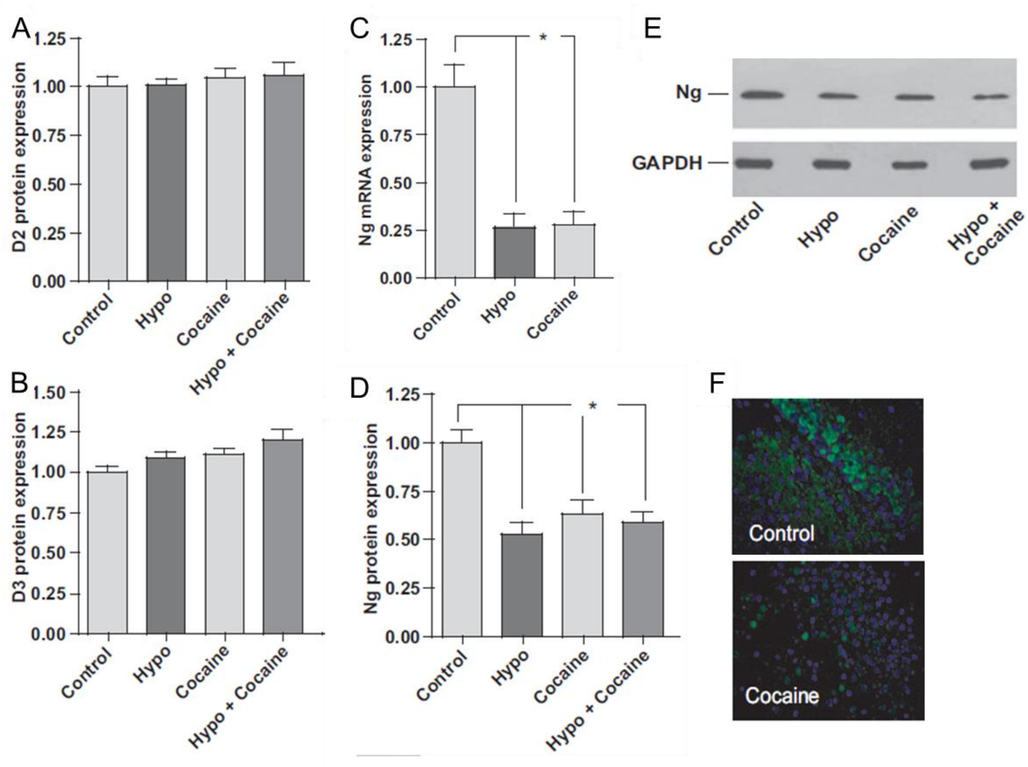


Figure 7. Chronic cocaine administration decreases neurogranin levels without affecting thyroid hormone processing enzymes
Densitometric analyses of protein levels of (A) D2, (B) D3, and (D) Ng were assessed after 14 days of PTU and/or cocaine administration. No significant changes in D2 or D3 were observed among groups. (C) Neurogranin mRNA and (D) protein levels were significantly decreased in hypothyroid and cocaine-treated mice compared to control mice. The effects of cocaine and PTU treatment were not additive. (E) Representative western blot of Ng expression. (F) Neurogranin (green) immunofluorescent labeling of hippocampal tissue from representative control and cocaine-treated mice. Nuclei are labeled with DAPI (blue). Magnification 400x. D2, deiodinase 2 enzyme; D3, deiodinase 3 enzyme; Ng, neurogranin; Hypo, hypothyroid; SCH, subclinical hypothyroid; DAPI, 4',6-diamidino-2-phenylindole. Data were analyzed by one-way ANOVA with Tukey's multiple comparisons *post hoc* test. n ≥ 6 mice per group. *$p < 0.05$ (from [238]).

A second enzyme, D3, present in neurons processes T3 to inactive forms to prevent overstimulation of TH-responsive signaling pathways. Changes in levels of either D2 or D3 may result in alterations in T3 levels, thereby affecting expression of TH-regulated genes. Consistent with our previous findings that TH levels were not affected by cocaine, western analyses revealed no significant changes in either D2 or D3 protein levels among groups (Figure 7, A and B). Therefore, changes in levels of TH-responsive genes are not likely due to altered D2 and/or D3 levels in this model.

Changes in levels of the TH-responsive gene Ng were assessed in posterior forebrain sections of euthyroid, hypothyroid and cocaine-treated mice. Decreased Ng mRNA and protein expression were observed in both hypothyroid (mRNA 35% and protein 50% of euthyroid controls) and cocaine-treated animals (mRNA 28% and protein 67% of euthyroid, controls) (Figure 7, C-E). Neither additive nor synergistic effects on Ng protein levels were observed in mice receiving both PTU and cocaine (59% of euthyroid controls) (Figure 7, D and E). In SCH mice and SCH mice receiving cocaine, levels of Ng were not significantly different from control values (82% and 84% of controls, respectively) (data not shown). Interestingly, cocaine had less of an effect on Ng levels in the SCH group than in euthyroid controls (data not shown). Immunofluorescence labeling of representative hippocampal sections from control (euthyroid) and cocaine-treated mice (Figure 7F) reveals that Ng levels (green) were significantly decreased by cocaine within this brain region. To rule out the possibility that the decreased expression of Ng was due to withdrawal from cocaine and not to the 14 day cocaine administration, one group of mice ($n = 6$) was euthanized immediately following the final cocaine injection of the 14-day paradigm and not subjected to the 48 h cocaine withdrawal and challenge. Messenger RNA and protein levels of Ng in brain sections from not withdrawn mice did not significantly differ from those of the cocaine 48 h withdrawal group (data not shown), confirming that the observed alterations in Ng levels were the result of chronic cocaine administration and not from withdrawal.

Cocaine decreases posterior forebrain expression of RXR-γ

In addition to T3 availability, TR is dependent upon heterodimerization with RXR for displacement of transcriptional co-repressors, recruitment of co-activators, and subsequent transcriptional activation of TH-responsive genes. Because no changes were observed in T4 levels in response to cocaine, yet significantly decreased expression of the TH-responsive gene Ng was observed, we hypothesized that dysregulation may be occurring at the level of the nuclear receptor complex. Two types of TH nuclear receptors exist: TR-α and -β, however, TR-β is the main regulator of the HPT axis and levels of this receptor type have been shown to fluctuate in direct response to thyroid status [139, 242]. Analyses of both mRNA and protein levels of TR-α and -β in euthyroid control versus cocaine-treated mice showed that cocaine had no significant effects on the levels of TH nuclear receptors (data not shown).

Transcriptional activation of Ng is controlled by the thyroid hormone responsive element (TRE) in its promoter region. To induce transcription, RXR-γ binds to 9-*cis*-RA and dimerizes with the T3-TR-β complex and together these factors interact with the TRE (Figure 5). Therefore, even though levels of TR are not changed in response to cocaine, alterations in RXR-γ levels could affect Ng transcription. Analyses of RXR-γ in cocaine-treated mice indicated that both the message and protein levels were significantly decreased ($^*p < 0.001$) (Figure 8, A-C). Message levels were decreased by 40%, and protein levels by 26% in cocaine-treated euthyroid mice compared with euthyroid control mice. In euthyroid mice, abundant RXR-γ is observed in the neuronal nuclei (arrowheads) (Figure 8D). However, a small amount of RXR-γ can be observed in the cytoplasm, where it co-localizes with the RA transporter, CRABP (arrowheads) (Figure

8, E and F). In contrast, less immunoreactivity of RXR-γ could be detected in brain sections from cocaine-treated mice (not shown). These results indicate that cocaine may be acting primarily on the RA/RXR-γ signaling pathway, rather than on the thyroid hormone pathway.

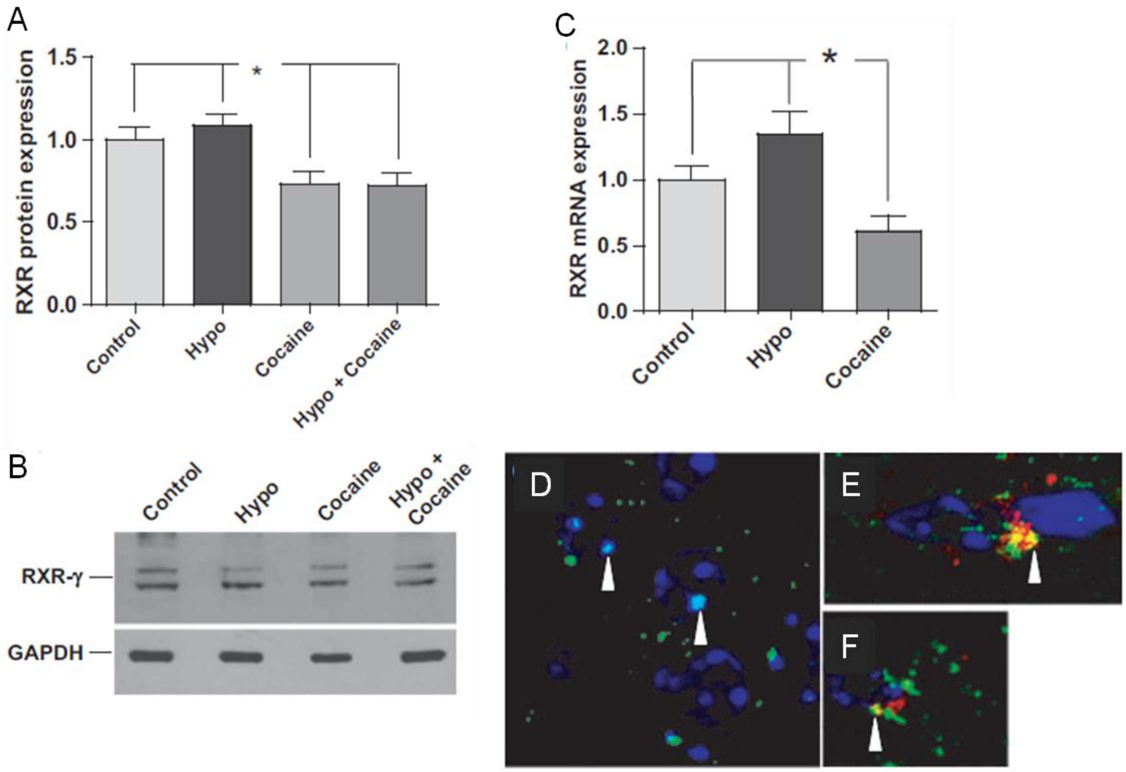


Figure 8. Chronic cocaine administration decreases expression of RXR-γ
(A) Densitometric analysis and (B) representative western blot of RXR-γ expression in posterior forebrain sections of PTU and cocaine-treated mice. RXR-γ protein levels are significantly decreased in cocaine-treated mice compared to control mice. (C) mRNA levels of RXR-γ as assessed by qRT-PCR. Retinoid X receptor-γ message levels are significantly decreased in the brains of cocaine-treated mice. (D) Retinoid X Receptor-γ immunofluorescence labeling (green) of a representative euthyroid control mouse brain demonstrating primarily nuclear localization of RXR-γ. Nuclei are labeled with DAPI (blue) (arrowheads). (E, F) Double-immunofluorescence labeling of CRBP-1 (red) and RXR-γ (green) showing some cytoplasmic co-localization (yellow) (arrowheads). Magnification 1000x. RXR-γ, retinoid X receptor-γ; Hypo, hypothyroid; DAPI, 4',6-diamidino-2-phenylindole. Data were analyzed by one-way ANOVA with Tukey's multiple comparisons *post hoc* test. n ≥ 6 mice per group. *p ≤ 0.001 (from [238]).

Chronic exposure to cocaine induces expression of CNS retinoic acid degrading enzymes in vivo

A number of mechanisms exist to regulate the amount of bio-available RA in CNS cells. One major contributor is the RA-degrading enzymes, including CYP26A1, the major degradatory enzyme within the CNS [120]. Increased levels of CYP26 and/or activity can lead to decreased bio-available RA. Drugs of abuse are known to stimulate up-regulation of a number of cytochrome P450 family enzymes, and studies in tobacco and alcohol demonstrate CYP26 is a target [243]. Additionally, chronic alcohol and nicotine abuse are associated with decreased levels of cellular RA in liver and lungs, respectively [153, 244]. We observed a small but non-significant decrease in serum retinol levels as assessed by ELISA analysis of RBP4 (Figure 9A). However, these findings do not rule out the possibility that increased metabolism of RA occurs at the cellular level.

In this context, we investigated levels of CYP26A1 following a chronic cocaine administration paradigm *in vivo*. Interestingly, CYP26A1 is expressed at low levels in adult brain, but has been shown to be up-regulated upon microglial activation [245]. Here, we show that chronic cocaine administration results in a significant increase in CYP26A1 protein levels in brain tissue (*$p < 0.001$, Figure 9B). Taken together, our data suggest that cocaine contributes to increased metabolism of RA by induction of CYP26A1 and, subsequently, a reduction in the level of 9-*cis* RA available to potentiate RXR signaling. This finding may account for the observed decrease in RXR-γ levels, as receptor levels are regulated by RA levels in a positive feedback system, as well as for the decreased transcription of Ng, which utilizes 9-*cis* RA for activation. Cell-specific expression of CYP26A1 in the adult murine brain in response to chronic cocaine exposure will be the topic of future investigations.

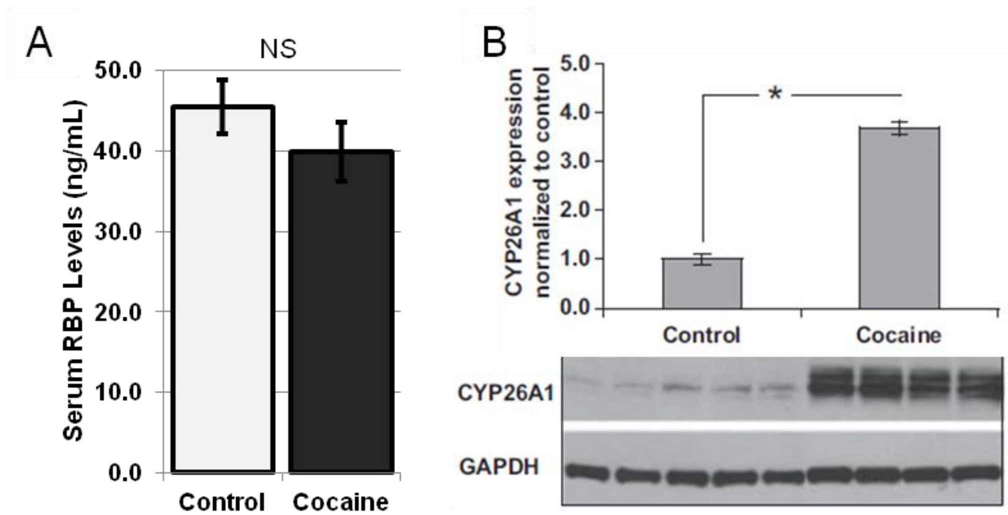


Figure 9. Chronic cocaine administration induces expression of the retinoic acid-degrading enzyme CYP26A1
(A) Enzyme-linked immunosorbent assay (ELISA) analysis of RBP4 revealed no significant difference in serum retinol levels between control and cocaine-treated mice. $n \geq 6$ mice per group. (B) Western analysis of euthyroid control mice ($n = 5$) and cocaine-treated mice ($n = 4$) indicated that cocaine significantly increased levels levels of CYP26A1 in posterior forebrain sections (top panel, quantification; lower panel, representative western blot). RBP, retinol binding protein; NS, not statistically significant. Data were analyzed by (A) Student's t-test and (B) one-way ANOVA with Tukey's multiple comparisons *post hoc* test. $^{*}p < 0.001$ (from [238]).

Cocaine induces a time-dependent decrease in hippocampal GAP-43 levels that parallels decrease in RXR-γ expression

Retinoic acid signaling directly affects the formation of synapses [162, 246]. Therefore, it is not surprising that disruption of RA signaling pathways may contribute to synapse dysfunction and/or loss. Similarly to Ng, GAP-43 is a PKC substrate involved in synapse formation and growth cone guidance during development [247]. Additionally, GAP-43 is transcriptionally regulated by RAR/RXR heterodimers, and is therefore sensitive to bioavailable RA levels [248]. In contrast to Ng, GAP-43 is localizes to the

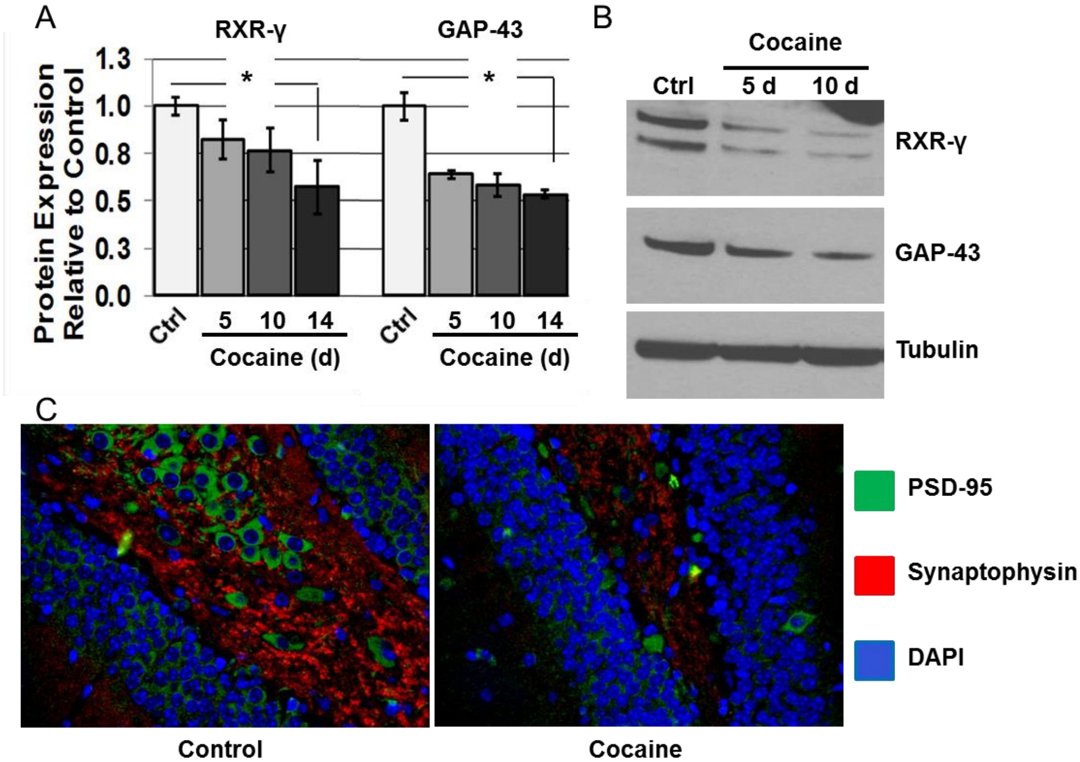


Figure 10. Cocaine administration decreases RXR-γ and GAP-43 levels in a time-dependent manner in the adult mouse hippocampus

(A) Densitometric analysis and (B) representative western blot of RXR-γ and GAP-43 expression in the hippocampi of control and cocaine-treated mice following a cocaine time course. Retinoid X receptor-γ and GAP-43 expression were significantly reduced within 5 d of daily cocaine administration and expression of both proteins decreased in parallel. Data were analyzed by one-way ANOVA with Tukey's multiple comparisons *post hoc* test. $n \geq 6$ mice per group. (C) Double-immunofluorescence labeling demonstrates decreased expression of neuroplasticity markers PSD-95 (green) and synaptophysin (red) in the hippocampus of cocaine-treated mice. Nuclei are labeled with DAPI (blue). Magnification 400x. RXR-γ, retinoid X receptor-γ; GAP-43, growth-associated protein-43; PSD-95, post-synaptic density protein-95; DAPI, 4',6-diamidino-2-phenylindole. *$p < 0.01$.

presynaptic terminal and is highly expressed during development. We evaluated GAP-43 and RXR-γ protein levels in the adult mouse hippocampus following a time course of cocaine treatment. Our results demonstrate a significant decrease in both hippocampal RXR-γ and GAP-43 within 5 days of cocaine treatment (Figure 10, A and B). Additionally, decreased levels of the synapse-related proteins synaptophysin and post-synaptic density-95 (PSD-95) were observed in the hippocampi of cocaine-treated mice compared to untreated controls, as assessed by double-immunofluorescence labeling (Figure 10C). These findings suggest that cocaine induces loss of neuronal plasticity-related proteins, and decreased RXR-γ expression could be a potential mechanism for this down-regulation.

Cocaine induces proteasome-dependent degradation of RXR-γ in neuronal cells

We have previously reported decreased levels of RXR-γ in specific brain regions following cocaine exposure in a murine model of chronic administration. To determine the mechanism(s) underlying this down-regulation in a cell-specific context, we exposed SH-SY5Y human neuroblastoma cells and human primary fetal neurons to a physiologically relevant dose of cocaine (5 μM) *in vitro* [43]. SH-SY5Y cells resemble striatal catecholaminergic neurons and, similarly to striatal neurons, readily express RARs and RXRs [249]. Additionally, SH-SY5Y cells produce TNF-α in response to cellular stressors and are sensitive to a number of compounds known to induce toxicity in dopaminergic neurons [250]. These properties make them a relevant model neuronal system in which to evaluate neurodegenerative processes. Significantly decreased levels of RXR-γ were observed in SH-SY5Y cells and in human primary neurons within 1 h of cocaine treatment (Figure 11, A-C). The levels of RXR-γ declined in a time-dependent manner, with maximal effect being observed at 24 h of cocaine treatment in SH-SY5Y cells and at 6 h of treatment in human primary fetal neurons (Figure 11, A-C).

To evaluate whether decreased protein expression of RXR-γ following exposure to cocaine was due to a reduction in mRNA levels, qRT-PCR analysis of message levels of RXR-γ was conducted in SH-SY5Y cells and in human primary fetal neurons in

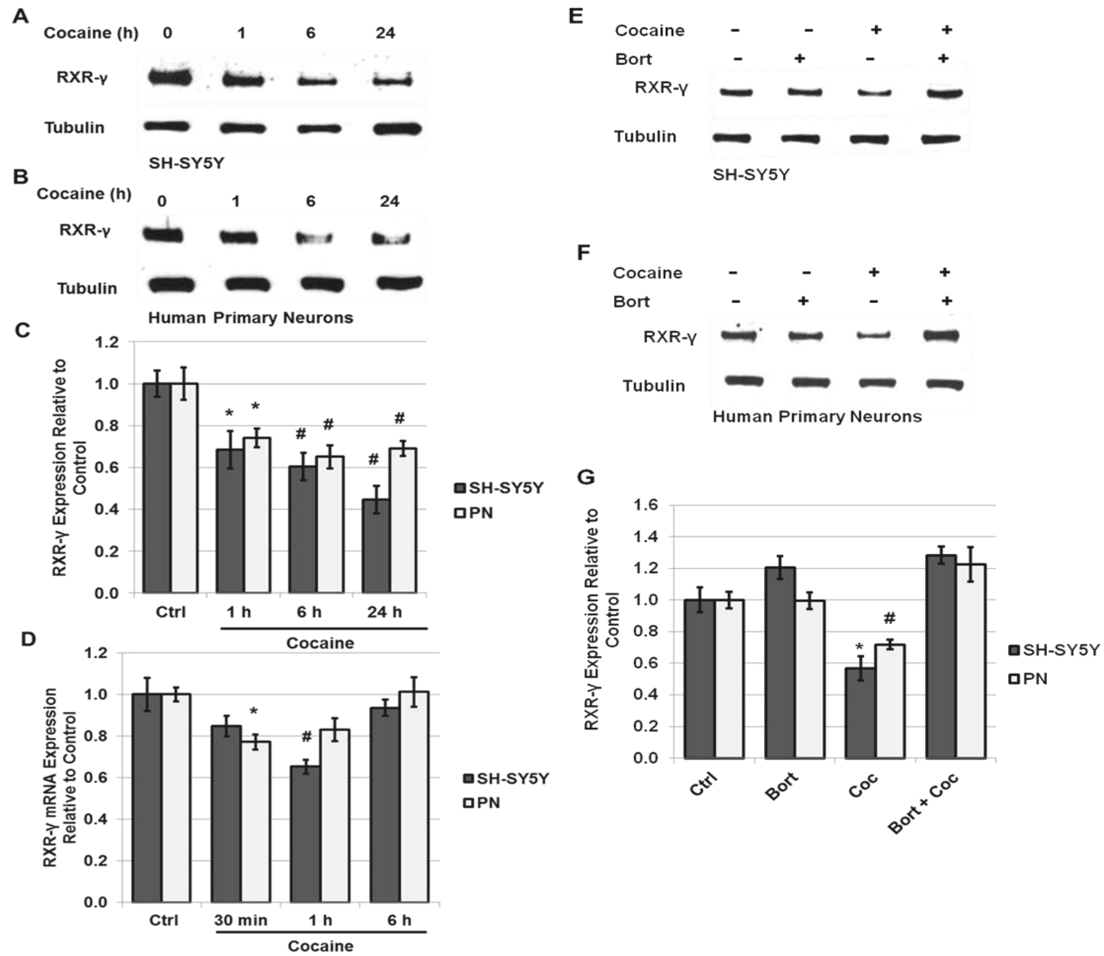


Figure 11. Cocaine induces proteasome-dependent degradation of neuronal RXR-γ *in vitro*

(A-C), SH-SY5Y neuroblastoma cells and human primary fetal neurons (PN) were treated with 5 μM cocaine (coc) for 1 h to 24 h. (A) Representative western blot of retinoid X receptor-γ (RXR-γ) expression in SH-SY5Y cells compared to tubulin loading control. (B) Representative western blot of RXR-γ expression in human primary fetal neurons compared to tubulin loading control. (C) Densitometric analysis of (A) and (B) demonstrates a time-dependent decrease in RXR-γ protein levels following cocaine exposure. (D) mRNA levels of RXR-γ following 30 min to 6 h of cocaine treatment in SH-SY5Y cells and human primary fetal neurons as assessed by qRT-PCR and normalized to the housekeeping gene GAPDH. (E-G) SH-SY5Y cells and human primary neurons were treated with 5 μM cocaine for 1 h in the absence or presence of the 26-S proteasome inhibitor, Bortezomib (Bort) (100 pM, 16 h pre-treatment). (E) Representative western blot of RXR-γ expression in SH-SY5Y cells compared to tubulin loading control. (F) Representative western blot of RXR-γ expression in human primary neurons compared to tubulin loading control. (H) Densitometric analysis of (E) and (F) demonstrating pre-treatment with Bort attenuates cocaine-induced decrease in RXR-γ protein levels. All experiments were performed in triplicate ($n \geq 3$) and analyzed by one-way ANOVA with Tukey-Kramer post-hoc testing where appropriate. Bar graphs depict mean ± S.E.M. *$p < 0.05$; #$p<0.01$. Ctrl, control.

response to cocaine. In both cell types, cocaine induced a rapid down-regulation of mRNA levels. A significant decrease was observed at 1 h in SH-SY5Y cells and at 30 min in human primary neurons, however, in both cell types levels began to recover shortly afterwards, and are not significantly different from vehicle-treated cells by 6 h (Figure 11D). These findings suggest that cocaine likely regulates RXR-γ expression by multiple mechanisms, but decreased mRNA production does not likely account for the sustained decrease in protein levels observed up to 24 h following treatment.

Because mRNA levels of RXR-γ decrease initially in response to cocaine treatment but then quickly recover to near baseline levels (Figure 11D), we sought to determine whether the observed sustained decrease in protein levels was due to protein degradation. Inhibition of the 26 S proteasome with Bortezomib (Bort) (100 pM; 16 h pre-treatment) completely blocked the decrease in RXR-γ protein levels following 1 h of cocaine treatment in SH-SY5Y cells and human primary neurons (Figure 11, E-G). Treatment with Bort alone did not induce a significant change in RXR-γ levels, suggesting that low amounts of protein are degraded under normal, non-stressed cellular conditions. These findings are in line with previous studies which demonstrated that cytokine-induced repression of nuclear receptor signaling is due, in part, to increased protein degradation [193].

Cocaine exposure induces production of TNF-α and JNK activation in SH-SY5Y cells and human fetal primary neurons in vitro

While RXRs are known to play a critical role in neuronal signaling, little information exists regarding how their levels are regulated. Our studies demonstrate that

RXR-γ levels do not change in response to treatment with either dopamine or forskolin, which mimics dopamine 1 receptor activation by activating adenylyl cyclase and raising levels of intracellular cAMP (data not shown). Therefore, the decrease seen in RXR-γ levels in response to cocaine is likely mediated primarily through a dopamine-independent mechanism.

Studies in other systems including cardiomyocytes, liver cells and lung carcinoma cells suggest that inflammatory cytokines such as IL-1β and TNF-α negatively regulate both RA signaling and levels of nuclear receptors [191, 194]. Evidence exists particularly for the TNF-α downstream effector JNK to play a role in nuclear receptor regulation in response to stress. For example, RXR-α is a known substrate of P-JNK and JNK-mediated phosphorylation at specific serine residues regulates the subcellular localization and ubiquitination state of RXR-α in hepatocytes and cardiomyocytes [193, 251]. Furthermore, previous studies demonstrate that exposure to cocaine induces production and secretion of TNF-α both *in vivo* and *in vitro* [43, 101, 185]. Therefore, we hypothesized that cocaine-induced production of TNF-α may contribute to alterations in RXR-γ levels. To confirm these findings in our culture system, and to characterize the time course of JNK activation in response to cocaine treatment, we assessed TNF-α levels following a time course of cocaine treatment by ELISA and qRT-PCR, and the ratio of P-JNK to total JNK by Western blotting. Our qRT-PCR results demonstrate that cocaine induced an approximate 3.5-fold change over control in TNF-α mRNA in SH-SY5Y cells within 30 min of treatment (Figure 12A). To evaluate whether TNF-α was similarly being secreted in response to cocaine at this time point, ELISA was performed to detect levels of the cytokine in the cell culture supernatant. The

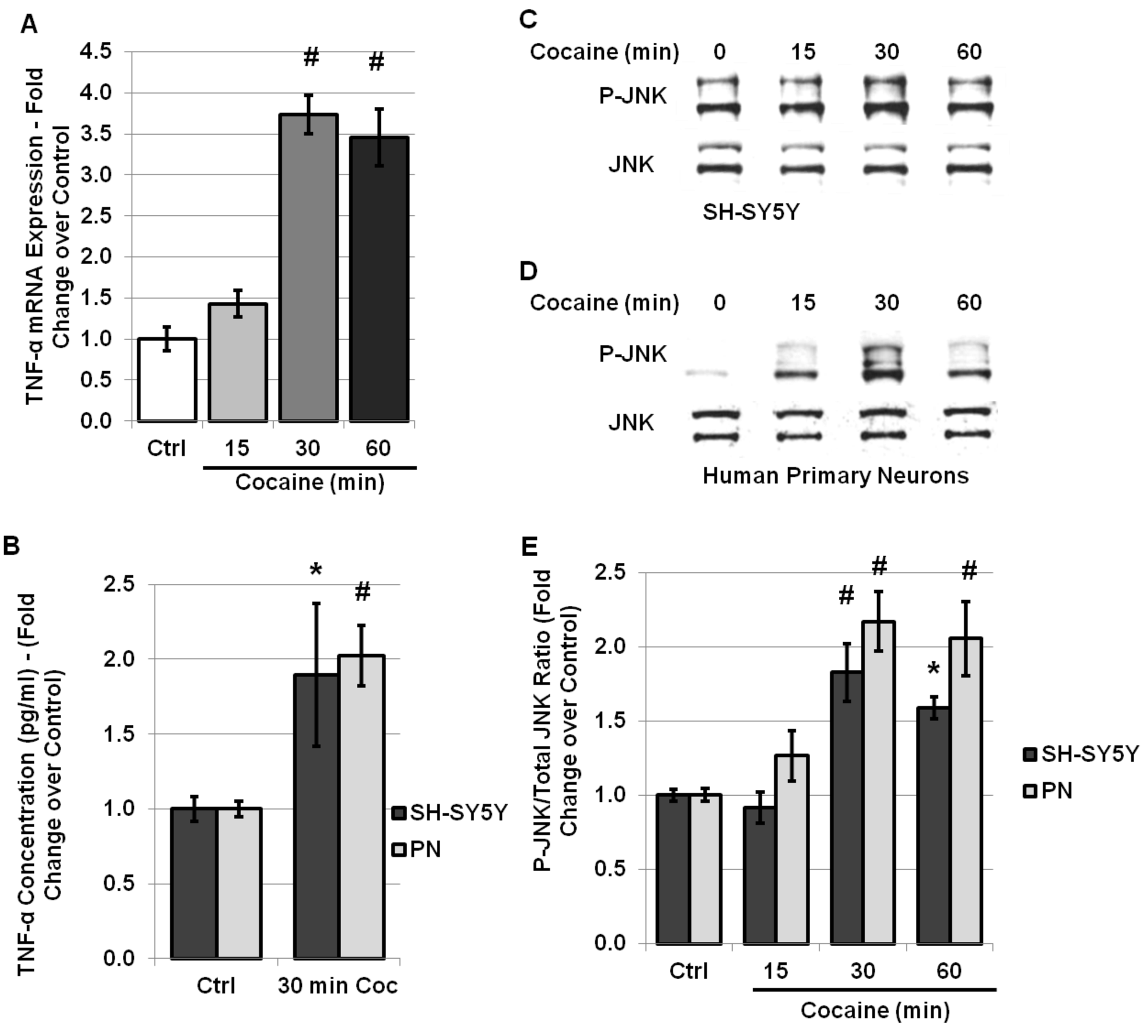


Figure 12. Cocaine induces neuronal TNF-α expression and JNK activation *in vitro*

(A) SH-SY5Y neuroblastoma cells were treated with 5 µM cocaine for 15 to 60 min and mRNA was harvested. qRT-PCR analysis of TNF-α mRNA demonstrated increased message levels at 30 min and 60 min of cocaine (Coc) exposure (B) ELISA analysis of TNF-α levels cell culture medium from SH-SY5Y cells and human primary neurons (PN). Cocaine induced significant increases in neuronal TNF-α secretion at 30 min of treatment. (C-E) SH-SY5Y cells and human primary neurons were treated with 5 µM cocaine for 15 to 60 min and analyzed by western blot for P-JNK immunoreactivity. (C) Representative western blot of P-JNK expression in untreated and cocaine-treated SH-SY5Y cells. (D) Representative western blot of P-JNK expression in untreated and cocaine-treated human primary neurons. (E) Densitometric analysis of (C) and (D) demonstrates a time-dependent increase in P-JNK/JNK ratio in response to *in vitro* cocaine treatment. All experiments were performed in triplicate ($n \geq 3$) and analyzed by one-way ANOVA with Tukey-Kramer post-hoc testing where appropriate. Bar graphs depict mean ± S.E.M. $*p < 0.05$; $\#p<0.01$. Ctrl, control.

results demonstrated increased TNF-α secretion at 30 min of cocaine treatment in both SH-SY5Y cells and in human primary fetal neurons (Figure 12B).

To determine the time course of JNK activation in response to cocaine exposure, SH-SY5Y cells and primary neurons were treated with drug and then harvested at various time points from 15 min to 6 h. Western blotting was performed to assess the ratio of activated P-JNK to total JNK. Our results demonstrated that the P-JNK/total JNK ratio was significantly increased within 30 min of cocaine treatment in SH-SY5Y cells and human primary fetal neurons compared to untreated cells (Figure 12, C-E). The P-JNK/JNK ratio returned to baseline by 6 h of cocaine treatment (data not shown). These findings demonstrate cocaine induces TNF-α expression and phosphorylation of JNK along a time course that parallels the observed decrease in RXR-γ expression (Figure 11).

TNF-α treatment mimics the effects of cocaine on RXR-γ levels, and inhibition of TNF-α signaling during cocaine exposure prevents decreases in RXR-γ

Since our findings demonstrate that cocaine treatment induces TNF-α expression and JNK activation at time points that shortly precede changes observed in RXR-γ levels in response to cocaine exposure, it was important to confirm that TNF-α treatment alone would mimic the effects of cocaine on neuronal RXR-γ levels. In both SH-SY5Y cells and human primary neurons, TNF-α treatment induced a time-dependent decrease in whole cell RXR-γ levels as assessed by western blot (Figure 13, A-D). Levels of RXR-γ were significantly decreased in both cell types within 30 min treatment (Figure 13, A-D). Immunofluorescent labeling of TNF-α-treated human primary neurons likewise

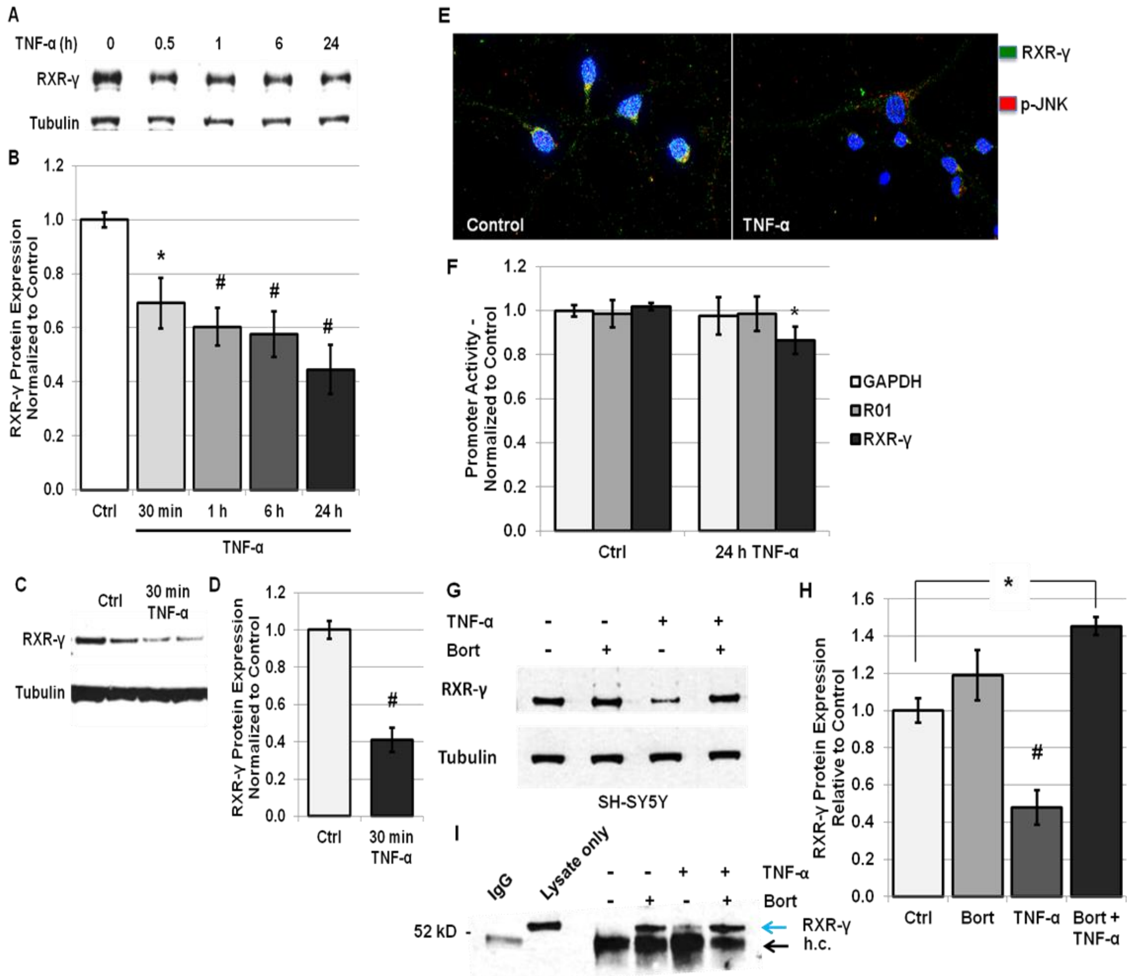


Figure 13. TNF-α Treatment mimics cocaine exposure in neurons *in vitro*
(A) Representative western blot and (B) densitometric quantification of RXR-γ following 30 min to 24 h of TNF-α treatment in SH-SY5Y cells. (C) Representative western blot and (D) densitometric analysis of RXR-γ following 30 min of TNF-α treatment in human primary neurons. (E) Double-immunolabeling of RXR-γ (green) and P-JNK (red) in TNF-α treated human primary neurons. (F) Assessment of RXR-γ promoter activity in control and TNF-α-treated N2a cells by luciferase expression. (G) Representative western blot and (H) densitometric quantification of RXR-γ expression following treatment with TNF-α +/- Bortezomib. (I) Immunoprecipitation of ubiquitinated proteins in TNF-α-treated SH-SY5Y cells +/- Bortezomib (Bort); western blot: RXR-γ. All experiments were performed in triplicate ($n \geq 3$) and analyzed by one-way ANOVA with Tukey-Kramer post-hoc testing where appropriate. Bar graphs depict mean ± S.E.M. *$p < 0.05$; #$p<0.01$. Ctrl, control; h.c., heavy chain.

demonstrated decreased RXR-γ expression (green), as well as positive P-JNK immunoreactivity (red) (Figure 13E).

Similar to cocaine exposure, treatment with TNF-α induced a rapid but transient down-regulation of RXR-γ mRNA levels (data not shown). To evaluate whether decreased promoter activity accounts for the slight decrease in RXR-γ mRNA levels in response to TNF-α, N2a cells were transfected with a human RXR-γ promoter luciferase construct and treated with TNF-α. Promoter activity was slightly but significantly reduced in TNF-α treated cells compared to untreated controls (87% of control) (Figure 13F). Luciferase expression in GAPDH- and R01-transfected cells did not change following TNF-α treatment, demonstrating specificity of TNF-α action at the RXR-γ promoter (Figure 13F). These findings suggest that multiple mechanisms likely contribute to the regulatory effects of TNF-α on RXR-γ levels, including a slight repression of promoter activity in addition to the robust increase in protein degradation. The increased protein degradation observed during cocaine treatment (Figure 11) was replicated with TNF-α treatment, as Bort pre-treatment completely blocked TNF-α-induced decreases in RXR-γ levels (Figure 13, G and H). Additionally, following treatment of SH-SY5Y cells with TNF-α for 30 min, immunoprecipitation with anti-ubiquitin antibody revealed RXR-γ immunoreactivity upon analysis by western blotting of immunoprecipitated lysate (Figure 13I). Treatment with Bort alone also led to the detection of ubiquitinated RXR-γ, suggesting that low levels of RXR-γ may be ubiquitinated under normal physiological conditions but are likely quickly cleared by the proteasome.

To evaluate whether cocaine induces changes in RXR-γ levels through induction of TNF-α signaling, SH-SY5Y cells and human primary fetal neurons were exposed to 1 h of cocaine treatment in the absence or presence of a TNF-α neutralizing antibody

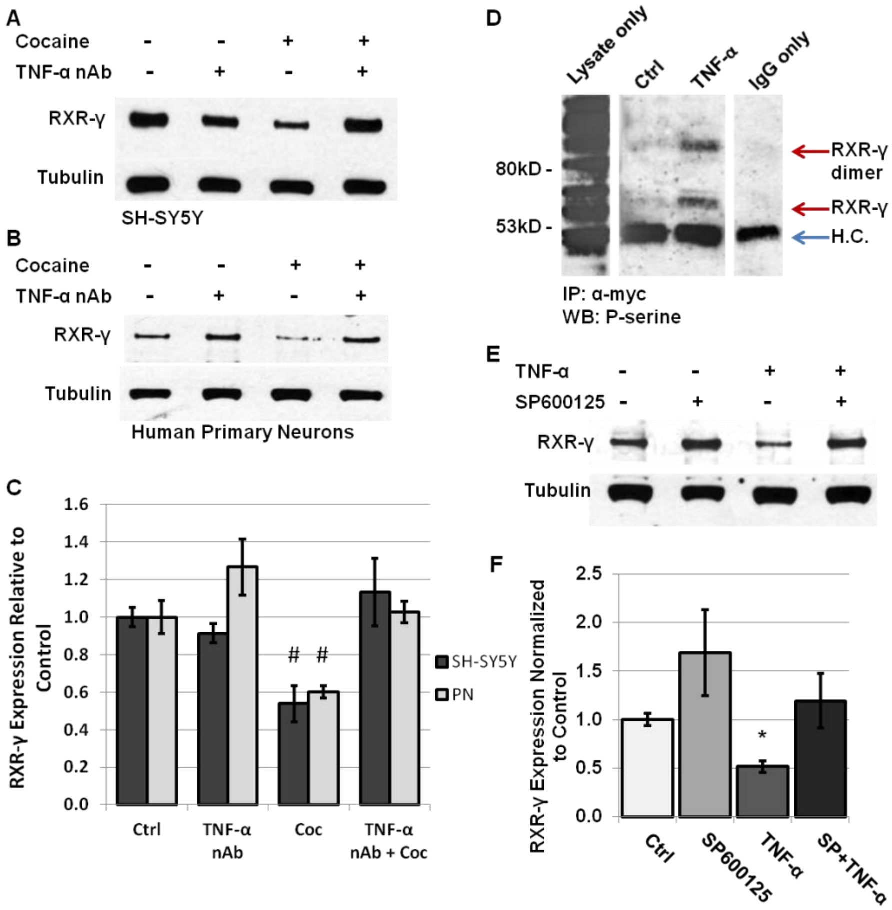


Figure 14. Inhibition of TNF-α signaling prevents cocaine-mediated decreases in neuronal RXR-γ levels

(A-C) SH-SY5Y cells and human primary fetal neurons (PN) were treated with cocaine (5 μM; 1 h) in the presence or absence of a TNF-α neutralizing antibody (TNF-α nAb). (A) Representative western blot of RXR-γ expression in SH-SY5Y cells compared to tubulin loading control. (B) Representative western blot of RXR-γ expression in human primary neurons compared to a tubulin loading control. (C) Densitometric quantification of RXR-γ levels following cocaine treatment +/- TNF-α nAb. (D) Immunoprecipitation of control and TNF-α-treated (50 ng/mL; 30 min) N2a cells transfected with myc-tagged RXR-γ demonstrates phospho-serine immunoreactivity in TNF-α-treated cells where RXR-γ was immunoprecipitated using myc antibody. The first lane demonstrates lysate only (no IP) and the last lane lane demonstrates IP using IgG and lysate only. Blue arrow indicates the heavy chain (H.C.). Red Arrow indicates RXR-γ with phosphorylated serine residues. (E-F) Human primary fetal neurons were treated with TNF-α (10 ng/mL; 30 min) in the presence or absence of the P-JNK inhibitor, SP600125. (E) Representative western blot showing RXR-γ expression compared to tubulin loading control. (F) Densitometric analysis of RXR-γ expression in human primary neurons. All experiments were performed in triplicate ($n \geq 3$) and analyzed by one-way ANOVA with Tukey-Kramer post-hoc testing where appropriate. Bar graphs depict mean ± S.E.M. *$p < 0.05$; #$p<0.01$.

TNF-α nAb) (0.5 μg/mL; 2 h pre-treatment). Following treatment with TNF-α nAb and cocaine, RXR-γ levels were not significantly different from control levels in either cell type (113% of control in SH-SY5Y cells and 103% of control in human primary neurons), while cocaine treatment alone induced an approximate 40% reduction, as previously observed (Figure 14, A-C). These data indicate that cocaine induces degradation of RXR-γ in a TNF-α-dependent manner. Furthermore, we demonstrate for the first time that inflammatory cytokines repress nuclear RA signaling in neurons *in vitro*.

A number of previous studies suggest that cytokines exert their effects on nuclear receptor signaling primarily through activation of JNK and subsequent P-JNK-mediated phosphorylation at specific residues of target nuclear receptors [188, 193, 251]. Therefore, we evaluated whether RXR-γ is phosphorylated following TNF-α exposure. Since there are no phospho-specific antibodies against RXR-γ, immunoprecipitation of myc-tagged RXR-γ in TNF-α-treated transfected N2a cells was conducted and resulted in phospho-serine immunoreactivity in immunoprecipitated lysate of treated cells, but not in control conditions (Figure 14D). Next, we evaluated whether inhibition of JNK signaling with the specific pharmacological inhibitor of P-JNK downstream kinase activity, SP600125, could attenuate the decrease of RXR-γ in response to TNF-α exposure. Our findings revealed that SP600125 (10 μM; 40 min pretreatment) attenuated the cocaine-mediated decrease in RXR-γ protein levels in human primary neurons (Figure 14, E and F), providing further support that JNK signaling is involved in the regulation of RXR-γ protein levels. Furthermore, treatment of human primary neurons with SP600125 alone led to an increase in basal levels of RXR-γ (Figure 14, E and F). These findings suggest that induction of TNF-α signaling and resultant P-JNK activation are key mediators in the reduction of RXR-γ protein levels.

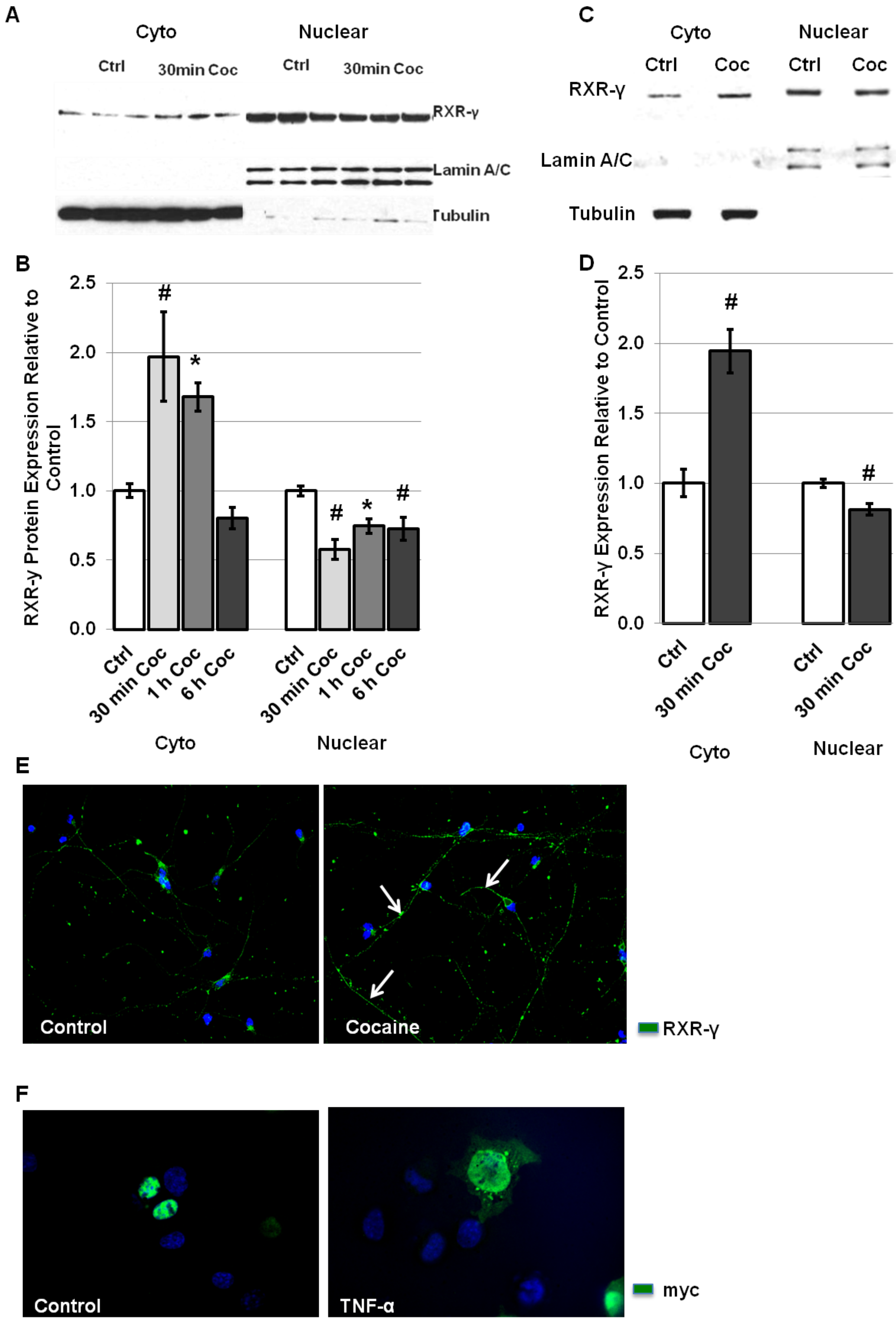
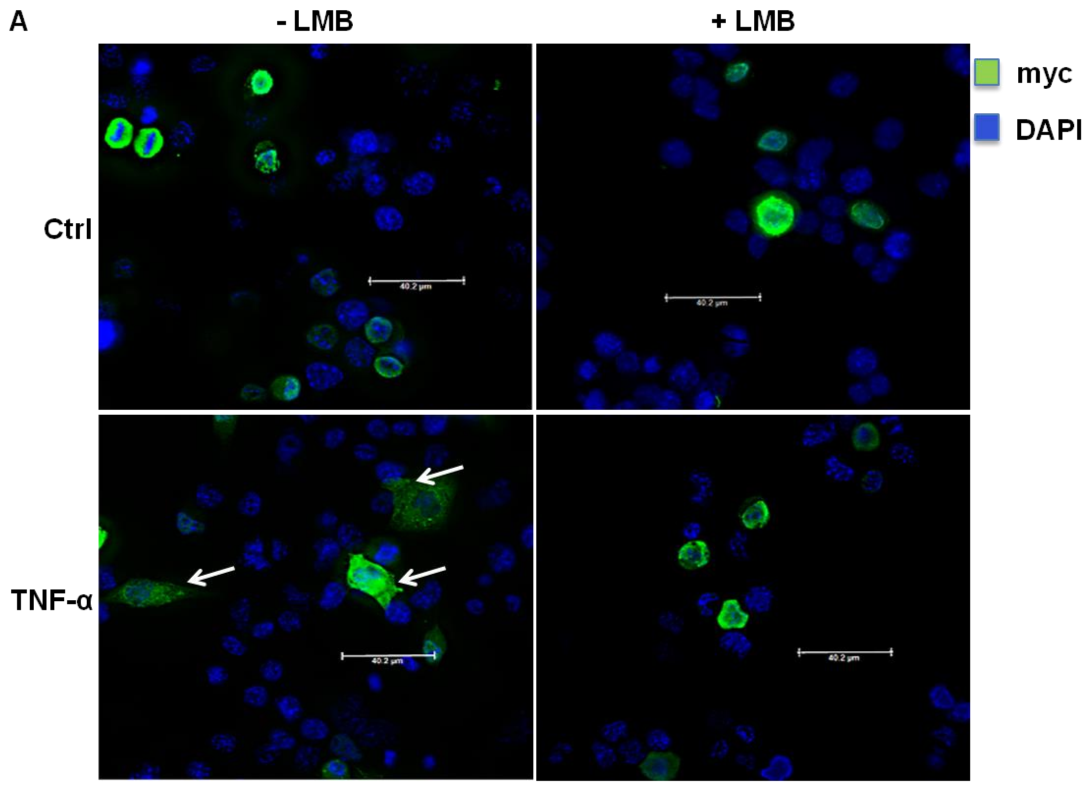


Figure 15. Cocaine and TNF-α induce nuclear export of RXR-γ
(A-B) SH-SY5Y cells were treated with 5 μM cocaine for 30 min to 6 h and harvested into nuclear and cytoplasmic fractions. (A) Representative western blot of RXR-γ expression in control and cocaine-treated (30 min) SH-SY5Y cells. A short exposure image of nuclear RXR-γ is juxtaposed alongside a longer exposure of cytoplasmic RXR-γ. Lamin A/C was used as a nuclear loading control and tubulin as a cytoplasmic loading control. (B) Densitometric quantification of cytoplasmic and nuclear RXR-γ levels in control and cocaine-treated cells. (C-E) Human primary neurons were treated with 5 μM cocaine for 30 min and harvested for nuclear and cytoplasmic fractions or fixed for immunocytochemical analysis. (C) Representative western blot of RXR-γ expression in control and cocaine-treated (30 min) human primary neurons. A short exposure of nuclear RXR-γ is juxtaposed to a long exposure of cytoplasmic RXR-γ. Lamin A/C was used as a nuclear loading control and tubulin as a cytoplasmic loading control. (D) Densitometric quantification of cytoplasmic and nuclear RXR-γ levels in control and cocaine-treated cells. All experiments were performed in triplicate ($n \geq 3$) and analyzed by one-way ANOVA with Tukey-Kramer post-hoc testing where appropriate. Bar graphs depict mean ± S.E.M. *$p < 0.05$; #$p<0.01$. (E) Immunolabeling of RXR-γ (green) in control and cocaine-treated human primary neurons. Arrows point to presence of RXR-γ immunoreactivity outside of the nucleus. Nuclei are labeled with DAPI (blue). Original magnification, x40. (F) N2a cells transfected with myc-tagged RXR-γ plasmid were treated with TNF-α (100 ng/mL; 15 min), fixed, and labeled with anti-myc antibody (green). Nuclei are labeled with DAPI (blue). Original magnification, x100.

Acute cocaine or TNF-α treatment induces nuclear export of RXR-γ in vitro

In hepatocytes, the inflammatory cytokine IL-1β induces nuclear export of RXR-α in a JNK-dependent manner, which leads to proteasomal degradation of the protein in the cytosol [193]. A shift in subcellular localization of RXRs to the cytoplasm suggests a loss of function, since these predominantly nuclear proteins act as DNA-binding transcription factors which bind to both target DNA recognition elements and to other nuclear receptors in the nucleus. To assess whether exposure to cocaine induces nuclear export of RXR-γ in neuronal cells, we treated SH-SY5Y cells with cocaine and assessed the subcellular localization of RXR-γ by nuclear/cytoplasmic fractionation followed by western blotting. Our results demonstrate that cocaine induces a decrease in nuclear levels of RXR-γ within 30 minutes, and a concomitant significant increase in cytoplasmic levels of RXR-γ compared to untreated cells (Figure 15, A and B). The cytoplasmic levels of RXR-γ remain significantly increased compared to controls for up to 1 h of treatment, while nuclear levels remain significantly decreased. By 6 h of cocaine treatment, cytoplasmic levels of RXR-γ returned to baseline while nuclear levels remained significantly lower than those of controls (Figure 15, A and B). Because significant changes were observed in RXR-γ subcellular localization within 30 min of cocaine treatment, this 30 min time point was selected to assess localization in human primary neurons. Similar to the results seen in SH-SY5Y cells, a shift from nuclear to cytoplasmic RXR-γ was observed in human primary neurons following 30 min of cocaine exposure (Figure 15, C and D). Likewise, immunolabeling experiments in human primary neurons following 30 min of cocaine treatment revealed detectable expression of RXR-γ in cell bodies and processes compared to untreated neurons, where RXR-γ expression was predominantly nuclear (Figure 15E). Finally, to assess if TNF-α treatment similarly produced a nuclear to cytoplasmic shift in RXR-γ, N2a cells, which do not express endogenous RXR-γ, were transfected with myc-DDK-tagged RXR-γ plasmid and treated with TNF-α for 15 min before fixation for immunocytochemical analysis. Immunofluorescence labeling using an anti-myc antibody revealed detection of myc-DDK-RXR-γ in the cytoplasm of N2a cells compared to untreated controls, where expression was predominantly nuclear (Figure 15F).



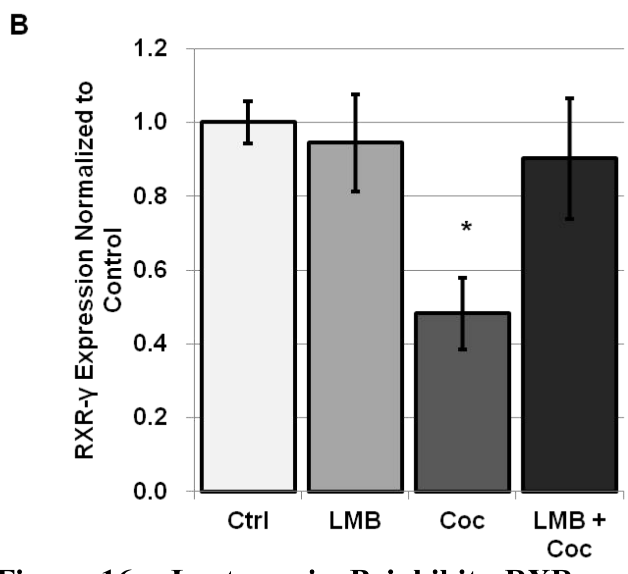


Figure 16. Leptomycin B inhibits RXR-γ nuclear export and inhibition of nuclear export prevents cocaine-mediated decreases in RXR-γ levels
(A) N2a cells were transfected with myc-tagged RXR-γ and treated with TNF-α (50 ng/ml) in the presence or absence of nuclear export inhibitor LMB (1 nM; 1 h pre-treatment). Immunocytochemical analysis of α-myc demonstrates TNF-α induces nuclear export of RXR-γ, which is blocked by pre-treatment with LMB. Original magnification x40, scale bar = 40.2 μm. (B) SH-SY5Y cells were treated with cocaine (5 μM; 1 h) in the presence or absence of LMB (1 nM; 1 h pre-treatment) and RXR-γ levels were assessed by western blot analysis. n = 3; $*p < 0.05$.

Inhibition of nuclear export prevents decrease in RXR-γ levels following cocaine treatment

To assess subcellular localization of exogenous RXR-γ in N2a cells in response to TNF-α, myc-tagged RXR-γ-transfected cells were treated with TNF-α for 15 min in the presence or absence of the CRM1-dependent nuclear export inhibitor, Leptomycin B (LMB) (1 nM; 1 h pre-treatment). Subsequent anti-myc immunolabeling revealed an increased number of cells exhibiting cytosolic labeling, compared to the untreated and TNF-α plus LMB conditions (Figure 16A). These results demonstrate that TNF-α treatment induces a nuclear to cytoplasmic shift in subcellular localization in RXR-γ. To the best of our knowledge, these findings are the first to confirm that RXR-γ is exported from the nucleus by a CRM1-dependent mechanism.

To evaluate whether inhibition of nuclear export prevents degradation of RXR-γ, we treated SH-SY5Y cells with cocaine (5 μM; 1 h) in the absence or presence of LMB (1 nM; 1 h pre-treatment). Inhibition of nuclear export attenuated the cocaine-mediated decrease in RXR-γ levels (Figure 16B), suggesting nuclear export is required for proteasomal-dependent degradation in response to cocaine exposure.

In vitro cocaine treatment decreases GAP-43 expression

Similar to Ng, GAP-43 expression is sensitive to bio-available RA levels. To assess whether *in vitro* cocaine treatment induces a decrease in GAP-43 expression, we treated SH-SY5Y cell with cocaine (5 μM) for 24 h and assessed protein levels of GAP-43. In accordance with our *in vivo* findings, cocaine treatment induced a significant decrease in neuronal GAP-43 levels (57% of control levels) (Figure 17, A and B). Again, these findings are in line with our hypothesis that cocaine disrupts expression of RXR-regulated genes in neurons. Whether cocaine-induced decreases in GAP-43 levels can be prevented and/or reversed by inhibition of TNF-α or JNK signaling will be the topic of continued research.

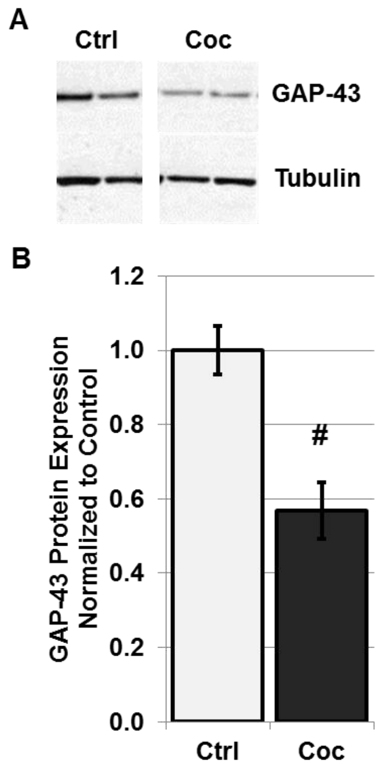


Figure 17. Cocaine decreases neuronal GAP-43 expression *in vitro*
(A-B) SH-SY5Y cells were treated with cocaine (5 μM) for 24 h and GAP-43 protein expression was assessed by western blot. (A) Representative western blot of GAP-43 expression in control and cocaine-treated cells compared to tubulin loading control. (B) Densitometric quantification of GAP-43 levels in control and cocaine-treated SH-SY5Y cells. $n \geq 3$; #$P < 0.01$. GAP-43, growth-associated protein-43; Coc, cocaine-treated.

Characterization of RXR-γ mutants

Previous studies in RXR-α suggest specific lysine residues may be post-translationally modified by ubiquitination or sumoylation to regulate protein levels and subcellular localization [207]. Therefore, we generated RXR-γ mutants through site-directed mutagenesis of plasmid DNA, to evaluate potential contributions of specific lysine residues in more depth. The first site we chose to mutate, K113, is highly predicted to be ubiquitinated by post-translational modification software [206], and resides within an identical motif ("DIKP") of a known sumoylation site in RXR-α (K108) [207]. The second lysine residue we elected to mutate, K249, is also highly predicted to be ubiquitinated by post-translational modification software analysis [206]. We performed single nucleotide substitutions using mutagenic primers to change the lysine residue to an arginine residue in each case. Selected clones were sequenced to confirm successful substitution (Genewiz) and large amounts of plasmid DNA were generated by maxiprep.

We evaluated expression levels and response to TNF-α of the mutant proteins in comparison to that of the wild-type (wt) RXR-γ plasmid. Interestingly, both mutants, K113R and K249R, were expressed at lower levels than wt plasmid following transfection of equal volumes into N2a cells (Figure 18, A and B). Furthermore, while TNF-α treatment (50 ng/ml; 24 h) induced a decrease in wt RXR-γ levels, TNF-α treatment had no effect on protein levels of either mutant (Figure 18, A and B). Subcellular localization of mutant proteins was evaluated by immunocytochemical labeling using a myc antibody. Though K249R-RXR-γ demonstrated primarily nuclear localization comparable to wt-RXR-γ, K113R-RXR-γ appeared to reside in both nuclear and cytoplasmic compartments, even in untreated cells (Figure 18C). Potential explanations for these findings are numerous. Sumoylation can serve as a nuclear localization signal in some protein systems. Therefore, ablating a potential sumoylation site at K113 might lead to a decrease in nuclear localization of the protein, and potentially a decrease in stability which accounts for significantly decreased expression. Interestingly, mutation of the potential ubiquitination site K249 rendered the mutant protein both unresponsive to TNF-α treatment but simultaneously expressed at a lower baseline level than wt plasmid protein. Expanded analyses of functional consequences resulting from K113R and K249R mutations are ongoing.

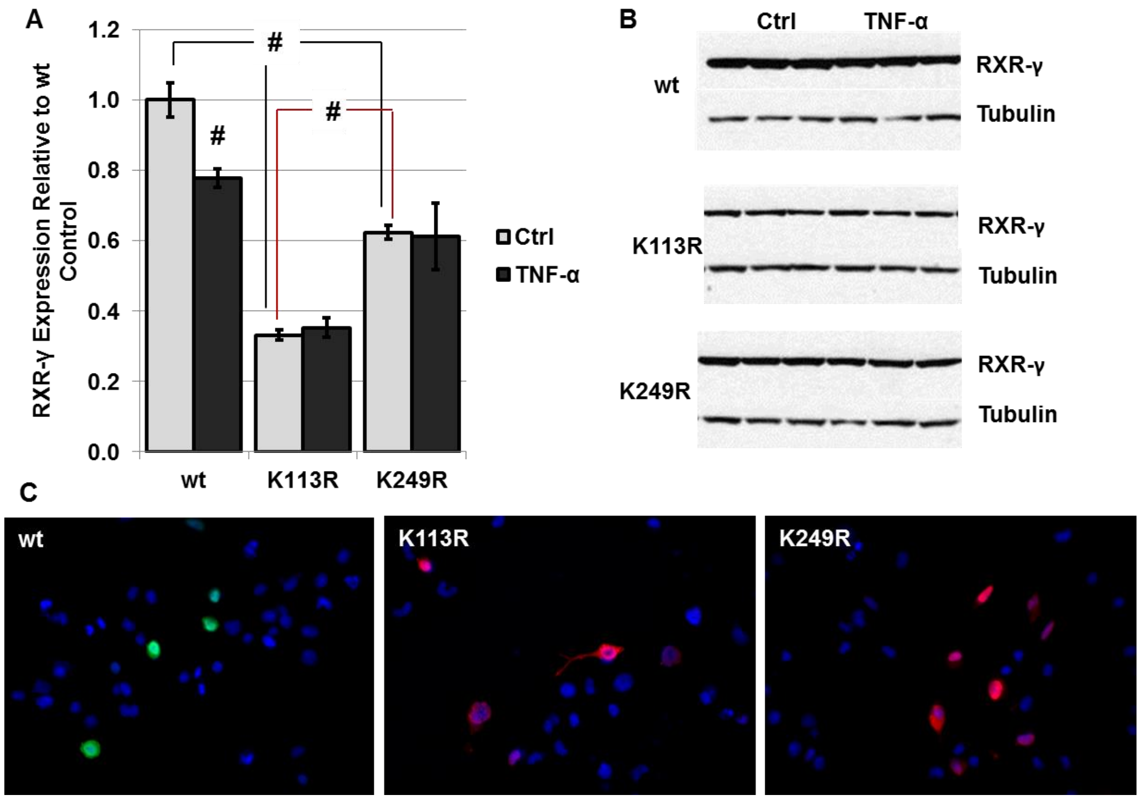


Figure 18. Characterization of RXR-γ mutants
(A-C) N2a cells were transfected with equal amounts of wt or mutant (K113R, K249R) RXR-γ plasmid and treated with TNF-α. (A) Quantification of RXR-γ expression in transfected N2a cells +/- TNF-α. $n \geq 3$; #$p < 0.01$. (B) Representative western blots of RXR-γ expression in wt or mutant-transfected N2a cells +/- TNF-α compared to tubulin loading control. All bands are from a single exposure of the same membrane (18 total samples). (C) Immunocytochemistry against myc antibody in wt and mutant-transfected N2a cells demonstrates predominant nuclear localization of wt and K249R RXR-γ, but both nuclear and cytoplasmic localization of K113R RXR-γ. α-myc is conjugated to a FITC-labeled secondary in wt-trasfected cells, and a TRITC-labeled secondary antibody in mutant-myc-RXR-γ-transfected cells. Nuclei are labeled with DAPI (blue). Original magnification, x40. wt, wild-type.

Discussion

In the present study, we evaluated the effects of chronic cocaine abuse on HPT signaling in euthyroid and PTU-induced hypothyroid and subclinical hypothyroid mice.

Previous studies have suggested cocaine may contribute to thyroid dysfunction but molecular mechanisms remain unknown. Furthermore, literature regarding the specific effects of cocaine on thyroid hormone responsive genes is lacking. Both chronic cocaine abuse and hypothyroidism have been associated with similar co-morbidities, including major depressive disorder and cognitive dysfunction; specifically, in areas related to memory and that are reliant on neuroplasticity [252-256]. Neurogranin is one of the only brain-specific genes involved in synaptic plasticity and its expression is TH-dependent in many brain regions [139]. Because of its sensitivity to T3 levels, Ng is decreased in hypothyroidism, particularly in the hippocampus [139, 214, 215]. Given that cocaine abuse can also result in deficiencies in hippocampal-related functions, we investigated the effects of chronic cocaine treatment on Ng levels and on several aspects of HPT signaling that could explain alterations in Ng expression. All male mice were used in this study to avoid confounding variables involving the effects of the female estrous cycle on TH processing. Furthermore, hypothyroidism has previously been shown to exert a greater effect on gene expression in male mice pups than in females [214, 229]. Gender-specific differences in the effects of cocaine on RA and Ng signaling are unknown and represent an area for future investigations.

Results from this study demonstrate that PTU-induced hypothyroidism and chronic cocaine abuse similarly reduce Ng protein levels (to 52% and 67% of control, respectively) as well as mRNA levels (to 35% and 28% of control, respectively) in posterior forebrain sections (Figure 7). We also chose to examine a subclinical hypothyroid state, induced by administering ½ dose of PTU, because this condition is relatively common in the human population and produces a number of cognitive and mood disturbances, yet few studies have used methods of manipulating thyroid status to mimic this state. Although we observed a downward trend in Ng levels in SCH groups compared with control levels, results did not achieve statistical significance. These findings do not rule out the possibility that SCH could disrupt neuroplasticity in human patients, and may be the subject of future studies. Previously, it has been demonstrated that Ng is decreased in the hippocampus of hypothyroid animals [139, 232]. Upon immunohistochemical analysis in this study, the greatest differences in Ng expression between control and cocaine treatment groups were observed within the hippocampus, as well (Figure 7F). Surprisingly, cocaine treatment did not alter serum T3 or T4 levels, or the expression levels of D2 or D3, indicating that the observed decrease in Ng was not a result of insufficient T3 levels, as is the case in hypothyroidism. However, it is possible that cocaine may alter the actual amount of T3 that reaches neurons via the disruption of normal astrocyte-neuron intercellular communication. Additionally, because SCH in humans is characterized by normal T3 and T4 levels but an abnormal TSH response, the results observed in cocaine-treated mice do not rule out the possibility that cocaine may have an effect on TSH that may ultimately lead to HPT alterations without directly impacting TH levels [257]. However, the lack of significant findings in TH levels and other components of the HPT signaling pathway in cocaine-treated animals prompted us to evaluate the effects of cocaine at the level of nuclear receptor signaling. Transcriptional activation of Ng requires the heterodimerization of ligand-activated TR and RXR-γ and binding of this complex to the TRE of the Ng gene. RXR belongs to a superfamily of nuclear receptors that bind the vitamin A derivative 9-*cis* RA, as well as certain long-chain fatty acids such as docosahexaenoic acid [118, 121]. Because VAD has previously been shown to decrease Ng expression at the level of the RXR, we evaluated whether cocaine abuse influences RXR signaling to similarly reduce Ng levels. Three RXRs exist – RXR-α, RXR-β, and RXR-γ – which are encoded by separate genes and differentially expressed. Retinoid X receptor-γ is primarily expressed in the hypothalamus, pituitary, hippocampus, and striatum and therefore serves as a likely target for involvement in Ng regulation [162, 258-261]. Furthermore, RXR-γ is known to be involved in dopaminergic signaling, as it is highly expressed in dopaminergic neurons in the striatum and RXR-γ mutant mice exhibit impaired locomotion and a blunted locomotor response to cocaine [143, 169]. Additionally, mice subjected to VAD or targeted disruptions of RXR-γ exhibit impaired long-term-potentiation and long-term-depression, reduced dopaminergic function, and depressive-like symptoms [169, 248, 262, 263]. Results from this study reveal for the first time that chronic exposure to cocaine decreases RXR-γ mRNA and protein levels in mouse posterior forebrain. The mechanism(s) by which cocaine regulates RXR levels and function will continue to be examined in future studies.

A number of possibilities exist for these observations. For instance, as RXR is biologically regulated by RA levels in a positive feedback system, cocaine exposure could result in increased metabolism of vitamin A or its derivatives, resulting in reduced bio-availability for RAR and RXR-dependent signaling. In support of this view, previous studies have shown that cocaine treatment in mice induces elevated levels of cytochrome p450 family members [126, 264]. We have expanded upon these studies by demonstrating that chronic cocaine administration significantly increases levels of CYP26A1, the main enzyme responsible for metabolism of cellular RA, in brain tissue

(Figures 5 and 9). Additionally, cocaine may alter the levels or function of CRBPI or CRABP I/II so that less retinol is taken up into cells, converted to RA, isomerized to 9-*cis* RA, bound to RXR, and transported successfully to the nucleus to initiate transcription of responsive genes. As RXR has been demonstrated to shuttle between the cytosol and nucleus, subcellular localization studies following cocaine treatment could yield valuable insight into the intra-cellular response to cocaine. Another point of potential regulation exists at the level of formation of the nuclear receptor complex. The TR/RXR complex unbound to its ligands is normally bound to the TRE of regulated genes where it associates with transcriptional repressors, such as SMRT and NCOR, to promote histone deacetylase activity and inhibition of active transcription [265]. Binding of T3 to the TR results in displacement of these repressors and results in recruitment of co-activators that possess histone acetylase activity to initiate and promote transcription of target genes [265]. Thus, TH-responsive genes may also be sensitive to levels of these co-factors, and down-regulation of these genes could result from increased repression or decreased activation factors. Cocaine abuse has previously been associated with chromatin remodeling and changes in specific histone modifications in the striatum, resulting in the regulation of gene transcription, and therefore may alter Ng transcription in a similar manner [261]. While RA signaling has recently been implicated in a number of physiological processes and its dysfunction may contribute to several neurodegenerative diseases, the effects of cocaine abuse on RA signaling have not been previously investigated. Our findings may shed light on a number of effects cocaine exerts for which no mechanisms have yet been elucidated. For instance, it has been well-documented that chronic cocaine abuse decreases D2R expression in a number of brain regions contributing to impaired dopaminergic signaling and a number of locomotor and psychological impairments attributed to cocaine abuse [169, 228]. Importantly, the D2R gene contains an RXR-response element within its promoter region [168]. Therefore, a cocaine-induced reduction in RXR signaling may account for altered D2R levels and function.

In conclusion, data presented in our *in vivo* studies highlight an effect of cocaine abuse that has not been previously reported, as well as a novel mechanism for the regulation of TH-responsive genes following cocaine exposure. In light of these findings, we demonstrate how separate conditions, chronic cocaine abuse and HPT dysfunction, converge on a single signaling pathway. These results contribute to the understanding of the underlying complexity of nuclear receptor signaling, and suggest that a delicate balance between signaling pathways in the heterodimeric nuclear receptor complex must be struck in order to transduce proper signaling and maintain a healthy neural environment.

To examine the observed cocaine-mediated reduction in RXR-γ levels at a mechanistic level in a cell-specific context, we utilized *in vitro* neuronal systems. While mechanisms underlying the regulation of RXR-γ in the CNS have been vastly unexplored, previous studies have demonstrated significant suppression of RXR-α signaling and levels in other cell types, including liver cells, keratinocytes, and cardiomyocytes in response to cellular stressors such as inflammatory factors and UV irradiation. While RXR-α is ubiquitously expressed and plays a vital role in proper function of a number of organ systems, RXR-γ exhibits much more restricted expression and little is known about its key functions. However, RXR-γ is highly enriched within the CNS including within the striatum, a key target of cocaine action. Furthermore, RXR-γ expression largely overlaps that of the TR/RXR-regulated synaptic plasticity protein, Ng, which we also found to be significantly down-regulated in response to chronic cocaine exposure *in vivo*.

In the present study, we report that *in vitro* cocaine treatment of SH-SY5Y neuroblastoma cells and human primary neurons induces a decrease in RXR-γ protein levels in both cell types, which can be prevented by proteasomal inhibition. Because cocaine is known to induce production of pro-inflammatory cytokines such as TNF-α both *in vivo* and *in vitro*, we evaluated whether cocaine-mediated induction of TNF-α was responsible for the observed alterations in RXR-γ expression. In this context, we found that cocaine treatment induced a time-dependent increase in TNF-α secretion and mRNA levels, and that inhibition of TNF-α with a function-blocking antibody during cocaine treatment attenuated the decrease in RXR-γ protein levels. Additionally, treatment of neurons with TNF-α alone mimicked the effects of cocaine, while inhibition of a key TNF-α downstream effector, JNK, with SP600125 attenuated both cocaine and TNF-α-mediated alterations in RXR-γ expression. Our overall proposed signaling pathway is depicted in Figure 19.

Retinoid-X-receptor-α is a known substrate of P-JNK, and its phosphorylation is believed to induce its nuclear export and degradation in non-CNS cell types, such as hepatocytes. Here, we provide the first evidence to date that RXR-γ may also serve as a substrate of P-JNK, and that phosphorylation likely negatively regulates levels of RXR-γ

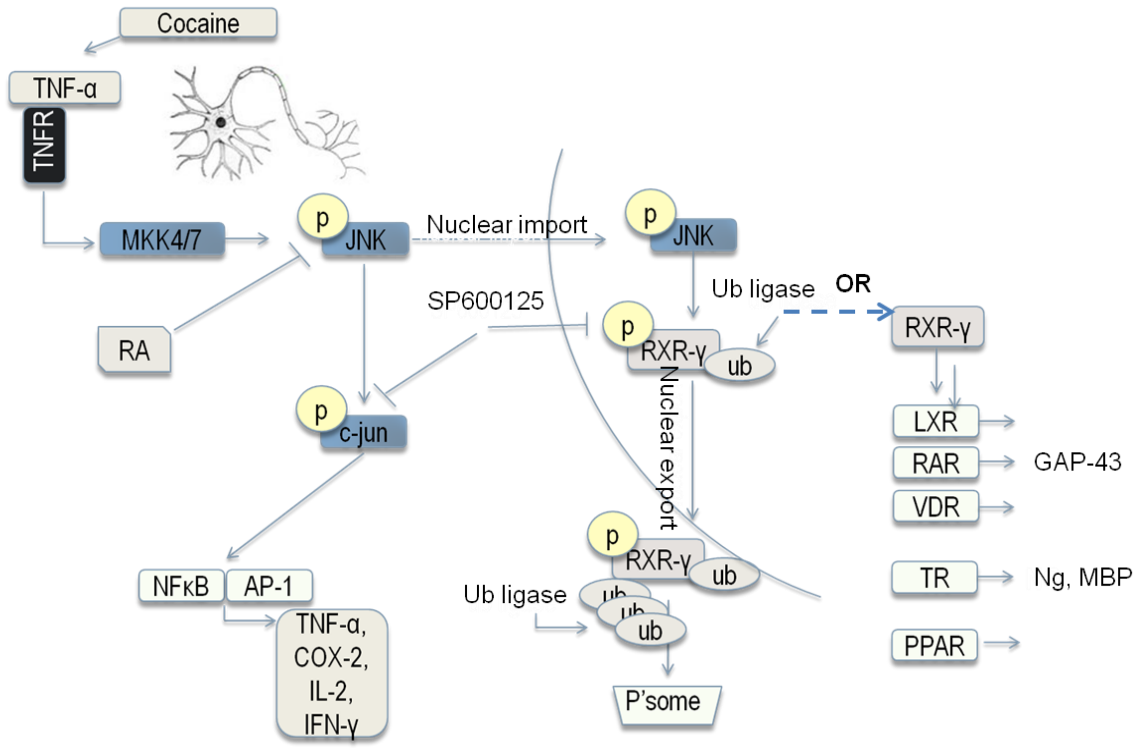


Figure 19. Proposed signaling pathway describing cocaine-mediated decrease in RXR-γ protein levels

Cocaine induces neuronal expression of TNF-α, which in turn activates cell stress kinase cascades, resulting in the activation of JNK by phosphorylation. P-JNK can in turn activate c-jun to induce transcription of NFκB and AP-1 regulated genes including TNF-α, COX-2, IL-2, and IFN-γ in the a well-characterized pathway. We propose that P-JNK phosphorylates nuclear RXR-γ, and phosphorylation of RXR-γ signals for its ubiquitination, nuclear export, and proteasomal degradation. In non-stressed cellular conditions, RXR-γ is not phosphorylated and/or ubiquitinated, and is free to heterodimerize with partner nuclear receptors and regulate transcription of target genes such as GAP-43, Ng, and MBP. Activation of JNK may be inhibited by RA, and downstream kinase activity of P-JNK can be blocked by the pharmacological inhibitor SP600125. RA, retinoic acid; RXR-γ, retinoid X receptor-γ; Ub, ubiquitin; P'some, proteasome; LXR, liver X receptor; RAR, retinoic acid receptor; VDR, vitamin D receptor; TR, thyroid hormone receptor; PPAR, peroxisome proliferator activated receptor; GAP-43, growth-associated protein-43; Ng, neurogranin; MBP, myelin basic protein.

in neurons. We demonstrate that inhibition of JNK signaling prevents RXR-γ degradation.

Cocaine treatment induced TNF-α production within 30 min, as assessed by ELISA. This time course coincided with downstream JNK activation, as assessed by ratio of P-JNK to total JNK by western blotting. Our results appeared to be independent of cocaine's effects on the dopaminergic system, as treatment of SH-SY5Y cells with dopamine alone did not produce a discernible effect on RXR-γ expression (data not shown). Interestingly, it has previously been reported that dopamine treatment induces TNF-α expression in SH-SY5Y cells, an effect not observed in our studies. This discrepancy could be attributed to differences in dosage, as we selected to use a physiological dose of dopamine, while previous studies instead utilized cytotoxic doses. Additionally, differences in treatment duration may have affected the observed outcomes. However, it remains a possibility that excessive synaptic dopamine resulting from cocaine-mediated inhibition of the dopamine transporter could induce cytotoxicity and inflammatory cascades through reactive breakdown products *in vivo* [82].

Our current findings may extend to other CNS cell types. For example, it was recently found that RXR-γ serves a critical role in oligodendrocyte maturation and remyelination following white matter lesion [182]. We have previously reported a reduction in white matter protein levels and increased oligodendrocyte cell death in response to cocaine exposure *in vivo* [266]. Additionally, this loss of white matter was attenuated by administration of the β-lactam antibiotic, Ceftriaxone (Ceft). While the protective effects of Ceft on white matter integrity were attributed primarily to maintenance of glutamate homeostasis via up-regulation of glial glutamate transporters, decreased cocaine-mediated neuroinflammation and subsequent effects on oligodendrocyte RXR-γ expression and signaling cannot be discounted from playing a role in the Ceft-mediated protection.

CHAPTER 4.

CHRONIC EXPOSURE TO COCAINE INDUCES WHITE MATTER LOSS, LIPID PEROXIDATION, AND DEATH OF OLIGODENDROCYTES

Introduction

Imaging studies and postmortem microarray data from human cocaine abuser populations suggest that chronic abuse disrupts white matter (WM) integrity and myelin gene expression in discrete brain regions, including the nucleus accumbens (NA) [22, 54, 56]. As human studies of abuse are often confounded by polydrug use and co-morbid conditions, *in vivo* animal studies are needed to delineate pathways affected by cocaine that may contribute to myelin loss to highlight potential treatment targets. In a recent magnetic resonance imaging study, WM abnormalities were reported in active cocaine users [85]. However, the WM abnormalities resolved to some degree in patients with prolonged abstinence, suggesting recovery potential, making results from our study relevant for adjuvant therapies during rehabilitation programs. The mechanisms involved in cocaine-induced myelin loss are unknown, but may involve glial excitotoxicity [21, 142, 267-272]. Even less clear are mechanisms that may contribute to white matter recovery or protection.

Some studies have evaluated the effects of maternal cocaine abuse on myelination defects in offspring [66], however, investigations of cocaine-mediated myelin disruption in adult animal models have not been previously described. Recent studies in our laboratory uncovered a new model by which cocaine significantly decreases WM proteins in adult mice. Chronic cocaine administration followed by withdrawal and challenge resulted in a significant decrease in a number of myelin-associated proteins, including MBP, PLP, MOG, and MAG in the NA. However, when ceftriaxone, a glutamate subtype transporter-1 (GLT-1) activator [22, 273, 274] was administered during the withdrawal period, no evidence of WM loss was observed. Ceftriaxone has displayed anti-addictive and neuroprotective properties in murine models of cocaine abuse and multiple sclerosis, respectively [22, 275, 276], although the exact mechanisms through which it acts are unknown. Additionally, recent studies by Sondheimer and Knackstedt reported that ceftriaxone prevented the induction of cocaine sensitization in a rat model of cocaine abuse [277].

Our results report two important observations in an adult mouse model: cocaine-induced myelin loss and attenuation of cocaine-induced WM protein loss in ceftriaxone-treated mice [266]. The observation that ceftriaxone negates cocaine-induced WM loss provides clues as to the mechanism through which cocaine may exert its effects on WM protein levels and points to alterations in glutamatergic homeostasis. Expanded studies are underway to address this hypothesis.

In addition to our results demonstrating WM loss in the NA, a detailed time course study revealed a time-dependent decrease in MBP levels within the hippocampus, as well. Demyelination in the hippocampus could have significant impact on cognitive function, especially in areas related to learning and memory.

Oligodendrocytes are highly vulnerable to lipid peroxidation events, which likely accounts for cell death and demyelination in some neuroinflammatory diseases [75]. Cocaine has been shown to induce lipid peroxidation in cardiac and liver systems [80], but little evaluation has been given to these events within the CNS. We provide evidence that cocaine increases lipid peroxidation in the hippocampus, and also in oligodendrocyte progenitor cell (OPC) culture systems. However, this effect could be attenuated by administration of 9-*cis*-RA in the OPC model. Taken together, these data suggest that cocaine-induced lipid peroxidation could contribute to oligodendrocyte death and demyelination, and antioxidative therapies that scavenge toxic byproducts may assist the restoration myelin integrity in cocaine abuse models.

Results

Chronic cocaine exposure in vivo causes loss of white matter in the nucleus accumbens of adult mice

Despite numerous clinical reports of altered myelination in cocaine abusers, very limited investigation of cocaine-mediated white matter damage has been performed in animal models. One study evaluated white matter integrity in cocaine-exposed rats by DTI and found decreased FA indicative of myelin defects [63]. Additionally, this study reported significantly decreased levels of MBP in the corpus callosum [63]. We have expanded upon these findings to investigate WM protein levels in brain regions known to be a direct target of cocaine, such as the NA, and regions involved in learning and memory that have previously been shown undergo alterations in response to chronic cocaine abuse, such as the hippocampus. Because clinical imaging studies suggest the degree of myelin integrity achieved during periods of cocaine withdrawal negatively correlates to probability of relapse, we chose to evaluate the effects of cocaine on WM levels after a two-week period of cocaine exposure (15 mg/kg, ip, 1 x daily), followed by a 30 day withdrawal period. We evaluated levels of WM proteins MBP and PLP, which additively constitute up to approximately 60-80% of the myelin sheath. Additionally, we evaluated another major protein, MOG, which was identified to be down-regulated in post-mortem brains of human cocaine abusers [54], as well as myelin-associated glycoprotein (MAG), which is critical regulator of myelin-axonal contact formation [278].

Chronic cocaine administration (15 mg/kg daily for 14 days) followed by 30-day withdrawal and cocaine challenge (15 mg/kg, once) resulted in significant decreases in myelin-associated proteins in the NA (Figure 20A). White matter deficits were not observed in the prefrontal cortex or other brain regions assessed (data not shown). During withdrawal, mice were given vehicle injections every day for 30 days, followed by a single dose cocaine challenge on the last day. Following the final challenge injection and tissue harvest, MBP levels in cocaine-treated animals were 38% lower than controls (P < 0.05), and PLP levels were 55% lower than controls (P < 0.001) (Figure 20A). Likewise, in cocaine-treated animals, the PLP alternate isoform DM20 was decreased by 33% compared to controls, but levels did not reach statistical significance (or were NS) (Figure20A). Levels of MOG and MAG were decreased significantly (P < 0.001) by 42 and 58%, respectively, compared to control levels (Figure 20A). Double-immunofluorescence labeling of NA tissue from a representative control mouse showed robust MBP immunoreactivity (green) and associated neurofilament (red) for axons (Figure 20B). On the other hand, in a cocaine-treated mouse, sparse MBP immunoreactivity (green) was observed in the NA (Figure 20C). Interestingly, neurofilament immunolabeling of the cocaine-treated mouse remained robust, suggesting the presence of unmyelinated axons (Figure 20C). These data indicate that 14 days of cocaine administration leads to significant loss of MBP, PLP, MOG, and MAG WM proteins even after 30 days of cocaine withdrawal and a single challenge cocaine injection.

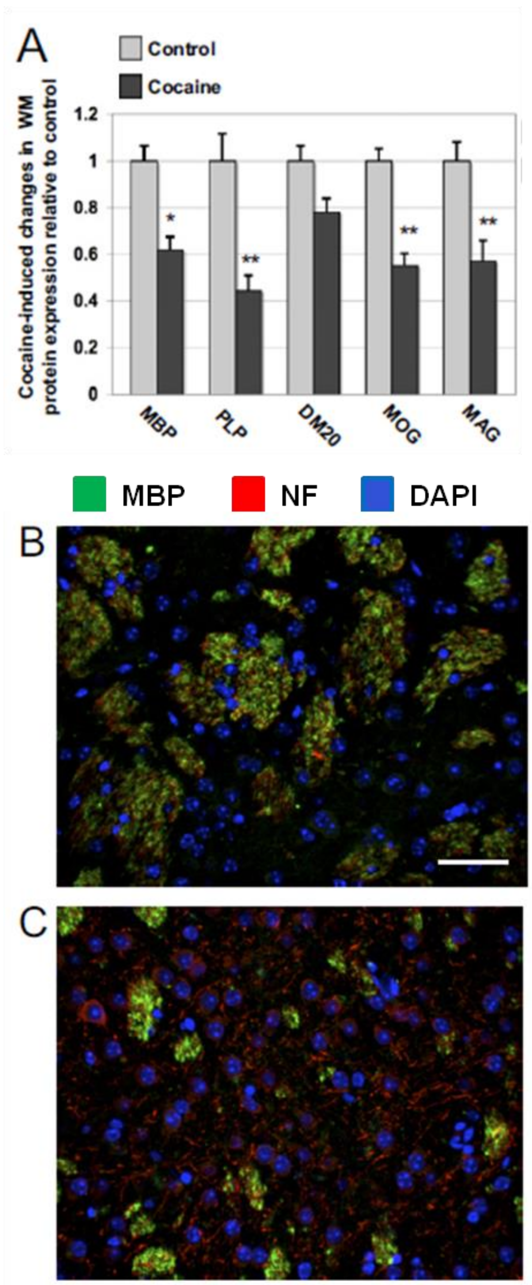


Figure 20. Cocaine decreases white matter proteins in the mouse nucleus accumbens (A) Protein levels of MBP, PLP, DM20, MOG, and MAG in the nucleus accumbens of mice administered cocaine (15 mg/kg) for 14 days, followed by 30 days of vehicle during withdrawal and a single challenge dose of cocaine (15 mg/kg). n = 8 to 10 mice per group. Data were analyzed by one-way ANOVA with Bonferroni's multiple comparisons post-hoc testing where appropriate. $*p < 0.05$; $**p < 0.001$). (B, C) Double immunofluorescence labeling of nucleus accumbens sections from (B) control and (C) cocaine-treated mice (14 days cocaine, 30 days withdrawal, followed by cocaine challenge). Tissues are labeled with MBP (green) for myelin and NF (red) for axons. Nuclei are labeled with DAPI (blue). Original magnification, x40. Scale bar = 10 µm. MBP, myelin basic protein; PLP, proteolipid protein; DM20, PLP alternate isoform; MOG, myelin oligodendrocyte glycoprotein; MAG, myelin-associated glycoprotein; NF, neurofilament; DAPI, 4',6-diamidino-2-phenylindole (from [266]).

Cocaine-induced white matter loss is ameliorated by administration of the β-lactam antibiotic, Ceftriaxone, during withdrawal

One hypothesis of cocaine-mediated WM damage is that chronic cocaine exposure disrupts glutamate homeostasis by inducing glutamate release following acute exposure to drug, but causes lowered synaptic glutamate levels in the NA during periods of withdrawal. Following cocaine abstinence, reintroduction to the drug elicits an increase in extracellular glutamate in the NA greater than that seen during initial cocaine exposures, even when the dosage remains the same [21]. Furthermore, cocaine has been shown to down-regulate expression of glial glutamate transporters, including excitatory amino acid transporter 2 (EAAT2, or GLT-1) [22], which is responsible for clearing greater than 80% of the synaptic glutamate. Mechanisms underlying this down-regulation remain largely unknown, but much concerted effort has been directed toward exploring mechanisms to increase GLT-1 levels, as this may aid in preventing and/or minimizing excitotoxicity in drug abuse models and also in a number of neurodegenerative diseases. In this context, the β-lactam antibiotic ceftriaxone has shown to be an activator of GLT-1 [274], and we therefore assessed the therapeutic potential of ceftriaxone in our animal model of chronic cocaine abuse.

Mice administered cocaine for 14 days followed by daily injections of ceftriaxone (200 mg/kg) during the 30-day withdrawal showed no significant loss of myelin proteins in the NA (Figure 21 A-F). In mice given ceftriaxone with cocaine for 14 days followed by vehicle during withdrawal, the loss of WM was similar to levels in the cocaine-only group (Figure 21, A–F) (compare the Coc group to the Coc with Ceft group). Likewise, mice given ceftriaxone only for 14 days, followed by injections of vehicle during

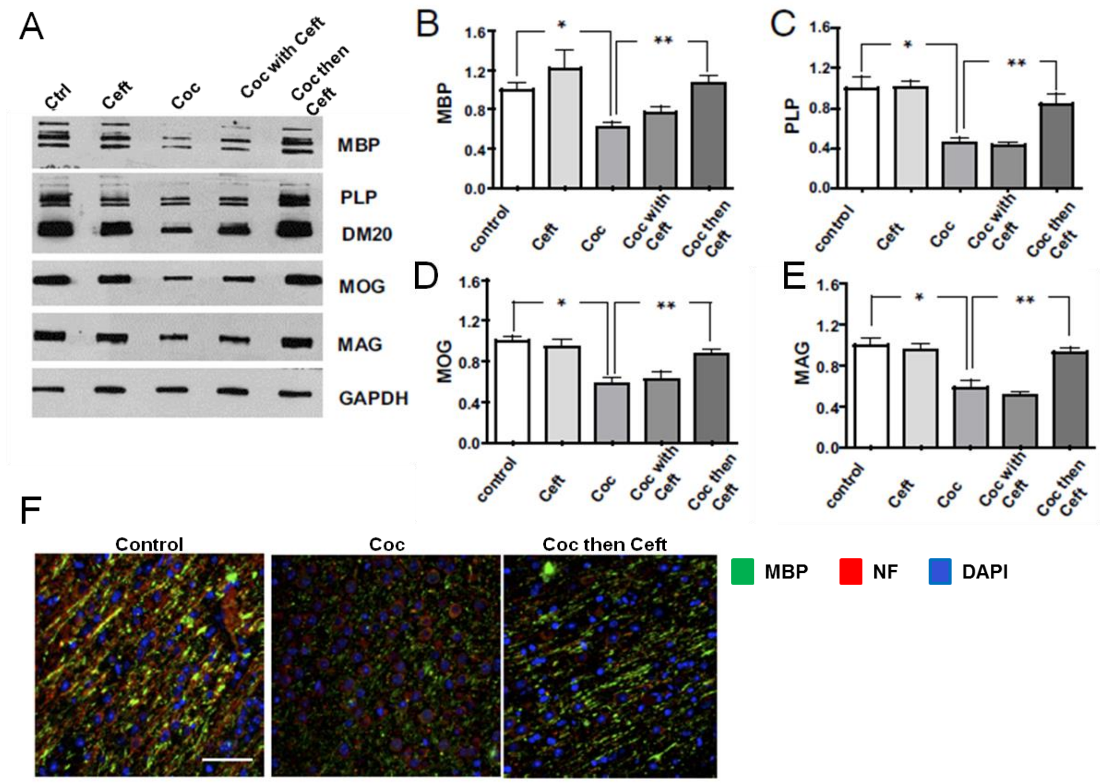


Figure 21. Administration of ceftriaxone during withdrawal attenuates cocaine-induced white matter loss

(A) Representative western blot of 10 µg protein from NA from one mouse from each group. GAPDH was used as a loading control. (B-F) WM protein levels in control; 17 days ceftriaxone followed by 30 days vehicle during withdrawal (Ceft); 14 days cocaine followed by 30 days vehicle during withdrawal (Coc); 3 days ceftriaxone pretreatment followed by 14 days of ceftriaxone and cocaine, and 30 days of vehicle during withdrawal (Coc with Ceft); 14 days of cocaine followed by 30 days ceftriaxone during withdrawal (Coc then Ceft). (B) Quantification of MBP (*$P < 0.05$, **$P < 0.01$), (C) PLP (*$P < 0.001$; **$P < 0.05$), (D) MOG (*$P < 0.001$, **$P < 0.01$), and (E) MAG (*$P < 0.001$, **$P < 0.01$) levels. n = 8-10 mice per group; analyzed by ANOVA with Bonferroni's multiple comparisons post-hoc testing. (F) Double immunofluorescence labeling of NA control, cocaine (15mg/kg) for 14 days followed by 30 days vehicle during withdrawal and a single challenge dose of cocaine, and cocaine (15 mg/kg) for 14 days followed by 30 days ceftriaxone (200 mg/kg) during withdrawal and a single challenge does of cocaine. Tissues are labeled with MBP in green, neurofilament in red, and nuclei are labeled in blue with DAPI. Original magnification, x40. Scale bar = 10 µm. MBP, myelin basic protein; PLP, proteolipid protein; MOG, myelin oligodendrocyte glycoprotein, MAG, myelin-associated glycoprotein (from [266]).

withdrawal had WM levels similar to those of the control group (Figure 21, A–F) (compare the control group to the Ceft group). These data show that administration of ceftriaxone during the withdrawal period after 14 days of cocaine administration prevents MBP, PLP, MOG, and MAG WM protein loss upon withdrawal and challenge.

Double immunofluorescence labeling of NA tissue from a representative control mouse showed robust MBP (green) and neurofilament (red) immunoreactivity (Figures 20B and 21F). On the other hand, in a cocaine-treated mouse, sparse MBP immunoreactivity (green) was observed in the NA (Figures 20C and 21F). In a mouse treated with cocaine for 14 days followed by ceftriaxone during withdrawal, MBP labeling appeared similar to levels in the control (compare green) (Figure 21F). However, co-localization (yellow) of neurofilament (red) with MBP (green) in mice given cocaine then ceftriaxone during withdrawal appears to be decreased (Figure 21F). This pattern may suggest that even though MBP levels are normal, association with axons may be altered in ceftriaxone-treated cocaine-administered (Coc then Ceft) mice.

Chronic cocaine exposure induces oligodendrocyte cell death in vivo, which can be blocked by administration of Ceftriaxone

Cocaine-mediated changes in glutamate signaling may induce oligodendrocyte cell damage or death, and may be responsible in part for the loss of WM proteins observed in cocaine-treated mice. Likewise, ceftriaxone (a GLT-1 activator) abolished cocaine-induced white matter protein loss, suggesting a contribution of glutamate excitotoxicity-mediated cell death. In this context, expression levels of GLT-1 and cleaved caspase-3, which is indicative of apoptosis, were assessed. GLT-1 levels in the cocaine-treated group were lower than levels in the control group, although changes did not reach statistical significance (Figure 22, A and B). Because GLT-1 is responsible for the clearance of more than 90% of glutamate in the synaptic cleft, even slight changes in GLT-1 expression could have a significant impact on synaptic glutamate levels [279]. In the cocaine-treated group, GLT-1 levels were significantly lower than those in ceftriaxone only and in cocaine then ceftriaxone groups ($P < 0.01$) (Figure 22, A and B). In the cocaine-treated group, caspase-3 levels were significantly greater than the control ($P < 0.05$) and cocaine then ceftriaxone groups ($P < 0.01$) (Figure 22, C and D) in which caspase-3 was nearly undetectable. In this context, other studies have shown that glutamate excitotoxicity induces apoptosis in oligodendrocytes in a caspase-dependent manner [72, 280]. To assess if oligodendrocytes in cocaine-treated mice without ceftriaxone showed increased caspase-3 cleavage, double immunofluorescence labeling for activated caspase-3 and an oligodendrocyte-specific marker, CC1, was conducted. CC1 immunoreactive oligodendrocytes (red) in cocaine-treated mice showed immunolabeling for caspase-3 (green) (Figure 22F, arrowheads), whereas, control mice (Figure 22E) and cocaine then ceftriaxone mice (Figure 22G) showed nearly undetectable caspase-3 immunolabeling.

Although detailed behavior assessments are beyond the scope of this report, we observed that ceftriaxone-treated mice and mice that received cocaine followed by ceftriaxone during withdrawal (Coc then Ceft) did not exhibit increased activity as observed in cocaine-treated mice (Coc) and in mice treated with cocaine and ceftriaxone simultaneously (Coc with Ceft) (data not shown). Thus, the prominent clinical signs observed in some of these groups included increased activity. Control, Ceft, and Coc then Ceft groups were not different from one another.

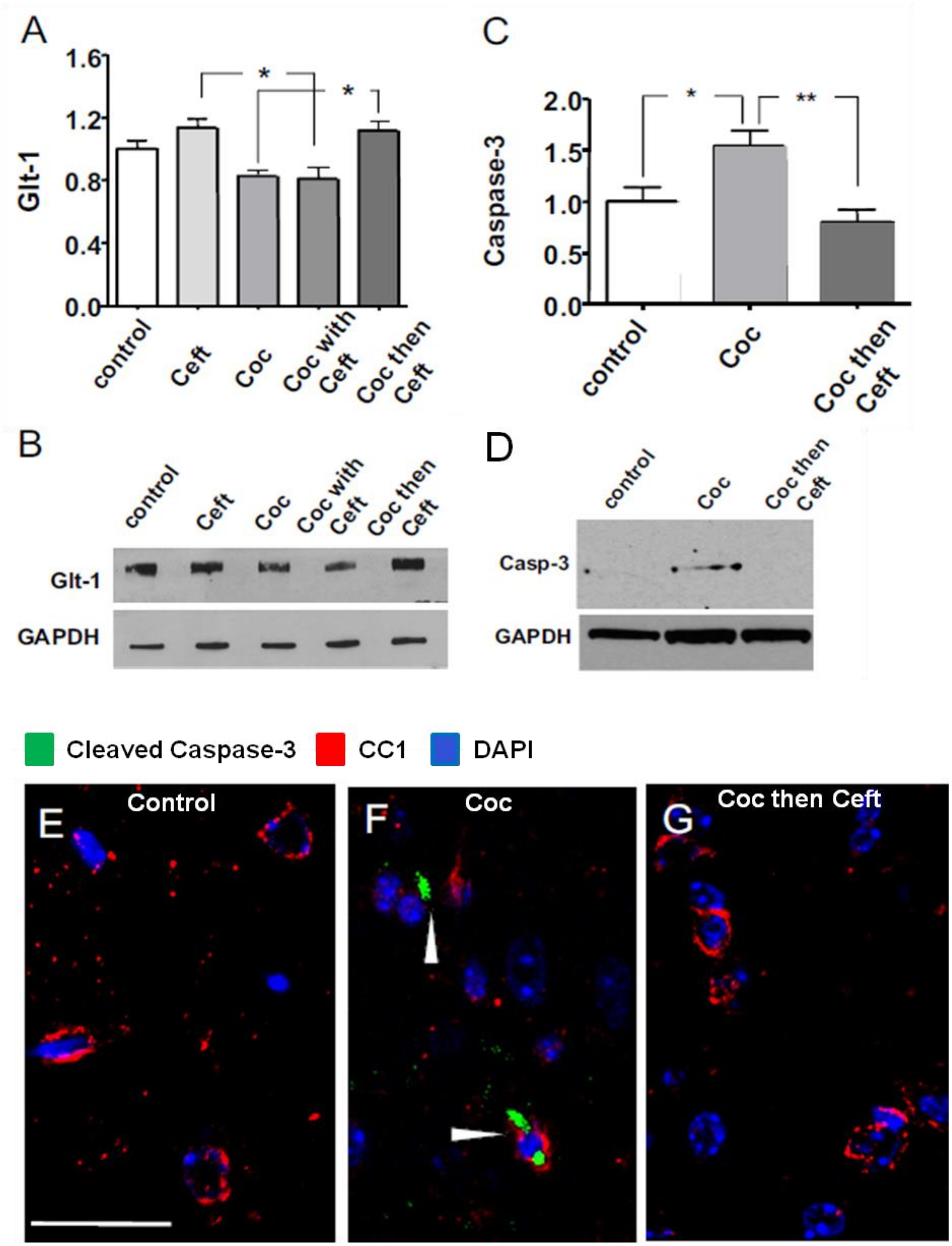


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Figure 22. Administration of ceftriaxone during withdrawal increases GLT-1 expression and prevents oligodendrocyte death in cocaine-treated mice
(A) GLT-1 levels in NA of control; 17 days ceftriaxone followed by 30 days vehicle during withdrawal (Ceft); 14 days cocaine followed by 30 days vehicle during withdrawal (Coc); 3 days ceftriaxone pretreatment followed by 14 days of ceftriaxone and cocaine, and 30 days of vehicle during withdrawal (Coc with Ceft); 14 days of cocaine followed by 30 days ceftriaxone during withdrawal (Coc then Ceft) (*$P < 0.01$). (B) Representative western blot of GLT-1 levels in 10 μg protein from NA from one mouse from each group. GAPDH was used as a loading control. (C) Quantification of cleaved caspase-3 levels in 40 μg protein from NA from one mouse from each group. GAPDH was used as a loading control (*$P < 0.05$, **$P < 0.001$). (D) Representative western blot of cleaved caspase-3 levels in control, 14 days cocaine followed by 30 days vehicle during withdrawal (Coc), 14 days cocaine followed by 30 days ceftriaxone during withdrawal (Coc then Ceft). n = 8-10 mice per group; data analyzed by ANOVA with Bonferroni's multiple comparison post-hoc testing. (E-G) NA tissue from representative mice labeled with antibody against cleaved caspase-3 (green), oligodendrocyte marker CC1 (red) and nuclei labeled blue with DAPI. (E) NA tissue from control show shows robust CC1 immunoreactivity (red) with no evidence of caspase-3 activity. (F) NA tissue from cocaine-treated mouse shows decreased CC1 immunoreactivity (red) with evidence of caspase-3 activity (green, arrowheads) associated with CC1+ oligodendrocytes. (G) NA tissue from cocaine treated mice that received ceftriaxone during withdrawal shows CC1 immunoreactivity (red) with undetectable caspase-3 activity. Original magnification, x100. Scale bar = 10 μm (from [266]).

Administration of ceftriaxone during cocaine treatment ameliorates cocaine-induced deficits in a novel object recognition task

Chronic cocaine abuse is associated with cognitive deficits such as impairments in working memory and executive function which are attributed to metabolic alterations in the PFC [47, 49]. Likewise, demyelination in frontal cortical circuitry can induce similar impairments and imaging studies suggest the degree of cortical WM integrity negatively correlates to the likelihood of relapse in cocaine-abstinent individuals [47, 86, 87]. To evaluate working memory in cocaine-administered mice, with or without co-administration of Ceft, we conducted a novel object recognition (NOR) task. Our results demonstrate that cocaine-administered mice are impaired in recognizing a novel object over a familiar object (47% of time spent exploring the novel object compared to 66% of time in vehicle-treated mice) (Figure 23). However, administration of Ceft during cocaine treatment prevented this effect, as Coc with Ceft mice spent 61% of time exploring the novel object, which was not significantly different from the time spent by vehicle-only and Ceft-only administered mice (66% and 63%, respectively) (Figure 23). These findings suggest that Ceft may be a useful therapeutic in the treatment of cocaine-induced behavioral and cognitive impairments.

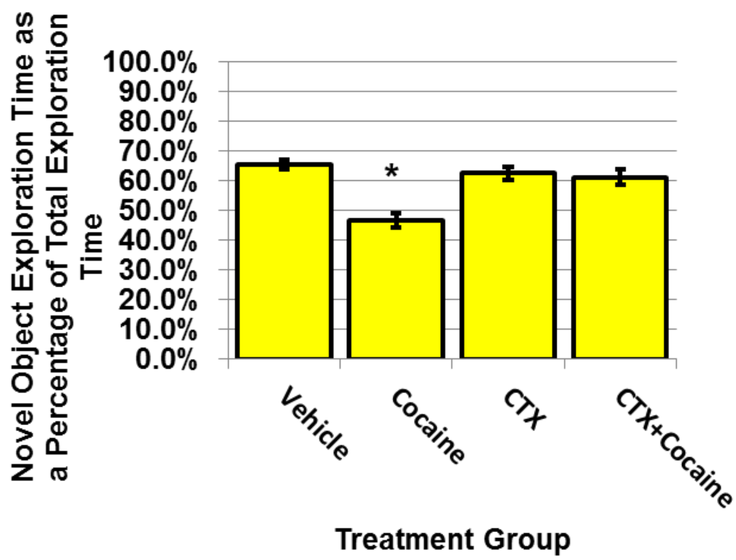

Figure 23. Ceftriaxone treatment during cocaine exposure attenuates novel object recognition deficits in cocaine-administered mice
Mice were treated with cocaine (15 mg/kg) for 14 days, with or without co-administration of ceftriaxone (CTX) (200 mg/kg), and were then subjected to a novel object recognition (NOR) task. Mice that were administered cocaine plus vehicle demonstrated deficits in NOR, while mice administered cocaine with CTX displayed novel object exploration time similar to control and CTX only treated mice. n = 8-10 mice per group, *$P < 0.05$.

Exposure to cocaine induces a time-dependent decrease in MBP levels in the adult mouse hippocampus

Hippocampal demyelination may contribute to significant learning and memory impairments. Since we saw significantly decreased levels of the TR/RXR-regulated gene neurogranin in cocaine-treated mice, we elected to evaluate whether cocaine would have a similar effect on hippocampal levels of MBP, which is similarly regulated transcriptionally by TR/RXR heterodimers [180]. In this context, we observed time-dependent reductions in hippocampal MBP levels in cocaine-treated mice (Figure 24). Quantitative densitometric analysis of MBP levels is provided (Figure 24A) with a representative western blot (Figure 24B). Additionally, immunolabeling of MBP in hippocampal sections from control and cocaine-treated mice (10 days of treatment) demonstrated significantly reduced MBP reactivity (green) in the hippocampus of cocaine-treated mice (Figure 24, C and D). Additionally, to confirm that the demyelination we saw following 14 days of cocaine abuse in the mouse NA is replicated at 10 days, we performed double-labeling with MBP (green) and SMI-32, a marker for hypo-phosphorylated neurofilament which is commonly used as a marker for demyelinated axons (red), demonstrates the presence of a number of unmyelinated axons in NA tissue sections from cocaine-treated mice (Figure 24E). Much less SMI-32 immunoreactivity was observed in the NA of control mice (data not shown).

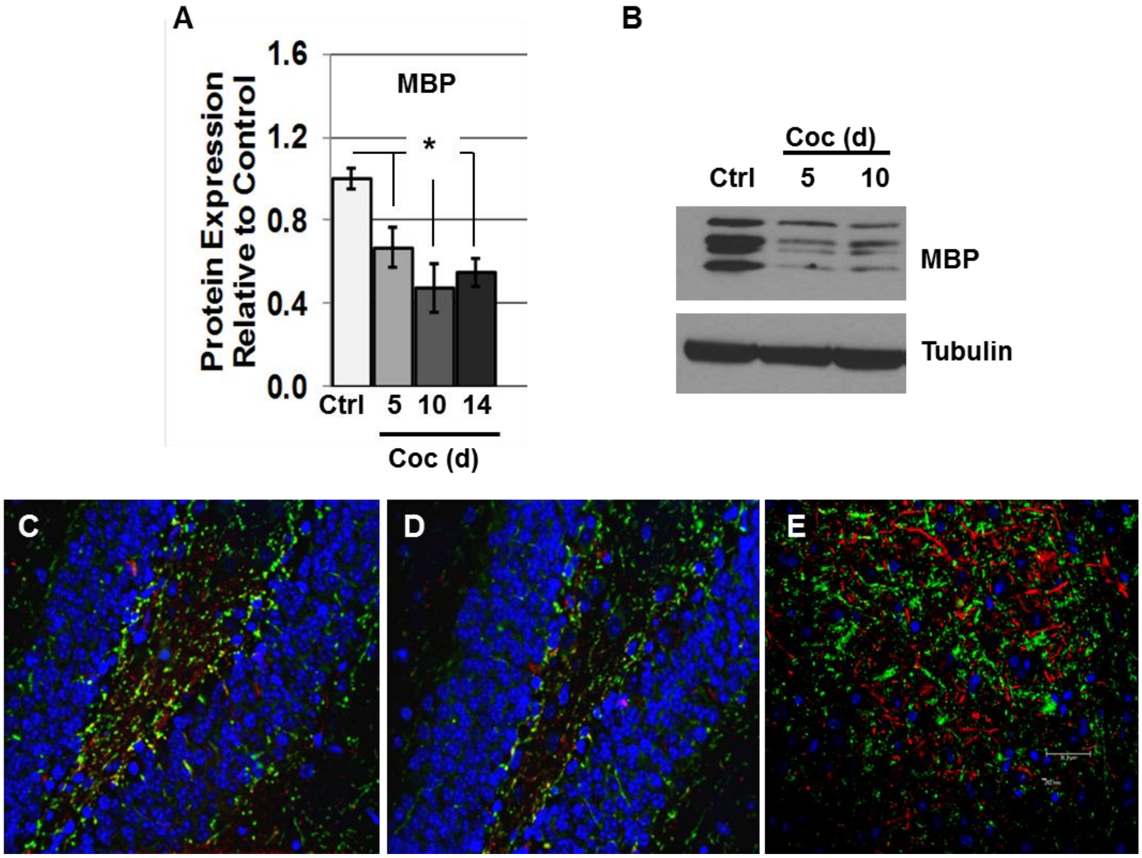

Figure 24. Cocaine decrease hippocampal white matter
(A) Quantification of MBP levels in hippocampal tissue from control and cocaine-treated (5, 10, and 14 days) mice (*P < 0.01), n = 4 mice per group. (B) Representative western blot of MBP levels in the hippocampus of control and cocaine-treated (5 and 10 days) mice. Tubulin was used as a loading control. (C-D) Double immunofluorescence labeling of MBP (green) and neurofilament (red) expression in the hippocampus of a (C) control and (D) cocaine-treated (10 days) mouse. (E) Double- immunofluorescence labeling of MBP (green) and SMI-32 (red), which detects hypophosphorylated neurofilament and is indicative of unmeylinated axons in the striatum of a cocaine-treated (10 days) mouse reveals the presence of numerous unmeylinated axons. Nuclei are labeled blue with DAPI, original magnification, x40. MBP, myelin basic protein; Ctrl, control; Coc, cocaine.

Exposure to cocaine induces lipid peroxidation in brains of mice in vivo

Because lipid peroxidation events are potential contributors to oligodendrocyte death and myelin damage, we assessed lipid peroxidation levels in the brains of cocaine-treated mice following 7 or 10 days of chronic, daily administration. We observed increased content of MDA, a byproduct of lipid peroxidation and cellular marker of oxidative stress, in hippocampal brain homogenate from cocaine-treated mice compared to vehicle-treated controls following 7 days of daily treatment (Figure 25A). Additionally, increased levels of 4-HNE, another lipid peroxidation byproduct that forms aldehyde protein adducts which may disturb normal protein functioning [78], were observed in hippocampi of cocaine-treated mice, as assessed by western blotting (Figure 25, B and C). Double immunofluorescence labeling of striatal tissue from control and cocaine-treated mice (10 days treatment), demonstrated increased co-localization of 4-HNE (green) in CC1+ (red) oligodendrocytes in cocaine-treated mice compared to control mice, where 4-HNE was nearly undetectable (Figure 25, D and E), suggesting increased lipid peroxidation also occurs in the striatum in response to cocaine exposure and oligodendrocytes are a viable target. Additionally, increased expression of the nitrative stress marker, 3-NT (red), was observed in the hippocampus of a cocaine-treated mouse compared to control, and increased expression and altered morphology of Iba1+ microglia (green) suggest immune activation in cocaine-treated mice (Figure 25, F and G).

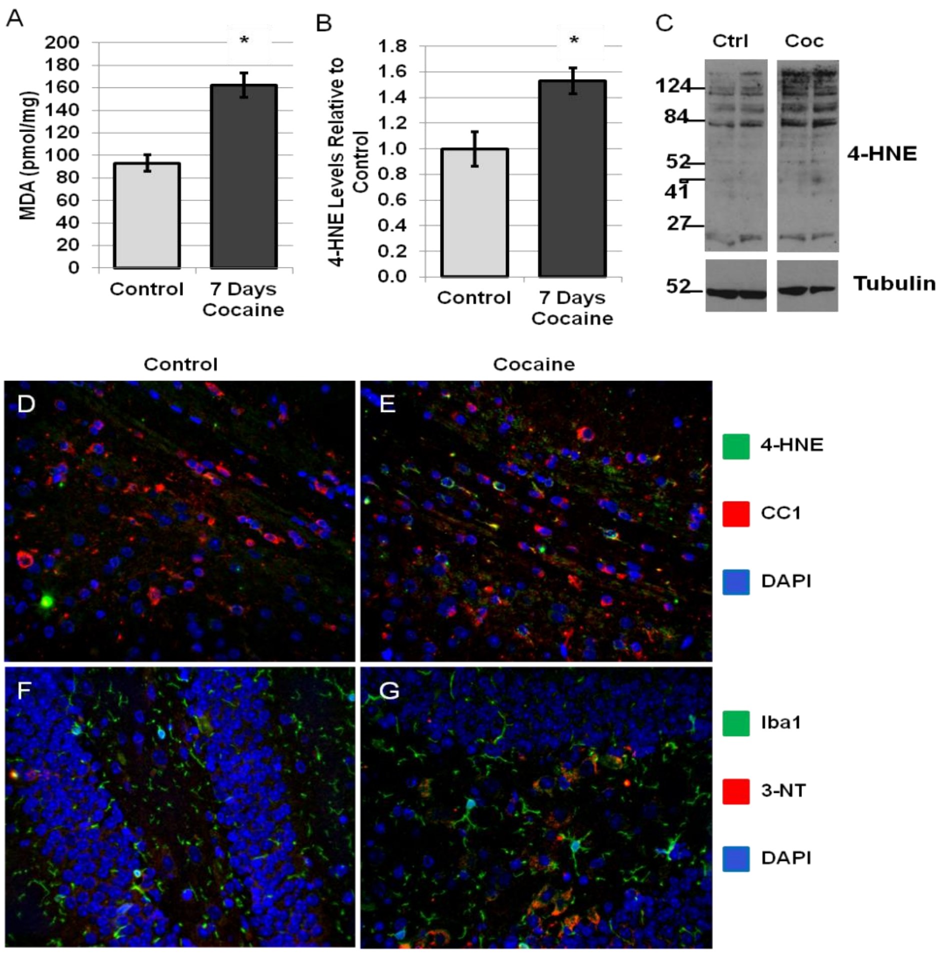

Figure 25. Cocaine induces lipid peroxidation *in vivo*
(A) MDA content from 10 mg hippocampal tissue from control and cocaine-treated (7 days) mice (*P < 0.01), n = 4 mice per group. (B) Quantification of 4-HNE levels from hippocampal tissue from control and cocaine-treated (7 days) mice (*P < 0.01), n = 4 mice per group. (C) Representative western blot of 4-HNE expression in hippocampal tissue from control and cocaine-treated mice. Tubulin was used as a loading control. (D-E) Double-immunofluorescence labeling for CC1 (red) and 4-HNE (green) in striatal tissue from a (D) control and (E) cocaine-treated (10 days) mouse. Increased lipid peroxidation is evidence by 4-HNE (green) immunoreactivity in CC1+ (red) cells in tissue of the cocaine-treated mouse. (F-G) Double-immunofluorescence labeling for microglial marker Iba1 (green) and nitrative stress marker 3-NT (red) in hippocampal sections of a (F) control and (G) cocaine-treated (10 days) mouse. Altered microglia morphology and increased 3-NT expression is observed in tissue from cocaine-treated mouse. Nuclei are labeled blue with DAPI, original magnification, x40. MDA, malondialdehyde; 4-HNE, 4-hydroxy-2-nonenal; 3-NT, 3-nitrotyrosine.

Exposure to cocaine induces lipid peroxidation and Bax expression in oligodendrocyte progenitor cells in vitro, while both are attenuated by administration of 9-cis-retinoic acid

To assess cell-specific effects of cocaine exposure in oligodendrocytes, an OPC *in vitro* system was utilized. CG4 cells were grown in conditioned medium (CM) from untreated or cocaine-treated B104 neuroblastoma cells. Following 48 h of exposure to cocaine CM, CG4 cells expressed significantly higher levels of the lipid peroxidation product 4-HNE compared to cells that were cultured in control CM (Figure 26, A and B). Additionally, expression of the pro-apoptotic protein Bax was increased in CG4 cells exposed to cocaine-CM compared to control CM (Figure 26, C and D). The increase in 4-HNE and Bax expression in cocaine-exposed cells could be attenuated by treatment with 1 μM 9-*cis*-RA during cocaine exposure (Figure 26). These findings suggest cocaine may contribute to WM loss through lipid peroxidation events, which oligodendrocytes are highly susceptible to due to their high lipid-to-protein ratio. Additionally, our studies highlight the potential therapeutic value of retinoid treatment and/or activation of RXRs to provide protection during times of increased cellular stress.

Discussion

Several pathways likely contribute to cocaine-mediated WM loss. Among potential contributors are changes in levels of dopamine, cellular retinoic acid, or glutamate. In this regard, acute central nervous system effects of cocaine administration include increased extracellular dopamine in the synaptic cleft due to the blockade of the dopamine transporter [281]. Activation of dopaminergic D3 receptors decreases

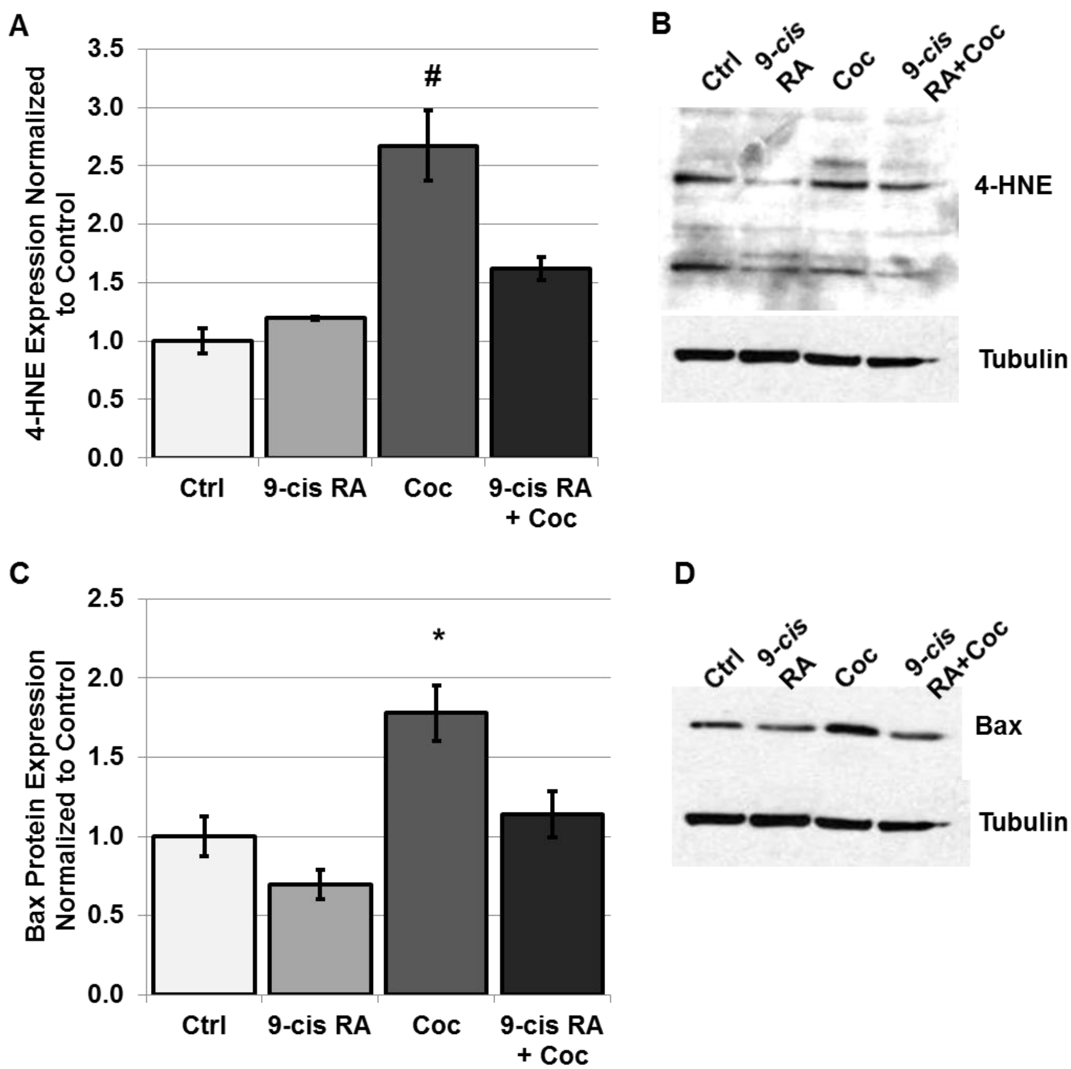

Figure 26. Cocaine induces lipid peroxidation in oligodendrocyte progenitor cells *in vitro*

(A-D) CG4 oligodendrocyte progenitor cells (OPCs) were treated with conditioned medium (CM) from cocaine-treated neurons for 48 h in the absence or presence of 9-*cis* RA (1 μM). (A) Densitometric quantification of 4-HNE levels in OPCs. (B) Representative western blot of 4-HNE expression in OPCs compared to tubulin loading control. (C) Densitometric quantification of Bax protein levels in OPCs. (D) Representative western blot of Bax expression in OPCs. $n \geq 3$; #$P < 0.01$, *$P < 0.05$.

differentiation of oligodendrocyte precursor cells into mature oligodendrocytes and inhibits myelin formation [74]. In addition, our laboratory has demonstrated that chronic cocaine abuse alters retinoic acid signaling and decreases expression of retinoid X receptor isoform-γ [238]. These findings are significant in the context of the current study, because cellular levels of retinoic acid regulate both MBP and PLP [180, 282]. Furthermore, Huang et al recently demonstrated the importance of retinoid X receptor isoform in oligodendrocyte maturation and remyelination after WM lesioning [182]. We have demonstrated that treatment of cocaine-exposed OPCs with 9-*cis*-RA attenuates increases in lipid peroxidation and Bax expression. Thus, cocaine-induced disruption of the retinoic acid pathway in oligodendrocytes may be responsible in part for the WM loss observed after paradigms of chronic abuse.

In our study, the cocaine-induced deficits in WM protein levels were normalized by the GLT1 activator ceftriaxone, suggesting a glutamate-related mechanism may be involved. Early studies reported that acute cocaine exposure increases extracellular glutamate in the NA [267, 268]. It is also reported that reintroduction to cocaine after forced abstinence elicits an increase in extracellular glutamate in the NA greater than increases observed after initial cocaine exposure [269, 283]. Moreover, recent studies by Sondheimer and Knackstedt report that in a rat model of cocaine abuse, ceftriaxone prevented the induction of cocaine sensitization [277]. In light of these findings, all mice received a single challenge dose of cocaine (15 mg/kg i.p.) before euthanasia 24 hours later. Numerous studies from Kalivas et al have expanded on these findings to show that decreased extracellular glutamate levels in the NA after withdrawal from chronic cocaine potentiates reinstatement of cocaine-seeking behavior [21-23, 269]. Studies from Kalivas et al also show that the effects can be prevented by restoring extracellular glutamate levels [22]. Thus, one potential explanation for the observations in our study is that the elevation in extracellular glutamate after cocaine challenge, coupled with the upregulation of *N*-methyl-D-aspartic acid (NMDA) and α-amino-3-hydroxy-5-methyl-4-

isoxazole-propanoic acid (AMPA) receptors, which likely occurred during cocaine withdrawal, may have resulted in glutamate-mediated toxicity that was manifested as oligodendrocyte apoptotic death and a reduction in WM proteins. It is also important to note that the deficits in WM protein levels became more pronounced during the course of withdrawal. WM protein levels after 30 days of withdrawal were only 50% of the control levels in some cases (Figure 20), whereas, a shorter withdrawal interval of 2 days resulted in significant WM loss, but to a lesser degree than a 30-day withdrawal (data not shown). These observations are supported by previous studies showing that temporal decreases in basal glutamate levels are more pronounced at later stages of withdrawal [267, 284]. Thus, repeated cocaine exposure followed by treatment with ceftriaxone during the withdrawal interval may increase the expression of GLT1, an effect that is hypothesized to offset the upregulation of NMDA and AMPA receptors.

Although neurons are reported to be most affected by increased extracellular glutamate on acute cocaine administration [285], the loss of WM proteins suggests damage to oligodendrocytes. In support of glutamate-mediated loss of WM, recent studies in multiple sclerosis, stroke, and cerebral palsy show that oligodendrocytes are damaged by glutamate excitotoxicity. However, other effects of ceftriaxone, such as activation of the GLT1 transporter, independent of changes in expression, reduction of T-cell activation by modulation of cellular antigen-presentation, or metal ion chelation cannot be excluded as contributors to our observations [276, 286]. Important studies from Hanlon et al report that when compared to cocaine users that had been abstinent for at least 1 month ($n = 24$), current cocaine users ($n = 24$) had significantly lower WM tissue densities [85]. Moreover, WM densities for abstinent cocaine users were similar to those of nondrug user controls, suggesting recovery of cocaine-induced WM loss upon prolonged abstinence. Given these findings, it is possible that with a longer withdrawal period in cocaine-treated mice, WM protein levels may have returned to normal in the absence of ceftriaxone. Expanded studies that include dose and time course responses are required to understand and characterize both the mechanisms through which cocaine decreases WM proteins and how ceftriaxone prevents this loss. However, the observation that ceftriaxone negates cocaine-induced WM loss provides clues as to the mechanism through which cocaine may exert its effects on white matter protein levels. One of the limitations of our study is the fact that a group of cocaine-treated withdrawal mice without challenge was not included. The NA plays an integral role in drug reward, as it receives dopaminergic projections from the ventral tegmental area and glutamatergic projections from the prefrontal cortex [287]. Dysfunction of the NA is associated with impulsive decision-making, anxiety, and major depressive disorder [197, 198]. Thus, future studies addressing WM loss and connectivity networks are required to understand cocaine-mediated WM loss and ceftriaxone-induced interventions in myelin disruptions more clearly.

CHAPTER 5.

CONCLUSIONS AND FUTURE DIRECTIONS

Cocaine-mediated neuroinflammation and nuclear receptor signaling

Cocaine abuse remains a significant societal problem for which no effective treatment currently exists. Chronic abuse is associated with behavioral abnormalities, mood disorders, and cognitive impairments, all of which exacerbate complications during rehabilitation and increase the likelihood of relapse. Exposure to cocaine can also evoke an immune response, both peripherally as well as localized within the CNS. This response, mediated primarily through activated microglia, can lead to induction of pro-inflammatory cytokines, decreased glial trophic support for neurons, and disruption of numerous signaling cascades. Our studies provide the first evidence that neuronal RXR signaling is one of these pathways impacted by chronic cocaine exposure. Furthermore, we present data which show cocaine-mediated disruption in RXR signaling is due, at least in part, to TNF-α induction and subsequent activation of cellular stress kinase cascades, namely the phosphorylation of JNK. Importantly, treatment of neurons with TNF-α alone mimics the dysregulation of RXR-γ signaling observed in response to cocaine exposure in multiple neuronal models. As TNF-α signaling is believed to be an integral mediator of neuronal damage in numerous neurodegenerative diseases, these findings may have far-reaching implications.

To expand upon our current studies, we will assess the role of TNF-α induction on RXR disruption *in vivo* by administering pharmacological inhibitors of TNF-α and/or its downstream effector JNK during cocaine treatment and during periods of cocaine withdrawal. Tumeric-derived curcumin is an inhibitor of phospho-JNK which can be administered orally, is well-tolerated, and readily crosses the BBB. Additionally, it provides powerful anti-oxidant and anti-angiogenic properties, leading to its use as a dietary supplement during treatment in many autoimmune disease and cancer clinical trials [288-290]. A number of the previously characterized protective properties of curcumin are summarized in Figure 27.

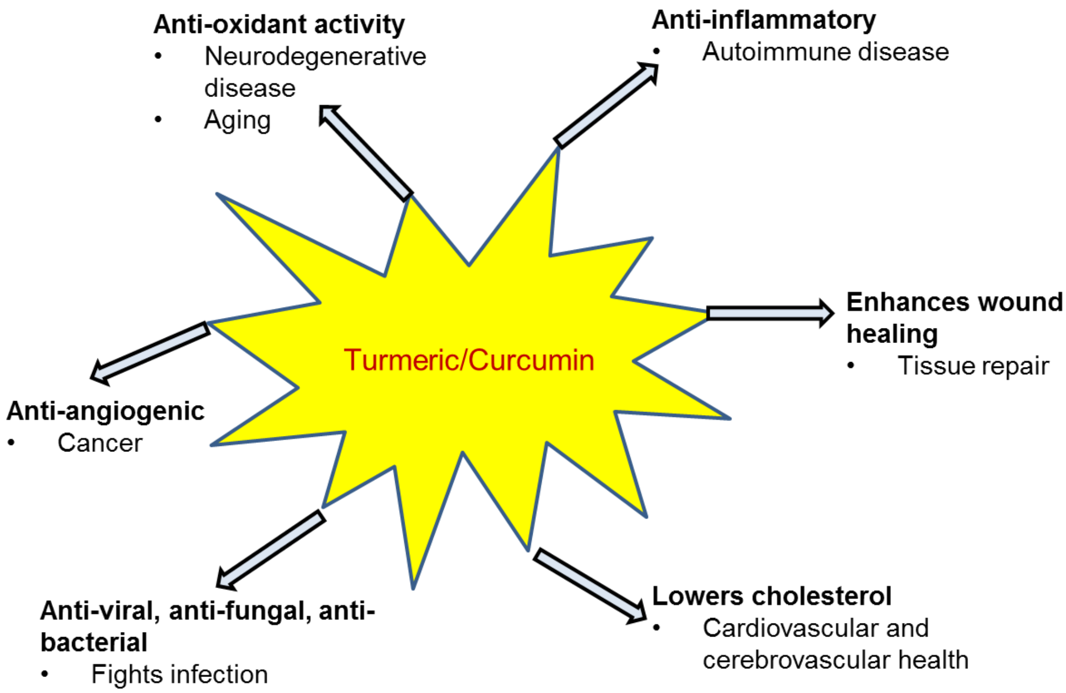

Figure 27. Protective effects of turmeric-derived curcumin
Previously characterized effects of curcumin include anti-inflammatory, anti-oxidant, anti-angiogenic, anti-bacterial, and cholesterol-lowering activity. Potential clinical applications for curcumin are listed (Adapted from [288]).

We will treat cocaine-administered mice with curcumin and assess striatal and hippocampal levels of RXR-γ and the plasticity-related proteins GAP-43 and Ng. Furthermore, we will assess cognitive function in curcumin-treated cocaine-administered mice compared to control and cocaine-only treated mice. Hippocampal-dependent spatial memory will be assessed by the Barnes maze, as studies in Ng-deficient mice demonstrate this aspect of cognitive function is severely impaired by lack of Ng expression. Additionally, striatal RXR-γ is required for dopaminergic neuron survival and proper D2R-mediated locomotor responses. Therefore, we will conduct assessment of dopamine neuron health by TUNEL/caspase labeling of tyrosine hydroxylase-positive neurons in the striatum by double-immunofluorescence labeling, and for dopamine-dependent locomotor activity by rotarod testing in all groups of mice. Levels of TNF-α in the serum as well in brain lysate will be assessed by ELISA analyses to confirm induction of TNF-α signaling in response to cocaine administration, and to confirm the anti-inflammatory effects of curcumin.

Cocaine-mediated white matter loss

Findings from our studies, along with those of previous imaging and microarray studies in human cocaine abusers, demonstrate significant loss of white matter as a result of chronic cocaine exposure. We also demonstrate that the β-lactam antibiotic ceftriaxone (Ceft) ameliorates loss of myelin proteins when administered during cocaine withdrawal. As RXR-γ has recently been identified as a critical regulator of oligodendrocyte maturation and remyelination following lesion [182], we will assess the effects of Ceft treatment on RXR-γ levels both *in vivo* and in cocaine-treated oligodendrocytes *in vitro*.

In a mouse model of chronic cocaine abuse, we will administer Ceft during active cocaine treatment and during withdrawal, and investigate RXR-γ levels at various time points. As chronic cocaine exposure, both in humans and in animal models, is associated with dysfunction of frontal cortical circuitry, we will utilize behavioral tests designed to investigate the function and integrity of the frontal cortical circuitry. It has previously been demonstrated that myelination of these pathways may be compromised in cocaine abusers, and this loss in proper conductivity likely contributes to cognitive impairment and also may confer enhanced vulnerability to relapse following periods of cocaine withdrawal. We will employ the alternating T-maze to test cognitive flexibility and executive function in control, cocaine-treated, and cocaine + Ceft-treated mice. The T-maze is designed to assess functions specifically mediated by frontal cortical neurons, including working memory and executive function [291]. Previously, we have touched upon this area of research and found that Ceft attenuated cocaine-induced deficits in a novel object recognition task (Figure 23), another assessment of working memory and executive function [200, 292].

Oligodendrocytes possess intrinsically low antioxidant defenses and are highly vulnerable to excitotoxic events. As cocaine-induced neuroinflammation may contribute to oligodendrocyte damage and myelin loss, we will assess whether Ceft treatment affects levels of TNF-α in serum and brain homogenate by ELISA. Furthermore, we may elect to assess white matter integrity in the frontal cortex, nucleus accumbens, and hippocampus of curcumin-treated cocaine-administered mice (described in section I).

In vitro, we will treat astrocyte/oligodendrocyte co-culture systems with conditioned medium from control and cocaine-treated neurons, in the presence and absence of Ceft. Because astrocytic glial transporters account for the majority of extracellular glutamate uptake, the presence of astrocytes in co-culture systems is likely required for Ceft-mediated effects. Cellular levels of RXR-γ will be assessed, along with measures for toxicity and cell death. Additionally, we will assess levels of the antioxidant glutathione in our *in vitro* culture model. Glutathione has shown to be extremely protective against white matter loss and may be upregulated by Ceft treatment [293]. Finally, we will evaluate the myelination capacity of cocaine-exposed oligodendrocytes using mixed glial and neuronal co-cultures. Rat primary glial cells and dorsal root ganglia neurons will be co-cultured in the absence and presence of cocaine plus/minus Ceft. Myelination will be assessed by levels of WM proteins, oligodendrocyte maturation as assessed by specific markers for stages of oligodendrocyte development, and *in vitro* myelination which will be assessed by electron microscopy.

Conclusions

As retinoid therapy is being considered for a number of clinical applications, and has been utilized successfully in a number of disorders, we may also consider this approach to treat cocaine-induced neuroinflammatory processes. First, RA has been shown to inhibit inflammation through transcriptional repression as well as by decreasing mRNA stability of a number of inflammatory gene products, including TNF-α [294, 295]. However, complications of utilizing retinoids include instability and rapid degradation, particularly since our studies demonstrate cocaine up-regulates expression of the RA-degrading enzyme CYP26. Our studies indicate that DHA, a polyunsaturated fatty acid and RXR ligand, is protective against TNF-α-mediated down-regulation of RXR-γ in SH-SY5Y cells (Figure 28). Therefore, we may assess the therapeutic potential of DHA and/or utilize synthetic RXR agonists which bypass normal RA metabolic pathways, such as Bexarotene. Bexarotene was recently demonstrated to reverse both biochemical and behavioral symptoms of Alzheimer's disease in a commonly used transgenic mouse model [173]. Additionally, as inflammatory mediators have been shown by our group and others to induce instability and degradation of RXRs, combined agonist and anti-inflammatory treatments may be necessary to activate signaling and prevent down-regulation of nuclear receptor levels.

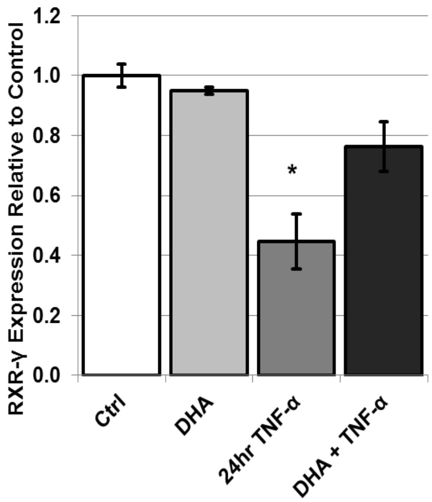

Figure 28. The RXR ligand DHA is protective against TNF-α-mediated decreases in neuronal RXR-γ protein levels Densitometric quantification of RXR-γ levels in SH-SY5Y cells treated with TNF-α (100 ng/ml; 24 h) in the absence or presence of DHA (1.5 μM; 2 h pre-treatment). $n \geq 3$; *$p < 0.01$. RXR, retinoid X receptor; DHA, docosahexaenoic acid.

In addition to cocaine abuse, a number of neurodegenerative diseases are characterized by increased production of pro-inflammatory cytokines such as TNF-α in the brain. For instance, HIV proteins are known to induce high levels of brain TNF-α during infection of the CNS. This leads to extensive neuronal damage and cognitive impairment, termed HIV-associated dementia (HAD) in its most severe form. Therefore, in future studies we may elect to utilize selected therapies such as curcumin treatment or retinoid therapy in animal models for HIV infection of the CNS.

Overall, our studies characterize two novel mechanisms: (1) cocaine-induced degradation of RXR-γ, and (2) cocaine-mediated white matter loss which is attenuated by Ceft administration. A better understanding of mechanisms underlying these events may lead to the development of more effective therapeutics in the treatment of cocaine abuse disorder, as well as in neurodegenerative diseases that are characterized by neuroinflammatory processes and myelin loss.

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