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Serotonin Receptor 2-Induced Mitochondrial Biogenesis as a Therapeutic Strategy for the Treatment of Multiple Pathologies

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Serotonin Receptor 2-Induced Mitochondrial Biogenesis as a Therapeutic Strategy for Treatment of Multiple Pathologies

By

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Approved by:

Chairman, Advisory Committee

DEDICATION

This is dedicated to my husband Joey, my parents and siblings, and to the rest of my family and friends. Their support throughout my time in graduate school has played a critical role in my accomplishments, and I deeply appreciate their encouragement.

It is also dedicated to the memory of Dr. Ryan Monfeli, who fostered my love of research at a critical time in my scientific development and who supported and encouraged me in my goals to enter graduate school. His thirst for knowledge and his dedication to his craft have been a constant inspiration as I have followed in his footsteps and will continue to inspire me as I continue in my career as a scientist.

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ABSTRACT

JENNIFER LINTON HARMON. Serotonin Receptor 2-Induced Mitochondrial Biogenesis as a Therapeutic Strategy for the Treatment of Multiple Pathologies. (Under the direction of CRAIG C. BEESON and RICK G. SCHNELLMANN)

Mitochondrial dysfunction is a common pathophysiological feature in many acute and chronic organ injury states. Often, this mitochondrial dysfunction is sub-lethal and persistent and is a major contributor to loss of cellular function in the absence of cell death. Mitochondrial biogenesis (MB) is the process by which new mitochondria are created, and studies have demonstrated that pharmacological induction of MB can reverse loss of mitochondrial content, improve mitochondrial function and reduce measures of acute organ injury.

Several classes of pharmacological agents that induce MB through divergent mechanisms have been identified. Previous studies demonstrated that 2,5-Dimethoxy-4-iodoamphetamine (DOI), a potent but non-specific serotonin receptor 2 (5-$HT_{2)}$ receptor agonist, was able to induce MB. Based on these findings, we screened a panel of 5-HT_2 receptor-specific agonists and antagonists and found that both the potent 5-HT_{2C} receptor agonist CP-809,101 and antagonist SB-242,084 were able to induce MB at nanomolar concentrations in RPTC and that these 5-HT_{2C} receptor ligands were able to induce MB in mouse renal cortex. Further work with these compounds using genetic manipulation of 5-HT_2 receptor expression in both knockout mouse models and treatment of primary RPTC with siRNA directed toward either the 5-HT_{2A} or 5-HT_{2C} receptor revealed that the observed ability of both of these compounds to induce biogenesis is dependent on the expression of the 5-HT_{2A} receptor.

After identifying the 5-HT_2 receptor responsible for the biogenic capacity of both ligands, we identified another drug, amoxapine, as a potent 5-$HT_{2A/2C}$ receptor antagonist and potential inducer of MB. Amoxapine increased cellular respiration, a marker of MB, in primary renal proximal tubule cells (RPTC) and induced an increase in PGC-1α mRNA expression; additionally, it increased peroxisome proliferator-activated receptor gamma co-activator (PGC-1a) mRNA expression in mouse renal cortex, indicating that it might be a potential pharmacological therapy for treatment of acute organ injury. However, daily amoxapine treatment of mice exposed to folic acid-induced acute kidney injury (FA-AKI) did not reverse mitochondrial deficits and did not improve renal function or survival in these mice.

Having identified the potential benefits of acute organ injury treatment with pharmacological inducers of MB, we observed that traumatic brain injury (TBI) caused the disruption of mitochondrial homeostasis in both the ipsilateral striatum and cortex after closed cortical impact (CCI), with concomitant increases in signaling through pathways associated with post-injury mitochondrial dysfunction. Future work characterizing the pattern of mitochondrial dysregulation and elucidating the signaling pathways that contribute to the suppression of mitochondrial function may reveal novel drug targets for pharmacological management of TBI as well as other acute organ injury states.

CHAPTER ONE

INTRODUCTION

MITOCHONDRIAL BIOLOGY

Structure and Function of Mitochondria

Mitochondria are subcellular organelles that are responsible for many processes that are critical to cellular function, including production of energy in the form of adenosine triphosphate (ATP), maintenance of calcium homeostasis, regulation of apoptosis, and generation of reactive oxygen species (ROS) in response to both physiological and pathological stimuli (1). These organelles have a unique double-membrane structure, which allows for sequestration of proteins and other mitochondrial contents into four locations: the outer mitochondrial membrane (OMM), the intermembrane space, the inner mitochondrial membrane (IMM) that contains the five electron transport chain (ETC) complexes and is organized into cristae to regulate ATP production rates, and the matrix, which is the innermost compartment in which many of the processes governing ATP production occur and is a major site for the generation of reactive oxygen species and free radicals (1-4) . Additionally, the mitochondrial matrix contains mitochondrial DNA (mtDNA) and the enzyme manganese superoxide dismutase (MnSOD; SOD2), which comprises the primary defense against oxidant damage to the ETC and mtDNA (4).

Mitochondria contain their own DNA, which has a double-stranded circular structure and encodes 13 proteins of the electron transport chain complexes, as well as 22 transfer RNAs and 2 ribosomal RNAs (1). Transcription and replication of mtDNA are performed by nuclear-encoded proteins which are, respectively, mitochondrial transcription factor A

(TFAM) and DNA polymerase γ (POLG) in concert with the TWINKLE helicase (1). A single mitochondrion can contain thousands of copies of mtDNA, and the relatively high rate of mtDNA mutation can result in populations of mtDNA with slightly different sequences—as well as populations of mitochondria with differences in mtDNA existing within one cell— which is called heteroplasmy (5). However, the sorting of mtDNA through mitochondrial fission and fusion, as well as the process of mitophagy, comprise a highly coordinated mechanism for the removal and degradation of mtDNA with deleterious mutations (2).

Mitochondrial Biogenesis

Mitochondria are highly dynamic organelles requiring tight regulation of mitochondrial number and function, which hinges upon mitochondrial biogenesis (MB), the process by which mitochondria grow and divide, resulting in alterations to mitochondrial size, number, mass and/or function (2, 6). This process is multifaceted, requiring the coordination of fission, fusion, mtDNA replication, transcription of genes from both mitochondrial and nuclear DNA, and the translation and import of proteins encoded in the nucleus (Fig. 1-1) (2, 6). Transcriptional regulation, including the major signaling mechanisms that control this process, is the best characterized and most easily pharmacologically-modulated facet of MB, although post-translational modulation of key regulatory proteins is also a viable target for therapeutic induction of MB (7).

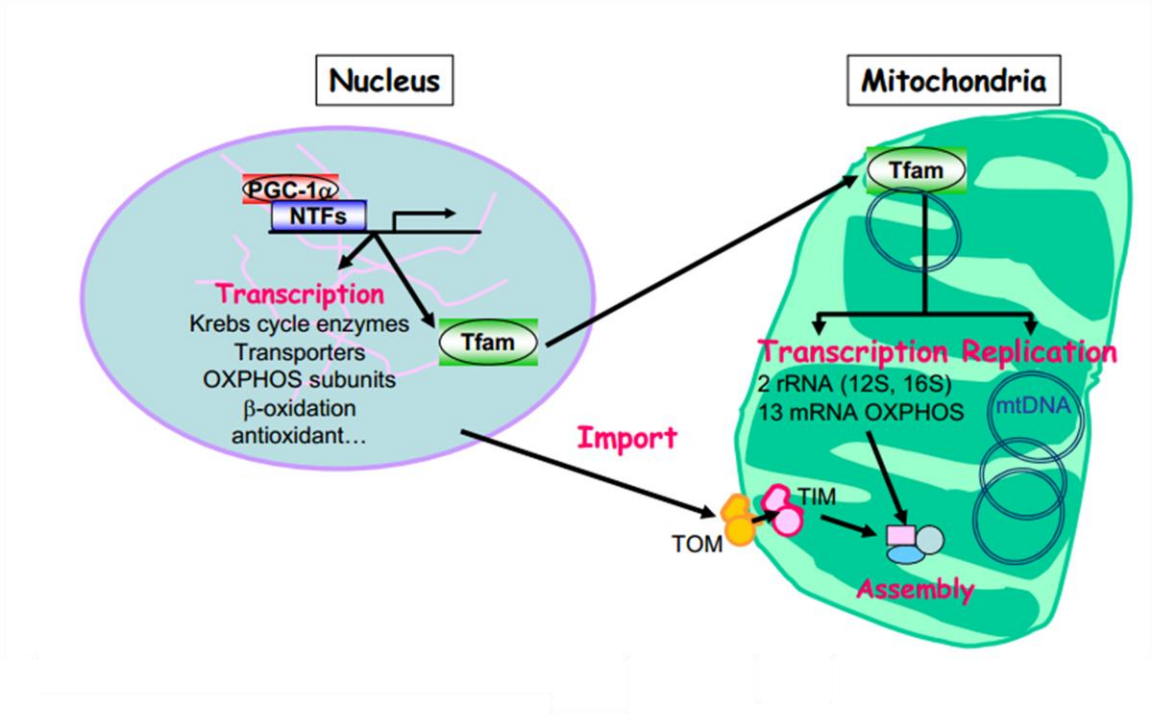

Fig. 1-1. Schematic Representation of Mitochondrial Biogenesis. Peroxisome proliferator-activated receptor gamma co-activator (PGC-1a) activates nuclear transcription factors (NTFs) leading to transcription of nuclear- encoded proteins and of the mitochondrial transcription factor Tfam. Tfam activates transcription and replication of the mitochondrial genome. Nuclear-encoded proteins are imported into mitochondria through the outer- (TOM) or inner (TIM) membrane transport machinery. Nuclear- and mitochondria-encoded subunits of the respiratory chain are then assembled (6).

Transcriptional Regulation of Mitochondrial Biogenesis

Peroxisome-Proliferator-Activated Receptor γ Co-activator α (PGC-1α)

PGC-1α, a non-DNA binding co-activator of a number of nuclear transcriptional factors, is a critical stimulator and the "master regulator" of MB that is responsive to a number of physiological and pathological stimuli in the cell (6, 8). First identified in muscle and brown adipose and highly expressed in tissues with high energy demands, such as kidney, liver, heart and brain, this protein both strongly induces expression of and co-activates nuclear respiratory factors (NRF 1 and 2) to promote the further transcription of mitochondrial proteins, which are then translated into precursor proteins in the cytosol and imported into the mitochondria by the translocases of the outer and inner membranes (6, 8-11). In the nucleus, NRFs are responsible for transcription of oxidative phosphorylation (ETC) proteins as well as TFAM, which translocates to the mitochondrial matrix to coordinate the transcription of the 13 proteins encoded by the mtDNA and stabilize the mitochondrial genome (5, 8-10).

Regulation of PGC-1α by Cell Signaling Pathways

Many cell signaling pathways have been implicated in the induction of PGC-1α, including cyclic AMP (cAMP) via the cAMP response element-binding protein (CREB), calcium via calcineurin and calcium/calmodulin-dependent protein kinase (CAMK), SIRT1, AMP kinase (AMPK), mitochondrial target of rapamycin (mTOR), myocyte enhancement factor-2 (MEF2), p38 MAPK, and nitric oxide (NO) via guanylyl cyclase activation and generation of cyclic GMP (cGMP) (Fig. 1-2) (2, 5-7, 12-23). Through these pathways, PGC-1α can respond to a physiological or pathological stress and can be manipulated by pharmacologic agents.

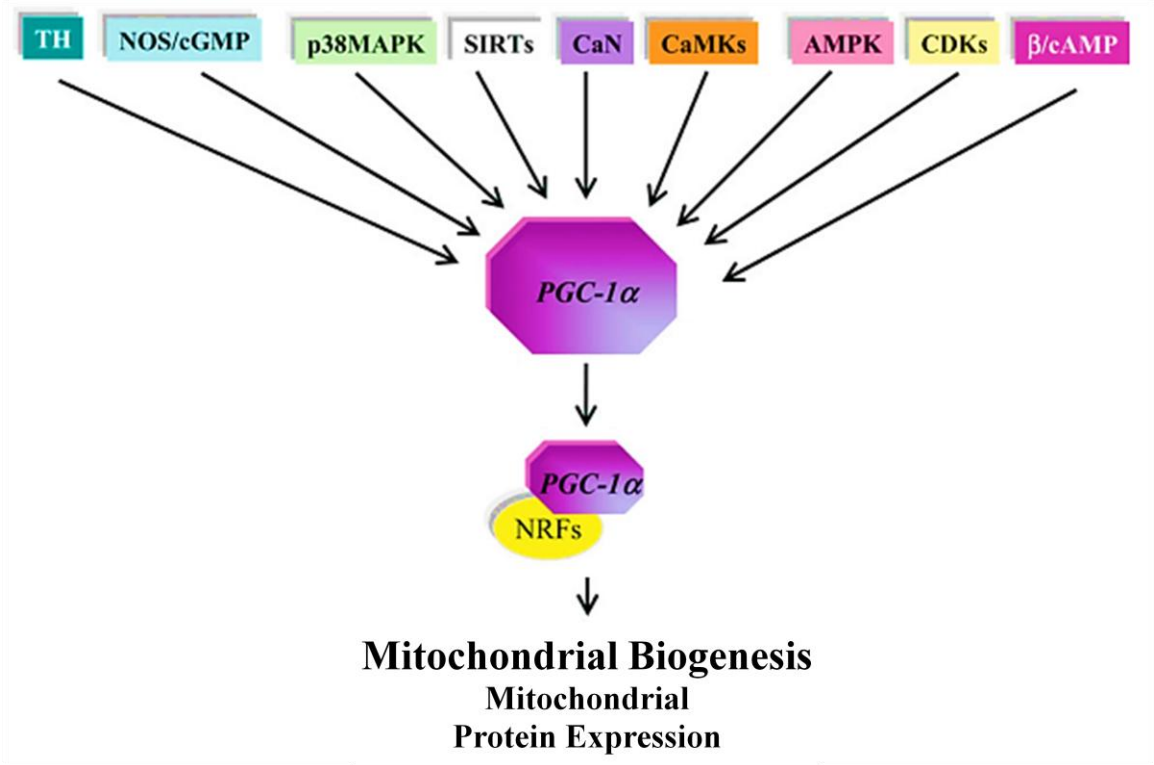

Fig. 1-2. PGC-1α Regulatory Cascade. Thyroid hormone (TH), nitric oxide synthase (NOS/cGMP), p38 mitogen-activated protein kinase (p38MAPK), sirtuins (SIRTs), calcineurin, calcium-calmodulin-activated kinases (CaMKs), adenosine-monophosphate-activated kinase (AMPK), cyclin-dependent kinases (CDKs), and β-adrenergic stimulation (β/cAMP) have been shown to regulate expression and/or activity of PGC-1α. PGC-1α then co-activates transcription factors such as nuclear respiratory factors (NRFs) known to regulate mitochondrial biogenesis.(6)

Physiological Induction of PGC-1α

PGC-1α induction is responsible for the cellular response to a number of physiological stressors, including caloric restriction, cold exposure and exercise. In situations in which caloric intake is restricted, PGC-1α is increased through a NO-induced increases in SIRT1 expression (24, 25). This increase in PGC-1α leads to mitochondrial adaptation and metabolic reprogramming toward glycogen and fat mobilization, gluconeogenesis and ketogenesis, the end result of which is a shift in energy production and increase in lifespan and longevity (25, 26).

PGC-1α is also responsible for thermogenesis in response to cold exposure; when exposed to cold for 12 h, an increase in PGC-1α mRNA was observed in mouse peripheral tissues, such as skeletal muscle and brown adipose tissue (27). Downstream of this increase in PGC-1α is an increase in uncoupling proteins (UCP 1 and 2) , which collapse the proton gradient established by electron transport chain, requiring increased flux through the ETC and resulting in a net increase in body temperature (27, 28).

Finally, exercise is also a potent inducer of PGC-1α in fat and in skeletal muscle (29-31). This increase in PGC-1α is an adaptation to both acute and chronic physical activity through independent signaling pathways. In periods of acute exercise, PGC-1α transcription is activated transiently by calcium signaling through calcineurin A; however, chronic exercise activates AMPK as well as an autoregulatory signaling loop that includes MEF2 and CAMK, the end result of which is an altered distribution of muscle tissue from fast-twitch type II to slow twitch type I, the oxidative fibers that promote endurance (30-33).

Induction of PGC-1α after Cellular Injury

Many organ pathologies that provoke cellular injury through hypoxia, reactive oxygen species, or inflammation can also induce cellular stress responses that result in the increase of mitochondrial biogenesis by induction of PGC-1α. Both C2C12 myoblasts exposed to hypoxic conditions and the skeletal muscle of mice that underwent arterial ligation-induced hypoxia demonstrated an increase in both PGC-1α mRNA and protein after 3 h of oxygen deprivation (34). Further examination of hypoxia in cultured primary cardiac myocytes demonstrated that this increase in PGC-1α is due to increased signaling through the AMPK pathway and results in increased uncoupled respiration, which suggests that PGC-1α participates in a compensatory mechanism to meet ATP demands during periods of hypoxia in the highly energy-dependent heart (35).

Reperfusion after hypoxia also initiates a cascade of events that leads to induction of PGC-1α as a result of increased oxidant stress. Multiple studies have demonstrated that cells treated with a non-lethal dose of an oxidant such as hydrogen peroxide or tert-butyl hydrogen peroxide (TBHP), which depletes the cellular antioxidant molecule glutathione, can induce an increase in PGC-1α and thereby mtDNA copy number and mitochondrial mass in both primary cells and immortalized cell lines isolated from a variety of different organs, including liver, kidney, heart and brain (36-38). Many signaling pathways have been implicated in the oxidant-induced increase in PGC-1α expression, including phosphatidylinositol 3'-kinase (PI3K)-Akt, NO/nNOS, p38 MAPK, Src, and epidermal growth factor receptor (EGFR) transactivation (36-39). Although oxidative stress can be propagated by increases in mitochondrial content, PGC-1α can also activate a number of antioxidant defenses through direct associate of PGC-1α with transcription factors at the promoters of genes encoding the mitochondrial antioxidant proteins SOD2 and

UCP2, indicating a functional role for its observed, though seemingly paradoxical, induction by oxidative stress (40).

Finally, inflammatory stress can activate signaling pathways leading to the induction of mitochondrial biogenesis, which has been identified as a survival factor in patients who fully recover from sepsis, a massive systemic inflammatory process (41). Lipopolysaccharide (LPS or endotoxin) is an inflammatory factor released from gram-negative bacteria; although it has been demonstrated to damage mitochondria, it has also been shown to increase expression of PGC-1α both indirectly through activation of the PI3K-Akt signaling pathway by ROS but also directly through an increase in circulating cytokines and subsequent activation of p38 MAPK tumor necrosis factor-α (TNF-α) (42, 43). Although this induction of PGC-1α is a likely mechanism to sustain ATP production in the face of mitochondrial injury due to inflammation and acute oxidative stress, it also contributes to cachexia, a negative consequence of cytokine release that features both maladaptive thermogenesis and excessive energy expenditure (22).

Pharmacological Induction of PGC-1α

Finally, there are many diverse classes of pharmacological agents that have been demonstrated to increase PGC-1α and induce mitochondrial biogenesis in a variety of organ systems. These pharmaceutical classes have three general mechanisms of action: duplication of the actions of PGC-1α on peroxisome proliferator activator receptor γ (PPARγ), alteration to its activating or deactivating post-translational modifications, and activation of one or more of the many signaling pathways that have been previously

implicated in increased transcription of PGC-1α as a result of exposure to either physiological or pathological stimuli.

One mechanism of pharmacological induction of MB is direct activation of PPARγ, which recapitulates the effects of its co-activator PGC-1α (44). Drugs in the thiazolidinedione (TZD) class are the best-characterized inducers of mitochondrial biogenesis by this mechanism (44-46). In human umbilical vein endothelial cells (HUVECs), treatment with the TZDs pioglitazone and ciglatazone resulted in increases in mRNA expression of PGC-1α, as well as its downstream targets NRF-1, TFAM and SOD2, and increased both mtDNA copy number and mitochondrial density (46). Further study in a line of human neuron-like cells recapitulated the increase in mtDNA copy number as a result of pioglitazone treatment, and further linked this drug to increases in ETC complex I and IV proteins and activity as well as to increased mitochondrial function indicated by increases in cellular oxygen consumption (44). Finally, pioglitazone was also demonstrated to restore both PGC-1α mRNA expression and mtDNA copy number in human adipose tissue from patients with diabetes, which at baseline were both decreased when compared to tissue from non-diabetic controls (45). These results indicate that TZDs have potential for induction of MB to treat a variety of pathological processes, including mitochondrial damage caused by chronic hyperglycemia.

PGC-1α can also be activated through post-translational modulation, such as deacetylation by SIRT1 and phosphorylation by AMPK (8). Several different drug classes have been demonstrated to activate SIRT1, which leads to deacetylation and subsequent activation of PGC-1α. Treatment of primary rabbit RPTC with common dietary isoflavones, including the soy phytoestrogens genestein and daidzein, increased SIRT1 expression; at the same doses that increased SIRT1, these compounds also

caused a decrease in acetylated PGC-1α and concomitant increase in PGC-1α protein expression, ETC complex protein expression, uncoupled oxygen consumption and increased ATP production (47). Finally, the SIRT1 activator SRT1720 was also shown to decrease acetylated PGC-1α in primary rabbit RPTC, which resulted in a dose-dependent increase in expression of proteins from ETC complexes I and V and uncoupled cell respiration; additionally, SRT1720 increased mtDNA copy number and ATP production (48). These studies strongly indicate that modulation of post-translational modifications of PGC-1α, particularly through deacetylation via SIRT1, is a promising strategy to induce MB across different highly energy-dependent tissues.

The final strategy for induction of MB is through pharmacological modulation of signaling pathways previously identified to increase transcription of PGC-1α. β_2 adrenergic receptor activation has also been demonstrated to induce MB through increased expression of PGC-1α (45). For example, the β_2 adrenergic receptor is a G-protein coupled receptor (GPCR), the activation of which mobilizes the G_s subunit to stimulate adenylyl cyclase, which results in the release of cAMP; subsequent CREB activation and binding to the PGC-1α promoter is a well-characterized signaling pathway linked to the induction of MB (1, 49). Systemic administration of a dose of norepinephrine exceeding physiological concentrations increased PGC-1α and TFAM mRNA in rat adipose tissue; the norepinephrine-induced increase in PGC-1α was blocked by the β_2 adrenergic receptor antagonist propranolol, indicating that the β_2 adrenergic receptor activation can induce MB (29). Additionally, administration of the β_2 adrenergic receptor-specific agonist formoterol was able to increase PGC-1α mRNA, as well as other molecular and functional markers of MB, in primary rabbit renal proximal tubule cells, adult feline cardiomyocytes and in mouse renal, cardiac, and skeletal muscle tissues (50, 51).

Inhibition of phosphodiesterase 5 (PDE5) has been shown to increase PGC-1α expression and other markers of MB in multiple tissues through an increase in cGMP, which has been identified as a pro-MB signaling molecule (19). PDE5 is an intracellular enzyme that selectively cleaves cyclic GMP to GMP, as opposed to other PDEs that either cleave both cGMP and cAMP or cAMP alone (52). Treatment of primary rabbit RPTC with a panel of drugs inhibiting all three types of cyclic nucleotide phosphodiesterases identified PDE inhibitors that increased intracellular levels of cGMP also increased uncoupled cellular respiration rates and expression of PGC-1α and other mRNAs encoding mitochondrial proteins (52). Additionally, treatment of mice with the PDE5 inhibitor sildenafil increased renal PGC-1α mRNA, as well as mRNA expression of other mitochondrial proteins, mtDNA copy number and ATP production (52). Similarly, tadalafil, a long-acting PDE5 inhibitor, increased PGC-1α protein expression and improved respiration in mitochondria isolated from mice in a model of type 2 diabetes; although cGMP was not directly implicated in this effect, it is likely responsible for the increased eNOS activity and subsequent Akt and AMPK activation observed as a result of tadalafil treatment (53).

The serotonin, or 5-hydroxytryptamine, receptor class 2 (5-HT_2) is another family of GPCRs that have been shown to induce MB (54). Serotonin signaling through the 5-HT_{2B} receptor was identified as a pro-survival pathway for cardiac mitochondria, so it was hypothesized that activation of this receptor would induce MB (55). Treatment of primary rabbit RPTC with DOI, a non-specific 5-HT_2 receptor agonist increased protein expression of PGC-1α, as well as expression of proteins from ETC complexes I and V; additionally, DOI increased both basal and uncoupled respiration and increased ATP production 24 h after initial treatment (56). These results indicate that induction of signaling through the 5-HT_2 class of receptors is a viable strategy to induce MB and that

identification of the specific receptor in this class responsible for DOI's effects is a necessary step to further identify more specific and potent biogenic agents.

5-HT_2 Receptor Biology

The biological roles of 5-HT are mediated by several families of receptors, which are classified according to structure and function. The 5-HT_2 family of receptors is composed of three receptor sub-types: 5-HT_{2A}, $_{2B}$ and $_{2C}$; canonical signaling through these receptors is G_q-coupled and their activation initiates phospholipase C (PLC) cleavage of phosphoinositol 4,5-bisphosphate (PIP2) to diacyl glycerol (DAG) and inositol triphosphate (IP3), the latter of which translocates to the endoplasmic reticulum to cause release of intracellular calcium, which can subsequently initiate signaling through a number of pathways, several of which have been demonstrated to increase expression of PGC-1α, as detailed below in Fig 1-3 (2, 19, 20, 56, 57). Although these receptors are in the same class and have shared characteristics in sequence, structure and pharmacology, they do have some differences in their distributions of expression, biological functions, distributions of expression, associated pathologies and signaling pathways (58).

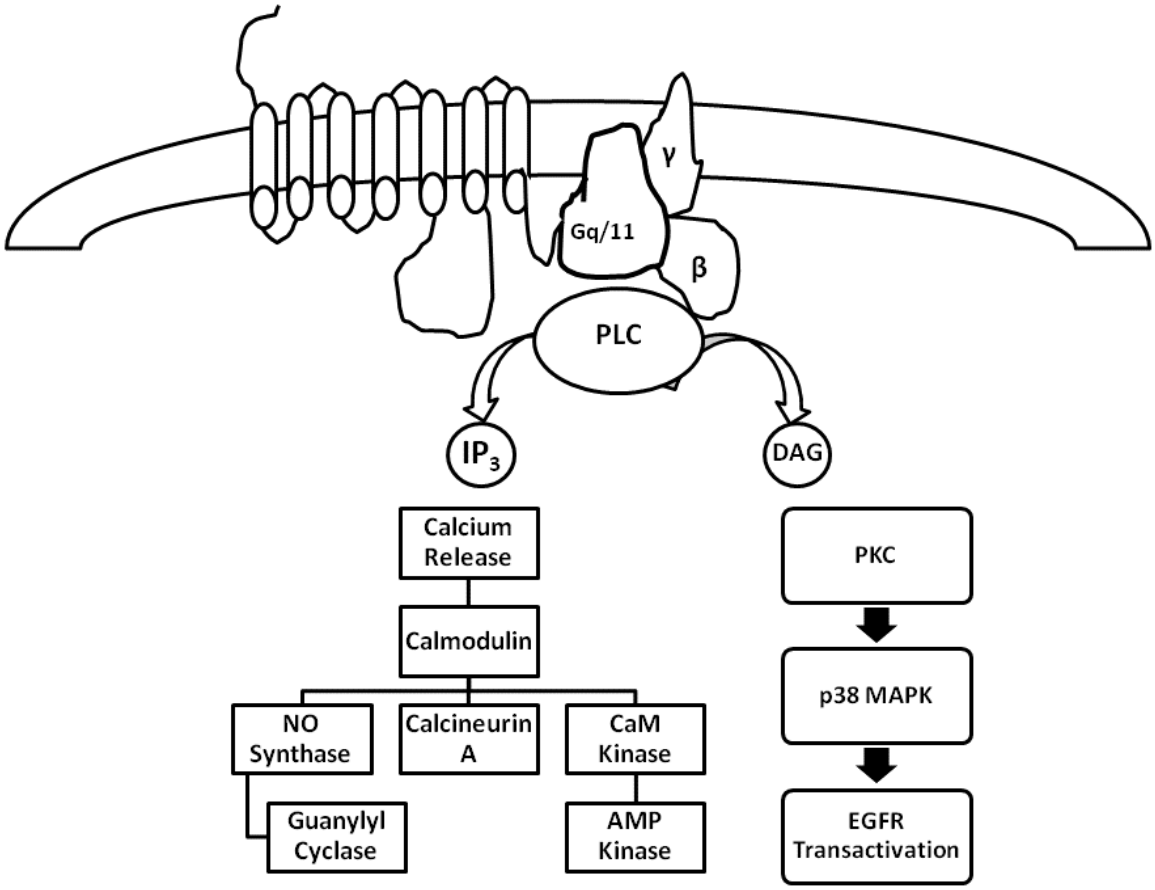

Fig 1-3. Schematic of Potential 5-HT$_2$ Receptor Mediated Mitochondrial Biogenesis Pathways. Ligand binding to 5-HT$_2$ receptors and subsequent canonical signal transduction through the $G_{\alpha q}$ protein can initiate a number of signal transduction pathways through the that have been previously demonstrated to induce mitochondrial biogenesis, including calmodulin/CamK, nitric oxide synthase/guanalyl cylase, AMPK and p38 MAPK (2, 19, 20, 56, 57).

The 5-HT$_{2A}$ Receptor

The 5-HT$_{2A}$ receptor is highly expressed in the tissues of the CNS, with prominent expression in the cerebral cortex; additionally, low levels of 5-HT$_{2A}$ receptor mRNA have been detected in peripheral tissues such as kidney, platelets and smooth muscle and it has been demonstrated to mediate smooth muscle contraction and platelet aggregation (57-60). In the CNS, this receptor plays a role in modulation of both aggressive behavior and sleep and is implicated in the pathogenesis of schizophrenia, anxiety, epilepsy, migraine and depression (57, 61, 62). Agonists for the 5-HT$_{2A}$ receptor tend to have hallucinatory effects, while its antagonists have been demonstrated to have atypical antipsychotic effects (63, 64).

The signaling pathways associated with the 5-HT$_{2A}$ receptor are complex and are activated differentially in response to a number of factors. Like all 5-HT$_2$ receptors, it is a G_q-coupled receptor and canonical signaling through this receptor is mediated by intracellular calcium release (58). However, the coupling of several other pathways—both G-protein-dependent and independent—has been described in the literature (58, 65, 66). In addition to G_q coupling, it has been reported that the 5-HT$_{2A}$ receptor can permissively couple to the $G_{\alpha i/0}$ protein in the kidney and that treatment of renal mesangial cells with 5-HT$_{2A}$ receptors with the endogenous agonist 5-HT resulted in both increased phosphoinositol turnover and decreased adenylyl cyclase activity, indicating that this receptor signals through concomitant coupling to multiple G proteins in this tissue (66). Additionally, there is evidence that the 5-HT$_{2A}$ receptor signals through G protein-independent coupling to phospholipase D (PLD) and phospholipase A$_2$ (PLA$_2$) and that agonists for this receptor can signal simultaneously through both canonical G-protein dependent phospholipase C activation and independent PLA$_2$ signaling (58, 65).

There is ample evidence that scaffolding proteins in the β-arrestin family are an important regulator of 5-HT_{2A} receptor signaling (63, 67-70). Although the traditional paradigm describes the role of these proteins in desensitization of the 5-HT_{2A} receptor through intracellular sequestration of the activated receptor, it is now recognized that these proteins can also mediate activation of Akt/GSK3β signaling by certain 5-HT_{2A} receptor ligands (63, 67). Interestingly, some of the physiological effects of endogenous 5-HT signaling are dependent on β-arrestin-mediated activation of Akt/GSK3β signaling, indicating that this "non-canonical pathway" is an important component of native 5-HT_{2A} receptor function (68-70).

Finally, constitutive activity of the 5-HT_{2A} receptor has been reported and is implicated in the neuronal processes mediating learning behavior (71). Given the accumulation of information about ligand activity that does not bifurcate neatly into agonist/antagonist categorization and the fact that most characterization of agonist or antagonist behavior at the 5-HT_{2A} receptor has been performed by assessing the ability of a ligand to either elicit or block calcium or IP_3 accumulation, it is possible that many ligands classically defined as antagonists may be better identified as inverse or biased agonists. Additionally, inverse agonism, which is the cessation of constitutive receptor activity upon binding of a ligand, must now be considered both as a complicating factor in the identification of drugs that antagonize the 5-HT_{2A} receptor but may also represent a novel therapeutic modality to treat several diseases (71).

The 5-HT_{2B} Receptor

The 5-HT_{2B} receptor has the broadest mRNA distribution in the 5-HT_2 receptor class and is expressed in almost every organ system in the body (59). Although it has been

implicated in smooth muscle contraction in the stomach, little else is known about its function due to the paucity of 5-HT_{2B} receptor specific agonists (72). Like the other 5-HT_2 receptors, its primary signaling mechanism is $G_{\alpha q}$ protein activation of PLC; pleiotropic signaling of this receptor is understudied, although some studies have indicated that this receptor can also activate $PLA_{2,}$ ERK and endothelial nitric oxide synthase (eNOS) (58).

Nebigil *et al.* identified the 5-HT_{2B} receptor as a novel target in survival signaling in cardiomyocytes and demonstrated that mice lacking this receptor had altered cardiac mitochondria structure and function (55). While ablation of this receptor led to a dilated cardiac phenotype, its overexpression led to a hypertrophic phenotype, which suggests that balanced signaling through this receptor is paramount for development and maintenance of proper cardiac structure as well as preservation of mitochondrial function (73).

The 5-HT_{2C} Receptor

The 5-HT_{2C} receptor has the most limited pattern of mRNA expression and has traditionally be described as being localized entirely to the CNS, with prominent expression in the choroid plexus, nucleus accumbens and hippocampus (58, 59). Although this receptor shares 80% transmembrane domain amino acid and 49% total amino acid homology with the 5-HT_{2A} receptor, agonism of these receptors results in physiologically opposite effects (*eg.* anxiogenic vs. anxiolytic) (60, 74). This receptor is implicated in motor function and in feeding behavior; agonists of this receptor suppress appetite, while 5-HT_{2C} receptor antagonists have anxiolytic properties (57, 75-77).

Additionally, the 5-HT_{2C} receptor is implicated in the pathophysiology of depression, schizophrenia and Parkinson's disease (78).

Like all 5-HT_2 receptors, the 5-HT_{2C} receptor is primarily coupled to the $G_{\alpha q}$ protein, the activation of which leads to release of calcium from the endoplasmic reticulum and the activation of downstream signaling pathways (58). In addition to $G_{\alpha q}$ protein-mediated signaling, the 5-HT_{2C} receptor can activate PLD through the $G_{\alpha 13}$ protein and PLA2 through an unknown signal transduction molecule (58, 78).

Mitochondrial-Nuclear Interactions and Retrograde Signaling

Although transcriptional control and nucleus-to-mitochondrial signaling is a critical facet of mitochondrial homeostasis, regulation of mitochondrial biogenesis through retrograde mitochondria-to-nucleus signaling also plays an important role in the cellular maintenance of mitochondrial function and number. Retrograde signaling allows for information regarding mitochondrial function to be relayed back to the nucleus and thus integrated into the genetic network therein, providing a mechanism for the cell to monitor several markers of mitochondrial health, including cellular metabolism and calcium dynamics (79). Furthermore, it allows for cells to assess their energetic state prior to cell proliferation, as a disruption in mitochondrial bioenergetics would preclude the cell from proliferating successfully (79). Current evidence suggests that retrograde mitochondrial signaling is regulated by a number of diverse mechanisms at both the transcriptional and translational levels.

Translational control of retrograde signaling is especially important for assessing mitochondrial health prior to cell proliferation and occurs through two processes:

mitochondrial ribosomal stalling and mitochondrial peptide export. Mitochondrial ribosomal stalling results in the accumulation of Opa1, a protein that is critical for IMM fusion and maintenance of the mitochondrial cristae; as a result of this accumulation, the mitochondrial membrane loses its potential, creating a stress signal that leads to the inhibition of cellular proliferation (79). In invertebrates, the translocation of the bZip transcription factor ATFS-1 from the mitochondria to the nucleus in response to protein stress results in the activation of a gene expression profile that is consistent with an unfolded protein response; this response then leads to the export of mitochondrial peptides into the cytoplasm, which may block peptide import into the mitochondrial and trigger a retrograde downregulation of gene expression (79).

A number of pathways have been implicated in the transcriptional control of retrograde signaling from the mitochondria to the nucleus. Mitochondrial stress induced in mammalian cells by either uncoupling of the proton gradient or depletion of mtDNA led to the activation of Ca^{2+}/calmodulin-responsive calcineurin, resulting in increased expression of genes involved in calcium transport and storage, leading to increased cytosolic free Ca^{2+} and alterations in cellular morphology (80). Furthermore, mitochondrial calcium stress has also been demonstrated to activate calcineurin, as well as protein kinase C, the JNK/MAPK pathways and CamKIV, all of which result in the activation of different nuclear transcription factors that have been shown to alter mitochondrial dynamics and function, including NFκB and CREB (80). Finally, the activation of the transcription factor CCAAT-enhancer-binding protein homologous protein (CHOP) by calcium stress provides another link between retrograde signaling and the unfolded protein response, and further indicate that this mitochondria-to-nuclear signaling is an important facet of mitochondrial quality control (81).

ACUTE KIDNEY INJURY

Definition of Acute Kidney Injury

Acute kidney injury (AKI) is defined broadly as a loss of renal function over hours to days; diagnosis of this loss of renal function is performed using the RIFLE (Risk, Injury, Failure, Loss of Function, End-stage renal disease) or AKIN (Acute Kidney Injury Network) criteria, which combine assessments of increasing serum creatinine and decreasing urine output to stratify patients into stages that correspond to severity of injury (Fig. 1-4) (82, 83). Since outcomes are directly correlated with the severity of AKI, this staging strategy is then used to determine further course of action for treatment of kidney injury (84). The treatment strategies for patients with AKI can vary significantly due to expected outcome and potential for recovery; for example, patients who are in Stage 1 AKI may benefit from early intensive therapy to prevent further damage to the kidney, whereas early and frequent renal replacement therapy can improve survival and promote recovery in patients diagnosed with later stage AKI (85).

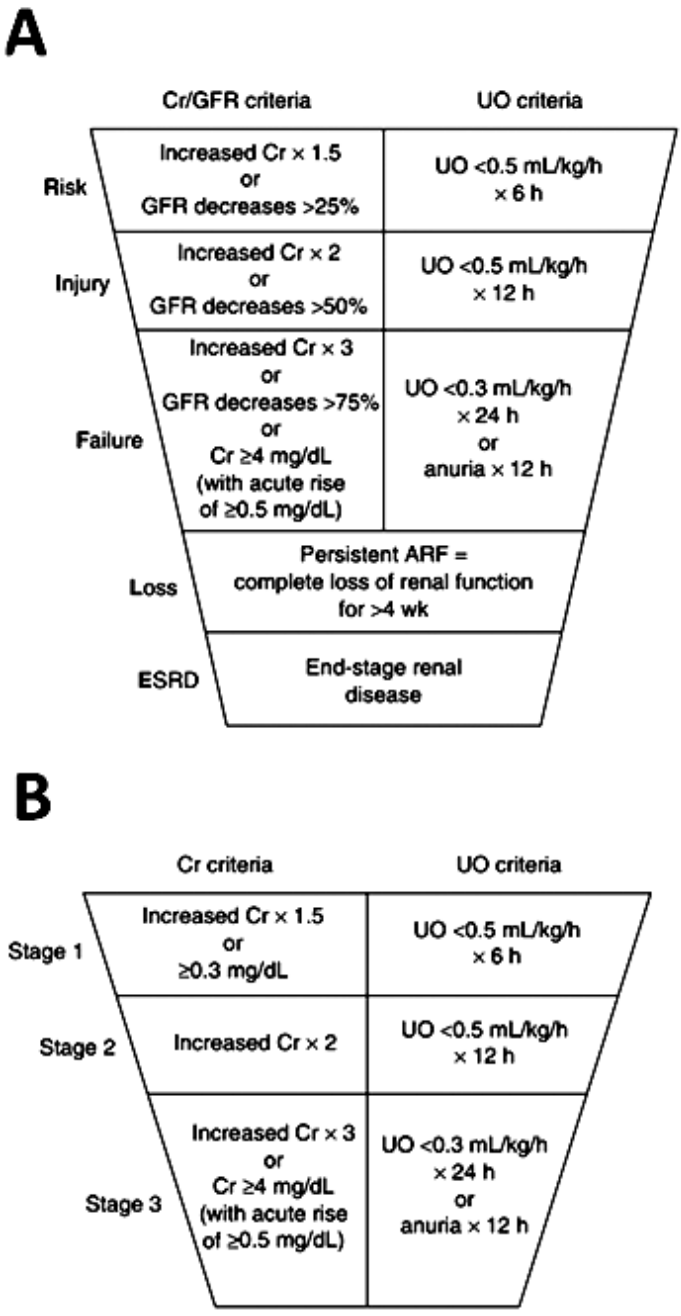

Fig. 1-4. The RIFLE and AKIN Criteria for Classification of AKI. (a) Risk, Injury, Failure, Loss of renal function and End-stage kidney disease (RIFLE) and (b) Acute Kidney Injury Network (AKIN) classifications for acute kidney injury (adapted from Bellomo et al.[8] and Mehta et al.,[9] with permission from BioMed Central). ARF= acute renal failure; Cr = creatinine; GFR= glomerular filtration rate; RRT = renal replacement therapy; UO= urine output (86).

The Impact of AKI on Population Health

AKI in the acute care setting can have a profound effect on morbidity and mortality of hospitalized patients, with in-hospital mortality of patients with AKI ranging from 24-75% and a 30-40% mortality rate for all patients with AKI (82). Additionally, 3-16% patients admitted to intensive care units have acute kidney injury, with a mortality rate that is 3x higher that of AKI patients outside of the ICU (87). Overall, AKI is estimated to result in 34 million hospitalizations per year, with associated expenditures attributed to hospital-acquired AKI exceeding $10 billion (84).

Causes of AKI

AKI has many diverse causes, which are broadly organized into three categories: prerenal, postrenal, and intrinsic/intrarenal. Prerenal causes of AKI comprise any primary injury or pathology, that restricts blood flow to the kidney while initially preserving tubular and glomerular function; common causes of prerenal AKI include cardiac failure or surgery, septic shock, and liver failure (85). Urinary outflow obstruction gives rise to postrenal AKI, which is the least common cause (85). Finally, intrinsic or intrarenal causes of AKI are defined by the renal structure that is primarily injured (*eg.* tubular or glomerular) and most often result from either ischemic or toxic insult (85). While these distinctions are made between the three causes of AKI, it should be noted that it is not uncommon for patients to experience multiple insults, and the presence of one cause of AKI can predispose the kidney to further damage; for example, sustained prerenal AKI is a risk factor for the development of intrinsic AKI, especially in vulnerable populations such as hospitalized patients and the elderly (85). This review will focus on intrinsic AKI caused by acute toxicity or ischemic and the pathogenesis thereof. The proximal tubular epithelial cells are highly vulnerable to both ischemic and toxic injury,

and the severity of tubular injury can usually be correlated with the severity of the insult (*eg.* dose of drug or length of ischemia) (88). Since the proximal tubular epithelium requires high energy production to maintain crucial transport functions and since mitochondrial dysfunction plays a major role in the pathophysiology of both toxic and ischemic AKI, emphasis will be placed on the complex and diverse mechanisms of mitochondrial pathology in these disease processes.

Pathogenesis of Cisplatin-Induced AKI

Cisplatin, a common anti-neoplastic agent, is a nephrotoxic compound that is rapidly absorbed in the kidney, especially the proximal straight tubule, via peritubular uptake in the first hour after its administration (89). Once inside the renal proximal tubule cells (RPTC), it is converted to several highly reactive species, which deplete glutathione, increase oxidative stress, and may cause DNA damage (89). Other mechanisms of cellular damage include mitochondrial swelling that likely results from increased oxidative stress, activation of mitogen activated protein kinases (MAPKs), activation of Caspase 3 leading to apoptosis, increased tumor necrosis factor α (TNFα) that propagates oxidative stress, and accumulation of inflammatory mediators that may mediate damage to surrounding renal structures (89). The mitochondria are responsible for the generation and propagation of reactive oxygen species (ROS) as well as major targets of these species; calcium (Ca^{2+}) dysregulation in the mitochondria has been linked to increased ROS following cisplatin administration (90). Additionally, increases in mitochondrial calcium concentrations can also trigger the mitochondrial permeability transition (MPT) and initiate the intrinsic apoptotic molecular cascade which results in the cleavage of Caspase 3. Finally, cisplatin administration causes significant decreases in renal adenosine triphosphate (ATP), the primary energy currency used by

cells and the final product of the electron transport chain in the mitochondria, indicating that there is a direct decrease in mitochondrial function (91). Therefore, mitochondria are central to several of the cellular damage mechanisms involved in cisplatin-induced AKI.

Pathogenesis of Myoglobinuric AKI

Rhabdomyolysis, the dissolution of skeletal muscle that results in the leakage of myoglobin into the blood, can be induced by a number of traumatic and non-traumatic events, including crush injuries, alcohol or illicit drug use, extreme strenuous exercise, or viral infection (92).

Myoglobinuric AKI develops in 13-50% of rhabdomyolysis cases and results from myoglobin circulation to the kidney, where it is filtered by the glomerulus and is endocytosed into tubular epithelial cells (92, 93). In the presence of an acidic urine environment, the ferrous iron in myoglobin can be oxidized to ferric oxide in the mitochondria via the Fenton reaction, generating ROS in the form of a hydroxyl radical (92). In the animal models of of myoglobinuric AKI, increased nitrosative stress in isolated renal mitochondria, decreased mitochondrial membrane potential, increased lipid peroxidation and decreased ATP production were observed, further demonstrating that myoglobinuria induces acute mitochondrial dysfunction in the kidney (93, 94).

Pathogenesis of Septic AKI

Sepsis is the pathological consequence of a systemic inflammatory response to severe infection, particularly to the presence of lipopolysaccharide (LPS) or endotoxin that is released by gram negative bacteria. While sepsis alone carries a high mortality rate,

septic AKI doubles the rate of mortality (95). The cellular pathophysiology of septic AKI is multifactorial and results from both prerenal and intrinsic renal injury. First, there is direct damage to the glomerular endothelium that is mediated by TNFα; this damage increases glomerular permeability and contributes to the leakage of protein, particularly albumin, into the urine (96). Tubular structural damage is not necessarily a feature of septic AKI, but both functional and molecular changes have been demonstrated to occur. One prominent observation is that mitochondria in the septic RPTC are susceptible to swelling; additionally, inactivation of the tubular mitochondrial electron transport chain complexes resulting in decreased renal ATP levels and decreased activity of manganese superoxide dismutase (MnSOD or SOD2), a mitochondrial antioxidant protein, further indicate that mitochondria play a central role in the development of septic AKI (95, 97). Finally, antioxidant therapy focused on the mitigation of mitochondrial damage was demonstrated to decrease oxidative stress and preserve peritubular capillary function, which suggests that mitochondrial-targeted therapies hold promise for the treatment of septic AKI (97).

Pathogenesis of Ischemic/Reperfusion (I/R)-Induced AKI

Vascular Changes in I/R-AKI

One prominent feature of the pathophysiology of I/R-AKI is changes in the renal vasculature after injury. Reduced response to vasodilatory signals by renal arterioles leads to persistent vasoconstriction and subsequent decreases in glomerular filtration rate (GFR), one measure of kidney function after AKI (98). This vasoconstriction can be attributed to a number of factors including decreased production of nitric oxide (NO) by damaged endothelial cells and the production of a number of vasoactive cytokines, including TNF-α, IL-1β and endothelin, by increased adhesion and activation of

leukocytes (99). Additionally, mitochondrial calcium accumulation may lead to a subsequent increase in cytosolic calcium that is implicated in an increased sensitivity to renal nerve-stimulated vasoconstriction (100).

Tubular Epithelium Damage and Structural Changes in I/R-AKI

RPTC are highly susceptible to damage following ischemic injury; under physiological circumstances, RPTC are attached to a basement membrane and maintain a polarity, with both an apical and a basolateral membrane. The most critical protein of the basolateral membrane is the Na^+/K^+-ATPase, which utilizes ATP to set up the sodium gradient that drives subsequent secondary and tertiary transport processes in both the apical and basolateral membranes; after ischemic injury, decreases in cellular ATP cause a loss of cellular polarity during which the Na^+/K^+-ATPase translocates from the basolateral membrane into the cytoplasm, which decreases transport of both sodium and other solutes across the cell membrane (100, 101). Death of RPTC by necrosis results from a number of insults, including acute oxidative stress and decreased ATP production, which will be discussed further in another section (98, 102). As a result, there is shedding of both necrotic cells as well as viable cells that have lost basolateral expression of integrin receptors, which causes obstruction of the tubular lumen; recovery of the proximal tubules, however, can be accomplished by the dedifferentiation, migration, proliferation and subsequent redifferentiation of the intrinsic surviving epithelial cells into a repopulated tubular epithelium (Fig. 1-6) (99, 100, 103-105).

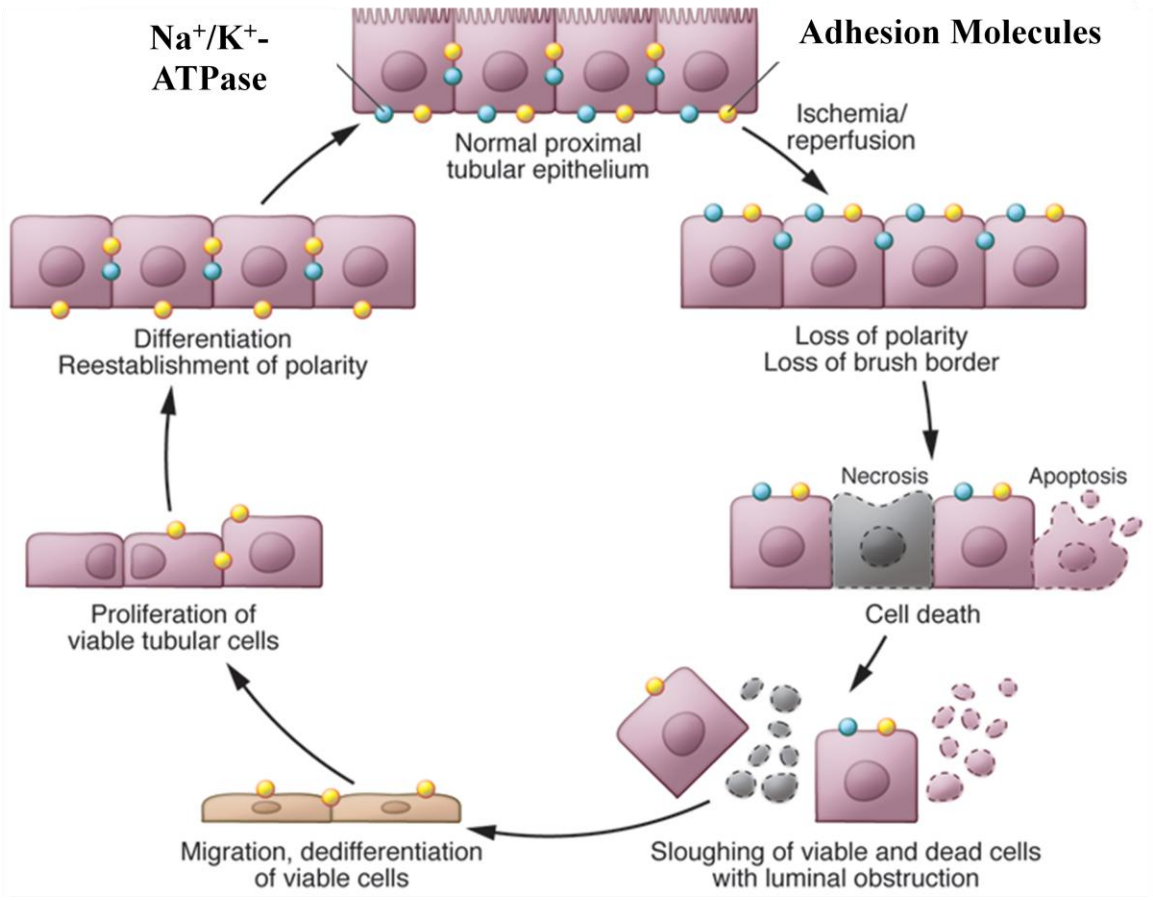

Fig. 1-5. Normal Repair in Ischemic AKI. The current understanding of tubular injury and repair after ischemic AKI. With IRI, the normally highly polar epithelial cell loses its polarity and brush border with proteins mislocated on the cell membrane. With increasing time/severity of ischemia, there is cell death by either necrosis or apoptosis. Some of the necrotic debris is released into the lumen. Viable epithelial cells migrate and cover denuded areas of the basement membrane. These cells undergo division and replace lost cells. Ultimately, the cells go on to differentiate and reestablish the normal polarity of the epithelium (99).

Mitochondrial Injury in I/R-AKI

The role of mitochondria in I/R-AKI has been extensively studied in a number of *in vitro* models, animal models and human disease states. One of the major roles of mitochondria in the pathogenesis of AKI is the generation of ROS after reperfusion, which is generally considered the most significant cause of injury to the renal tubular epithelium after an I/R insult (85, 102, 106, 107). Additionally, ischemia also causes a decrease in MnSOD activity that is more profound than the decrease in the cytosolic copper/zinc superoxide dismutase (Cu/ZnSOD or SOD1), which indicates that the increase in ROS is more localized to the mitochondrial matrix (106). Excessive ROS—especially in the mitochondrial matrix—can cause damage to the lipids of the inner mitochondrial membrane, mtDNA and the proteins of the electron transport chain; oxidative injury to the ETC then leads to the propagation of ROS at Complexes I and III (98, 108). The consequences of acute increases in ROS after I/R injury may be long-lasting. mtDNA has limited repair mechanisms and is susceptible to deletions and mutations that could result in inactivation of the electron transport chain; this could further propagate ROS generation and inhibit ATP production past the acute phase (106).

ATP depletion is another facet of mitochondrial dysfunction in I/R-AKI; decreased flux through the ETC as a result of decreased oxygen to serve as the final electron acceptor in the chain results in decreased ATP levels, which contributes to loss of epithelial cell polarity and inability of RPTC to maintain transport gradients across their membranes, leading to increased intracellular calcium concentration (85, 109). Decreases in ATP production in renal mitochondria after I/R were correlated with decreases in mitochondrial membrane potential; protection against this decrease in ATP by the addition of citric acid cycle metabolites preserved mitochondrial membrane potential

(110). Finally, depletion of ATP in an *in vitro* model of AKI led to increased fragmentation of mitochondria and disruption of mitochondrial dynamics, associated with the activation and translocation of dynamic-related protein 1 (Drp1), a mitochondrial fission protein (111). Transfection of RPTC with a dominant-negative Drp1 lead to a preservation in mitochondrial morphology as well as decreased cytochrome c release, indicating that ATP levels may be linked to apoptosis following I/R injury through disrupted mitochondrial dynamics (111).

Therefore, mitochondrial induction of apoptosis also contributes to the pathophysiology of AKI following I/R injury. One mechanism by which mitochondria contribute to apoptosis after I/R injury is via the mitochondrial permeability transition (MPT), which can arise as a result of reduced mitochondrial membrane potential or mitochondrial calcium dysregulation and leads to the collapse of ATP production (98, 106, 112). The result of the MPT is the release of cytochrome c, which is usually sequestered in the mitochondrial inter-membrane space; when cytochrome c is released into the cytosol, it activates Caspase 3 and triggers the intrinsic apoptotic pathway (113).

Treatment of Acute Kidney Injury

Experimental Treatments for Vascular Injury in AKI

Several experimental treatments for vascular causes of AKI have been studied in both AKI patients as well as in animal models of AKI. Several catecholamine treatments were suggested for the pharmacological management of AKI, though the benefit of these drugs is controversial. Norepinephrine, which increases systemic blood pressure, was administered to patients who have hypotensive vasodilation as a result of AKI; since most patients who have AKI are already in a vasoconstricted state, this therapy would

have limited use for the general population of AKI patients (86). Dopamine, which dilates renal arterioles and improves renal blood flow, has been administered as both a prophylactic agent for AKI as well as in patients who have already developed AKI (85). However, increased risks of systemic side effects such as tachycardia and gut necrosis, as well as a lack of evidence of clinical efficacy, have limited the use of this drug as a therapeutic for AKI (85, 86). Other non-catecholamine vasodilatory therapeutics have been suggested for treatment of AKI, though none of these drugs have been approved for this use in patients. For example, calcium-channel blockers, which prevent the increase in intracellular calcium in vascular smooth-muscle cells, promote vasodilation after AKI and have been shown to improve outcomes and reduce tubular necrosis after renal transplantation; however, these drugs pose the risk of overcorrecting vascular tone, causing hypotension and decreased renal perfusion in the already-damaged kidney (85). Furthermore, vasodilatory atrial natriuretic peptide (ANP) reduces the severity of AKI in animals, as well as to reduce need for dialysis in these animals, and is the most promising vasodilatory agent to date (85). Small randomized control trials (RCT) have duplicated these effects in patients after cardiac surgery, but ANP needs further examination in larger trials to become an established pharmacological modality (86).

Experimental Therapeutics for Tubular-Cell Injury

Most experimental pharmacological therapeutics to treat tubular-cell injury have focused on the use of osmotic agents and diuretics to restore the solute balance that is disrupted by the intracellular translocation of the Na^+/K^+-ATPase after an acute ischemic event, the purpose of which is to reduce cellular swelling and tubular obstruction caused by this osmotic imbalance (85). Mannitol, an osmotic agent, prevents the delay in kidney graft

function that results from ischemia as well as to treat early myoglobinuric renal failure (85). Loop diuretics, such as furosemide and bumetanide, protect against renal ischemia, especially when used in conjunction with an osmotic agent; however, these agents are likely to only have clinical benefit for patients with mild renal ischemic damage (85, 86).

Limitations on Treatments for Vascular or Tubular-Cell Injury

As demonstrated in previous discussion, many therapies designed to reduce vascular or tubular-cell injury have shown limited promise in clinical trials. Some minor factors that contribute to this limitation are poor patient recruitment, poor optimization of drug dose or administration schedule, poor attention to both disease and patient heterogeneity and poor recognition of the reality that combination therapy is most likely necessary for a treatment to be viable and clinically relevant (86). However, the most limiting factor for developing pharmacological interventions for AKI is poor early diagnosis of patients and a lack of good biomarkers to measure both the onset of renal injury and the severity of the injury; for example, the window for detection of AKI using the current standard of diagnosis, serum creatinine, is 24-48h after injury (86). As a result of the lag between injury and ability to detect this injury, patients with less severe disease who could benefit from early intervention are not identified or provided treatment that could prevent further damage and disease progression; furthermore, there are no current pharmacological agents that could prevent ischemic cellular damage, although there have been some preclinical advancements in discovery of drugs that can prevent or reverse this cellular damage. As a result, current drug discovery is focused on developing agents that address the pathways of cellular injury, including inflammation cascades, both intrinsic and extrinsic apoptosis pathways, and the multifaceted aspects of mitochondrial

dysfunction addressed previously in this review (86). Therefore, the most promising avenues of further research are improved development of biomarkers that predict injury or identify early stages of AKI and further development of agents that modulate intracellular mechanisms of ischemic tubular cellular injury. Current research in both of these areas of interest will be reviewed shortly.

Biomarkers of AKI

Traditional biomarkers of AKI

The current biomarkers most frequently used in the clinical setting are serum creatinine (SCr) and blood urea nitrogen (BUN); although these biomarkers have limited utility in identifying early AKI, they are popular because they are easy to measure and are inexpensive (114). Because both of these markers are measures of protein metabolism, they are significantly dependent on muscle mass and tubular secretion and have a wide range of normal values that vary with non-renal factors such as body weight, age, sex, and liver function (114, 115). This variability negatively affects the ability of clinicians or researchers to accurately assess severity of AKI or to predict an individual patient's likely clinical outcome, which increases the risk of failure for clinical trials and drug development (115).

Novel Biomarkers of AKI

Recent research has prioritized the development of novel biomarkers of tubular injury AKI, which may better predict both development and severity of disease; the goal of this research is to identify markers that have the same benefits of existing markers, such as ease and affordability, while improving the specificity and early detection over those

achievable by either SCr or BUN (115). Two promising novel biomarkers for AKI detection and stratification of injury severity are neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (KIM1). NGAL is a protein that is expressed at low concentrations in the epithelial cells of several organs, including the lung and stomach, but injury to epithelial cells greatly increases its expression (115). NGAL has the positive feature of being increased in both blood and urine after AKI and is one of the earliest biomarkers discovered (115). Translational studies with this biomarker have demonstrated that it correlates highly with SCr in the later phases of AKI and that increases in NGAL in the period of time 2-6 h after cardiac surgery were predictive of subsequent development of AKI; therefore, studies characterizing patterns of NGAL expression show that it has promise as a very specific and robust measure for development of AKI, although the fact these studies were small and largely performed in patients with uncomplicated AKI and few comorbidities necessitates further demonstration of its utility (115).

KIM-1 was initially discovered as a tubular protein that is undetectable in normal kidneys but is greatly increased in dedifferentiated proximal tubule cells; in both human renal injury and animal models of AKI, expression of KIM-1 was positively predictive of and correlative with degree of renal injury while correlating negatively with renal function (116). In addition to increases of KIM-1 in renal cells measured after kidney biopsy, there is urinary detection of one proteolytically-cleaved domain of the protein after ischemic AKI, which differentiates this pathology from both prerenal AKI and chronic kidney disease and may allow clinicians to identify patients who could benefit from certain therapies (115). The discovery of these molecules yields promise for the improvement of early and specific clinical detection of ischemic AKI, but further development is needed to achieve the ease of use and affordability of current markers.

Furthermore, the individual strengths of these biomarkers, as well as those of SCr and BUN in later stages of AKI, indicate that these markers might best function as part of a serum or urine panel approach for AKI detection and risk stratification (115).

Laboratory Models of Mitochondrial Dysfunction in Acute Kidney Injury

Development of pharmacological treatment for AKI necessitates both *in vitro* and *in vivo* models that accurately mimic the patterns of cellular injury seen in clinical cases of AKI. A number of models that duplicate various aspects of molecular damage after AKI have been developed and are well-characterized in the literature.

In Vitro Model: Tert-butyl Hydroperoxide-induced Oxidant Injury in Primary Rabbit RPTC

Because oxidant damage is the most significant contributing factor to tubular cell injury after renal I/R injury, a cellular model that duplicates this oxidant injury in renal tubular cells is critical to the development of drugs to treat AKI. Although immortalized renal tubular cells are commercially available, their relative lack of reliance on oxidative phosphorylation to generate ATP makes them an undesirable candidate for *in vitro* models of oxidative damage. Thus, an *in vitro* model to duplicate the physiology of the ischemic renal tubule was required. To address this need, an isolation procedure for primary RPTC, a growth medium with supplements to promote oxidative phosphorylation, and an incubation procedure that duplicates the shear stress of the renal tubule to promote differentiation of these cells were developed and have provided researchers with an optimized cell population with a similar metabolic profile to *in vivo* RPTC (117). To model the oxidant damage from renal I/R injury, tert-butyl hydroperoxide (TBHP) was used as a model hydroperoxide that undergoes catalysis by glutathione peroxidase and cytochrome P-450s to respectively cause glutathione oxidation and formation of hydroxyl radicals (118). This initial oxidant injury provokes a

cascade of cellular damage, including lipid peroxidation by hydroxyl radicals, dysregulation of sodium transport, and glutathione depletion (118). As this cascade is very similar to that seen *in vivo* in renal I/R injury, this model has proven utility in examining mitochondrial dysfunction in the context of subcellular tubular epithelial damage caused by acute oxidative stress (48, 56).

In this model system, it was demonstrated that treatment with 200 μM TBHP was sufficient to disrupt several markers of mitochondrial homeostasis in primary rabbit RPTC (Fig. 1-7). 24h after a 6h treatment with TBHP, RPTC demonstrated decreases in both FCCP-uncoupled respiration and ATP content, indicating that acute oxidant injury is sufficient to cause significant mitochondrial dysfunction (48, 56, 118). These decreases in functional markers were correlated with cell death and changes in cellular morphology at the same time point after injury, indicating that there is a temporal relationship between acute oxidative stress, mitochondrial dysfunction and cell injury (48). However, additional work in our laboratory demonstrated that there is an increase in PGC-1α protein expression 1-3 days after acute oxidant injury that is dependent on both p38 MAPK and EGFR activation, but that PGC-1α protein levels drop precipitously at 4 days after injury and remain suppressed through 6 days after TBHP treatment (38). It was further demonstrated that both basal and FCCP-uncoupled respiration return to control levels by 6 days after TBHP exposure, and that the recovery of these markers of mitochondrial function preceded improvements in cellular function as measured by recovery in activity of the Na^+/K^+-ATPase (38, 119).

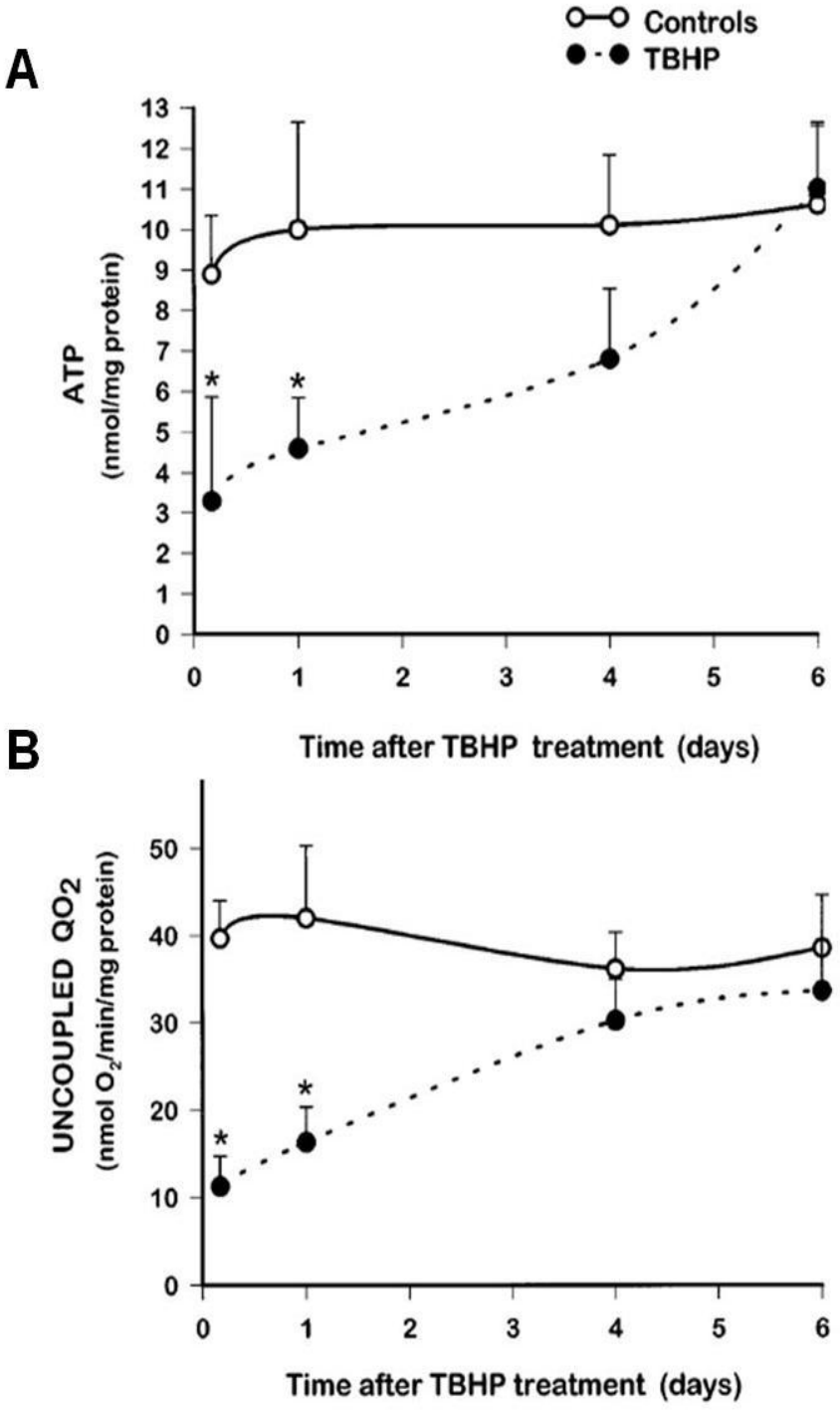

Fig. 1-6. Disruption of Mitochondrial Function after TBHP Treatment. 200 μM TBHP treatment causes early dysfunction in RPTC ATP content (A) and uncoupled respiration (B) that recover over four days post-injury. Data are presented as means ± SE, n ≥ 4. *P< 0.05, significantly different from controls (119).

In Vivo Model: Bilateral Renal I/R Injury

To duplicate the patterns of ischemic injury in AKI, a model of mouse I/R injury was developed. In this model, the renal pedicle is clamped for 20 minutes and then allowed to reperfuse (120, 121). Some of the positive features of this model in the duplication of clinical AKI are the presence of acute tubular necrosis, reproducibility of the procedure, the similarity of its injury and recovery process to that seen in humans and the recapitulation of the inflammatory response demonstrated in human disease; however, as a pure ischemia process, it does not completely model the vascular effects of AKI and therefore simplifies the demands for therapeutic support in acute disease (121).

One of the additional benefits of this model in the development of pharmacological agents that prevent or reverse patterns of molecular damage in AKI is the extensive characterization of mitochondrial damage in the first week after I/R injury (120, 122). Ligation of the renal pedicle for 20 minutes was sufficient to significantly decrease kidney function and disrupt mitochondrial homeostasis (Fig 1-8). 24 h after initiation of renal reperfusion, there was a significant increase in the renal injury biomarker SCr; this marker, as well as the tubular injury marker KIM-1, remained significantly increased through 6 days after injury (120, 122). These increases in AKI biomarkers were correlated with significant decreases in mRNA expression of nuclear-encoded NDUFB8 and ATP synthase β, as well as mitochondrial-encoded ND6 and COXI (122). These decreases in mRNA for electron transport chain proteins was accompanied by a decrease in the respective protein levels for NDUFB, COXI and ATP synthase β; the decreases in both mRNA and protein for these mitochondrial markers were detectable at 24 h and persisted through 6 days after initial reperfusion (120, 122). These disruptions in mitochondrial homeostasis were accompanied by decreases in tubular FCCP-uncoupled oxygen consumption through 6 days after treatment, and this decrease in

mitochondrial function was coincident with increases in tubular necrosis, further indicating that mitochondrial dysfunction is a major contributing factor to persistent tubular cell injury after AKI (120).

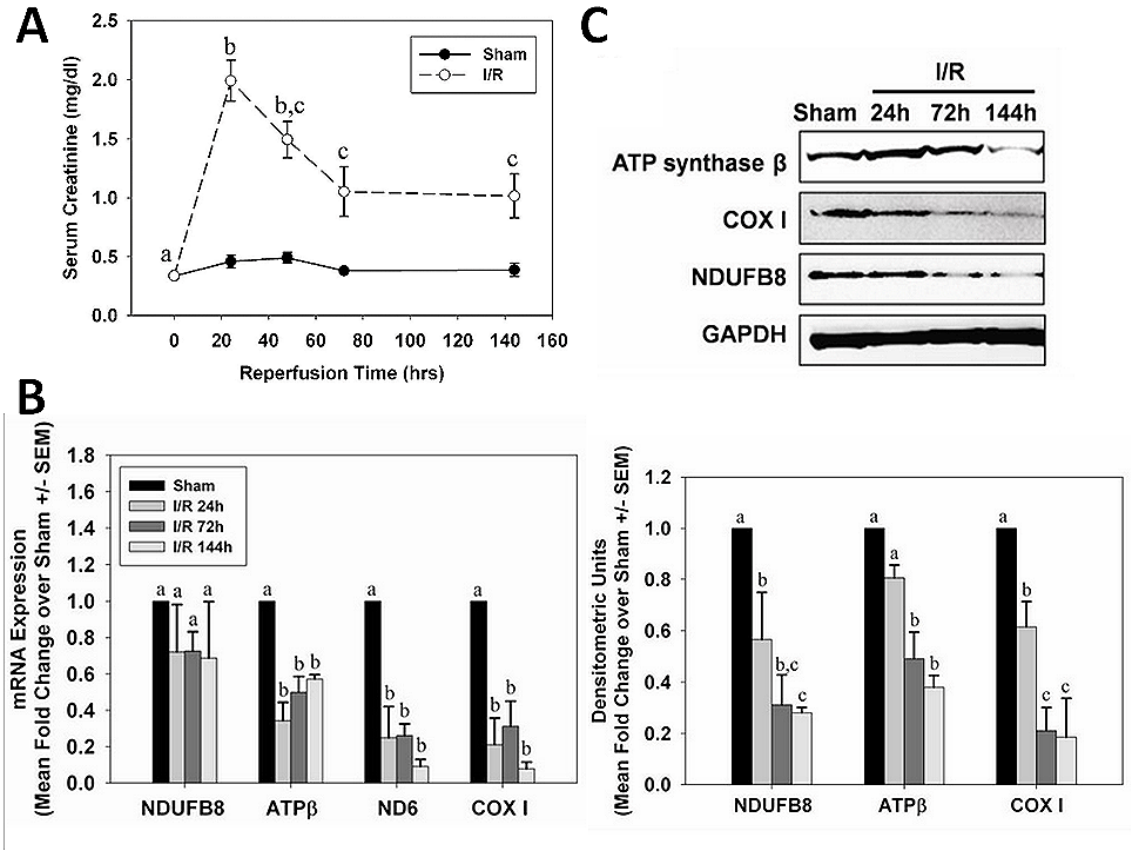

Fig. 1-7. Suppression of Renal Function and Depletion of Mitochondrial Proteins After I/R-AKI. Serum creatinine levels were significantly elevated 24 h after reperfusion, and then slowly decreased between 24 and 144 h without returning to normal levels (A). mRNA from sham and I/R mice was analyzed by qRT-PCR for expression of nuclear-encoded respiratory genes NDUFB8 and ATP synthase β and the mitochondrial-encoded genes ND6 and COXI at 24, 72, and 144 h after injury (B). Expression of mitochondrial respiratory proteins from kidneys of sham and I/R mice was examined by immunoblot analysis (C). Bars with different superscripts are significantly different from one another ($P < 0.05$) (122).

Mitochondrial Strategies for the Treatment of AKI

Inhibition of Apoptosis

Several promising pharmacological therapies for treatment of AKI focus on inhibition of apoptosis. Since caspase-3 is a protease that is involved in both the intrinsic and extrinsic pathways of apoptosis and has been demonstrated to play a role in hypoxic kidney injury, inhibiting its activation is an attractive strategy for blocking pro-apoptotic signals from either inflammatory mediators or mitochondrial dysfunction; preventing its activation in a rat I/R model of AKI did improve SCr, but tubular injury remained unchanged (86). Another anti-apoptotic therapeutic that has been investigated is minocycline, a second-generation tetracycline antibiotic; in addition to its antibiotic mechanisms of action, minocycline has been demonstrated to shift the expression of the Bcl-2 family of proteins toward anti-apoptotic signaling in the central nervous system and to inhibit the MPT in an animal model of liver ischemia (123, 124). Early work with minocycline in a rat model of renal I/R injury found that it was able to reduce tubular cell necrosis, indicating that further exploration of this compound may be warranted in clinical trials (86). Finally, it should be noted that administration of cyclosporine A, which is a traditional inhibitor of the MPT, would be an attractive treatment strategy and has been demonstrated to be effective in reducing I/R injury in other organ systems, but it is not a candidate drug for renal I/R injury due to its vasoconstrictive effects (125-128). Conclusions from current work indicate that some inhibitors of apoptosis may be good candidates for pharmacological treatment of AKI and warrant further research; however, an argument can be made that inhibiting apoptosis in cells that would otherwise undergo this process would result in persistent renal injury due to survival of cells that would otherwise be too damaged to remain intact. Therefore, addressing the mechanisms of reversible sub-lethal cellular damage that are upstream of apoptosis may be a more

high-yield strategy to inhibit cell death, improve cellular repair and preserve tubular structure after an acute ischemic kidney injury.

Treatment with Antioxidants

Since oxidant injury from reactive oxygen species such as superoxide and hydroxy radicals, as well as downstream oxidative species such as lipid peroxides, is considered the primary mediator of kidney injury arising from an acute I/R insult, scavenging or inactivating these species is an attractive therapeutic strategy to target early initiators of cell damage and consequent death. Treatment with the antioxidant U83836E, a 2-methylaminochroman that protects against oxidant injury by interacting with ferrous iron to prevent the Fenton reaction and by scavenging peroxyl radicals, decreased oxidized glutathione and significantly preserved ATP production in a rat I/R model of AKI, resulting in decreased tubular necrosis and increased tubular regeneration (129). Treatment with this antioxidant also reduced SCr and BUN in animals exposed to I/R to sham levels as early as 24h after reperfusion, indicating that this is a viable therapeutic strategy to improve renal function after AKI (129). Additional recent research has focused on antioxidant therapy that is specifically targeted to the mitochondria, since these organelles are exquisitely sensitive to oxidant damage and therefore responsible for propagation of ROS. In a cecal ligation puncture (CLP) model of septic AKI, in which acute oxidant damage is an important pathophysiological process, treatment with the mitochondrial-targeted antioxidant Mito-TEMPO significantly decreased mitochondrial superoxide production by preserving Mn-SOD activity, which is pathologically inactivated in the early phases of AKI (97). The effects of this antioxidant therapy were preservation of ETC function, as measured by oxygen consumption and kidney ATP levels, significant improvement in BUN levels, and a significantly increased survival rate of injured animals

(97). The results of these studies strongly suggest the utility of antioxidant therapy after AKI, especially in the context of combination therapy with other therapeutics.

Treatment with Mitochondrial Biogenic Agents

Another strategy to preserve renal function and support tubular cell recovery after AKI is to increase mitochondrial number and improve function of existing mitochondria through the induction of mitochondrial biogenesis. Prior to examination of pharmacological induction of mitochondrial biogenesis as a therapeutic strategy for treatment of AKI, proof-of-concept studies were performed utilizing adenoviral over-expression of PGC-1α, the "master regulator of mitochondrial biogenesis," in primary RPTC. While over-expression of PGC-1α prior to treatment with the oxidant TBHP potentiated resultant decreases in mitochondrial function as measured by cellular oxygen consumption, over-expression of PGC-1α after the induction of injury by TBHP preserved mitochondrial function and ATP production and restored decreased mitochondrial ETC proteins ATP Synthase-β and ND6 to baseline levels (130). The results of this study thereby supported the hypothesis that induction of mitochondrial biogenesis is a viable treatment option for the preservation of mitochondrial function after AKI.

Following the success of genetic modulation of mitochondrial biogenesis in improving RPTC mitochondrial function after acute oxidant injury, work was done to examine if pharmacological inducers of mitochondrial biogenesis would likewise improve mitochondrial function and, furthermore, improve cellular recovery in both *in vitro* and *in vivo* models of ischemic kidney injury. In the primary RPTC treated with 400 μM TBHP, the SIRT1 activator SRT1720, which had been previously demonstrated to increase both total PGC-1α protein and activated (deacetylated) PGC-1α in naïve RPTC, preserved

ATP production and significantly recovered uncoupled cellular respiration (48). Although approximately 50% of RPTC in a previously confluent monolayer have died and sloughed off 6h after TBHP treatment, the improvements in mitochondrial function observed after addition of SRT1720 to the cell media improved recovery, migration and regeneration of the remaining cells when compared to those that received only vehicle control treatment (48). Similarly, 24h treatment with 10 μM DOI, a 5-HT_2 receptor pan-agonist that had been demonstrated to increase ATP levels and nuclear-encoded mitochondrial proteins in naïve RPTC, promoted recovery of uncoupled respiration in cells that had been treated with 400 μM TBHP (56).

While improvements in mitochondrial function and cell recovery in the *in vitro* TBHP model of acute oxidant injury with mitochondrial biogenic agents supported the hypothesis that biogenic agents have utility in the treatment of AKI, the homogenous nature of this model and its inability to recapitulate multifactorial nature of human AKI reduced the impact of these findings. Therefore, it was necessary to examine the effects of these drugs in a more complex animal model of AKI.

To examine the effects of pharmacologically-induced mitochondrial biogenesis on mitochondrial and disease progression in a more clinically relevant model of AKI, mice that had been exposed to 20 min of renal pedicle ligation were treated daily for five days after initial reperfusion with 0.3 mg/kg formoterol, a β_2-adrenergic agonist that had been previously demonstrated to increase uncoupled respiration and mtDNA copy number in primary RPTC at nanomolar concentrations (50, 120). Treatment of these mice was initiated 24 h after the initial injury, which is a highly clinically relevant timeline due to delayed identification and stratification of patients with AKI in the hospital setting. Six days after I/R injury, expression of both nuclear- and mitochondrial-encoded proteins

comprising subunits of different ETC complexes were decreased in I/R mice that had only received vehicle treatment but were returned to baseline levels by formoterol treatment (Fig 1-9) (120).

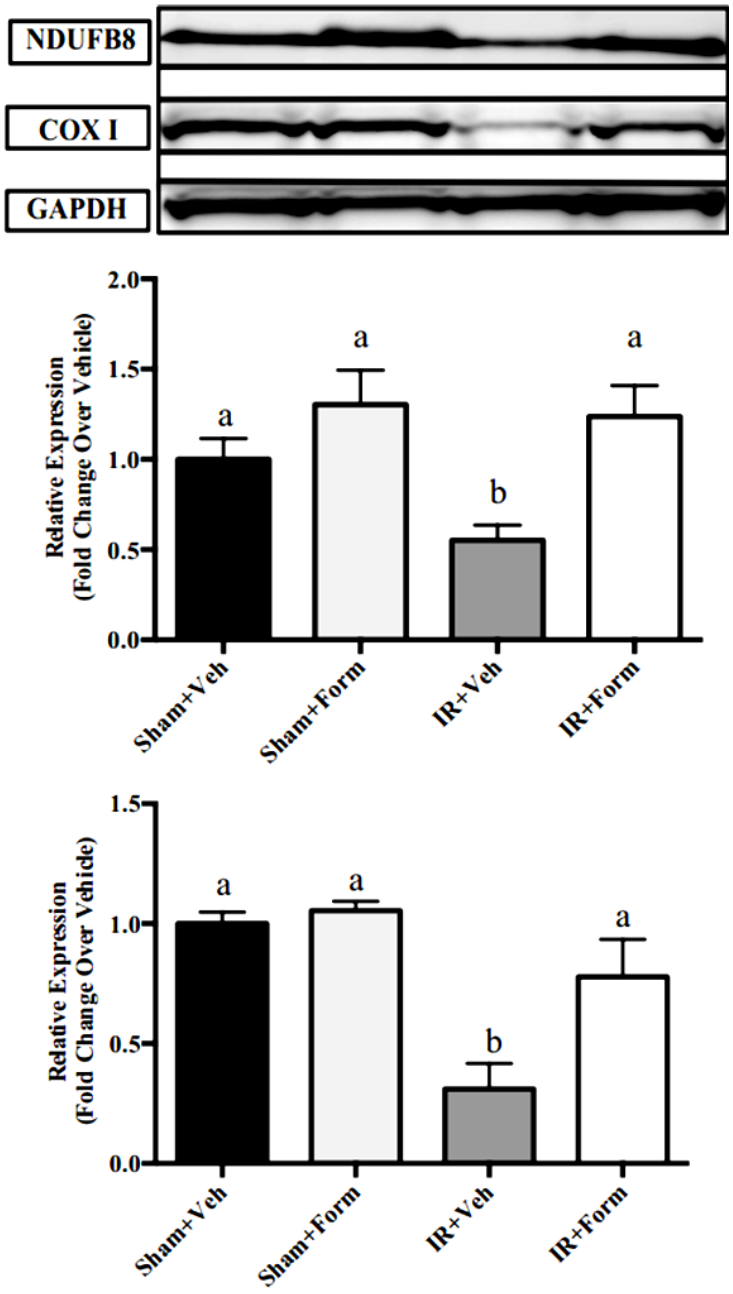

Fig. 1-8. Formoterol (Form) restored mitochondrial protein expression after I/R-induced AKI. Mice were subjected to sham or I/R surgery and subsequent treatment with vehicle (Veh) or formoterol. Markers for MB were evaluated via immunoblot 144 hours after surgery. Shown are renal cortical lysate and mitochondrial ETC proteins NDUFB8 (middle graph) and COX I (bottom graph). Densitometric semiquantification is shown below the representative blots. Samples were analyzed *via* one-way ANOVA followed by a Student–Newman–Keuls post-hoc test to evaluate differences between groups. Bars with different superscripts are significantly different from one another. Data points are mean±SEM and are relative values compared with control (n=6, $p < 0.05$) (120).

Coincident with these improvements in mitochondrial protein content were preserved uncoupled respiration in I/R mice treated with formoterol in comparison to the decreased respiration observed in I/R mice treated with only vehicle; furthermore, these improvements in mitochondrial function were correlated with improved markers of renal function, including the return of SCr in formoterol-treated I/R mice to baseline levels six days after initial injury, compared to the persistent increase in SCr in vehicle-treated SCr, and a significant reduction in the renal expression of KIM-1 in formoterol-treated I/R mice (Fig 1-10) (120). These results strongly suggest that pharmacologically-induced mitochondrial biogenesis is a viable treatment for the reversal of mitochondrial dysfunction and the promotion of tubular cell recovery after ischemic AKI, which holds promise in further reduction of organ damage and improved outcomes for human AKI patients. Furthermore, these results indicate that formoterol, which is an FDA-approved drug currently used for the treatment of asthma, may be a good candidate for further exploration and consequent transition to human clinical trials.

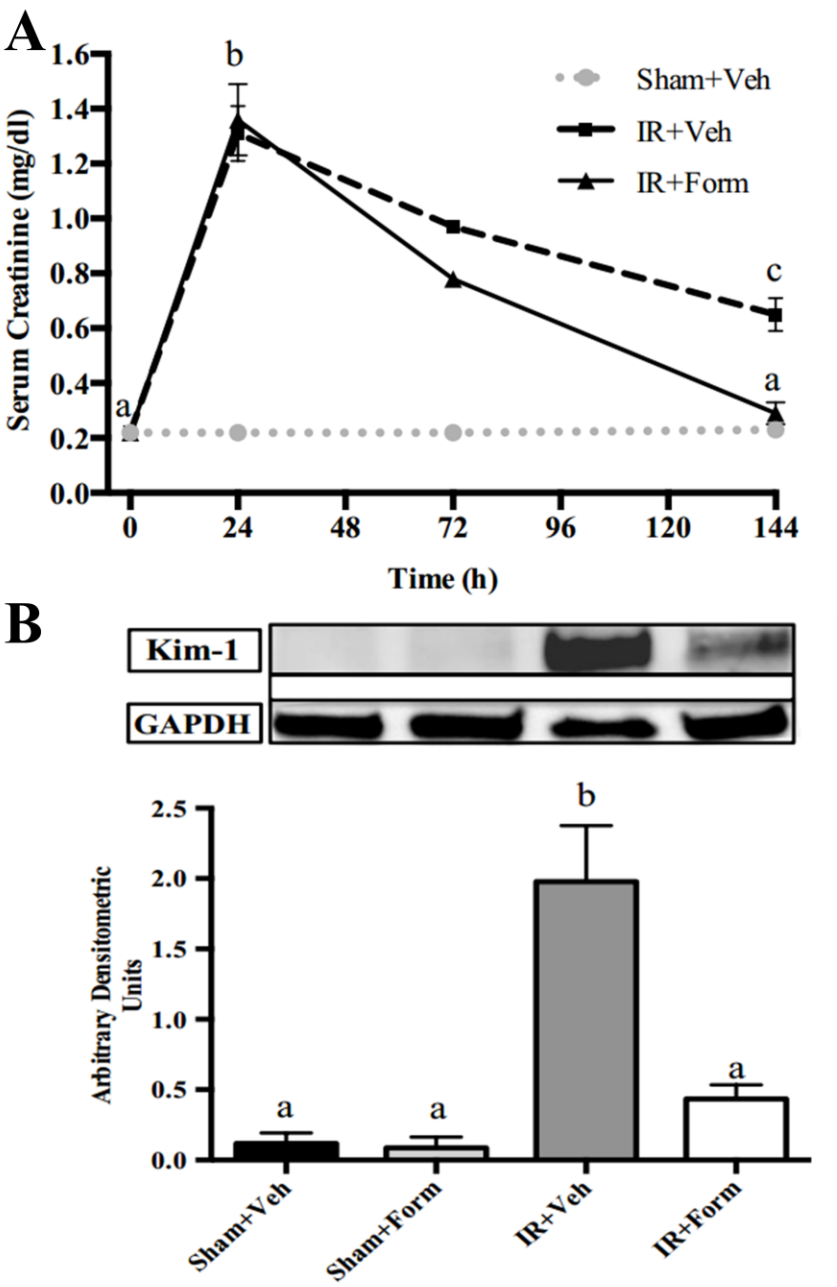

Fig. 1-9. Treatment with Formoterol Restored Kidney Function and Mitigated Proximal Tubule Injury. Mice were subjected to sham or I/R surgery and subsequent treatment with vehicle (Veh) or formoterol (Form). Kidney function was assessed via serum creatinine (A) and tubular injury *via* KIM-1 immunoblot analysis (B). KIM-1 protein was measured in kidneys from mice 144 hours after injury and quantified by densitometry. Samples were analyzed *via* one-way ANOVA followed by a Student–Newman–Keuls post hoc test to evaluate differences between groups. Data points are mean ±SEM; with bars with different superscripts are significantly different from one another (n=5, $P < 0.01$). GAPDH, glyceraldehydes 3-phosphate dehydrogenase (120).

TRAUMATIC BRAIN INJURY

Definition of Traumatic Brain Injury

Traumatic brain injury (TBI) is broadly defined as any injury "affecting brain function resulting from non-penetrating loading of the contact and non-contact type;" in this context, contact loading is an injury resulting from physical impact to the head, while non-contact loading is an injury sustained from force exerted from a distance, the prototypical examples of which are acceleration/deceleration injuries as well as explosive force from a blast (131). TBI can be further categorized into focal or diffuse injuries; in focal injury, the primary damage is localized to a particular brain region, while diffuse injuries are distributed throughout the brain and are associated with global axonal damage or swelling of brain substructures (131, 132). Although the patterns of injury differentiate these two types of TBI, they share similar pathophysiological characteristics which will be described later in this review.

The Impact of TBI on Population Health

TBI is a serious global health problem and is the leading cause of global morbidity and mortality in people younger than 45 (132, 133). The United States averages 1.7 million TBI cases per year, 50,000 of which result in death (133, 134). TBI is twice as common in males as in females, and most likely to be experienced by children aged 0-4 and adolescents aged 15-19 (133). Additionally, there are subpopulations of Americans who are at higher risk of sustaining a TBI; primarily, these patients are involved either in active military service or participate in sports and other high-contact recreation activities (133). Although TBI is considered an acute injury, it can result in lifelong deficits in motor, cognitive, behavioral and emotional function; these deficits can range from minor inabilities to perform daily activities to debilitating life-long disability requiring constant

care (133). Additionally, TBI renders patients more susceptible to further neurological complications and has been linked to increased risk of addiction, depression and Alzheimer's disease, among other chronic conditions (133). Finally, the financial burden of TBI is multifaceted and comprises direct medical costs of hospitalizations and associated medical visits as well as lost productivity and inability to return to work; with all factors considered, the lifelong cost of TBI is estimated at $60-221 billion per year (133, 134). At this point in time, treatment for TBI is non-curative and focused on symptom management, with no efficacious approved therapeutic options to directly address the underlying cellular pathophysiology of the disease, and public health efforts focused on prevention of TBI through education and risk-reduction programs (134). The paucity of neuroprotective pharmacological therapies thus far is due to multiple factors, including the narrow therapeutic window for intervention, difficulties in identifying the best route of administration (eg. Intravenous vs. intrathecal), consideration of BBB function, and clinical heterogeneity of injury profile and underlying conditions that influence recovery and are difficult to duplicate in commonly used animal models (135). Although shortcomings in these models have been clearly identified, development of more complicated and relevant models is a complex task, thus current rodent models of TBI will continue to predominate in the foreseeable future (135).

Animal Models of TBI

Several animal models of TBI have been developed to provide opportunities for mechanistic study and drug discovery through recapitulation of the pathophysiology of either focal or diffuse injury, although it should be noted that no singular animal model is sufficient to duplicate the full panel of acute and chronic patterns cellular injury after TBI (136).

Focal TBIs are usually modeled through local skull distortion and direct contact of an impacting injury to the brain (136). The most common and best characterized animal model of focal TBI is the fluid percussion model, in which a craniotomy is performed and a fluid pressure pulse is delivered through a saline-filled reservoir (131, 136). The benefit of this model is that the impact is able to be modulated to recapitulate a range of severities of injury that yield experimentally quantifiable motor and cognitive deficits, but it is disadvantageous because it is not directly clinically relatable (136). Another model of focal TBI is controlled cortical impact (CCI), an invasive method that uses an impactor to direct mechanical injury to the exposed dura of an area of the brain exposed by craniotomy (136). This model is beneficial because the parameters of injury are easily controlled and modified to model a spectrum of injury severity, although the use of craniotomy in this model reduces its clinical relevance to human injury, in which skull fracture is not necessarily well-correlated with severity of damage (131, 136). A final model for focal brain injury is the weight drop method in which a guided weight impacts either the closed skull or exposed dura, though this model is limited by lack of control over the impact velocity and risk of rebound impact, both of which lead to unpredictability and lack of consistency in the resulting brain deformities (136).

Diffuse injury is best modeled through restrained head acceleration models; these models are desirable because they are able to accurately recapitulate the diffuse axonal injury that is a critical pathological consequence of human diffuse TBI (136). However, these models have only been successfully performed in higher order mammals such as miniature pigs and several species of primates, because their brain structure and relative brain mass compared to weight is more similar to that of humans (136). Therefore, the expense and logistical requirements of these models limits their usefulness and

precludes their use for early phases of drug discovery, in which high numbers of animals are required to mitigate the effects of drug attrition due to toxicity or inability to elicit the desired therapeutic response.

Pathophysiology of TBI

The development of neurological injury after an initial traumatic event is classified into two phases. The first stage is primary damage at the immediate moment of injury; it is a irreversible result of mechanical force on the brain and can occurs as a contusion, laceration, intracranial hemorrhage or diffuse axonal injury (132, 136). Secondary injury is a result of the activation of a physiological cascade characterized by toxicity from excessive release of excitatory neurotransmitters, which results in increased calcium flux, hypoxia, and the consequent increase in ROS and protease activation (132, 136, 137). Other features of secondary injury include increased inflammation and cytokine signaling, as well as deficits in respiration and mitochondrial function (Fig 1-11)(132, 136).

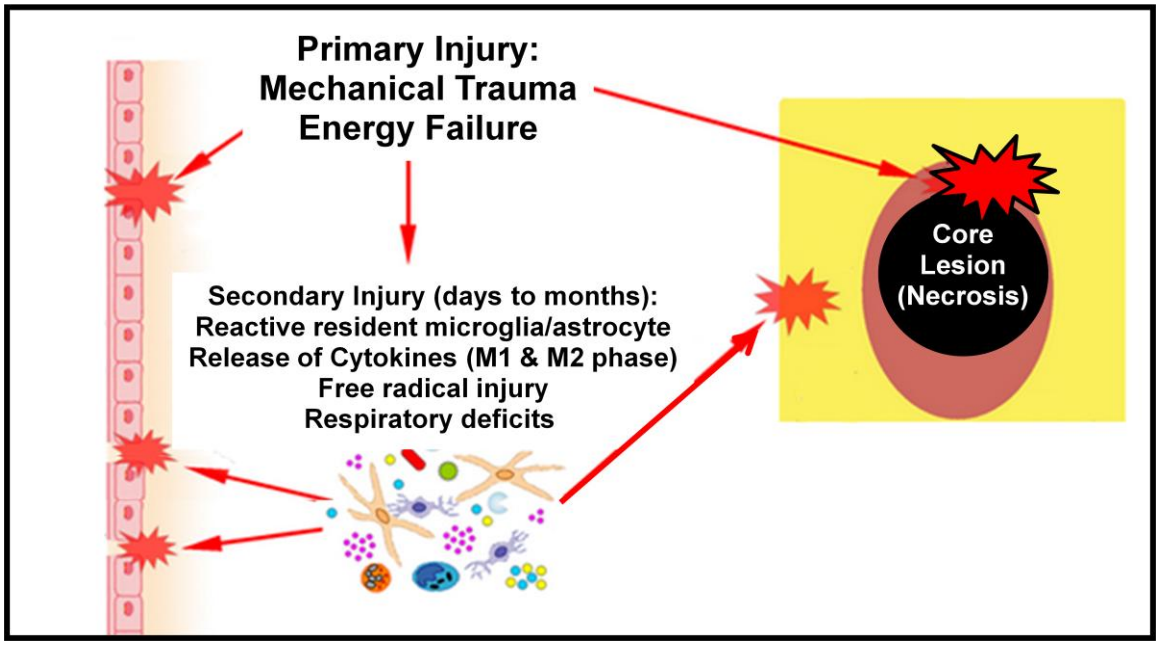

Fig. 1-10. Mechanisms of Cell Injury Following TBI. Acute traumatic injury to the brain or spinal cord causes mechanical damage and energy failure in parenchymal cells and endothelia that comprise the blood-brain and blood-spinal cord barriers (BBB and BSCB, respectively). Secondary injury mechanisms exacerbate tissue damage and BBB/BSCB dysfunction (138).

Inflammation and TBI

Robust immune activation is a common molecular cascade observed in both the acute and chronic temporal phases after TBI; this immune response includes local infiltration of peripheral inflammatory cells, activation of microglia, reactive astrocytosis and release of many cytokines into the cerebrospinal fluid (CSF) and blood (135, 139-141). The relationship of cytokine release to brain injury, function, and recovery is complicated, with some cytokines designated as pro-recovery markers and others identified as drivers of common complications of TBI (140, 142). Although there are many cytokines expressed after TBI, this review will focus on the pro- and anti-inflammatory cytokines that have been demonstrated to have the most profound effects on tissue damage and clinical outcome.

One of the maladaptive consequences of inflammatory activation is increased blood-brain barrier (BBB) permeability, which contributes to subsequent life-threatening increases in intracranial pressure, one of the leading contributors to neuronal death and the best predictor of poor outcome after TBI (137, 140). One factor that has been demonstrated to contribute to BBB permeability is increased accumulation of ROS-releasing peripheral leukocytes in damaged tissue; in both experimental animal models as well as analysis of CSF in patients who have experienced severe TBI. Increases in intercellular adhesion molecule (ICAM-1), which mediates leukocyte adhesion and subsequent infiltration, have also been positively correlated with degradation of the BBB (140, 142). Additional work suggests that matrix metalloproteinase-9 (MMP-9) also contributes to BBB permeability, and that MMP is activated by the cytokine TNF-α, which is released by activated microglia after TBI (141, 143-145).

Cytokine release from activated microglia is resolved into temporal phases, with pro-inflammatory cytokines released in the M1 phase (1-2 d), followed by the reflexive release of regulatory anti-inflammatory cytokines in a M2 (2-5 d) (139, 144). Cytokines that are prominent in this secondary M2 phase are IL-10 and transforming growth factor β (TGF-β) (139, 144, 146). IL-10 actively suppresses TNF-α and attenuates other non-inflammatory pathways, such as production of ROS; this increased expression of IL-10 is associated with neurogenesis and recovery after TBI (147-149). Additionally, administration of exogenous IL-10 as late as 1 h after experimental fluid percussion injury resulted in decreases in pro-inflammatory cytokines and improved neurological function, indicating that the anti-inflammatory response is critical to recovery after acute injury and that pharmacologically mimicking the M2 phase at an earlier time point is a promising strategy to reduce the severity of tissue damage after TBI (147). These results were supported by evidence that administration of IL-10 after excitotoxic spinal cord injury, which shares several pathological features with TBI, mitigated cell loss and prevented advanced gray matter damage in the post-acute phase, which further suggests that IL-10 is a long-term pro-survival factor that can positively affect chronic outcomes after acute neurological injury states (148).

Another prominent anti-inflammatory mediator in acute TBI is TGF-β, which is released in the delayed M2 phase of microglia activation (144, 145). TGF-β is responsible for several facets of recovery after acute neurological trauma and is capable of promoting neurogenesis through both anti-inflammatory signaling and stimulation of neural stem cell proliferation, migration and growth (150). Although its promotion of this stem cell growth has a direct effect on neurogenesis, the indirect effects of its anti-inflammatory properties in neurogenesis are less understood, but its suppressive effect on TNF-α expression is likely one contributor to its protective effects (140).

IL-6 is unique in that it has both pro- and anti-inflammatory effects; although it peaks in the early M1 phase with other cytokines that have been linked to increased severity and damage after TBI, it is observed to promote cellular recovery and tissue regeneration and is positively correlated with better Glascow Coma Score after acute neurological injury(140, 145). The nature of this duality in its inflammatory effect is still being explored, but current evidence suggests that low levels of IL-6 after injury may be protective, while increased plasma concentrations, especially those above 100 pg/mL, correlate with more severe injury and poor outcome after TBI (145, 149). Additionally, the patterns of expression between cell types may contribute to its paradoxical effects; for example, astrocyte overexpression of IL-6 leads to a maladaptive chronic reactive astrocytosis and neuronal loss, but its secretion by microglia leads to inhibition of TNF-α may therefore exert a more beneficial effect (140, 149).

Mitochondrial Dysfunction and TBI

Overview of Mitochondrial Dysfunction after TBI

Post-TBI, neurons are susceptible to both necrosis and apoptosis (132, 151). Necrosis, or uncontrolled cell death, occurs due to total metabolic failure, ATP depletion, and irreversible cell injury and is the most likely fate of cells directly affected by the primary mechanical and most severe ischemic injury (132, 151). Apoptosis, or programmed cell death, is likely to affect cells in the penumbra of the initial injury, which are cells that initially undergo only sub-lethal injury; these cells are still structurally intact and partially functioning to produce some ATP, and their injury is potentially reversible, making these cells the target of therapeutic intervention (132, 151). There is increasing evidence that initial sub-lethal mitochondrial dysfunction in the secondary phase after TBI (and other

ischemic cerebral events) is highly associated with the changes in cellular and tissue structure and function, as well as the lasting behavioral, cognitive and motor deficits observed after these injuries (152). These patterns of dysfunction after TBI are still being elucidated, but current research on these mechanisms of dysfunction can be broken into several subsets, including acute oxidative stress, mitochondrial calcium dysregulation, ETC and associated metabolic enzyme dysfunction, and altered modulation of autophagy and apoptosis (152). When these aspects of mitochondrial dysfunction become sufficiently severe, altered mitochondrial structure and diminished function are observed. Because each of these mechanisms can adversely affect other aspects of mitochondrial function, and because any of these mitochondrial pathways can be therapeutically modulated to contribute to improved mitochondrial health after TBI, it is necessary to further elucidate the contributions these factors make to overall mitochondrial and cellular injury.

Increased Oxidative Stress after TBI

Ischemia is a pathological feature common to many acute neurological injury processes; the effects of ischemia are very serious, and even a lesion as small as 10% of the total brain volume is sufficient to result in a severe neurological outcome, such as coma or persistent vegetative state (132). Oxidative stress created by the influx of ROS generated by these patterns of acute ischemia and subsequent reperfusion, as well as those generated by the increased inflammatory mediators and glutamate-mediated excitotoxicity, is one of the most important determinants of secondary molecular injury after TBI (135, 153).

In physiological circumstances, molecular oxygen, the final electron receptor in the ETC, is fully reduced to water by Complex IV; however, reperfusion after ischemia causes an inflow of excessive molecular oxygen, which is only partially reduced to superoxide (152). Under basal conditions, small amounts of ROS are generated, but the redox balance of the mitochondrion is tightly controlled by modulation of expression of the mitochondrial antioxidant proteins SOD2 and UCP2 by two mechanisms: 1) increased transcription through a PGC-1α-mediated pathway and 2) protein kinase D (PKD)/transcription factor nuclear factor κB (NF- κB) mitochondrion-to-nucleus signaling that results in increased SOD2 expression as a result of a compensatory mitochondrial ROS detoxification program (153-155). After acute ischemia, these antioxidant detoxification mechanisms are overwhelmed by the influx of molecular oxygen and resultant increased ROS, disrupting the redox balance and shifting toward an increased oxidative state (156). Furthermore, this increased presence of ROS can create other oxidative species such as peroxynitrite, a highly reactive anion that further propagates oxidative damage and disrupts the structure and function of a variety of mitochondrial components through oxidation of lipids, proteins and mtDNA (135, 151, 152, 157, 158).

One mitochondrial component easily disrupted by lipid peroxidation as a result of increased ROS is the IMM; with increased oxidation of lipids, the fluidity and structure of the IMM is disrupted, which affects the structure and efficacy of the ETC components, further inducing generation of ROS at Complexes I and III (108, 152, 159). Oxidation of mitochondrial proteins further contributes to the dysfunction of the ETC; additionally, the disruption of the structure of these proteins as a result of oxidation makes them susceptible to degradation by proteases, causing decreased protein content and subsequent dysfunction of the mitochondria (152). Finally, ROS can cause oxidative damage to mtDNA, which is located in the mitochondrial matrix where ROS are highly

induced after acute ischemic injury and which has poor quality control mechanisms to remove damaged bases or repair lesions (151, 152). The end result of the oxidative insults on mtDNA in the setting of acute brain injury is deletion of portions of the mtDNA and depletion of mtDNA copy number, a common result of increased oxidative stress on the mtDNA; susceptibility to these mtDNA deletions increases with age and may correlate with decreased post-injury survival of particular cell populations and prognosis of patients after an acute ischemic event (160, 161). Finally, acute CCI in mice increased molecular markers of autophagy in ipsilateral hippocampus, and ultrastructural analysis of this tissue through transmission electron micrography (TEM) discovered increased autophagic vesicles, indicating that increased oxidative stress leads to increased organelle recycling after TBI (162). However, current literature on the effects of this increased autophagy conflict in its assessment of autophagy as a pro-survival or pro-death mechanism in cells affected by TBI (162, 163).

Calcium Dysregulation after TBI

Another facet of mitochondrial injury after TBI is excessive intracellular calcium concentration as a result of excitotoxic glutamate signaling through NMDA receptors on cellular membranes in both the directly affected tissue as well as the surrounding affected penumbra of injury (164). Mitochondria are responsible for cellular calcium homeostasis and can sequester calcium through the mitochondrial calcium uniporter to buffer cytosolic calcium concentrations, alterations of which can induce activation of calpains and other proteases (135, 156, 158, 165). Additionally, damage to the endoplasmic reticulum further shifts the onus of calcium buffering to mitochondria and exacerbates the increases in calcium concentration caused by increased glutamate signaling (152). The effects of mitochondrial calcium dysregulation are multifaceted and

include increased ROS generation, cytoskeletal damage due to increased protease activity, and initiation of apoptosis (135, 152, 153).

Initiation of Apoptosis after TBI

The end result of uncontrolled oxidative stress and overwhelming calcium concentrations after TBI is apoptosis through mitochondrial signaling (135, 152). This process is the intrinsic, or mitochondrial, pathway of apoptosis and occurs in three phases: an initiation phase during which cells activate both pro-apoptotic and pro-survival pathways as the result of increased stress, the integration phase in which these signals all converge at mitochondria, and the post-mitochondrial phase (151, 152, 166). Although activation of the initiation phase does not necessarily result in apoptosis, the tipping point comes in the integration phase, when lethal signaling exceeds the simultaneous pro-survival signaling; these "death signals" lead to the MPT, and the permeability of the mitochondrial membranes results in initiation of the post-mitochondrial phase, a process that includes loss of membrane potential, subsequent deficits in ATP synthesis due to dissipation of the proton gradient, further uncontrolled ROS production, and release of cytochrome c into the cytosol (152, 166, 167). After translocation to the cytosol, cytochrome c interacts with the apoptosome to activate the "initiator" caspase 9, which subsequently activates the "executioner" caspase 3, the most abundant caspase in the CNS, and results in apoptosis (137, 166).

Alterations in Mitochondrial Protein Content, Structure and Function

Damage that is not severe enough to provoke the MPT after oxidant- and calcium-induced molecular stress can still yield mitochondrial damage with decreased protein content, altered structure and diminished function. Acute and sub-chronic changes in

mitochondrial protein, especially the proteins comprising the complexes of the ETC, is one of the least studied facets of post-TBI mitochondrial damage, despite the fact that these changes in ETC content directly influence mitochondrial function and ability to produce ATP. However, a few studies have demonstrated changes in mitochondrial protein content in experimental models of TBI. In *in vitro* mechanical stretch model of mild TBI, gene expression for complexes I, III, IV and V of the ETC was significantly decreased 24 h after injury; however, gene expression had returned to baseline at 72 h post-injury, indicating that the mechanical damage used for this model may not be significant enough to recapitulate the complex post-injury cellular milieu that is present in the secondary phase of TBI (168). Glutamate oxaloacetate transaminase, a mitochondrial protein that participates in the glutamate pathway used as an alternative energy-production mechanism by neurons, was demonstrated to be decreased in a blast injury model of TBI; the decrease of this protein is significant, because it indicates that TBI has a global effect on the energy production capabilities of the cell and leaves it more susceptible to cell death as a result of diminished ATP production (169). Finally, animals that were exposed to a mild TBI followed by repeated stress exposure had decreased ETC proteins in both ipsilateral hippocampus and cortex 7 d after injury, which correlated with decreased behavioral outcomes in these animals and indicates that changes in mitochondrial protein content may play a role in chronic deficits in neurological function after TBI (170). This relationship between ETC function and chronic neurological dysfunction is supported by the finding that many chronic neurodegenerative diseases share dysfunction of ETC complex I as a common pathophysiological feature (171).

Changes in mitochondrial structure are another pathological feature of TBI and can result from altered fission/fusion signaling as well as membrane fluidity caused by lipid

peroxidation (151, 152). In a CCI model of TBI, ipsilateral cortical tissue demonstrated minor alterations in mitochondrial structure as early as 30 minutes after injury; 12 h after injury, almost all mitochondria in this tissue had altered morphology, including mitochondrial matrix condensation, cristae disruption, and swelling, which is indicative of increased mitochondrial membrane permeability (157). Even at 24 h post-injury, not all mitochondria in a single field presented with the same degree of structural dysfunction, indicating that heteroplasmy may contribute to differential mitochondrial susceptibility to damage(157, 172).

Diminished function, as measured by decreases in the oxygen consumption/cellular respiration and/or decreased ATP production, has been demonstrated in several models of TBI. Persistent decreases in ATP production were observed in both *in vitro* and *in vivo* models of TBI and were correlated with other mitochondrial damage markers, including lipid peroxidation, decreased ETC gene expression and mitochondrial enzyme activity (135, 168, 169, 173). In addition to its role in both necrosis and apoptosis, ATP depletion in both the cortex and hippocampus following CCI caused an early disruption of synaptic homeostasis due to an inability to maintain ion transport through ATP-dependent channels, which further exacerbates calcium dysregulation and subsequent ROS production as well as reduces the cellular pool of glutamate available to feed into the neuron's secondary energy production mechanisms (173).

In a mouse CCI model, the respiration of cells isolated from both the ipsilateral cortex and hippocampus was decreased as early as 30 minutes after injury; this was followed by a reflexive recovery of respiration at 1 h post-injury, indicating that an initial severe irreversible mitochondrial dysregulation may result from the stress of the primary injury and that these critically dysfunctional mitochondria are rapidly cleared, leaving

mitochondria that are sub-lethally injured and capable of temporarily maintaining ETC function (157). However, by 3 h post-injury, the oxygen consumption by these cells is once more significantly decreased in both of these tissues and remains suppressed through 72 h after initial insult, suggesting that there is an ongoing loss of mitochondrial function that is likely the result of the many mitochondrial damage patterns initiated in the secondary damage phase of TBI (157, 165). Additionally, the measured respiration of mitochondria isolated from debrided cortical tissues of human TBI patients was generally positively correlated with Glascow Coma Score, suggesting that early preservation of mitochondrial function is an important factor in recovery after TBI (174).

Mitochondrial Strategies for Treatment of TBI

Antioxidant Administration and ETC Bypass to Reduce Oxidative Stress

Because increased oxidative stress is the major contributing factor of mitochondrial dysfunction after TBI and many antioxidants have been used to successfully treat other acute injury states, decreasing oxidative species is an attractive treatment strategy to treat early mitochondrial dysfunction after TBI (135, 153). To combat the increase in superoxide production that results from ischemia and reperfusion, as well as mechanical injury, scavengers such as modified SOD have been tested; however, these interventions have a very narrow therapeutic window, which has limited their success in clinical trials (153).

Although the increase in ROS after injury is rapid and transient, those acute oxidative species react with other biomolecules to create highly reactive aldehyde, carbonyl, and nitrosyl groups on lipids and proteins that participate in the propagation of oxidative stress and inflammation; because these reactive molecules are abundant in the 3-12 h

window after TBI in both the cortex and hippocampus, they are an attractive target for mitigation of mitochondrial damage (137, 153, 157). In a mouse CCI model, the lipid peroxide scavenger U-83836E significantly reduced both cortical expression of both the lipid peroxide-associated reactive aldehyde 4-hydroxynonenal (4-NHE) and its downstream protein nitration product; in these same animals, a concomitant improvement in mitochondrial calcium buffering capacity and recovery of RCR was observed, suggesting that reducing the secondary byproducts of early oxidative stress may confer significant protection against mitochondrial dysfunction (175). Similarly, drugs used in animal models to scavenge reactive carbonyl groups, such as D-penicillamine, carnosine, and hydralazine, have also demonstrated post-TBI neuroprotective effects, including decreased cell permeability, decreased neuron necrosis, reduced infarct size, preservation of BBB function, and improved neurological recovery (153). Another strategy that has been tested in animal models is removal of reactive biomolecules through support of the cell's native antioxidant defenses; for example, drugs that replenish stores of glutathione, such as n-acetylcysteine (NAC) and γ-glutamylcysteine, decreased protein carbonyls, improved respiratory capacity and calcium homeostasis and decreased autophagy after TBI, resulting in preserved BBB structure, reduced cerebral edema and decreased lesion volume (153). Following its successful use in animals, NAC was administered to patients with mild blast-induced TBI, resulting in improved behavioral consequences, such as preserved memory function (153).

Although most antioxidant strategies employ the use of drugs that removed existing reactive molecules, another proposed strategy to decreased oxidative stress is to prevent the formation of ROS. Mitochondrial uncoupling, which collapses the mitochondrial protein gradient and increase electron transport chain flux, depletes the

pool of molecular oxygen that is susceptible to oxidation, and both genetic overexpression of uncoupling proteins and administration of the pharmacological uncouplers 2,4-DNP and FCCP were able to decrease cortical ROS production, support mitochondrial calcium buffering, reduce cortical tissue damage, and improve behavioral outcome after TBI (176, 177). Another proposed strategy to decrease ROS production is electron chain bypass, a method that uses mild redox agents to decrease ROS at ETC complexes I and III, which are the major sites of ROS generation in the mitochondria (108, 178).

Inhibition of Mitochondrial Permeability Transition after TBI

Another common mitochondrial target for the treatment of TBI is reduction of apoptosis through inhibition of the MPT that results from mitochondrial calcium dysregulation caused by glutamate toxicity. Two inhibitors of the MTP, Cyclosporin A (CsA) and NIM811, have been used in numerous animal studies and human trials of TBI. Use of CsA in rodent models of TBI demonstrated decreased mitochondrial swelling in traumatically injured neurons, increased mitochondrial membrane potential, and decreased cortical lesion volume (126-128, 152, 179). In early human trials, however, CsA has demonstrated limited efficacy in improving long-term neurological outcome (135). The disparity in efficacy of CsA in animals versus humans may be due to differences in time of administration; animals were treated anywhere from 30 min before to 1 h after injury, but humans were treated anywhere up to 8-12 h after injury, which may be too late to efficiently prevent a significant amount of mitochondrial permeabilization (126-128, 135). Additionally, CsA is an immunosuppressant drug, so a non-immunosupressive MPT inhibitor, NIM811, was developed and tested in rodent CCI models. Treatment with NIM811 after TBI recapitulated the effects of post-TBI CsA

treatment, including increased mitochondrial respiration, cortical tissue sparing, and improved spatial memory; however, it has similar initiation time requirements to CsA, indicating that it may have the same limitations for clinical utility (180, 181).

Induction of Mitochondrial Biogenesis as a Potential Treatment for TBI

Because mitochondrial biogenesis is a necessary and beneficial response to cellular stress, induction of mitochondrial biogenesis has been identified as a potential novel treatment modality for TBI (137). Although no pharmacological inducers of mitochondrial biogenesis have yet been used to treat TBI, it has been demonstrated that both hypothermia and fasting are potential neuroprotective treatments for acute ischemic brain injury (182, 183). Since cold exposure and calorie restriction are well-characterized inducers of mitochondrial biogenesis, it is likely that this response is at least partially responsible for the neuroprotective effects of these treatments (24, 27). Additionally, the ability of the mitochondrial biogenic drug formoterol to preserve mitochondrial protein expression, improve mitochondrial respiration, promote cell recovery and support organ function after acute ischemic insult to the kidney, a disease state that mirrors many of the pathophysiological features of acute brain ischemia, further supports the hypothesis that pharmacological induction of mitochondrial biogenesis is a viable treatment strategy for TBI (120).

Chapter Two

5-HT_2 Receptor Regulation of Mitochondrial Genes: Pharmacological Effects of Agonists and Antagonists

ABSTRACT

Mitochondrial biogenesis (MB) is an important physiological process that occurs under both basal and physiological stress conditions, such as cold exposure and fasting. In both acute and chronic injury settings, mitochondria are often dysfunctional, which leads to further secondary deficits in cell function, including increased production of reactive oxygen species (ROS) and decreased energy production. Deficits in energy production are especially deleterious for those organs that require levels of ATP to conduct transport activities and critical cell signaling through ion flux. Both recent and current research have revealed that recovery of mitochondrial function is necessary for recovery of cellular function and that induction of mitochondrial biogenesis through activation of peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α) is a novel strategy for the treatment of a variety of acute and chronic diseases. In this study, we tested the ability of the 5-HT_{2C} receptor agonist CP-809,101 and the 5-HT_{2C} receptor antagonist SB-242, 084 to induce mitochondrial biogenesis and found that both agonist and antagonist increased both respiration rates and PGC-1α mRNA expression in primary cultures of renal proximal tubules cells (RPTC). Additionally, both CP-809,101 and SB-242,084 increased mRNA expression of both PGC-1α and the mitochondrial proteins ND1 and NDUFB8. Although these compounds are classically defined as 5-HT_{2C} ligands, we found that they still increased MB mRNA expression in both RPTC in which the 5-HT_{2C} receptor has been down-regulated by siRNA and in the kidney cortex of mice lacking the 5-HT_{2C} receptor. Interestingly, the ability of these compounds to

increase PGC-1α mRNA in RPTC was determined to be dependent on the 5-HT_{2A} receptor, for which both of these compounds have a 100 nM affinity. These results indicate that modulation of 5-HT_{2A} receptor signaling results in the induction of MB and that treatment with these drugs may be an effective therapeutic option for treatment of both acute and chronic organ disease. Since these drugs were screened in renal tissue, they may be particularly useful in the treatment of acute kidney injury (AKI), a pathology for which few reparative treatments currently exist.

INTRODUCTON

Mitochondrial dysfunction is a pathological state underlying many diseases, including chronic diseases such as diabetes, Alzheimer's disease, Huntington's disease and amyotrophic lateral sclerosis (ALS or Lou Gehrig's syndrome) and acute injuries to the heart, liver and kidney (151, 164, 172, 184-186). The organ systems most susceptible to mitochondrial dysfunction are those with high energy requirements. The renal system, which requires high levels of ATP to drive the transport processes necessary for active tubular transport, is at risk for mitochondrial dysfunction caused by acute ischemia/reperfusion, drugs, toxicants and diabetes (187). It has been demonstrated that recovery of mitochondrial function precedes recovery of cellular structure and function in an RPTC model of oxidant injury (112, 188-190). Although recovery of mitochondrial function after acute kidney injury is critical to recovery of both cellular and organ function, our laboratory has previously reported that mitochondrial proteins are suppressed up to 144 h after initial ischemic injury, which indicates that promoting the recovery of mitochondrial function is a viable therapeutic strategy for the treatment of AKI (122).

One potential strategy for improving mitochondrial function is promotion of mitochondrial biogenesis (MB), an intricate process that drives the coordinated transcription of both mitochondrial DNA- and nuclear-encoded genes to increase cellular mitochondrial content. Mitochondrial health and homeostasis is maintained through MB, mitochondrial fission and fusion, and mitophagy; under physiological conditions, these mechanisms function together to remove unhealthy mitochondria (171). Central to this process is the induction of peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α), the "master regulator of MB," (191). Increases in PGC-1α lead directly and indirectly to

the transcription of genes necessary for mitochondrial function, including nuclear respiratory factor (NRF) and mitochondrial transcription factor A (TFAM), and genes that encode proteins of ETC complexes (192). The net result of increased PGC-1α is the upregulation of the proteins of the ETC, which results in greater electron flux and an increase in ATP production. PGC-1α can be induced by a number of both physiological and pathological stimuli, including cold exposure, caloric restriction, exercise and acute injury, and both pharmacologically- and genetically-induced increases in PGC-1α ameliorate mitochondrial dysfunction in several model systems, including acute oxidant injury in renal proximal tubular cells (RPTC), ALS and Huntington's disease ((158, 186, 193-196).

Many signaling pathways have been implicated in the upregulation of PGC-1α transcription, including cAMP through CREB, AMP-kinase, calcineurin A/MEF-2, calcium/calmodulin-dependent protein kinase (CAMK-IV), NO/cGMP, and mTOR (16, 18-21, 24, 32, 33, 193). Additionally, our laboratory has identified the roles of Src, p38 MAPK and EGFR transactivation in the signaling pathway that leads to increased PGC-1α expression in response to oxidant injury in RPTC (197). Our group has previously identified several pharmacological targets that activate these signaling pathways and increase PGC-1α activity, including the β_2 adrenergic receptor, phosphodiesterases and the 5-HT_2 class of receptors (54, 56, 198, 199). Although the roles of several of these targets in the induction of renal MB have now been fully characterized, little work has been done to fully understand the role of the 5-HT_2 receptor in mitochondrial signaling.

The 5-HT_2 family of receptors is composed of three receptor sub-types: 5-HT_{2A}, $_{2B}$ and $_{2C}$. All 5-HT_2 receptors are G-protein-coupled receptors (GPCRs) traditionally described as being coupled to $G_{q/11}$ protein for their signal transduction, though some groups have

suggested that at least some of the receptor subtypes in this class may also couple to $G_{i/0}$ (200-202). Signaling through these receptors through $G_{q/11}$ or the $G_{i/0}$ second messengers can lead to activation of pathways previously implicated in the regulation of PGC-1α. Additionally, 5-HT was identified as a survival factor for mitochondria in cardiomyocytes through 5-HT_{2B} receptor signaling (203). Several studies examining 5-HT_2 receptor signaling in the kidney demonstrated expression of 5-HT_{2A}, $_{2B}$ and $_{2C}$ mRNA in primary renal tubule cells; additionally, 5-HT_{2A} protein was found to be expressed in renal mesangial cells (66, 204).

Subsequently, our laboratory investigated the role of renal 5-HT_2 mitochondrial signaling and demonstrated that the non-specific 5-HT_2 receptor agonist DOI induced MB and ameliorated mitochondrial dysfunction caused by acute *tert*-butyl hydrogen peroxide (tBHP)-induced oxidant injury in RPTC These data suggest that this family of receptors is involved in mitochondrial homeostasis in the kidney (54). Since DOI is a pan-agonist, we could not assign the MB activity to a specific 5-HT_2 receptor.

The availability of potent and specific $5HT_2$ receptor ligands have made it possible to investigate receptor specificity for the induction of MB. In this study we demonstrate the expression of the $5HT_{2C}$ receptor in primary RPTC and describe the ability of both a classically-defined 5-HT_{2C} agonist and an antagonist to induce MB in the kidney. Furthermore, we used both a 5-HT_{2C} knockout mouse and siRNA directed to 5-HT_{2C} and 5-HT_{2A} mRNA to study the role of these receptors in MB in the kidney.

MATERIALS AND METHODS

Reagents

CP-809,101 and SB-242,084 were purchased from Tocris Bioscience (Ellisville,MO). All other chemicals were purchased from Sigma Aldrich (St. Louis, MO).

Animal Care and Use. All experiments were performed in strict accordance with the guidelines in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All protocols were approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina, or Columbia University and appropriate efforts were made to reduce animal suffering.

Isolation and Culture of Proximal Tubules

Female New Zealand white rabbits (1.5-2.0 kg) were purchased from Charles River Laboratories (Wilmington, MA). Renal proximal tubule cell (RPTC) isolation was performed using the iron oxide perfusion method described previously and cultured under improved conditions as previously described (205). Three days after initial plating, de-differentiated RPTC were trypsinized and re-plated on XF-96 polystyrene cell culture microplates (Seahorse Bioscience, North Bellerica, MA) at a density of 18,000 cells/well and maintained at 37^{o}C for 3 days before experimentation (206). For other RPTC experiments, isolated renal proximal tubules were plated in 35-mm dishes used 8 days after initial plating. RPTC were treated with experimental compounds for 24 h.

Oxygen Consumption

The oxygen consumption rate (OCR) of RPTC was measured using the Seahorse Bioscience XF-96 Extracellular Flux Analyzer as previously described (206). Each 96-well assay plate was treated with vehicle control (DMSO <0.5%), and 1, 10 and 100 nM concentrations of the experimental compounds. Basal OCR was measured before injection of carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, 0.5 uM),

which allows for the measurement of uncoupled OCR (FCCP-OCR), a marker of MB (117). Compounds that increased FCCP-OCR by 15% were considered positive for induction of MB.

5-HT_{2C} Receptor Protein Expression

5-HT receptor proteins were isolated from RPTC as previously described (207). A 30 µg sample of the resulting protein was then treated with either N-Glycosidase F (PNGase; New England BioLabs, Ipswich, MA) according to the manufacturer's instructions or temperature-matched control conditions for 2 h, then loaded onto an SDS-PAGE gel. After electrophoretic transfer to nitrocellulose, $5HT_{2C}$ protein levels were determined by immunoblot analysis using an anti-$5HT_{2C}$ receptor monoclonal antibody (1:250, Santa Cruz Biotechnology, Santa Cruz, CA; SR-2C (D-12)) and an anti-β-actin antibody (1:1000, Santa Cruz Biotechnology), respectively, followed by an HRP-labeled anti-mouse secondary antibody (1:1000, Santa Cruz Biotechnology). The secondary antibody was detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL).

In vivo Mouse Studies

Male C57/Bl6 mice (6-8 weeks old) were purchased from the National Cancer Institute (Bethesda, MD). Mice were individually housed in a temperature-controlled room under a 12-h light/dark cycle and randomly assigned to either vehicle control group or one of two treatment groups. Mice were administered a single intraperitoneal dose of either diluent (40% (2-Hydroxypropyl)-β-cyclodextrin in 0.9% saline), CP-809,101 (1 mg/kg) or SB-242,084 (1 mg/kg) and euthanized 24 h later. Kidneys were isolated and snap frozen for quantitative PCR (qPCR) analysis.

Generation of 5-HT_{2C} Transgenic Mice

5-HT_{2C} transgenic mice on a 129SvEv/Tac background were bred as described previously (208). Briefly, 5-HT_{2C} is an X-linked gene; therefore, female mice heterozygotic for the transgene were bred with wild -type (WT) male mice, generating WT and heterozygote female mice, and WT and 5HT_{2C} null male offspring. Tail clips were taken and lysed overnight for PCR genotyping identification of the animals. Mice were weaned at 3-4 weeks single sex group house under standard conditions until adulthood.

5-HT_{2C} Transgenic Mouse Experiments

Animals were housed on a 12 h light/dark cycle with food and water available *ad libitum*. At 6-9 weeks of age, 5-HT_{2C} WT and KO mice were randomly assigned to vehicle control, CP-809,101 (1 mg/kg) or SB-242,084 (1 mg/kg) treatment groups and treated as described above. 24h following a single injection of either diluents or drug, mice were euthanized, and kidneys were isolated and snap-frozen for quantitative PCR (qPCR) analysis.

Generation of 5-HT_{2A} Transgenic Mice

5-HT_{2A} KO mice andWT littermate controls on a 129SvEv/Tac background were originally obtained from Taconic (Taconic Farms). Mice heterozygous for a transgenic STOP cassette flanked by

LoxP sites located upstream of the htr2a gene were crossed, generating WT, KO, and heterozygous littermates, identified by PCR genotyping (209, 210). Mice were weaned at 3–4 weeks of age and were single sex group house under standard conditions until adulthood.

5-HT_{2A} Transgenic Mouse Experiments

Animals were housed on a 12 h light/dark cycle with food and water available *ad libitum*. At 6-9 weeks of age, 5-HT_{2A} WT and KO mice were randomly assigned to vehicle control, CP-809,101 (1 mg/kg) or SB-242,084 (1 mg/kg) treatment groups and treated as described above. 24h following drug treatment mice were euthanized, and dissections were performed to obtain the organs and tissues of interest for quantitative PCR (qPCR) analysis.

5-HT_{2C} and 5-HT_{2A} Knockout Experiments in RPTC

Rabbit mRNA sequences for 5-HT_{2A} and 5-HT_{2C} receptor were obtained from Ensembl. The BLOCK-iT™ RNAi Designer (Invitrogen) was used to design siRNA to these sequences. Two days past confluency, RPTC were treated with either 200 nM siGenome non-targeting siRNA #3, 100 nM of both 2C1 and 2C2 siRNA or 100 nM of both 2A1 or 2A2 siRNA (Table 2). 72 h after siRNA treatment, RPTC were treated for 24 h with the experimental drugs and harvested for RT-PCR analysis.

Real-Time Reverse Transcription-PCR

Total RNA was extracted from RPTC or renal cortex samples using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized via reverse transcription using the RevertAid First Strand cDNA kit (Thermo Fisher Scientific, Waltham, MA) with 1-2 ug of RNA. PCR products were amplified from 5 uL of cDNA template using 2x Maxima SYBR green qPCR master mix (Thermo Fisher Scientific) and 400 nM concentrations of each primer (Integrated DNA Technologies, Inc., Coralville, IA). Primer sequences for PGC-1α, ND1, NDUFB8, and β-actin were described previously (50, 122).

Statistics

Data are presented as means ± SEM. Single comparisons for normal data were performed using a Student's t-test, whereas data found to not have a normal distribution were subjected to a Mann-Whitney U-test. Multiple comparisons for normal data were performed using one-way analysis of variance (ANOVA) with an appropriate post-hoc test to compare multiple means. Kruskal-Wallis one-way analysis of variance was used to do multiple comparisons for non-normal data, and a Holm-Sidak's post-test was used to compare multiple means. Single and multiple comparison data were considered statistically significantly different at $P \leq 0.5$. RPTC isolated from a single rabbit represented an individual experiment (n=1) and were repeated until $n \geq 4$ was obtained. Rodent studies were repeated until $n \geq 3$ was obtained.

RESULTS

The 5-HT_2 Receptors are Expressed in the Kidney and in RPTC

5-HT_{2A} , $_{2B}$ and $_{2C}$ receptor mRNAs were identified in RPTC (Fig. 1A). The reported molecular weight of the 5-HT2c receptor is 48 kDa; this molecular weight corresponds to the receptor after it has undergone one N-glycosylation. Additionally, it has been reported that the receptor is also expressed as a 60 kDa protein N-glycosylated at two sites and as a 38 kDa protein with no N-glycosylations. In mouse frontal cortex, the receptor is more prominently expressed as the 48 kDa receptor, although the 38 kDa receptor is also expressed (Fig. 1B). Conversely, the most prominent isoforms detected in the mouse kidney is the 38 kDa non-glycosylated protein, with lower expression of the 60 kDa and 48 kDa glycosylated proteins (Fig. 1B).

In both rat and rabbit frontal cortex, the 5-HT_{2c} receptor is expressed as both the 48 kDa and 60 kDa glycosylated receptors, but there is little or no expression of the 38 kDa non-glycosylated protein. In RPTC, all three isoforms of the receptor are expressed, with the 60 kDa receptor expressed most prominently. Treatment of the isolated receptor protein with PNGase for two hours decreased the quantity of the 60 kDa receptor and increased the quantity of the 38 kDa receptor (Fig. 1C).

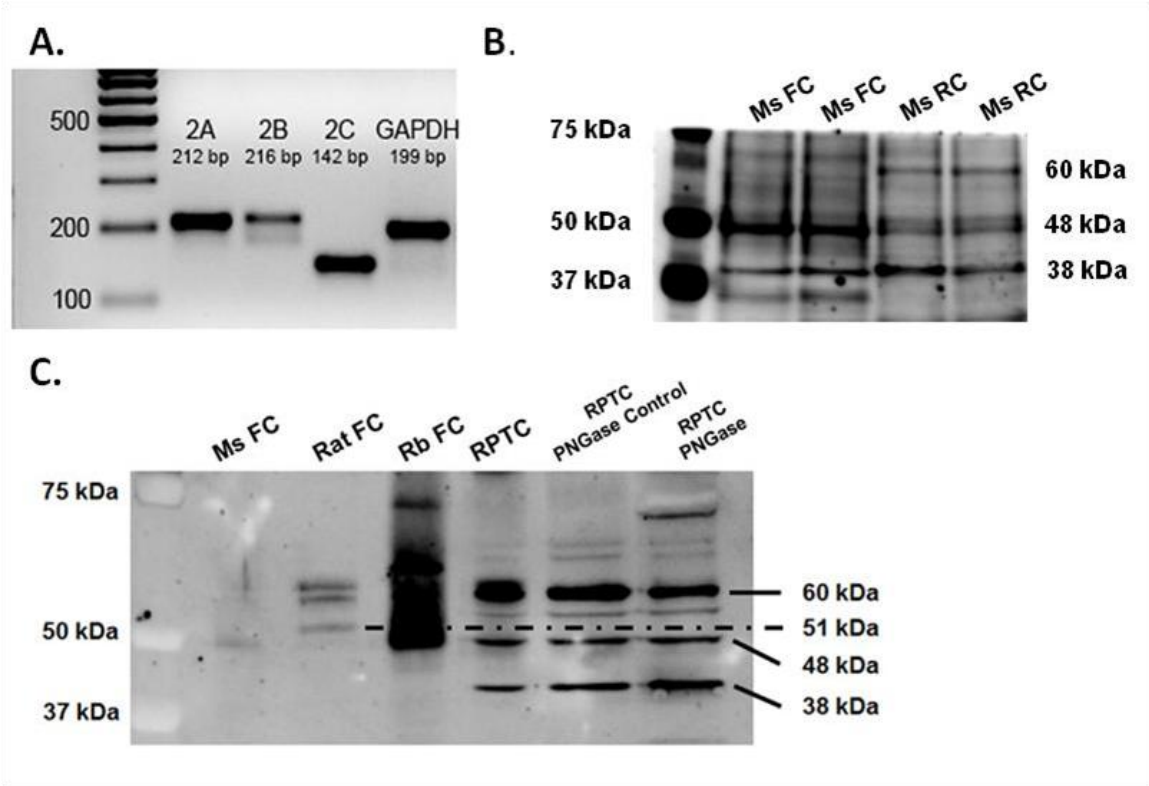

Fig. 2-1. 5-HT$_2$ Receptors are Expressed in Renal Tissue. 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptor mRNA expression were measured in RPTC using GAPDH as a control gene (A). 5-HT$_{2C}$ receptor protein expression was analyzed by immunoblot in mouse frontal cortex and renal cortex (B) and in RPTC both in the presence and absence of Peptide N-Glycosidase F (C). Ms FC = Mouse Frontal Cortex; Ms RC = Mouse Renal Cortex; RPTC = Renal Proximal Tubule Cell.

Both a Classical 5-HT_{2C} Agonist and Antagonist Induce MB in RPTC

RPTC were plated in XF-96 plates and grown under improved culture conditions that maintain polarity, differentiated function and respiration rates similar to *in vivo* renal proximal tubule cells (198, 205). RPTC were treated for 24 h with either vehicle control (0.5% DMSO), CP-809,101 (selective 5-HT_{2C} agonist; 0.1, 1, 10 and 100 nM) or SB-242,084 (selective 5-HT_{2C} antagonist; 0.1, 1, 10 and 100 nM). CP-809,101 increased FCCP-OCR relative to vehicle controls at 100 nM (Fig. 2A). SB-242,084 increased FCCP-OCR relative to vehicle controls at 1, 10 and 100 nM (Fig. 2B). It should be noted that the Kd for CP-809,101 and SB-242,084 for the 5-HT_{2C} receptor are 0.1 nM and 10 nM, respectively (Table 1). CP-809101 is1000-fold more potent at the 5-HT_{2C} receptor compared to 5-HT_{2A} and 5-HT_{2B} receptors, respectively (Table 1) (211). Similarly, SB-242,084 is 100-fold more potent at the 5-HT_{2C} receptor compared to 5-HT_{2A} and 5-HT_{2B} receptors, respectively (Table 1) (77).

To demonstrate that these increased FCCP-OCR rates resulted from increased MB, PGC-1α mRNA were measured using quantitative PCR. PGC-1α mRNA was increased at 10 and 100 nM CP-809,101 and at 10 and 100 nM SB-242,084 (Fig. 2C and 2D). These results indicate that 5-HT_{2C} agonists and antagonists potently increase some markers of MB in RPTC.

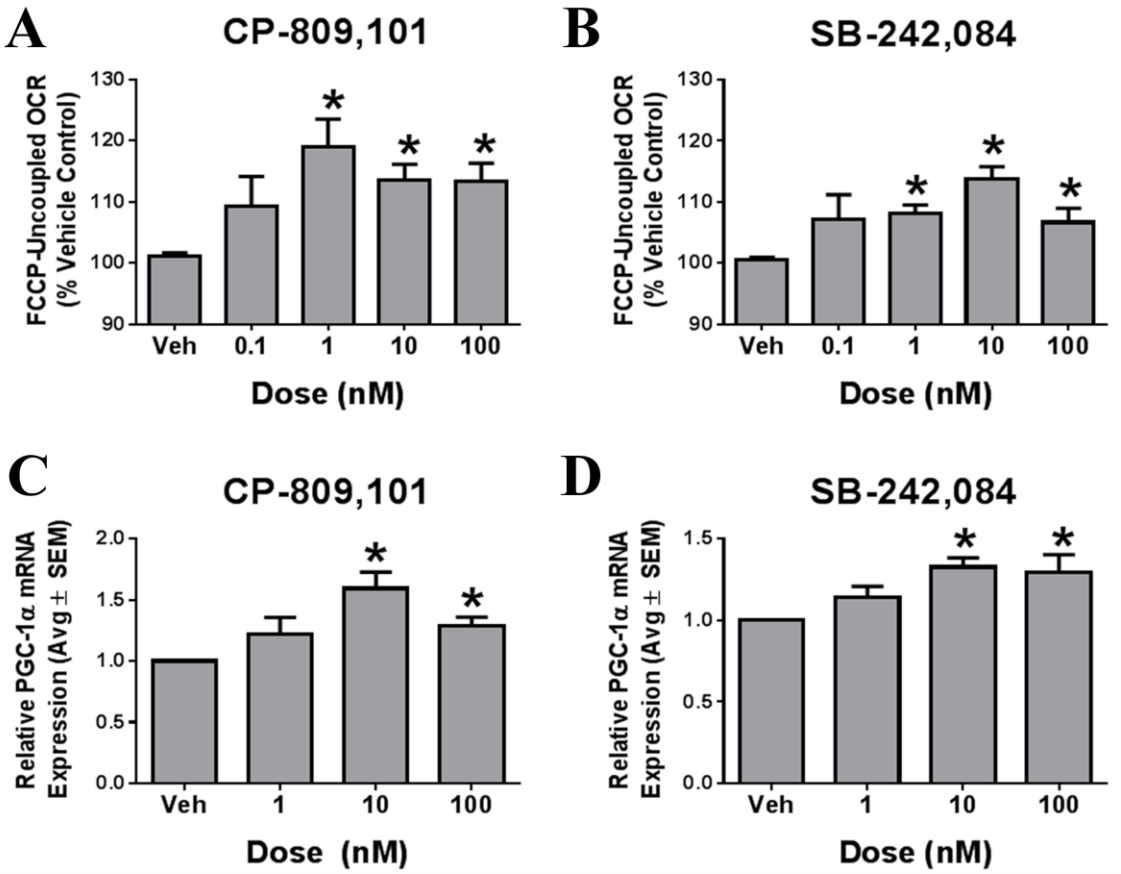

Fig. 2-2. Both a 5-HT$_{2C}$ Agonist and Antagonist Induce MB in RPTC. RPTC were treated with either CP-809,101 (A) or SB-242,084 (B) for 24 h. FCCP-uncoupled mitochondrial respiration was measured using the Seahorse XF-96 instrument. RPTC PGC-1α mRNA expression was measured using tubulin as a control gene following 24 h of treatment with either CP-809,101 (C) or SB-242,084 (D). Data were analyzed using Kruskal-Wallis with Dunn's multiple comparison test. Data are represented as mean ± S.E.M., n≥5.*, $p < 0.05$ vs. vehicle control. $n \geq 3$, * $p < 0.05$;

5-HT_{2C} Agonist and Antagonist Induce MB *in vivo*

In kidney cortex of male C56/Bl6 mice treated with 1 mg/kg of CP-809,101 for 24h, PGC-1α mRNA increased 1.6-fold, while mRNA expression of the mitochondrial-encoded gene NADH dehydrogenase 1 (ND1) and nuclear-encoded gene NDUFB8 increased 1.9- and 1.9-fold, respectively (Fig 3). ND1 and NDUFB8 are mitochondrial proteins. Mice treated with 1 mg/kg of SB-242,084 for 24 h demonstrated a 1.9-fold increase in renal cortical PGC-1α mRNA, with a concomitant 1.4- and 1.9-fold increase in ND1 and NDUFB8 mRNA, respectively (Fig. 3). There were no changes in mtDNA copy number or mitochondrial protein expression between vehicle and either 1 mg/kg CP-809,101 or 1 mg/kg SB-242,084 at this time point (Fig. 1-3).

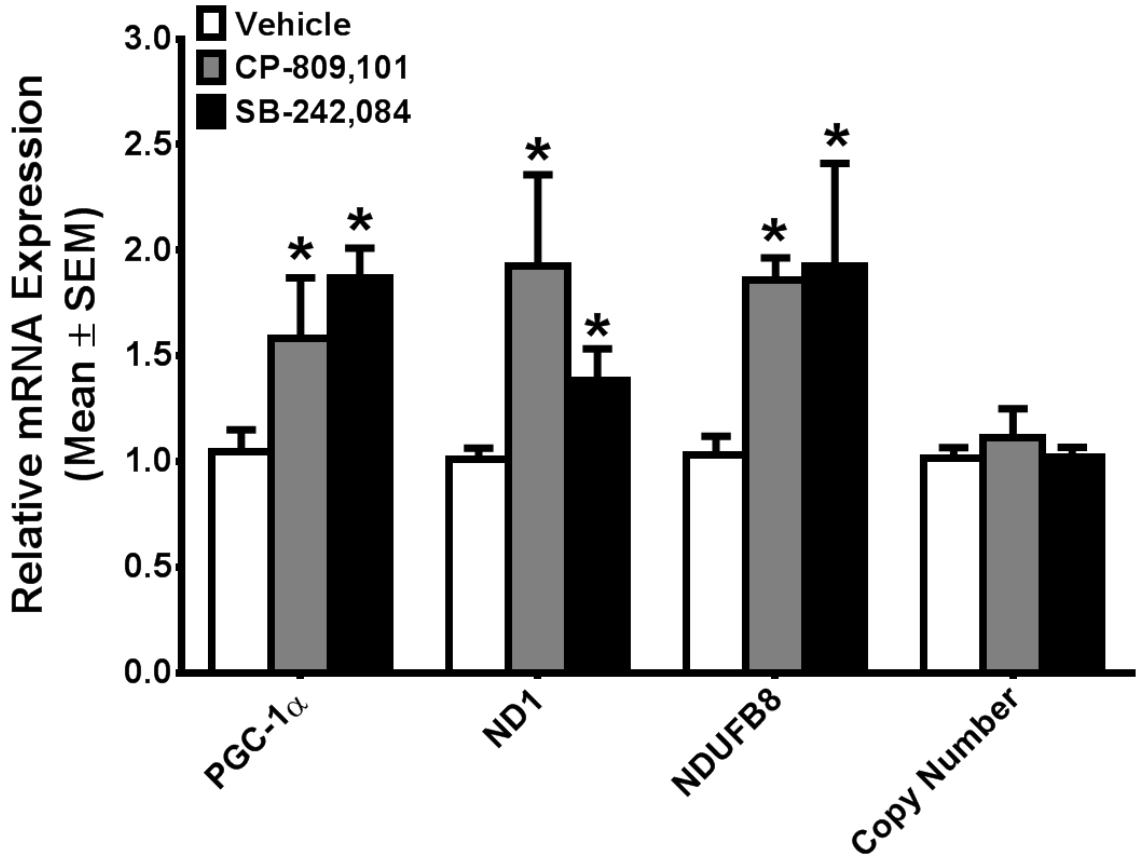

Fig. 2-3. Both a 5-HT_{2C} Agonist and Antagonist Induce Mitochondrial Gene Expression in Naïve Mouse Kidney Cortex. C57/Bl6 mice were treated with a single intraperitoneal dose of CP-809,101 or SB-242,084. PGC-1α, ND1 and NDUFB8 mRNA expression in renal cortex were determined by RT-PCR using actin as a control gene. mtDNA copy number was determined by qPCR, using ND1 for the mtDNA gene and actin for the nuclear control gene. Values reported as mean ± SEM. Student's t- test was used to determine significance. $n \geq 3$, * $p < 0.05$.

Both a Classical 5-HT_{2C} Agonist and Antagonist Induce Renal MB *in vivo* in Mice Lacking the 5-HT_{2C} Receptor

At baseline, mice lacking the 5-HT_{2C} receptor have 20% less PGC-1α mRNA compared to WT control mice, but there is no difference in either ND1 or NDUFB8 mRNA between the two groups (Fig. 2-4A-F).

Treatment of WT mice with CP-809,101 did not increase PGC-1α, ND1 or NDUFB8 mRNA over vehicle (Fig. 2-4A-C), while treatment of these mice with SB-242,084 increased ND1 mRNA 1.5-fold but did not increase PGC-1α or NDUFB8 mRNA over vehicle treatment (Fig. 2-4D-4F).

Treatment of the 5-HT_{2C} knockout mice with CP-809,101 induced a 1.5- and 1.4-fold increase in ND1 and NDUFB8 mRNA, respectively, though PGC-1α mRNA expression remained unchanged (Fig. 2-4A-C). Treatment with SB-242,084 increased PGC-1α mRNA 1.5 fold over vehicle-treated 5-HT_{2C} knockout mice (no change over vehicle-treated WT 5-HT_{2C} mice), with 1.5-fold changes in both ND1 and NDUFB8 mRNA (Fig. 2-4D-F).

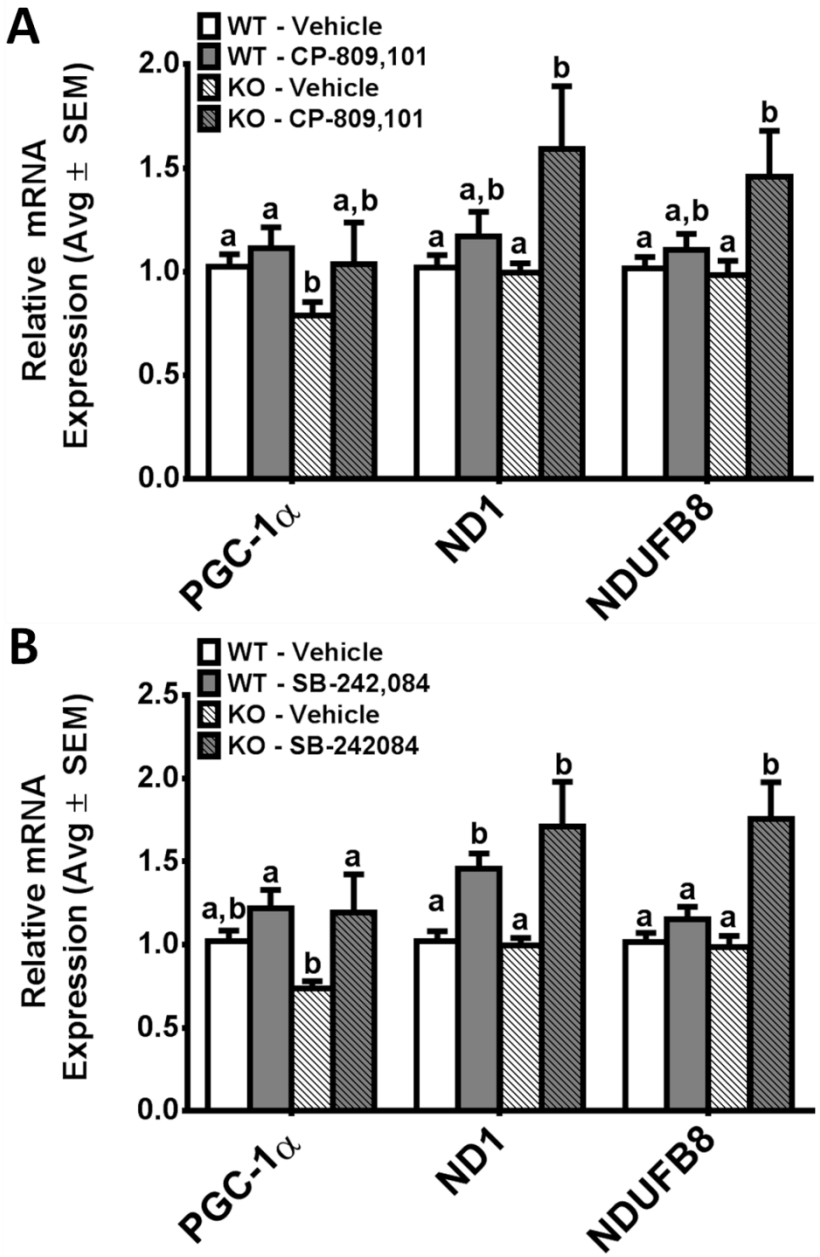

Fig. 2-4. Both a Classical 5-HT$_{2C}$ Agonist and Antagonist Induce Renal MB *in vivo* in Mice Lacking the 5-HT$_{2C}$ Receptor. 5-HT$_{2C}$ receptor WT and KO 129Sv mice were treated with a single intraperitoneal dose of CP-809,101 or SB-242,084 and euthanized 24 h post-injection. PGC-1α (A, D) ND1 (B, E) and NDUFB8 (C, F) mRNA expression in renal cortex were determined by RT-PCR using actin as a control gene. Fold expression of change in each group are reported relative to the WT – Vehicle group. Values reported as mean ± SEM; bars with different superscripts are significantly different from one another. Mann-Whitney U test was used to determine significance. n ≥ 5, $p < 0.05$.

Both a Classical 5-HT_{2C} Agonist and Antagonist Induce MB in RPTC Treated with 5-HT_{2C} siRNA But Not in RPTC Treated with 5-HT_{2A} siRNA

In RPTC pretreated for 72 h with 200 nM scramble siRNA, treatment with 10 nM CP-809,101 increases PGC-1α mRNA PGC-1a mRNA 1.5-fold; similarly, RPTC pretreated with 100 nM concentrations of two pooled siRNA directed toward 5-HT_{2C} mRNA have a 1.5-fold increase in PGC-1α mRNA after 24 h treatment with 10 nM CP-809,101 compared with matched siRNA- and vehicle-treated RPTC (Fig. 2-5A). However, RPTC pretreated with 100 nM concentrations of two pooled siRNA directed toward 5-HT_{2A} mRNA demonstrate no increase in PGC-1α mRNA after 24 h treatment with 10 nM CP-809,101 compared with matched siRNA- and vehicle-treated RPTC (Fig. 2-5A).

Similarly, 24h treatment of RPTC with 10 nM SB-242,084 after 72 h pretreatment with either 200 nM scramble siRNA or 100 nM concentrations of two pooled siRNAs directed toward 5-HT_{2C} mRNA increased PGC-1α mRNA by 1.2-fold compared to matched siRNA- and vehicle-treated RPTC (Fig. 2-5B). However, 24h treatment of RPTC with 10 nM SB-242,084 after pretreatment with 100 nM concentrations of two pooled siRNAs directed toward 5-HT_{2A} mRNA does not increase PGC-1α mRNA compared with matched siRNA- and vehicle-treated RPTC (Fig. 2-5B).

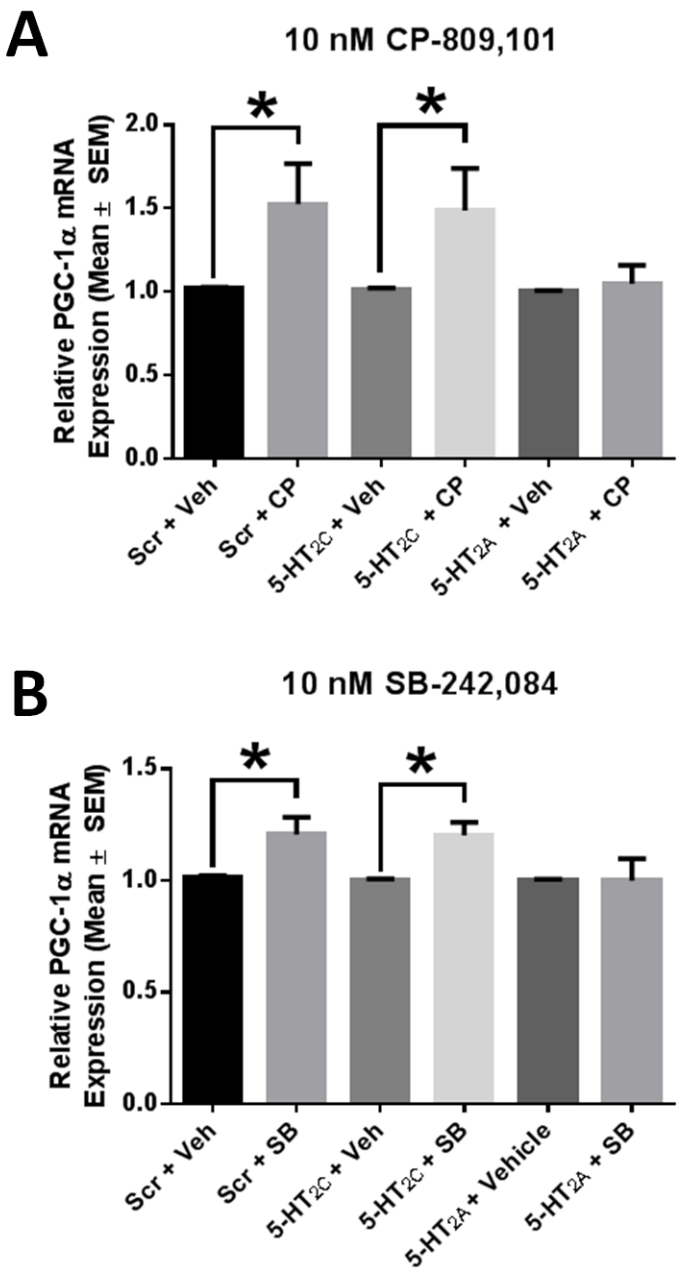

Fig. 2-5. Both a Classical 5-HT$_{2C}$ Agonist and Antagonist Induce MB in RPTC Treated with 5-HT$_{2C}$ siRNA But Not in RPTC Treated with 5-HT$_{2A}$ siRNA. RPTC were pretreated with negative control or siRNA directed toward 5-HT$_{2C}$ or 5-HT$_{2A}$ receptor for 72 h then treated with vehicle, CP-809,101 (A) or SB-242,084 (B) for 24 h. PGC-1α mRNA expression was measured using tubulin as a control gene. Data were analyzed using a Mann-Whitney U test between each siRNA + vehicle group and its corresponding amoxapine treatment group. Data are represented as mean ± S.E.M., n≥ 5.*, $p < 0.05$ vs. siRNA-matched vehicle control.

DISCUSSION

Mitochondrial homeostasis is an exquisitely controlled cellular process which requires the constant coordination and interaction of both nuclear- and mitochondrial DNA-encoded proteins to maintain energy production and includes the dynamic processes of fission, fusion and mitophagy (171). Disruption of mitochondrial homeostasis is a common pathological process to a multitude of diseases that span almost every organ system, especially those that require high energy production for proper tissue function. Moreover, mitochondria can propagate initial oxidative stress caused by toxicant exposure or acute ischemic insult and are often serve as both a target and a cause of acute oxidant injury. Because mitochondrial damage is often the inciting pathological feature of acute injury and can contribute to overall tissue damage via release of ROS and induction of apoptosis, early intervention to correct mitochondrial dysregulation is an attractive therapeutic strategy for a variety of acute injuries. Additionally, mitochondrial dysregulation is a common pathological feature of many chronic illnesses, and approaches that support mitochondrial health have been shown to improve to overall function in animal models of chronic diseases (185, 196, 212). Therefore, pharmacological inducers of MB represent a novel class of therapeutic agents to preserve or restore mitochondrial and overall cellular function in multiple models of both acute and chronic disease.

Although overwhelming evidence suggests that induction of MB represents a robust opportunity for treatment of a variety of diverse diseases, attempts to identify pharmacological inducers of MB have not yielded clinical options for treatment of mitochondrial dysfunction in the setting of acute or chronic disease. The recognition of a number of pathways implicated in the induction of MB has allowed our laboratory to identify a series of existing drug classes that may influence and promote biogenic

signaling. Identification of these drug classes allows us to focus our discovery approach on existing pharmaceuticals, leading to accelerated characterization of safe and clinically-established drugs to treat severe illnesses that are currently only treated with supportive measures.

The 5-HT_2 family of receptors act through both canonical and non-canonical signaling pathways previously identified to be important in the induction of MB. Additionally these receptors have an established library of potent and specific agonists and antagonists that are currently prescribed for clinical use in the treatment of a variety of neurological and psychiatric illnesses. Therefore, we determined that established $5HT_2$ ligands represented a potential opportunity for the discovery of a safe and effective treatment to induce MB in a clinical setting. Briefly, the 5-HT_2 receptors are classically defined to be coupled to $G_{q/11}$ proteins, which activate the phospholipase C second-messenger pathway and leads to release of intracellular calcium (141, 201). Increased intracellular calcium can activate a number of additional pathways, including the CaMK, NOS and cGMP pathways, which have established roles in the induction of MB. Early examinations of these classes of compounds by our laboratory demonstrated that DOI, a 5-HT_2 pan-agonist, induced MB at relatively high concentrations that were non-specific for a single 5-HT_2 receptor. Though this class of receptors—especially the 5-HT_{2A} and 5-HT_{2C} receptors—have high degrees of structural and functional homology, we hypothesized that it was likely that DOI was interacting with one specific receptor in this family to induce MB (213).

To probe the 5-HT_2 receptor specificity for the induction of MB, we used a preliminary respirometric screen to identify several specific 5-HT_2 ligands that induced MB. Our most potent ligands were specific for the 5-HT_{2C} receptor, so we next verified that this

receptor was expressed in both mouse renal cortex and in primary rabbit RPTC. With RT-PCR, we demonstrated that mRNA for all three 5-HT_2 receptors was expressed in the kidney. A protein isolation previously shown to enrich the quantity of 5-HT_2 receptors detectable in a sample was performed, and Western blot analysis of these isolates identified that the 5-HT_{2C} protein was expressed in our cells in a 60 kDa band. This 60 kDa band was determined to be 5-HT_{2C} receptor with two N-glycosylations, which was verified by treatment with PNGase, which decreased the 60 kDa band and increased a 38 kDa band that conforms with a reported non-glycosylated 5-HT2C receptor (214). The presence of the 60 kDa N-glycosylated band, previously identified *in vivo* in the hippocampus and choroid plexus, is significant because it indicates the presence of a mature 5-HT_{2C} receptor. The presence of mature 5-HT_{2C} receptor, previously thought to be expressed almost exclusively in the CNS, in the kidney introduces a novel drug target for renal MB. Moreover, we suggest this mature 5-HT_{2C} receptor may play a novel role in renal cellular homeostasis that differs from its function in the CNS.

After verifying that the mature 5-HT_{2C} receptor was present in primary RPTC, we used secondary *in vitro* assays to further characterize our most promising drug leads from our preliminary respirometric screen: CP-809,101 and SB-242,084. CP-809,101 is a potent 5-HT_{2C} agonist, with a pEC_{50} of 9.96 for 5-HT_{2C} and 1000-fold selectivity over the 5-HT_{2A} and 5-HT_{2B} receptors (215). SB-242,084 is a potent 5-HT_{2C} antagonist, with a pK_i of 8.2-9.0 for 5-HT_{2C} and 100-fold selectivity over the 5-HT_{2A} and $_{2B}$ receptors (77, 216). We expected that CP-809,101 but not SB-242,084 would induce MB. However, both of these compounds increased FCCP-OCR at nanomolar concentrations and increased PGC-1α mRNA at 10nM concentrations *in vitro*, which indicates that these compounds are inducing MB at concentrations specific for the 5-HT_{2C} receptor. Because of the paradoxical nature of both an agonist and an antagonist for the same receptor producing

the same biological outcome, we decided to further characterize their potential to induce MB *in vivo*.

Vehicle, CP809,101 and SB-242,084 were administered ip to naïve C57/Bl6 mice at 1 mg/kg. These doses were consistent with those previously administered to animals and reported in the literature by other groups, with CP-809,101 reported to be given at doses from 0.3-56 mg/kg and SB-242,084 reported to be given at doses from 0.01-10 mg/kg (215). Both CP-809,101 and SB-242,084 significantly increased PGC-1α mRNA, as well as mRNA for the mitochondrial DNA-encoded gene ND1 and the nuclear DNA-encoded gene NDUFB8. These results demonstrate that CP-809,101 and SB-242,084, a 5-HT_{2C} agonist and antagonist, respectively, both increase mRNAs associated with MB in the renal cortex.

We next sought to verify that these two compounds were inducing MB through the 5-HT_{2C} receptor, for which both compounds have 100-1000x specificity over the 5-HT_{2A} and 5-HT_{2B} receptors. To probe this, we treated either WT or 5-HT_{2C} KO mice with 1 mg/kg CP-809,101 and 1 mg/kg SB-242,084, which had been demonstrated to induce renal MB in C57/Bl6 mice. Our first observation was that 5-HT_{2C} KO mice had a 20% decrease in PGC-1α mRNA compared to WT mice, which we suggest indicates a role for the 5-HT_{2C} receptor in physiological renal mitochondrial homeostasis. Treatment of WT 129sv mice with 1 mg/kg CP-809,101 did not increase any mRNA measures of MB in the kidney, whereas treatment with 1 mg/kg SB-242,084 increased ND1 mRNA expression, with no changes measured in PGC-1α or NDUFB8 mRNA.

The difference between the molecular response of these naïve WT mice and naïve C57/Bl6 mice to treatment with both CP-809,101 and SB-242,084 may reflect a diverse

pattern of 5-HT_2 receptor expression between these two inbred mouse strains. Differences in genetics between inbred mouse strains has been linked to diversity in response to both pharmacological therapies and disease agents (*e.g.* LPS) and is identified as a limiting factor in the use of mouse models to perform research on disease mechanisms as well as drug discovery (217). Furthermore, it has been reported that there are differences in 5-HT_{2C} RNA editing that leads to strain-specific differences in the expression of 24 different 5-HT_{2C} receptor isoforms, which display different levels of constitutive activity and functional response to stimulation with 5-HT (132, 218). It is reasonable to expect that these strain differences in expression patterns may be extrapolated to the 5-HT_{2A} and 5-HT_{2B} receptors as well and may require generation of separate dose-response analyses for induction of MB by our lead compounds in individual mouse strains.

After characterizing the response of WT 129sv mice to our two drugs of interest, we next sought to compare the response of WT and KO mice to these agents. Interestingly, treatment of 5-HT_{2C} KO mice with CP-809,101 led to increases in both ND1 and NDUFB8 mRNA and treatment of 5-HT_{2C} KO mice with 1 mg/kg SB-242,084 increased PGC-1α, ND1 and NDUFB8 mRNA expression. These results indicate that these compounds, though classically identified as a specific 5-HT_{2C} agonist and antagonist, respectively, do not increase mRNA markers of MB through their actions at the 5-HT_{2C} receptor.

Because these compounds induced MB in mice lacking the 5-HT_{2C} receptor, we hypothesized that biogenic signaling by these compounds is dependent on the 5-HT_{2A} receptor. We then utilized siRNA-directed knockdown of either the 5-HT_{2A} or 5-HT_{2C} receptor in our primary RPTC model to further probe the receptor specificity of the

biogenic response to our lead compounds. Similar to our studies with 5-HT_{2C} KO mice, treatment of RPTC with either CP-809,101 or SB-242,084 after pre-treatment with siRNA directed toward the 5-HT_{2C} receptor increased PGC-1α mRNA to the same magnitudes as those observed after treatment of these compounds in negative control siRNA-treated cells. However, no increases in PGC-1α mRNA were detected in RPTC treated with these either of these compounds after pre-treatment with 5-HT_{2A} receptor siRNA. The most likely explanation for these results is that the ability of these compounds to induce MB is via signaling through the 5-HT_{2A} receptor, the expression of which has been previously reported in the kidney (144, 200, 213, 219, 220).

Our observations that both a classically identified agonist and antagonist for the same pharmacological target receptor are contradictory, but there are several potential explanations for the paradoxical effect we see with both of these compounds. The first potential explanation is that the action of these compounds on the 5-HT_2 class of receptors is not identical in the CNS and the kidney. Most of the compounds defined as either 5-HT_{2A} agonists or antagonists have been characterized as such in the CNS or in cell lines overexpressing the 5-HT_{2A} receptor. Thus, it is possible that physiological signaling via this receptor in the kidney diverges from that in the CNS and that these two ligands both act as agonists in this system, signaling through a calcium-mediated pathway to increase cGMP, which has been demonstrated by our laboratory to be important for pharmacological induction of renal MB (199). Another explanation is that these two drugs act through divergent pathways, which later converge on PGC-1α to induce MB. In this case, it is still likely that CP-809,101, a 5-HT_{2C} agonist, is signaling through a calcium-mediated pathway to increase cGMP and induce MB. However, it is possible that SB-242,084 serves as an inverse agonist in the kidney, acting upon a constitutively active 5-HT_{2A} receptor to induce MB. The 5-HT_{2A} receptor in the kidney has

been shown to couple to the $G_{i/0}$ protein, which inhibits adenyl cyclase. Furthermore, it has been suggested that constitutive activity of these receptors has an important physiological role and that inverse agonists for these receptors may have broad therapeutic potential (181, 200). Therefore, we suggest that SB-242,084 may serve as an inverse agonist for a constitutively active $G_{i/0}$-coupled 5-HT_{2A} receptor. Since the $G_{i/0}$ protein inhibits adenylyl cyclase, inverse agonism would increase intracellular cAMP, which regulates PGC-1α expression and activity via the PKA/CREB signaling pathway (135). Future studies are focused on further elucidating the signaling pathways by which these drugs induce MB.

Our results indicate that both 5-HT_{2C} receptor agonists and antagonists can result in the induction of MB both *in vitro* and *in vivo*, though these effects appear to be independent of the 5-HT_{2C} receptor. Although neither of our compounds induces biogenesis through 5-HT_{2C} signaling, we see a reduction of PGC-1α mRNA in 5-HT_{2C} KO mice as compared to WT and suggest that the 5-HT_{2C} receptor is important for maintenance of mitochondrial homeostasis in the kidney. Therefore, more work should be done to investigate the contribution of renal 5-HT_{2C} and 5-HT_{2A} receptor signaling to maintenance of mitochondrial homeostasis and promotion of MB in the kidney. Furthermore, we suggest that both agonists and antagonists of 5-HT_2 receptors are viable candidates for the treatment of kidney disease, and future studies will explore the ability of these compounds to rescue or support mitochondrial function after an established model of acute kidney disease. The use of 5-HT_{2A} antagonism is an attractive approach, because it avoids possibility of non-mitochondrial cardiotoxicity through agonism of the 5-HT_{2B} receptor (73, 221). This 5-HT2B receptor-mediated cardiotoxicity is a serious side effect that has limited drug discovery and safety of 5-HT_2 class agonists, since most of these compounds do activate the 5-HT_{2B} receptor at

increasing concentrations, a factor that resulted the Food and Drug Administration's approval of the appetite suppressant fenfluramine being rescinded in (222, 223). Additionally, these compounds were developed for use in the CNS, and we therefore suggest that these drugs may be novel drug candidates for the treatment of mitochondrial dysfunction in both acute and chronic neurological pathologies, including stroke, Alzheimer's disease, Huntington's disease, Parkinson's disease, and ALS.

CHAPTER THREE

AMOXAPINE, A NON-SELECTIVE POTENT 5-$HT_{2A/2C}$ RECEPTOR ANTAGONIST, INDUCES RENAL MITOCHONDRIAL BIOGENESIS

ABSTRACT

Acute kidney injury (AKI) is a critical disease process that accounts for a significant number of hospitalizations, has a high associated mortality rate and accounts for approximately $10 billion in medical spending each year. Many disparate mechanisms of AKI have common pathophysiological features, which converge on mitochondrial dysfunction and associated membrane disruption, respiratory deficits and loss of energy production. Recent studies have examined patterns of mitochondrial dysfunction following AKI and have demonstrated that mitochondrial content and function are rapidly lost after an acute ischemic event and that these mitochondrial deficits persist through at least 6-14 d after the initiating injury. Further studies have demonstrated that pharmacological stimulation of mitochondrial biogenesis (MB) can reverse or mitigate mitochondrial dysfunction after AKI, resulting in improvement in renal tubular function. Additionally, previous work identified that both an agonist and an antagonist of the 5-HT_{2A} receptor were capable of inducing MB in the kidney. In this study, we further tested the ability of amoxapine, another potent but non-specific 5-$HT_{2A/2C}$ receptor antagonist, to induce renal MB. We found that amoxapine increased both cellular respiration and PGC-1α mRNA expression in primary renal proximal tubules at nanomolar concentrations. Through the use of siRNA, we determined that increased PGC-1α mRNA expression stimulated by amoxapine in RPTC depends on expression of the 5-HT_{2A} receptor. Additionally, daily injections of 0.3 mg/kg amoxapine induced PGC-1α mRNA expression in mouse kidney cortex at 48 h post-initial injection, but interestingly, amoxapine had no effect on MB in three CNS tissues: frontal cortex, hippocampus, or

striatum. Finally, despite its promise as a biogenic agent, amoxapine did not increase mitochondrial mRNA expression or mtDNA copy number and failed to restore renal function following folic acid-induced AKI (FA-AKI). These results indicate that amoxapine induces renal MB through antagonism of the 5-HT_{2A} receptor, but that the use of this therapeutic may be limited by other cellular injury factors present in the acute phase after initial insult.

INTRODUCTION

Acute kidney injury (AKI), the loss of renal function over hours to days, is a severe pathological state with high percentages of mortality, especially in patients who develop this disease in a hospital setting, resulting in more than $10 billion of associated costs (82-84). Despite the serious consequences of this disease state, current therapies to treat AKI are largely supportive, and there are no current therapies that reverse the molecular mechanisms of cellular damage that are prominent pathophysiological features common to all causes of AKI, including toxicant and ischemia/reperfusion AKI.

One significant cause of AKI is ischemia/reperfusion, the most serious consequence of which is increased renal tubular oxidative stress (85, 102, 106, 107). Oxidative stress resulting from an acute increase in ROS induced by the sudden influx of oxygen following reperfusion is propagated by the generation of secondary reactive species, such as lipid peroxides and protein carbonyls, which disrupt the structure of the inner mitochondrial membrane and electron transport chain and cause mtDNA damage and loss (159, 161).

The consequence of acute oxidant stress in the mitochondria is a loss of mitochondrial function. Our laboratory has demonstrated that 20 minutes of renal pedicle ligation followed by reperfusion induced a decrease of both mRNA and protein expression of ETC components, including mitochondrial-encoded COXI and nuclear-encoded NDUFB8 and ATP synthase β, which persisted through 6 d post-injury and were accompanied by a persistent decrease in cellular respiration (120, 122). These markers of mitochondrial dysfunction were correlated with increased renal injury markers, including increased SCr and KIM-1, which did not return to control levels by 6 d post-injury (120, 122). Additionally, our laboratory determined that restoration of

mitochondrial function after acute oxidant injury to RPTC was required for recovery of cellular function (38, 119). Therefore, mitochondria are an important pharmacological target for treatment of AKI and improving mitochondrial function could promote tubular recovery and proliferation, which no current clinical treatment modality accomplishes.

An attractive strategy to improve mitochondrial function after acute injury is the pharmacological induction of MB, one of the processes by which mitochondrial homeostasis in maintained. MB requires coordination of transcription of both nuclear- and mitochondrial-encoded genes and is under the exquisite control of peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α), the “master regulator of MB” (6). Our laboratory determined that overexpression of PGC-1α in RPTC after TBHP treatment promotes recovery of mitochondrial protein expression, cellular respiration, and ATP content, indicating that induction of MB can reverse the mitochondrial dysfunction after acute oxidant injury (130). Additionally, our laboratory demonstrated that pharmacological induction of MB through stimulation of the β2-adrenergic receptor with the agonist formoterol recovers expression of both nuclear-encoded protein NDUFB8 and mitochondrial-encoded COXI 6 d after renal pedicle ligation (120). Additionally, treatment with formoterol promoted recovery of renal cortical mitochondrial respiration, mitigated tubular necrosis, and reduced renal injury markers SCr and KIM-1 (120). These results provide strong evidence that pharmacological induction of MB is a high-yield strategy to prevent persistent decreases in mitochondrial content and function and to improve tubular cell recovery after acute I/R injury.

PGC-1α is under tight physiological control, and a number of signaling pathways have been implicated in its induction, including cAMP through CREB, AMP-kinase, calcineurin A/MEF-2, calcium/calmodulin-dependent protein kinase (CAMK-IV), NO/cGMP, and

mTOR (16, 18-21, 24, 27, 32, 33). Our group has identified a number of compounds that induce mitochondrial biogenesis through diverse mechanisms using several different pharmacological classes, including β2-adrenergic agonists, phosphodiesterase inhibitors, SIRT1 activators, isoflavones, and 5-HT_2 receptor agonists and antagonists (47, 48, 50, 52, 56).

Our laboratory previously characterized the ability of a number of 5-HT_2 receptor agonists and antagonists to induce MB in RPTC and mouse renal cortex. The 5-HT_2 family of receptors is composed of three receptor sub-types (2A, 2B and 2C), all of which are G-protein-coupled receptors (GPCRs) traditionally characterized to signal through $G_{q/11}$ protein, leading to the release of intracellular calcium and downstream activation of several pathways associated with MB, including CAMK/calmodulin signaling and NO/cGMP production (200-202). DOI, a non-selective 5-HT_2 receptor agonist, was shown to increase PGC-1α expression as well as expression of nuclear-encoded ETC proteins NDUFB8 and ATP synthase β (56). Furthermore, DOI promoted recovery of cellular respiration after TBHP treatment, indicating that 5-HT_2 receptor agonism is a promising strategy to reverse AKI through promotion of MB (56).

Further investigation of this class of receptors demonstrated that both classically-defined 5-HT_{2C} agonist CP-809,101 and antagonist SB-242,084 were capable of increasing cellular respiration and PGC-1α mRNA expression in RPTC as well as mRNA expression of PGC-1α, mitochondrial-encoded ND1 and nuclear-encoded NDUFB8 in mouse kidney cortex. Through the use of mice lacking the 5-HT_2 receptor as well as RPTC treated with siRNA directed toward either the 5-HT_{2A} or 5-HT_{2C} receptor, our laboratory further determined that both CP-809,101 and SB-242,084 induced MB through the 5-HT_{2A} receptor. Although the effect of these drugs is not mediated by the 5-

HT_{2C} receptor, mice that do not express this receptor have a 20% decrease in PGC-1α mRNA expression under baseline conditions, indicating that the 5-HT_{2C} may play a novel role in regulation of renal mitochondrial homeostasis.

Induction of MB through antagonism of the 5-HT_{2A} receptor is a more promising strategy for treatment of AKI because it avoids the risk of off-target 5-HT_{2B} receptor-mediated non-mitochondrial cardiotoxicity common to 5-HT_2 receptor agonists (73, 221-223). A number of selective serotonin reuptake inhibitors (SSRIs) currently approved by the FDA for use as antidepressants are also potent non-specific 5-$HT_{2A/C}$ receptor antagonists, the investigation of which may lead to the expedited development of a safe effective drug to induce renal MB and treat AKI (224). In this study, we demonstrate the ability of the non-specific 5-$HT_{2A/2C}$ receptor antagonist amoxapine to induce MB in the kidney through the 5-HT_{2A} receptor. Furthermore, we sought to identify the effects of amoxapine on MB in other organ systems and examined its effect on mitochondrial homeostasis following folic acid-induced AKI (FA-AKI).

MATERIALS AND METHODS

Reagents

Amoxapine and all other chemicals were purchased from Sigma Aldrich (St. Louis, MO).

Animal Care and Use

All experiments were performed in strict accordance with the guidelines in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All protocols were approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina, and appropriate efforts were made to reduce animal suffering.

Isolation and Culture of Proximal Tubules

Female New Zealand white rabbits (1.5-2.0 kg) were purchased from Charles River Laboratories (Wilmington, MA). Renal proximal tubule cell (RPTC) isolation was performed using the iron oxide perfusion method described previously and cultured under improved conditions as previously described (205). Three days after initial plating, de-differentiated RPTC were trypsinized and re-plated on XF-96 polystyrene cell culture microplates (Seahorse Bioscience, North Bellerica, MA) at a density of 18,000 cells/well and maintained at 37oC for 3 days before experimentation (206). For other RPTC experiments, isolated renal proximal tubules were plated in 35-mm dishes used 8 days after initial plating. RPTC were treated with experimental compounds for 24 h.

Oxygen Consumption.

The oxygen consumption rate (OCR) of RPTC was measured using the Seahorse Bioscience XF-96 Extracellular Flux Analyzer as previously described (206). Each 96-well assay plate was treated with vehicle control (DMSO <0.5%), and 1, 10 and 100 nM concentrations of the experimental compounds. Basal OCR was measured before

injection of carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, 0.5 uM), which allows for the measurement of uncoupled OCR (FCCP-OCR), a marker of MB (117). Compounds that increased FCCP-OCR by 15% were considered positive for induction of MB.

In vivo mouse studies

Male C57/Bl6 mice (6-8 weeks old) were purchased from the National Cancer Institute (Bethesda, MD). Mice were individually housed in a temperature-controlled room under a 12-h light/dark cycle and randomly assigned to either the vehicle control or the drug treatment group. Mice were administered a daily intraperitoneal dose of either diluent (40% (2-Hydroxypropyl)-β-cyclodextrin in 0.9% saline) or amoxapine (0.3 mg/kg) and euthanized 24, 48 or 120 h after the initial dose. Kidneys were isolated and snap frozen for quantitative PCR (qPCR) analysis.

$5\text{-}HT_{2C}$ and $5\text{-}HT_{2A}$ Knockout Experiments in RPTC

Rabbit mRNA sequences for 5-HT2A and 5-HT2C receptor were obtained from Ensembl. The BLOCK-iT™ RNAi Designer (Invitrogen) was used to design siRNA to these sequences. Two days past confluency, RPTC were treated with either 200 nM siGenome non-targeting siRNA #3, 100 nM of both 2C1 and 2C2 siRNA or 100 nM of both 2A1 or 2A2 siRNA (Table 2). 72 h after siRNA treatment, RPTC were treated for 24 h with experimental drugs and harvested for RT-PCR analysis.

Folic Acid Animal Model

Male C57BL/6 mice (8-10 weeks of age) were given a single intraperitoneal injection of 250 mg/kg folic acid dissolved in 250mM sodium bicarbonate or saline control as described previously (52, 225). Mice were given intraperitoneal injections of either

diluents or amoxapine (0.3 mg/kg) every 24 hours beginning 1d after FA injection. Mice were euthanized at 7d, and kidneys were removed and snap-frozen for qPCR analysis.

Real-Time Reverse Transcription-PCR

Total RNA was extracted from RPTC or renal cortex samples using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized via reverse transcription using the RevertAid First Strand cDNA kit (Thermo Fisher Scientific, Waltham, MA) with 1-2 ug of RNA. PCR products were amplified from 5 uL of cDNA template using 2x Maxima SYBR green qPCR master mix (Thermo Fisher Scientific) and 400 nM concentrations of each primer (Integrated DNA Technologies, Inc., Coralville, IA). Primer sequences for PGC-1α, ND1, NDUFB8, and β-actin were described previously (50, 122). The previously described ΔΔ-Ct analysis method was used to calculate fold changes in expression (50).

Mitochondrial DNA Content

Relative quantity of mtDNA in mouse renal cortical tissue samples was measured using real-time PCR. After treatment, DNA was isolated from tissues using the DNEasy Blood and Tissue Kit (Qiagen, Valencia, CA), and qPCR was performed using 5 ng of cellular DNA. Expression of NADH dehydrogenase 1 (ND1), a mitochondrial gene, was measured and normalized to nuclear-encoded β-actin. The ΔΔ-Ct analysis method was used to calculate fold changes in expression (50).

Immunoblot Analysis

Mouse kidney cortex was homogenized in 500 µL of protein lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4; 1 mM EDTA; 1 mM EGTA; 2 mM sodium orthovanadate; 0.2 mM phenylmethylsulfonyl fluoride; 1 mM HEPES, pH 7.6; 1 µg/ml leupeptin; and 1 µg/ml aprotinin) using a Polytron homogenizer. The homogenate was

stored on ice for 10 min and then centrifuged at 10000 g for 10 min at 4°C. The supernatant was collected, and protein was determined using a bicinchoninic acid kit (Sigma, St. Louis, MO) with bovine serum albumin as the standard. Proteins (50–75 μg) were separated on 4 to 20% gradient SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked either in 5% dried milk or BSA in TBST (0.1% Tween 20 in 1× Tris-buffered saline) and incubated with 1:1000 antibody dilutions of anti-PGC-1α (EMD, Billerica, MA); COX-1 (Abcam, Cambridge, MA); anti-NDUFS1 (Abcam, Cambridge, MA); and anti-GAPDH (Fitzgerald, Acton, MA) overnight at 4°C. After incubation for 2 h at room temperature with secondary antibodies (1:2000) conjugated with horseradish peroxidase, membranes were detected by chemiluminescence. Densitometric analysis was performed using ImageJ (226).

Statistics

Data are presented as means ± SEM. Single comparisons for normal data were performed using a Student's t-test, whereas data found to not have a normal distribution were subjected to a Mann-Whitney U-test. Multiple comparisons for normal data were performed using one-way analysis of variance (ANOVA) with an appropriate post-hoc test to compare multiple means. Kruskal-Wallis one-way analysis of variance was used to perform multiple comparisons for non-normal data, and a Holm-Sidak's post-test was used to compare multiple medians. Single and multiple comparison data were considered statistically significantly different at $P \leq 0.05$. RPTC isolated from a single rabbit represented an individual experiment (n=1) and were repeated until $n \geq 4$ was obtained. Rodent studies were repeated until $n \geq 3$ was obtained.

RESULTS

Amoxapine, a 5-$HT_{2A/2C}$ Antagonist, Induces MB in RPTC

RPTC were plated in XF-96 cell plates and incubated under previously described conditions that preserve the polarity, differentiation, function and respiratory capacity of *in vivo* RPTC (50, 205). RPTC were treated for 24 h with vehicle control or amoxapine (0.1, 1, 10, 100 nM). A significant increase in FCCP-OCR was observed at all doses relative to vehicle controls (Fig. 3-1A). It should be noted that the K_D for amoxapine is 1 nM for both the 5-HT_{2A} and 5-HT_{2C} receptors, but it is not reported to act as an antagonist on the 5-HT_{2B} receptor.

Because pharmacological agents can increase respiration through mechanisms other than mitochondrial biogenesis, we treated 35 mm plates of RPTC grown under optimized conditions with either vehicle control or amoxapine (30 and 100 nM) and measured PGC-1α mRNA expression. Both 30 and 100 nM concentrations of amoxapine increased PGC-1α mRNA expression by 1.2 and 1.25-fold respectively (Fig. 3-1B). These results indicate that amoxapine, a 5-HT_2 receptor antagonist, induces MB in RPTC.

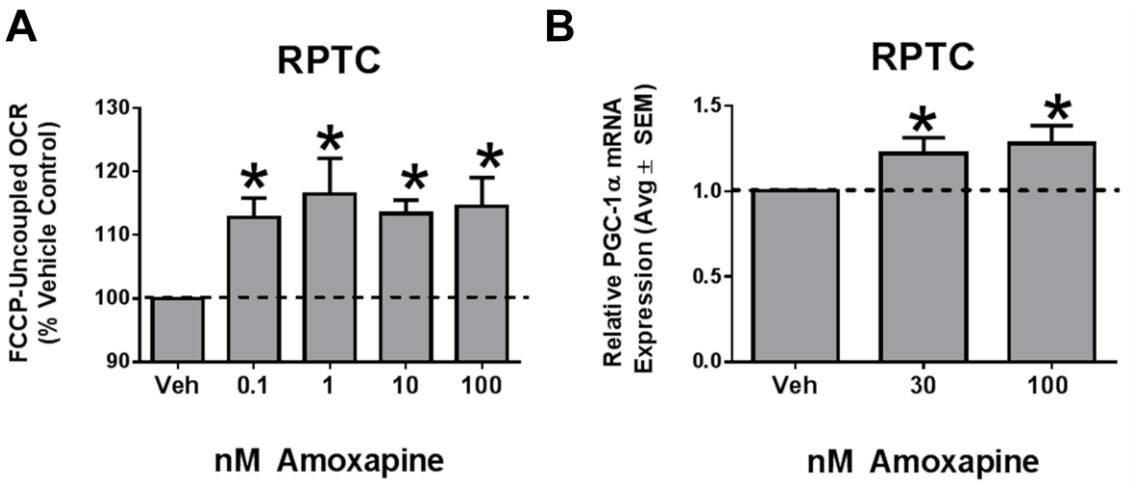

Fig. 3-1. Amoxapine, a 5-HT$_{2A/2C}$ Antagonist, Induces MB in RPTC. RPTC were treated with amoxapine for 24 h. FCCP-uncoupled mitochondrial respiration was measured using the Seahorse XF-96 instrument (A). PGC-1α mRNA expression was measured using tubulin as a control gene (B). Data were analyzed using Kruskal-Wallis test followed by Dunn's Multiple Comparison Test. Data are represented as mean ± S.E.M., n≥5.*, $p < 0.05$ vs. vehicle control.

siRNA Knockdown of the 5-HT$_{2A}$ Receptor Prevents Induction of MB by Amoxapine

Because amoxapine is a potent, but non-selective, antagonist of both the 5-HT$_{2A}$ receptor and the 5-HT$_{2C}$ receptor, we pretreated RPTC for 72 h with negative control siRNA or siRNA directed toward either the 5-HT$_{2A}$ receptor or 5-HT$_{2C}$ receptor. We then measured PGC-1α mRNA expression after 24 h of treatment with either diluent or 30 nM amoxapine, a dose that was previously demonstrated to effectively increase this MB marker in RPTC. Consistent with the results in naïve RPTC, PGC-1α mRNA expression was increased 1.2 fold in RPTC pretreated with negative control siRNA (Fig. 3-2). Similarly, 72 h pretreatment with siRNA directed toward the 5-HT$_{2C}$ receptor did not abrogate the biogenic capacity of amoxapine and even potentiated its ability to stimulate MB, resulting in a 1.6-fold increase of PGC-1α mRNA expression 24 h after treatment (Fig. 3-2). Conversely, pretreatment with siRNA directed toward the 5-HT$_{2A}$ receptor for 72 h completely blocked an increase in PGC-1α mRNA expression after 24 h of 30 nM amoxapine treatment (Fig. 3-2). These results strongly suggest that amoxapine induces MB through 5-HT$_{2A}$ receptor signaling.

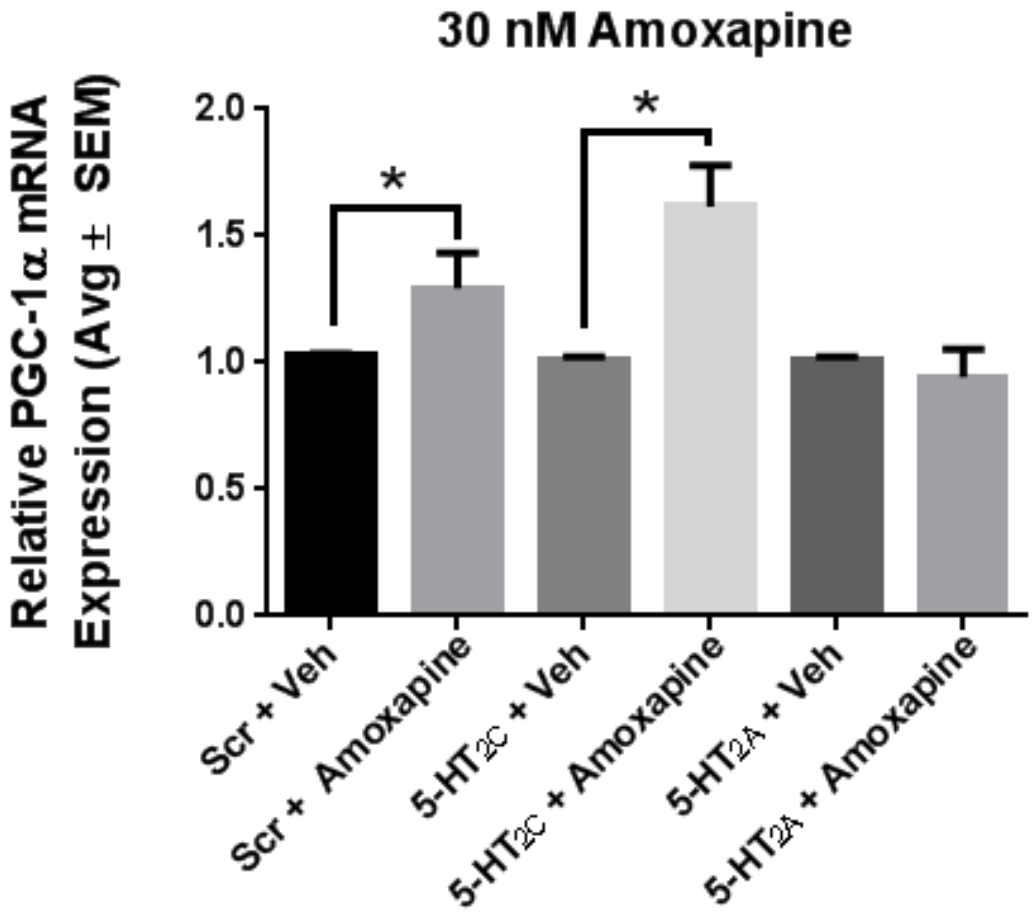

Fig. 3-2. siRNA Knockdown of the 5-HT_{2A} Receptor Prevents Induction of MB by Amoxapine. RPTC were pretreated with negative control or siRNA directed toward 5-HT_{2C} or 5-HT_{2A} receptor for 72 h then treated with either vehicle or amoxapine for 24 h. PGC-1α mRNA expression was measured using tubulin as a control gene. Data were analyzed using a Mann-Whitney U test between each siRNA + vehicle group and its corresponding amoxapine treatment group. Data are represented as mean ± S.E.M., $n \geq 5$, *$p < 0.05$ vs. siRNA-matched vehicle control.

Amoxapine Induces PGC-1α *In Vivo*

Male C57/Bl6 mice were treated with daily intraperitoneal doses of either amoxapine (0.3 mg/kg) or diluent and euthanized 24, 48 or 120 h after the initial injection. In kidney cortex, amoxapine increased PGC-1α mRNA expression 1.25 fold 48 h after the initial dose of amoxapine, but no difference in PGC-1α mRNA expression was observed at 24 (d.n.s.) or 120 h after amoxapine treatment (Fig. 3-C). No change in mtDNA copy number was observed at 24, 48 or 120 h after amoxapine treatment. We further investigated the 0.3 mg/kg amoxapine treatment and found that it did not increase renal cortical expression of PGC-1α protein, nor expression of either COXI, a mitochondrial-encoded protein, or NDUFS1, a nuclear-encoded protein at 48 h (Fig. 3-2D and F) or 120 h (Fig 3-2E and G) after initial injection.

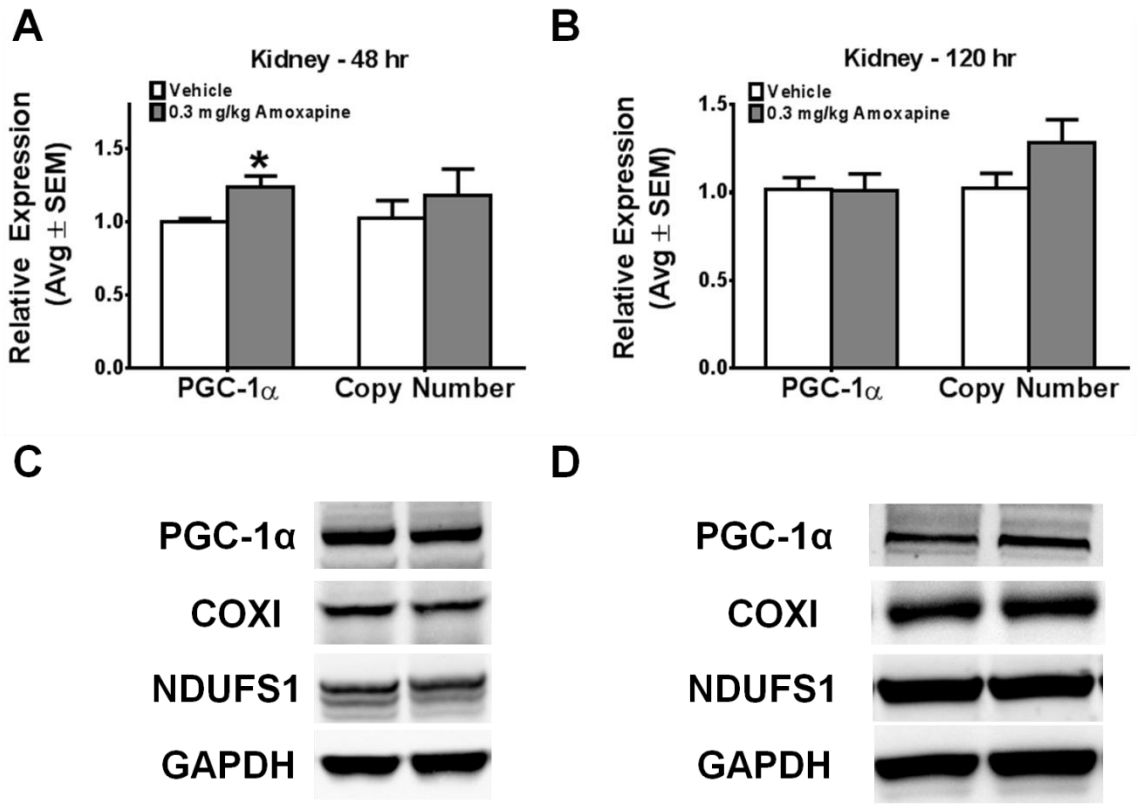

Fig. 3-3. Amoxapine Induces PGC-1α mRNA *In Vivo.* C57/Bl6 mice were treated with daily intraperitoneal doses of amoxapine and euthanized at 24, 48 and 120 h after initial amoxapine injection. PGC-1α mRNA expression was determined by RT-PCR using actin as a control gene. mtDNA copy number was determined by qPCR, using ND1 for the mtDNA gene and actin for the nuclear control gene. PGC-1α, COXI and NDUFS1 protein expression were determined with immunoblot analysis using GAPDH as a loading control. PGC-1α mRNA expression and mtDNA copy number were measured in renal cortex at 24 (d.n.s.), 48 (A) and 120 h (B) after initial dose of amoxapine. PGC-1α, COXI and NDUFS1 protein expression were measured at 48 (C) and 120 (D) h after initial amoxapine injection. Values reported as mean ± SEM. Mann-Whitney U test was used to determine significance. n =4-9, *$p < 0.05$.

Amoxapine Does Not Induce MB in CNS Tissue *In vivo*

Because amoxapine was first characterized and developed as an anti-depressant drug and is designed to cross the blood-brain barrier (BBB), we then examined its ability to induce MB in three CNS tissues. We measured PGC-1α mRNA expression and mtDNA copy number, two markers of MB, after 0.3 mg/kg amoxapine treatment in the frontal cortex, hippocampus and striatum at 48 h after the initial dose because this is the time point at which MB was increased in the kidney cortex. However, at this time point, no increase in either of those MB markers was demonstrated in frontal cortex, hippocampus or striatum (Fig 3-4A-C).

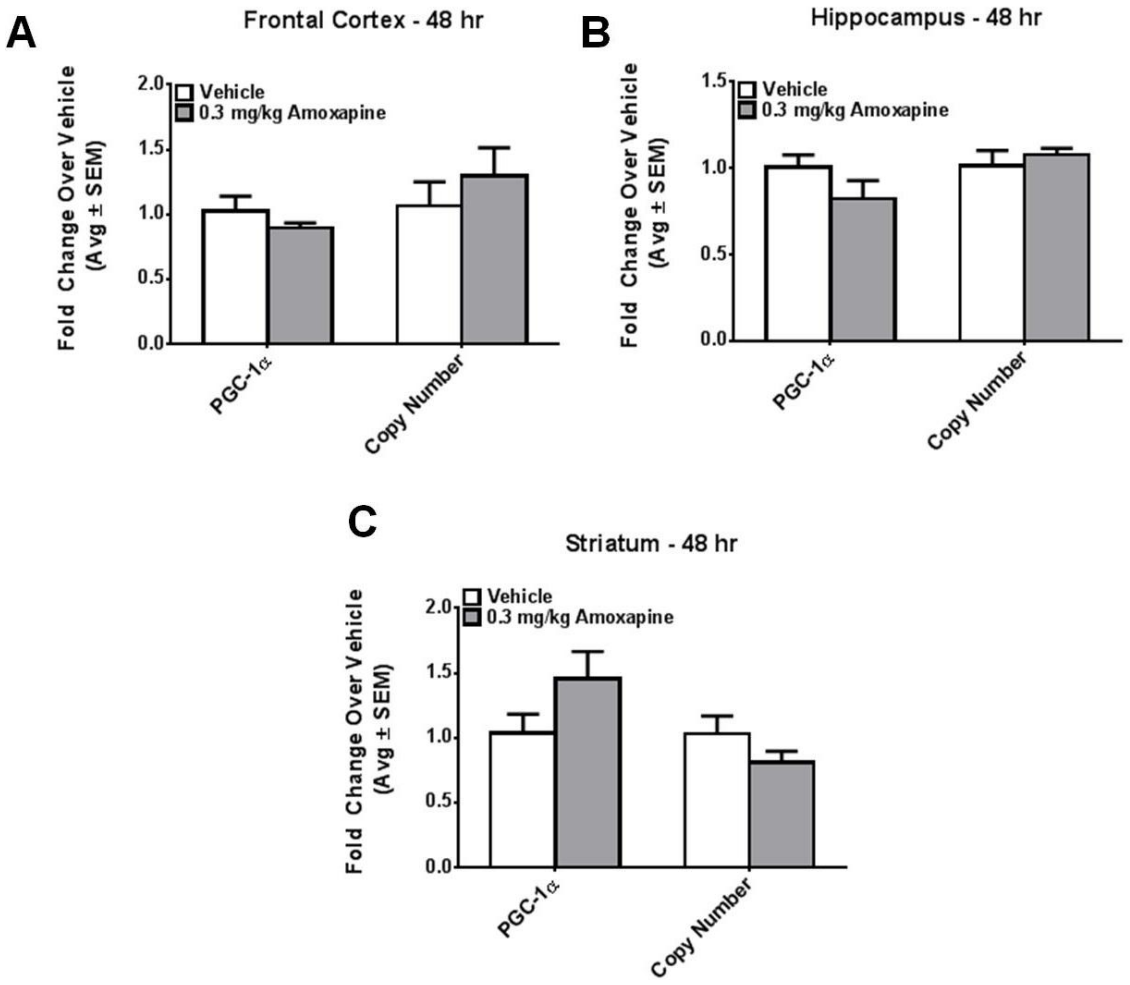

Fig. 3-4. Amoxapine Does Not Induce MB in CNS Tissue *In Vivo*. PGC-1α mRNA expression was determined by RT-PCR using actin as a control gene. mtDNA copy number was determined by qPCR, using ND1 for the mtDNA gene and actin for the nuclear control gene. PGC-1α mRNA expression and mtDNA copy number were at 48 h after initial amoxapine injection in frontal cortex (A), hippocampus (B) and striatum (C). Values reported as mean ± SEM. Mann-Whitney U test was used to determine significance. n =4-9.

Amoxapine Does Not Promote Recovery of MB after FA-induced AKI

Because 0.3 mg/kg amoxapine increased MB markers in the kidney cortex of naïve mice, we hypothesized that this drug would promote recovery of MB after AKI induced by injection of FA, which our laboratory previously demonstrated to severely decrease both mitochondrial biogenesis and renal function (227). FA-treated mice given diluent alone had reduced PGC-1α, TFAM and COXI mRNA expression at ~50% and ~30% of control, respectively. Folic acid also decreased mitochondrial-encoded COXI mRNA expression and mtDNA copy number to ~25% and ~30% of control, respectively. Amoxapine treatment did not significantly change expression of any of these MB markers (Fig. 3-5A-D).

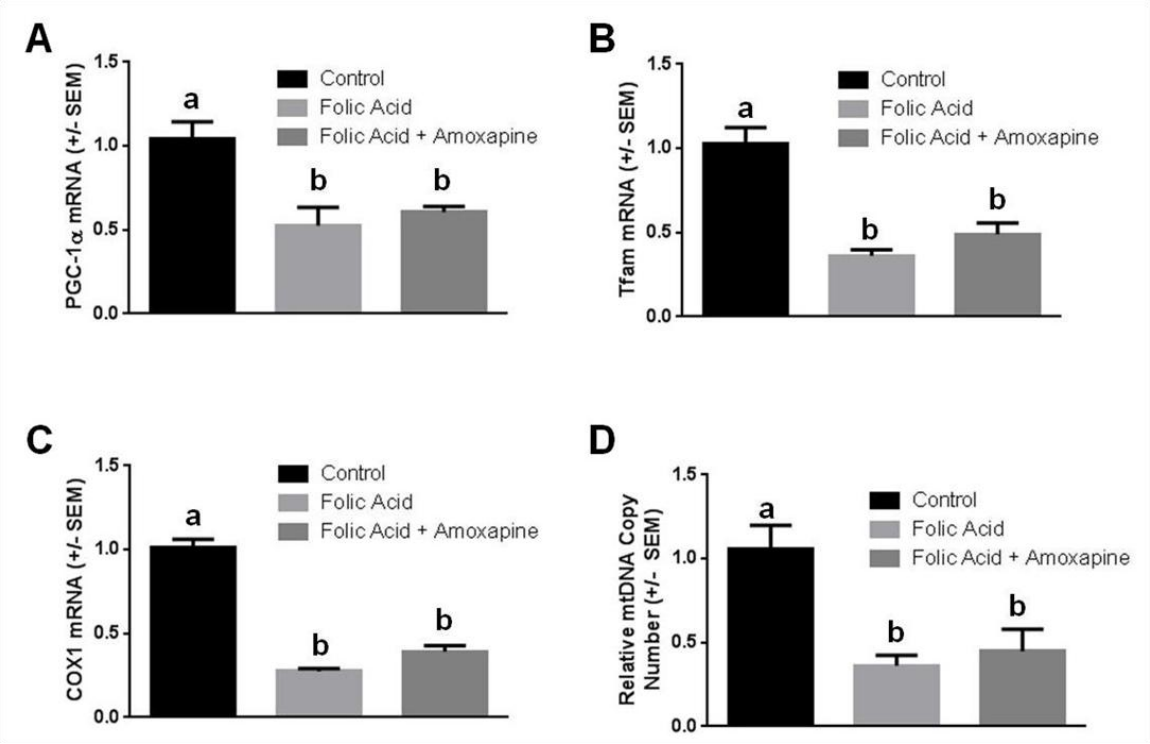

Fig. 3-5. Amoxapine Does Not Promote Recovery of MB after FA-induced AKI. PGC-1α (A), TFAM (B) and COXI (C) mRNA expression were determined by RT-PCR using actin as a control gene. mtDNA copy number was determined by qPCR, using ND1 for the mtDNA gene and actin for the nuclear control gene (D). These markers were measured in control, FA-AKI and FA-AKI + 0.3 mg/kg amoxapine groups in renal cortex 7 d post-injury. Values reported as mean ± SEM; bars with different superscripts are significantly different from one another. One-way ANOVA with Bonferroni *post-hoc* test was used to determine significance. *$p < 0.05$, $n \geq 4$..

Amoxapine Does Not Restore Renal Function or Promote Survival after FA-induced AKI

To test the hypothesis that amoxapine treatment would promote renal recovery, BUN was measured in serum collected 1, 2, 4 and 7 d after FA injection. At all time points measured after injury, FA mice treated with diluent alone had a ~8-fold increase in BUN over control (Fig. 3-6A). There was no significant difference in BUN in FA mice treated with amoxapine from those treated with diluent control (Fig. 3-6B). Additionally, survival 7 d after FA injection was 76% of control, and treatment with amoxapine did not significantly alter that survival percentage (Fig. 3-5C). These results suggest that FA-induced AKI is a severe injury and that the failure of amoxapine treatment to significantly induce MB in this model prevents it from promoting recovery of renal function and improving survival in FA-treated mice.

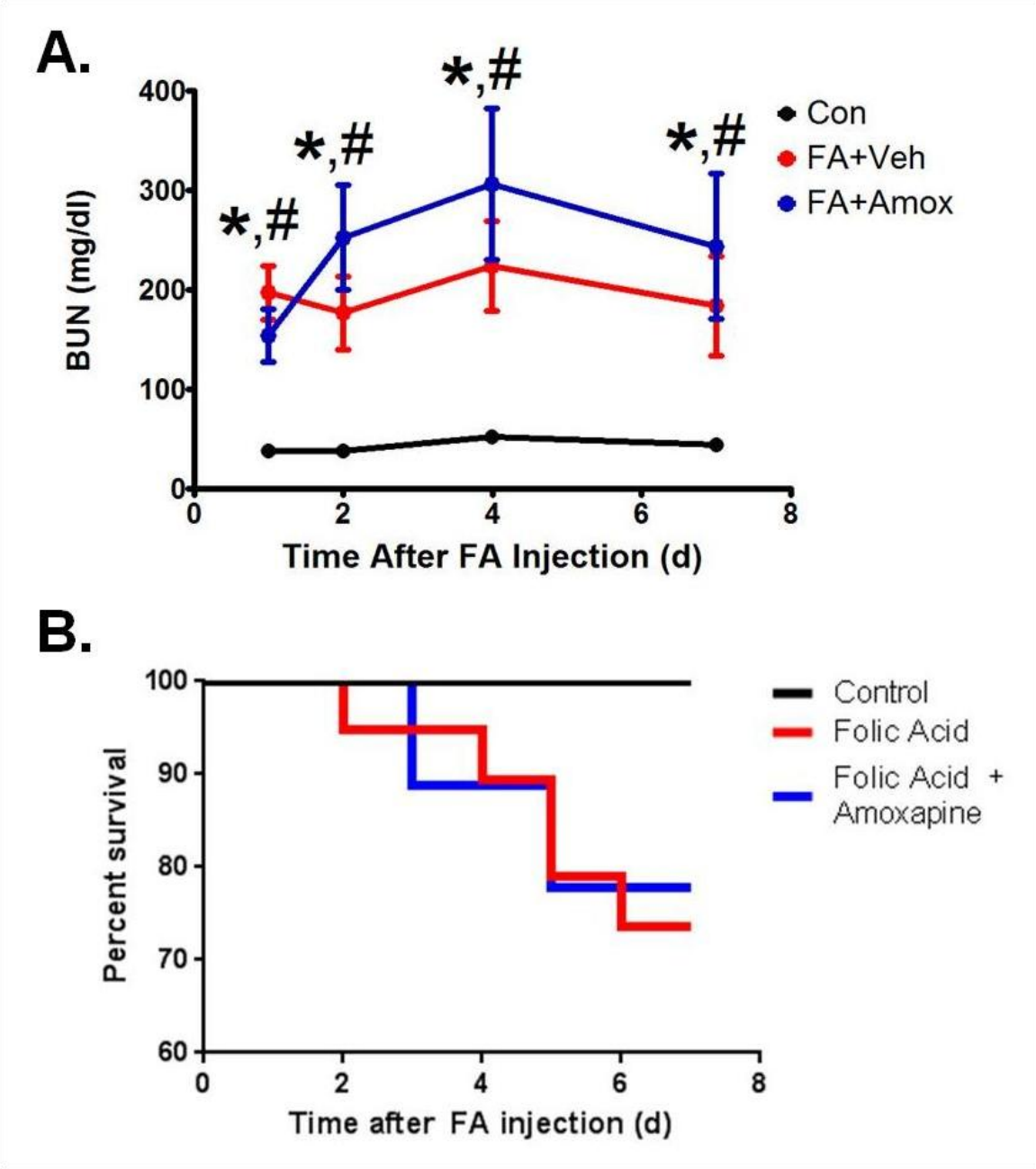

Fig. 3-6. Amoxapine Does Not Restore Renal Function or Promote Survival after FA-induced AKI. AKI was induced in C57BL/6 by a single intraperitoneal injection of FA. Mice received daily injections of amoxapine (0.3 mg/kg) or saline vehicle beginning 24h after FA. Kidney function was assessed via serum creatinine at 1, 2, 4 and 7 d post-injury (A). A Kaplan-Meier Survival Curve was generated using % survival of each group on each day after FA-AKI (B). Differences between FA and control (*) and FA + amoxapine and control (#) groups were analyzed using a Mann-Whitney U test. N=4-14, *, # $p < 0.05$ compared to control.

DISCUSSION

Mitochondrial dysfunction is a well-characterized pathophysiological consequence and initiator of acute kidney injury, and previous work demonstrated that pharmacological induction of MB promoted recovery of tubular structure and decreased markers of renal injury after I/R-AKI (120). Additionally, recent work in our laboratory characterized a pattern of mitochondrial dysfunction after FA-AKI and demonstrated that treatment with the PDE5 inhibitor sildenafil partially restored mtDNA copy number as well as expression of TFAM and COXI mRNA; the mitigation of these mitochondrial damage markers was correlated with a decrease in the renal injury marker KIM-1 (52). The conclusion drawn from these studies is that stimulation of MB is a promising novel opportunity to treat cellular damage in the face of AKI. In this study, we examined the ability of amoxapine, a 5-$HT_{2A/2C}$ receptor antagonist, to induce MB in RPTC and in both naïve and FA-AKI mouse renal cortex by measuring PGC-1α mRNA expression, mtDNA copy number and expression of mRNAs for mitochondrial proteins 24-120 h after drug administration.

Previous studies in our laboratory demonstrated that CP-809,101, a 5-HT_{2C} receptor agonist, and SB-242,084, a 5-HT_{2C} receptor antagonist, were both capable of inducing MB in both RPTC and mouse renal cortex but, contrary to our expectations, biogenic signaling by these compounds was dependent on the expression of the 5-HT_{2A} receptor. We found antagonism of the 5-HT_{2A} receptor to have more potential for drug development, because agonists of any member in the 5-HT_2 receptor class frequently exerts non-specific effects on other 5-HT_2 receptors at increasing doses; the possibility of off-target effects makes these agonists less attractive drug leads due to the risk of non-mitochondrial cardiotoxicity mediated by the 5-HT_{2B} receptor (73, 221-223). Additionally, 5-HT_{2A} receptor specific agonists are not candidates for clinical use because most potent agonists of this receptor are hallucinogenic (228). Finally, it was found that many FDA-

approved SSRIs are also potent antagonists at the 5-HT_{2A} and 5-HT_{2C} receptors, which led to the identification of a panel of readily available and clinically safe drugs with potential to induce MB in the kidney (224). An initial screen of this panel of drugs suggested that the antidepressant amoxapine was a candidate drug lead and warranted further analysis of its mitochondrial biogenic capabilities.

Amoxapine is a non-specific but potent 5-$HT_{2A/2C}$ receptor antagonist, with a K_D of 1 nM for both receptors (224). Amoxapine has no reported antagonist effects on the 5-HT_{2C} receptor, but it inhibits the norepinephrine, serotonin and dopamine transporters at 60 nM, 16 nM, and 4 µM, respectively (229). Therefore, it has 60-fold selectivity for the 5-$HT_{2A/2C}$ receptors over the serotonin transporter, which we found acceptable to avoid potential competing effects of increased native serotonin signaling on MB.

In RPTC, amoxapine was demonstrated to increase FCCP-uncoupled respiration at 0.1, 1, 10 and 100 nM doses, which is consistent with its K_d values for both the 5-HT_{2A} and 5-HT_{2C} receptors. To verify that this increase in respiration was due to increased MB, we measured PGC-1α mRNA and found it to be increased at 30 and 100 nM concentrations of amoxapine.

Because we previously found that biogenic signaling at the 5-HT_2 class of receptors was dependent on the expression of the 5-HT_{2A} receptor, we hypothesized that this was also the mechanism by which amoxapine induced increases in respiration and PGC-1α mRNA expression. However, the relatively low K_d value of amoxapine for the norepinephrine transporter introduced the possibility that its biogenic effect was secondary to β_2 adrenergic receptor agonism by increased extracellular norepinephrine concentrations. RPTC were pretreated for 72 h with negative control siRNA or siRNA

directed against either the 5-HT_{2A} and 5-HT_{2C} receptors and then treated for 24 h with 30 nM amoxapine. Amoxapine increased PGC-1α mRNA expression in both RPTC pretreated with negative control siRNA as well as siRNA directed against the 5-HT_{2C} receptor, but no increase in PGC-1α mRNA was observed in RPTC pretreated with siRNA against the 5-HT_{2A} receptor. These results indicate that amoxapine induces MB through the 5-HT_{2A} receptor, which is consistent with our findings using other drugs in this class of biogenic agents.

Given the ability of amoxapine to induce MB in RPTC, we decided to further characterize its biogenic capability *in vivo*. We administered daily injections of amoxapine (0.3 mg/kg, ip) into C57/Bl6 mice and collected kidneys 24, 48 and 120 h after initial injection to screen for MB. PGC-1α mRNA was increased in renal cortex compared to vehicle control at 48 h after initial injection, but no significant difference was found between vehicle and amoxapine-treated kidney cortex at 24 or 120 h after injury. Additionally, no change in mitochondrial DNA copy number was detected at any time point examined in this study, and PGC-1α, COXI and NDUFS1 protein expression was unchanged at 48 and 120 h after initial injection of amoxapine. The increase of PGC-1α mRNA in absence of secondary markers of MB, such as increased mtDNA copy number or mitochondrial protein expression, may indicate the activation of negative regulatory mechanisms that oppose PGC-1α. Increased mitochondrial production in the absence of physiological necessity can be maladaptive and lead to increased production of ROS, so PGC-1α and its downstream effectors can be negatively modulated by several mechanisms, including the negative regulatory proteins RIP140 and GCN5-L1 (230, 231).

Because the 5-HT_{2A} receptor is highly expressed in the CNS and because amoxapine is designed to cross the BBB, we sought to determine if 0.3 mg/kg amoxapine could also induce MB in CNS tissues. We detected a maximal effect on renal MB at 48 h post-initial injection of amoxapine, so at this time point, we screened for increases in PGC-1α mRNA expression or mtDNA copy number in the frontal cortex, hippocampus and striatum, but found that neither of these markers of MB was increased in any of these tissues. Due to the differences in receptor expression levels, it is interesting that the biogenic capacity of the 5-HT_{2A} receptor antagonist amoxapine is specific to the kidney. However, Garsnovskaya *et al.* demonstrated that the 5-HT_{2A} receptor displays both canonical G_q and non-canonical $G_{i/0}$ protein signaling in the kidney, and these differences in tissue- and cell type-specific signaling profiles may explain the specificity of its biogenic effects.

Given this renal specificity for the induction of MB and previous work in our laboratory that demonstrated the ability of pharmacologically-induced MB to promote recovery of renal function after AKI, we next sought to determine if treatment with amoxapine would mitigate mitochondrial dysfunction and decrease renal damage markers in folic acid-induced AKI. Unlike the results achieved with sildenafil, 0.3 mg/kg amoxapine was not sufficient to induce MB in FA-AKI kidneys, as demonstrated by the persistent decrease in mtDNA copy number and expression of mitochondrial mRNAs in FA-AKI kidneys treated with both vehicle and amoxapine. Additionally, amoxapine did not significantly alter renal function after FA-AKI, as determined by persistently increased BUN in both the vehicle-treated and amoxapine-treated groups though seven days after initial injury. Finally, amoxapine failed to significantly increase survival of FA-AKI mice over those treated with vehicle.

Given the utility of sildenafil to induce MB and promote recovery after FA-AKI, it is surprising that amoxapine exerts no effects on the mitochondrial markers studied in this experiment. However, there are several possibilities that may explain these results. The first is that the 5-HT_{2A} receptor is expressed at the membrane, whereas PDE5 is intracellular cytosolic protein. One well-characterized pathophysiological effect of AKI is the loss of tubular cell polarity and the loss or relocation of membrane-bound proteins, such as Na^+/K^+-ATPase (100, 101). It is therefore possible that the disorganization of the tubular epithelial cell membrane leads to decreased expression of the 5-HT_{2A} receptor, decreasing the intended target of amoxapine and inhibiting its ability to induce MB. Additionally, dedifferentiation, proliferation, migration and re-differentiation of epithelial cells to repopulate the renal tubular epithelium is a critical process in the recovery of renal function. In our *in vitro* model, we have observed that RPTC need to continue in culture for 2 d past confluency to differentiate sufficiently for expression of the 5-HT_{2A} receptor. This lag in 5-HT_{2A} receptor expression in differentiating cells, which populate the recovering tubular epithelium, especially when compared to the expression of an intracellular target such as PDE, may also explain the disparity in response of FA-AKI to these two pharmacological agents.

Although the utility of amoxapine in treatment of acute injury does appear to be limited, more research with this compound is warranted in mitochondrial dysfunction observed in chronic models of renal injury, which are less likely to have the membrane deficits or heterogeneous differentiation profile characteristic of AKI.

CHAPTER FOUR

DISRUPTION OF MITOCHONDRIAL HOMEOSTASIS FOLLOWING SEVERE TRAUMATIC BRAIN INJURY

ABSTRACT

Numerous studies have demonstrated that traumatic brain injury (TBI) results in oxidative stress and calcium dysregulation in mitochondria, indicating that mitochondrial damage is a major component of TBI pathology. However, little work has examined the time course of molecular mitochondrial damage during the first week post-injury. We examined markers of mitochondrial homeostasis after closed cortical impact (CCI), a rat model of severe TBI. In ipsilateral striatum, CCI caused a reduction in PGC-1α mRNA at 3 and 6 d post-injury and reduced mtDNA copy number at 3 d post-injury that recovered by 6 d. In ipsilateral cortex, CCI reduced PGC-1α mRNA in ipsilateral cortex at 6 d post-injury. Additionally, expression of mitochondrial-encoded mRNAs COXI and ND1 were decreased at 3 and 6 d after injury in ipsilateral striatum and at 6 d post-injury in ipsilateral cortex, while corresponding mitochondrial protein expression was not decreased. Treatment of CCI rats with amoxapine, which was demonstrated previously to induce mitochondrial biogenesis (MB), did not promote recovery of markers of mitochondrial homeostasis in either ipsilateral striatum or cortex. The inability of a mitochondrial biogenic agent to effectively mitigate mitochondrial dysregulation after TBI suggests the persistent activation of multiple signaling pathways that may continue to suppress mitochondrial homeostasis in the sub-acute phase of days to weeks post-TBI. We detected increased antioxidant mRNA and well as increased cytokine mRNA expression as well as an induction of microRNAs demonstrated in other tissues to disrupt markers of mitochondrial homeostasis. These findings reveal that there may be differential susceptibilities of different brain structures to mitochondrial injury. These

results further indicate that molecular pathways demonstrated to interfere with mitochondrial homeostasis and function are continually activated after TBI and that this persistent suppressive signaling may yield novel drug targets for the treatment of mitochondrial dysfunction following TBI.

INTRODUCTION

Traumatic brain injury (TBI) is a serious public health concern that contributes significantly to morbidity and mortality in people younger than 45 both globally and in the United States, which averages 1.7 million TBI cases per year (132-134). Although TBI can result from a number of traumatic events that inflict a non-penetrating injury, certain populations are considered at high-risk, particularly those in active military service who are at risk for blast injury or those who participate in sports and other high-contact recreational activities (133).

Although TBI is an acute insult, it has chronic and lifelong sequelae, including functional deficits in multiple brain structures that lead to decreased ability to complete motor and memory tasks and inhibition of behavior and emotional control (133). When costs incurred for direct medical care are combined with the lifelong costs of diminished productivity and loss of ability to work, they total between $60-221 billion per year (133, 134). Despite the serious burden of this disease both on individual patients and to society at large, treatment options for TBI in the acute to sub-acute phase are largely supportive and are designed to treat the symptoms of the injury without addressing the underlying molecular pathways that contribute to or cause those symptoms.

TBI can be classified into two phases: the primary phase which results immediately and irreversibly due to mechanical force and the secondary phase, a delayed phase in which the activation of multiple physiological cascades results in ischemia, swelling and dysfunction in several neuronal cell types (136, 137). Traditionally, the physiological features that comprise the secondary phase of TBI are separated into two distinct categories: increased inflammatory signaling and glutamate excitotoxicity (136, 137).

The release of cytokines by microglia, the resident macrophages in the brain, in the secondary phase of TBI has several consequences, some of which are in direct opposition to each other (232). The first phase of cytokine release involves the generation of increased pro-inflammatory cytokines such as TNF-α, which activates metalloproteinase-9 (MMP-9) and contributes to blood-brain barrier (BBB) permeability, indirectly leading to neuronal death after TBI (141, 143-145). IL-6, which is also released in the first phase, has both pro-inflammatory and anti-inflammatory effects and may be protective at low concentrations of release by microglia but becomes maladaptive at high concentrations and when expressed by astrocytes (140, 145, 149). The second phase involves the release anti-inflammatory mediators, including IL-10 and TGF-β, both of which suppress TNF-α (139, 140, 142, 144-146, 148, 149).

Glutamate toxicity results in increased calcium flux into neuronal cells and hypoxia, which then leads to mitochondrial dysfunction (132, 136, 137). Mitochondrial dysfunction is a prominent feature of TBI and has been linked to broad changes in cell and tissue structure and function and contributes prominently to the chronic neurological deficits experienced by TBI patients (152). These disruptions in mitochondrial function are multi-factorial, and include the increased production of reactive oxidative species (ROS), calcium dysregulation leading to initiation of the mitochondrial permeability transition and, if mitochondrial injury is sufficiently severe, initiation of apoptosis (135, 152, 153).

Sub-lethal mitochondrial dysfunction involves patterns of damage that are not severe enough to induce mitochondrial permeability and resultant apoptosis but still contribute strongly to decreased cellular function post-TBI. Although increased oxidative stress,

disrupted calcium homeostasis and downstream apoptotic signaling after TBI have been well-characterized and targeted for pharmacological intervention, little work has been performed to characterize deficits in mitochondrial biogenesis and the maintenance of mitochondrial homeostasis.

Mitochondrial biogenesis (MB) is a process that contributes to maintenance of mitochondrial homeostasis by increasing mitochondrial number or content through the coordination of mitochondrial dynamics, mtDNA replication and transcription of both nuclear- and mitochondrial-encoded genes that encode mitochondrial proteins, including components of the ETC (2, 6). MB occurs as the result of activation of peroxisome proliferator-activated receptor γ co-activator 1-α (PGC-1α), the “master regulator” of MB, which binds to and co-activates a number of nuclear transcription factors implicated in maintenance of mitochondrial content, structure and function, resulting in increased flux through the ETC and a net increase in ATP production (2, 6). Disruption of MB has been identified in the pathophysiology of both acute and chronic disease of several organs with high energy demands, including heart, liver and kidney (122, 225, 234-237). Furthermore, pharmacological induction of MB has been demonstrated to promote recovery in several models of acute organ injury (52, 120).

Here, we report that controlled cortical impact, a model of focal TBI, disrupts mitochondrial homeostasis in the ipsilateral striatum and cortex and describe a putative relationship between cytokine release and mitochondrial disruption through induction of miRNAs.

MATERIALS AND METHODS

Reagents

Amoxapine and all other chemicals were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise noted.

Animal Care and Use

All experiments were performed in strict accordance with the guidelines in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All protocols were approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina, and appropriate efforts were made to reduce animal suffering.

Controlled Cortical Impact (CCI)

Male Long-Evans Rats (3-4 months old) were anesthetized with ketamine/xylazine (100mg/10 mg/kg i.p.), placed into a stereotaxic frame and a 4mm craniotomy (0.5mm anterior and 4mm lateral to bregma) was created centered over the right forelimb area of the sensorimotor cortex (Fl-SMC). A controlled cortical impact (CCI) was induced using a Benchmark Stereotaxic Impactor (Leica, Buffalo Grove, IL) with a 3mm diameter impact tip angled 18o away from the vertical, permitting the tip to impact perpendicular to the surface of the brain, 1.7 D.V. The impactor tip penetrated the exposed brain at 3.0m/s for 300ms. After the impact, the wound was covered with gel film and dental acrylic and the incision was sutured. Topical antibiotics were applied to the incision and Buprenorphin (0.05mg/ml) was administered subcutaneously. Sham animals received all drugs and were placed into a stereotaxic frame, after which an incision and fascia retraction are performed and then closed with sutures. In the 6 day injury studies, one group of rats was given daily intraperitoneal injections of 0.3 mg/kg amoxapine. Euthanasia was performed 24 h after the final dose of amoxapine.

Euthanasia /Tissue Extraction

Animals were overdosed with of sodium pentobarbital (Euthasol, 100mg/kg, i.p.). When animals were non-responsive to toe and tail pinch, an incision was made to reveal the cisterna magna and CSF fluid was extracted through a small puncture via 25 gauge syringe. Animals were then decapitated and brain areas were quickly dissected to isolate ipsi- and contra-injury sensory and motor cortices, hippocampus, and striatum.

Motor Behavior Assessment: Ladder Task

The ladder task was used to assess coordinated forelimb use, stepping accuracy, and limb placement. The ladder apparatus is made of two plexiglass walls, with 3mm pegs spaced 1cm apart from each other. The ladder is raised ~20cm off the ground with a neutral start cage and the animal's home cage at the end. Animals were allowed to walk freely across the ladder and videotaped for three trials. Scoring of the steps was done with slow motion video replay and based on a previously established rating scale (238). The total number of steps and missteps were calculated and scored on a scale (0-6) based on how the animal places the forelimb on the rungs of the ladder. Errors were counted when an animal completely missed the ladder rung and a fall occurred (score of 0) or placed the limb but when weight bearing either fell (score of 1) or slipped (score of 2). Percent error was calculated as: (#0+1+2)/total steps.

Real-Time Reverse Transcription-PCR – Mitochondrial and Inflammatory Gene Expression

Total RNA was extracted from cortex, hippocampus and striatum samples using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized via reverse transcription using the RevertAid First Strand cDNA kit (Thermo Fisher Scientific, Waltham, MA) with 0.5-2 ug of RNA. PCR products were amplified from 5 uL of cDNA template using 2x Maxima SYBR green qPCR master mix (Thermo Fisher

Scientific) and 400 nM concentrations of each primer (Integrated DNA Technologies, Inc., Coralville, IA) (Table 1). Fold changes in expression were calculated using the ΔΔ-Ct analysis method detailed previously by Wills *et al.* (50).

Mitochondrial DNA Content

Relative quantity of mtDNA in rat CNS tissue samples was measured using real-time PCR. After treatment, DNA was isolated from tissues using the DNEasy Blood and Tissue Kit (Qiagen, Valencia, CA), and qPCR was performed using 5 ng of cellular DNA. Expression of NADH dehydrogenase 1 (ND1), a mitochondrial gene, was measured and normalized to nuclear-encoded β-actin (Table 1). The previously described ΔΔ-Ct analysis method was used to calculate fold changes in expression (50).

Immunoblot analysis

Cortex or striatum tissue was homogenized in 500 µL of protein lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4; 1 mM EDTA; 1 mM EGTA; 2 mM sodium orthovanadate; 0.2 mM phenylmethylsulfonyl fluoride; 1 mM HEPES, pH 7.6; 1 µg/ml leupeptin; and 1 µg/ml aprotinin) using a Polytron homogenizer. The homogenate was then centrifuged at 10000g for 10 min at 4°C. The supernatant was collected, and protein was determined using a bicinchoninic acid kit (Sigma, St. Louis, MO) with bovine serum albumin as the standard. Proteins (50–75 µg) were separated on 4 to 20% gradient SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked either in 5% dried milk or BSA in TBST (0.1% Tween 20 in 1× Tris-buffered saline) and incubated with 1:1000 antibody dilutions of anti-PGC-1α (EMD, Billerica, MA); COX-1 (Abcam, Cambridge, MA); anti-ND1 (Abcam, Cambridge, MA); anti-NDUFB8 (Invitrogen, Grand Island, NY); anti-NDUFS1 (Abcam, Cambridge, MA); and anti-GAPDH (Fitzgerald, Acton, MA) overnight at 4°C. After incubation for 2 h at room temperature with secondary antibodies (1:2000) conjugated with horseradish

peroxidase, membranes were detected by chemiluminescence. Image analysis and densitometry were performed using Image-J software**.**

Real-Time Reverse Transcription-PCR – miRNA Expression

Total RNA was extracted from cortex and striatum samples using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized via reverse transcription using the Kit iScript Advanced cDNA (Bio-Rad, Hercules, CA) with 0.5-2 ug of RNA and 400 n M of the specific RT primer (Table 2) for each miRNA following the stem-loop pulsed RT-PCR method previously described by Varkonyi-Gasic *et al. (239)*. PCR products were amplified from 5 uL of cDNA template using 2x Maxima SYBR green qPCR master mix (Thermo Fisher Scientific) and 400 nM concentrations of primers (Integrated DNA Technologies, Inc., Coralville, IA) (Table 2). Target miRNAs were amplified with a specific forward primer and a universal reverse primer. U6 was used to normalize target miRNA expression and was amplified with a specific forward primer and a reverse primer that is identical to its RT primer. Fold changes in expression were calculated using the ΔΔ-Ct analysis method previous described (50).

Statistics

Data are presented as means ± SEM. Single comparisons for normal data were performed using a Student's t-test, whereas data found to not have a normal distribution were subjected to a Mann-Whitney U-test. Multiple comparisons for normal data were performed using one-way analysis of variance (ANOVA) with an appropriate post-hoc test to compare multiple means. Kruskal-Wallis one-way analysis of variance was used to perform multiple comparisons for non-normal data, and an appropriate post-hoc test was used to compare multiple medians. Single and multiple comparison data were considered statistically significantly different at $P \leq 0.05$. Rodent studies were repeated until $n \geq 3$ was obtained.

RESULTS

CCI to the SMC Causes Unilateral Forelimb Motor Deficits

To first examine the gross effect of TBI on motor function, both sham and CCI rats were assessed using the ladder task. One day after injury, CCI rats had a 10-fold increase in impaired limb placement error compared to that of sham rats (Fig. 4-1). The forelimb motor deficit slightly recovers at 6 d post-injury, but a 5-fold increase in impaired limb placement error in CCI rats over sham rats persists to this time point (Fig. 4-1). No significant alteration in foot slip was observed in the unimpaired limb (data not shown). These results indicate that motor function is significantly altered in this model of TBI and provide evidence that the injury sustained to the SMC by CCI is severe, specific to the site of injury, and sustained in the sub-acute phase after initial insult.

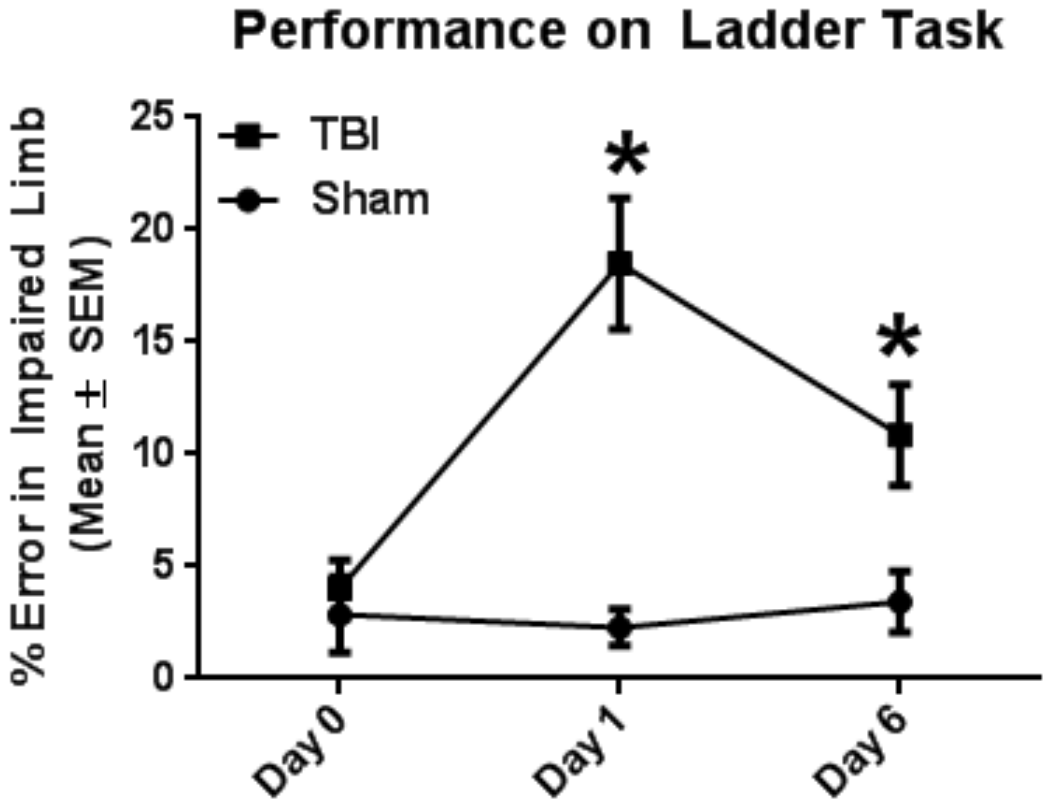

Fig. 4-1. CCI to the SMC Causes Unilateral Forelimb Motor Deficits. Rats were subjected to either sham or CCI treatment. Their performance on the ladder task for forelimb motor function was assessed pre-injury and at 1 and 6 d post injury by measuring the percent error in impaired limb foot slip. Differences between sham and TBI were analyzed using the Mann-Whitney U test. Data are reported as mean ± SEM. n = 7-8, *$p < 0.05$ between sham and TBI groups at each day.

CCI Results in Altered Mitochondrial Homeostasis in Tissues Ipsilateral to Injury

To screen for mitochondrial damage in the sub-acute phase after injury, PGC-1α mRNA expression and mtDNA copy number were measured in both ipsilateral cortex, hippocampus and striatum at 1, 3 and 6 d after CCI. Neither PGC-1α mRNA expression nor mtDNA copy number was changed in the ipsilateral cortex at 1 or 3 d after injury (Fig 4-2A and B). Six days after CCI, there was a 20% decrease in ipsilateral cortical PGC-1α mRNA expression compared to sham control but mtDNA copy number remained unchanged (Fig. 4-2C). One day after injury, there was no change in either PGC-1α mRNA expression or mtDNA copy number in ipsilateral hippocampus (Fig. 4-2D). Three days post-CCI, mtDNA copy number in the hippocampus was decreased to 65% of control, but PGC-1α mRNA expression remained unchanged (Fig. 4-2E). Six days after injury, mtDNA copy number had returned to sham control levels in the hippocampus, and no change in PGC-1α mRNA expression was detected (Fig. 4-2F). No change in either of our mitochondrial homeostasis markers was observed in the ipsilateral striatum 1 d after initial CCI, but both PGC-1α mRNA expression and mtDNA copy number were decreased to 60% of sham control by 3 d post-injury (Fig. 4-2G and H). Ipsilateral striatal mtDNA copy number had recovered to sham control levels by 6 d after initial injury, but PGC-1α mRNA expression remained significantly suppressed to 75% of sham control expression at this time point (Fig. 4-2I). These results are suggestive of a pattern of disrupted mitochondrial homeostasis in tissue ipsilateral to CCI that is initiated in the secondary phase (post-1 d) of acute injury and indicate that injury to the cortex has associated deleterious effects on diverse structures of the affected brain, including the hippocampus and striatum. The differential time courses in injury between these three structures and the delay observed before detection of cortical mitochondrial dysfunction may indicate disparate susceptibilities to and defenses against mitochondrial injury between different tissues of the brain.

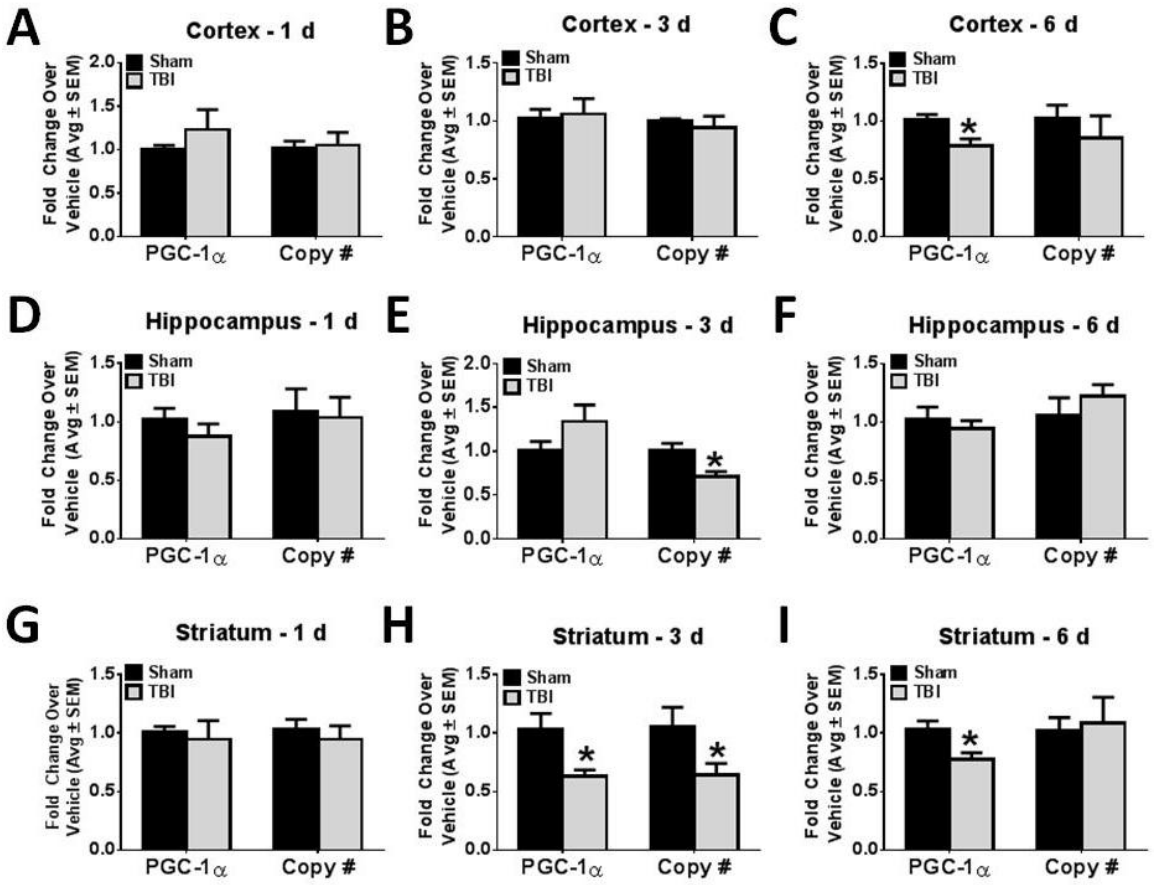

Fig. 4-2. CCI Results in Altered Mitochondrial Homeostasis in Tissues Ipsilateral to Injury. PGC-1α mRNA expression was determined by RT-PCR using tubulin as a control gene. mtDNA copy number was determined by qPCR, using ND1 for the mtDNA gene and actin for the nuclear control gene. These markers were measured in the ipsilateral cortex at 1 d(A) 3 d (B) and 6 d (C) 6 d post-injury; in the ipsilateral hippocampus at 1 d (D) 3 d (E) and 6 d (F) post-injury and in the ipsilateral striatum at 1 d (G) 3 d (H) and 6 d (I) post-injury . Values reported as mean ± SEM. Either Student's T-test or Mann-Whitney U test was used to determine significance. *$p < 0.05$, n =4-16.

CCI Does Not Result In Altered Mitochondrial Homeostasis in Tissues Contralateral to Injury

To determine if unilateral CCI resulted in only unilateral mitochondrial dysfunction, PGC-1α mRNA expression and mtDNA copy number were measured in contralateral cortex, hippocampus and striatum at 1, 3 and 6 d after injury. No change in either of these markers was detected in any tissue studied at any time point after injury (Fig. 4-3A-I). These results demonstrate that unilateral impact to the SMC produces a unilateral pattern of disrupted mitochondrial homeostasis in the acute through sub-acute phase.

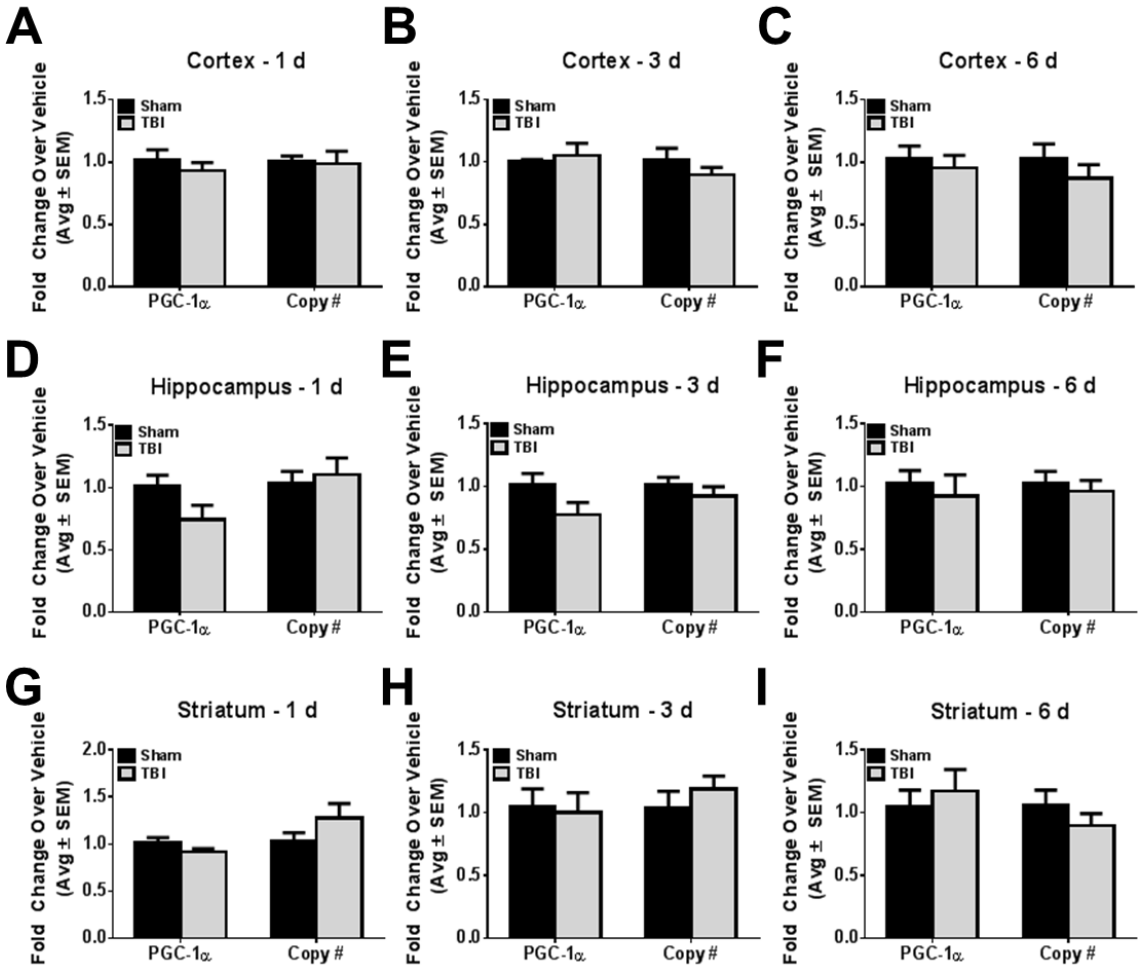

Fig. 4-3. CCI Does Not Result In Altered Mitochondrial Homeostasis in Tissues Contralateral to Injury. PGC-1α mRNA expression was determined by RT-PCR using tubulin as a control gene. mtDNA copy number was determined by qPCR, using ND1 for the mtDNA gene and actin for the nuclear control gene. These markers were measured in the contralateral cortex at 1 d (A) 3 d (B) and 6 d (C) 6 d post-injury; in the contralateral hippocampus at 1 d (D) 3 d (E) and 6 d (F) post-injury and in the contralateral striatum at 1 d (G) 3 d (H) and 6 d (I) post-injury . Values reported as the mean ± SEM. Either Student's T-test or Mann-Whitney U test was used to determine significance. n =4-8.

CCI Results in Decreased mtDNA Transcripts in Ipsilateral Striatum and Cortex

To further characterize mitochondrial deficits following CCI seen in the ipsilateral striatum and cortex, we measured an additional panel of mRNAs associated with mitochondrial homeostasis, including the nuclear-encoded mitochondrial transcription factor A (TFAM) and NDUFS1 and mitochondrial-encoded COXI and ND1. In addition to the previously described decreases in PGC-1α mRNA expression and mtDNA copy number in ipsilateral striatum at 3 d post-injury, mitochondrial-encoded COXI and ND1 mRNAs are both decreased to 70% of those in sham control animals, but no differences in nuclear-encoded mRNAs was observed (Fig. 4-4A). Similar to PGC-1α, both COXI and ND1 mRNA remain suppressed to 80% at 6 d post-injury, while TFAM and NDUFS1 mRNA expression remain unchanged (Fig. 4-4B). Similarly, in ipsilateral cortex 6 d post-injury, which is the first time point at which we detected a decrease in PGC-1α mRNA expression in our initial screen, we observed a decrease in COXI and ND1 mRNA expression to 60% that of sham control rats (Fig. 4-4C). These results point to the possibility that the secondary phase of damage following acute CCI initiates multiple mechanisms of injury with independent causes and points of impact along the pathways that comprise mitochondrial homeostasis.

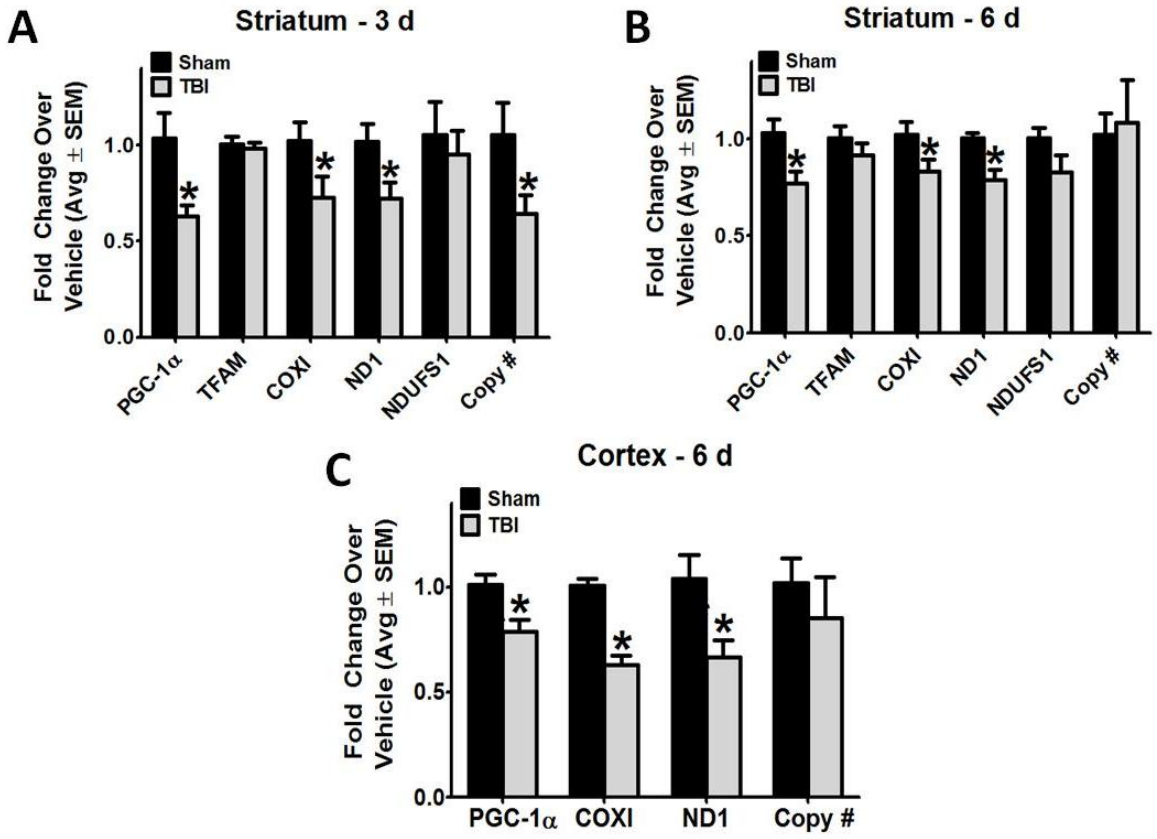

Fig. 4-4. CCI Results in Decreased mtDNA Transcripts in Ipsilateral Striatum and Cortex. PGC-1α, TFAM, COXI, ND1 and NDUFS1 mRNA expression were determined by RT-PCR using tubulin as a control mRNA. These markers were measured in the ipsilateral striatum at 3 d (A) and 6 d (B) post-injury and in the ipsilateral cortex at 6 d (C) post-injury. Values reported as mean ± SEM. Student's T-test or Mann-Whitney U test was used to determine significance. *$p < 0.05$, n =4-16.

CCI Does Not Affect Mitochondrial Protein Expression In the First Six Days After Injury

To assess if decreases in mRNA expression observed in the ipsilateral and striatum and cortex 6 d after injury had further consequences, we measured the expression of three mitochondrial proteins at this time point. We observed no change in PGC-1α, ND1 or NDUFS1 protein expression in either the ipsilateral striatum (Fig. 4-5A and B) or cortex (Fig. 4-5C and D), despite the decrease we had observed in PGC-1α and ND1 mRNA expression in both tissues. These results are interesting, because decreased mRNA expression is expected to result in coordinating decreased protein expression, especially when mRNA expression is suppressed over days, as we observed in the ipsilateral striatum.

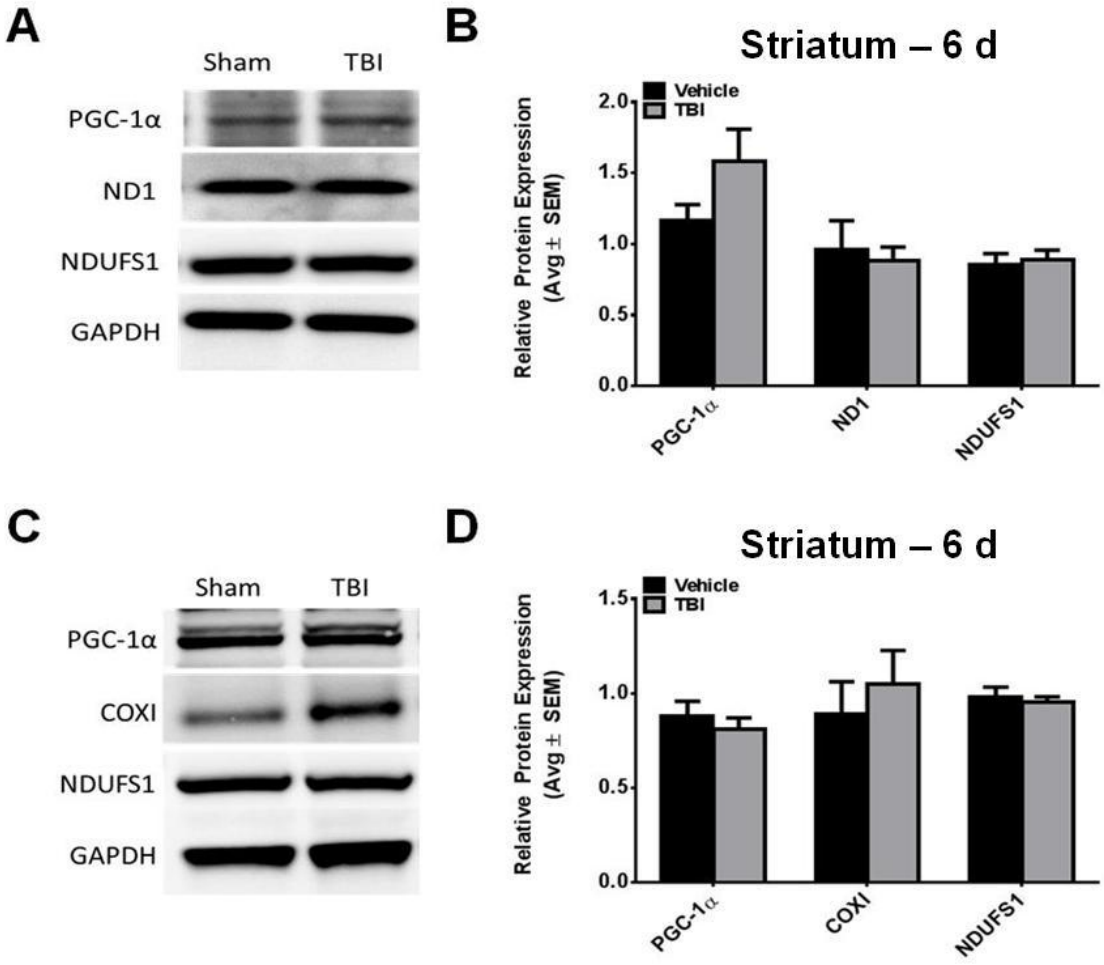

Fig. 4-5. CCI Does Not Affect Mitochondrial Protein Expression In the First Six Days After Injury. PGC-1α, COXI, ND1 and NDUFS1 protein expression were determined using immunoblot analysis using GAPDH as a loading control. These markers were measured 6 d post-injury in the ipsilateral striatum (A) and ipsilateral cortex (B). Values reported as mean ± SEM. n = 5-6.

Amoxapine Does Not Promote Recovery of Mitochondrial Content after CCI

Our laboratory had previously demonstrated that amoxapine, a potent 5-$HT_{2A/2C}$ antagonist, was capable of inducing MB in mouse kidney cortex. Because it is used clinically as an antidepressant and is thus designed to penetrate the BBB, we hypothesized that amoxapine would reverse patterns of mitochondrial disruption and promote recovery of motor function in CCI rats 6 d after initial injury. In ipsilateral cortex, PGC-1α and COXI mRNA expression was decreased to 75% and 60% of sham controls, respectively in both CCI rats given diluent and those given amoxapine (Fig. 4-7A). Although significance wasn't achieved, there was a strong trend toward decreased ND1 mRNA expression in the cortex of CCI rats given either diluent or amoxapine (Fig. 4-7A). Similarly, PGC-1α and ND1 mRNA in CCI rats given diluent were both expressed at 75% of sham control in the striatum and remained similarly suppressed in CCI rats treated with amoxapine (Fig. 4-7B). COXI mRNA was not significantly decreased in the striatum of these CCI rats, although its expression in both diluent and amoxapine-treated animals trended toward a decrease (Fig. 4-7B). These results indicate that amoxapine is not an effective inducer of MB in this model and has limited utility as a pharmacological treatment for TBI.

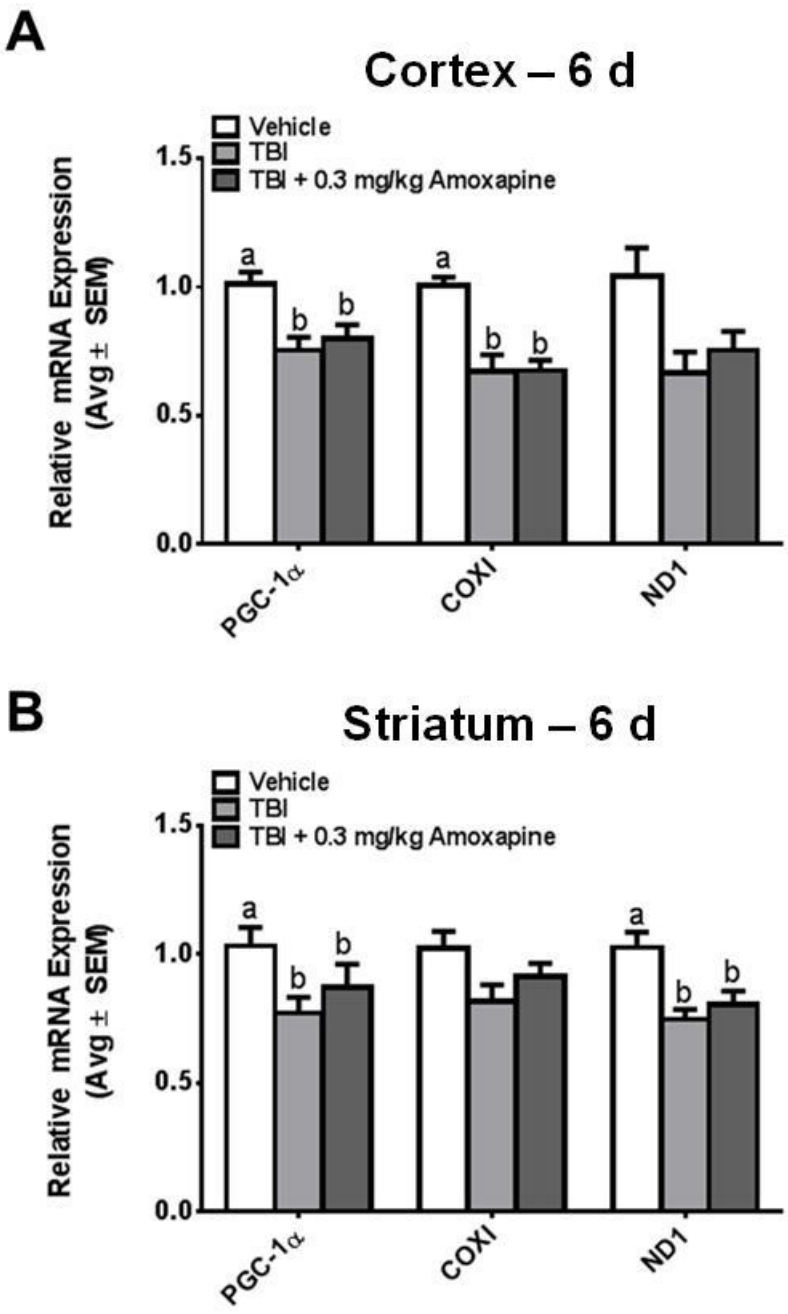

Fig. 4-6. Amoxapine Does Not Promote Recovery of Mitochondrial Content after CCI. PGC-1α, COXI and ND1 mRNA expression were determined by RT-PCR using tubulin as a control gene. These markers were measured in sham, TBI and TBI + 0.3 mg/kg amoxapine groups in ipsilateral cortex (A) and striatum (B) at POD 6. Values reported as mean ± SEM; with bars with different superscripts are significantly different from one another. Kruskal-Wallis test with Dunn's post-test was used to determine significance. *$p < 0.05$, n =14-16.

CCI Induces Antioxidant Mechanisms in Ipsilateral Striatum and Cortex

Because amoxapine did not reverse the observed disruptions in mitochondrial DNA and mRNA expression, we next hypothesized that there were ongoing mechanisms of injury in the first 6 days after TBI that were not able to be overcome by stimulation of mitochondrial biogenesis. We hypothesized that one such mechanism of injury was increased oxidative stress, a hallmark of TBI pathophysiology that disrupts mitochondrial function and propagates mitochondrial injury, so we next sought to identify potential markers of acute oxidant stress in our CCI model. To measure potential oxidative stress signaling, we measured mRNAs for two antioxidant proteins: superoxide dismutase 2 (SOD2) and uncoupling protein 2 (UCP2). In the ipsilateral striatum, SOD2 mRNA expression was increased 1.4-fold over sham control at 1 d after injury but had returned to sham control levels by 3 d (Fig. 4-6A). Conversely, ipsilateral striatal UCP2 expression remained unchanged at 1 d after CCI, but increased 3-fold over sham control at 3 d and remained elevated 2-fold at 6 d post-injury (Fig. 4-6B). Cortical SOD2 mRNA expression followed an identical pattern to the striatum, with a 1.5-fold increase observed at 1 d post-CCI that returned to baseline levels at 3 d after injury (Fig. 4-6C). Expression of UCP2 mRNA in ipsilateral cortex remained at baseline levels 1 d post-injury, but increased 3-fold above sham control levels at 3 d after CCI; unlike the striatum, cortical UCP2 mRNA continued to increase and was expressed >4-fold over sham controls at 6 day after cortical impact (Fig. 4-6D). These results are suggestive of a multi-phase antioxidant program following injury and indicate that oxidative stress is a consequence of acute CCI that begins early after injury, affects multiple brain structures simultaneously and persists through the first six days after initial insult.

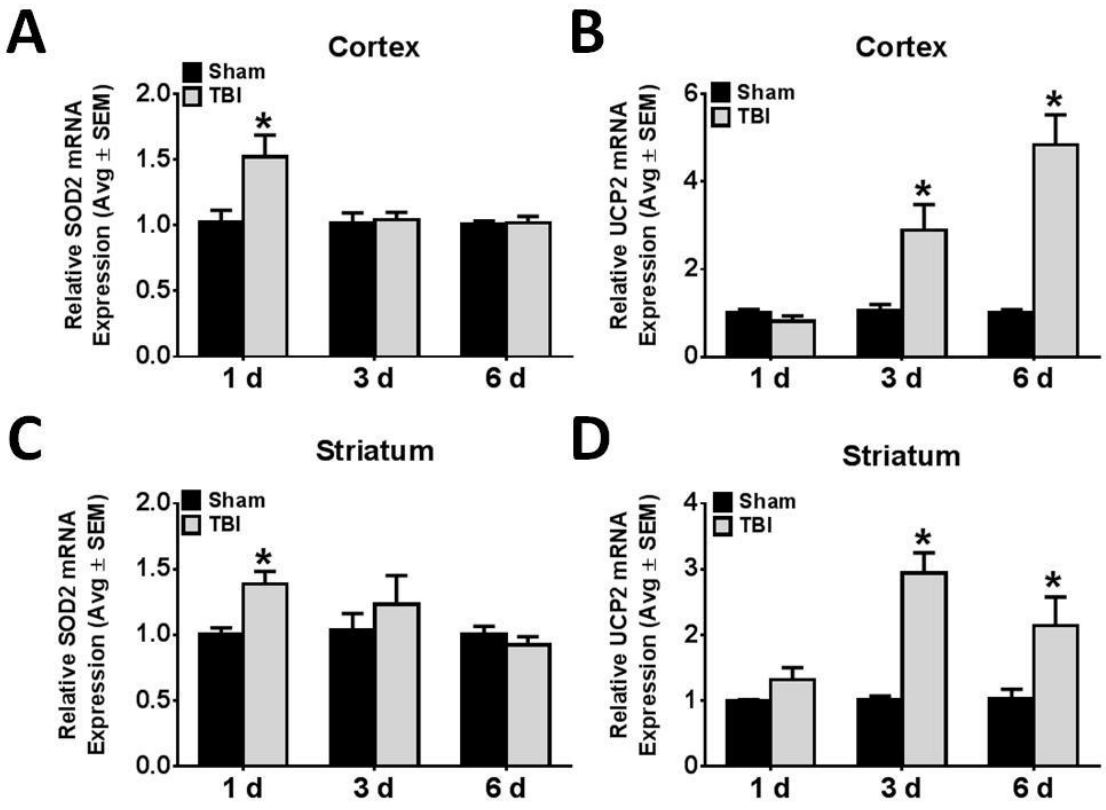

Fig. 4-7. CCI Induces Antioxidant Mechanisms in Ipsilateral Striatum and Cortex. SOD2 and UCP2 mRNA expression were determined by RT-PCR using tubulin as a control mRNA. In ipsilateral striatum, SOD2 mRNA expression was measured at 1, 3 and 6 d post-injury (A) and UCP2 mRNA expression was measured at 1, 3 and 6 d post-injury (B). In ipsilateral cortex SOD2 mRNA expression was measured at 1, 3 and 6 d post-injury (C) and UCP2 mRNA expression was measured at 1, 3 and 6 d post-injury (D). Values reported as mean ± SEM. Student's T-test or Mann-Whitney U test was used to determine significance. *$p < 0.05$, n =4-16.

CCI Induces Expression of Pro-Inflammatory and Anti-Inflammatory Cytokines

Based on the inability of amoxapine to induce MB in this model, we hypothesized that persistent activation of suppressive pathways by diverse effector molecules may limit pharmacological induction of MB. In light of increasing evidence that inflammation can modulate PGC-1α mRNA expression and contribute to mitochondrial dysfunction in acute injury states, we measured mRNA expression for IL-6 and TGF-β, which have been demonstrated to upregulate miRNAs that interfere with mitochondrial homeostasis. In ipsilateral striatum of CCI rats, IL-6 mRNA expression was increased 2-fold at 1 d post-CCI but returned to baseline at 3 and 6 d (Fig. 4-8A). Conversely, ipsilateral striatal TGF-β mRNA was decreased to 60% of sham control levels at 1 d post-injury, returned to baseline levels at 3 d, and was increased 1.25-fold at 6 d after CCI (Fig. 4-8B). Interestingly, ipsilateral cortical expression of IL-6 mRNA compared to sham control was increased 4.5-fold at 1 day after injury, returned to baseline at 3 d, and increased 2-fold at 6 days post-injury (Fig. 4-8C). TGF-β mRNA expression in ipsilateral cortex was unchanged at 1 and 3 d post-injury and significantly increased 1.3-fold over sham at 6 d after initial insult. These results reveal complex patterns of cytokine expression in both ipsilateral striatum and cortex that may contribute to the dysregulation of mitochondrial homeostasis following CCI.

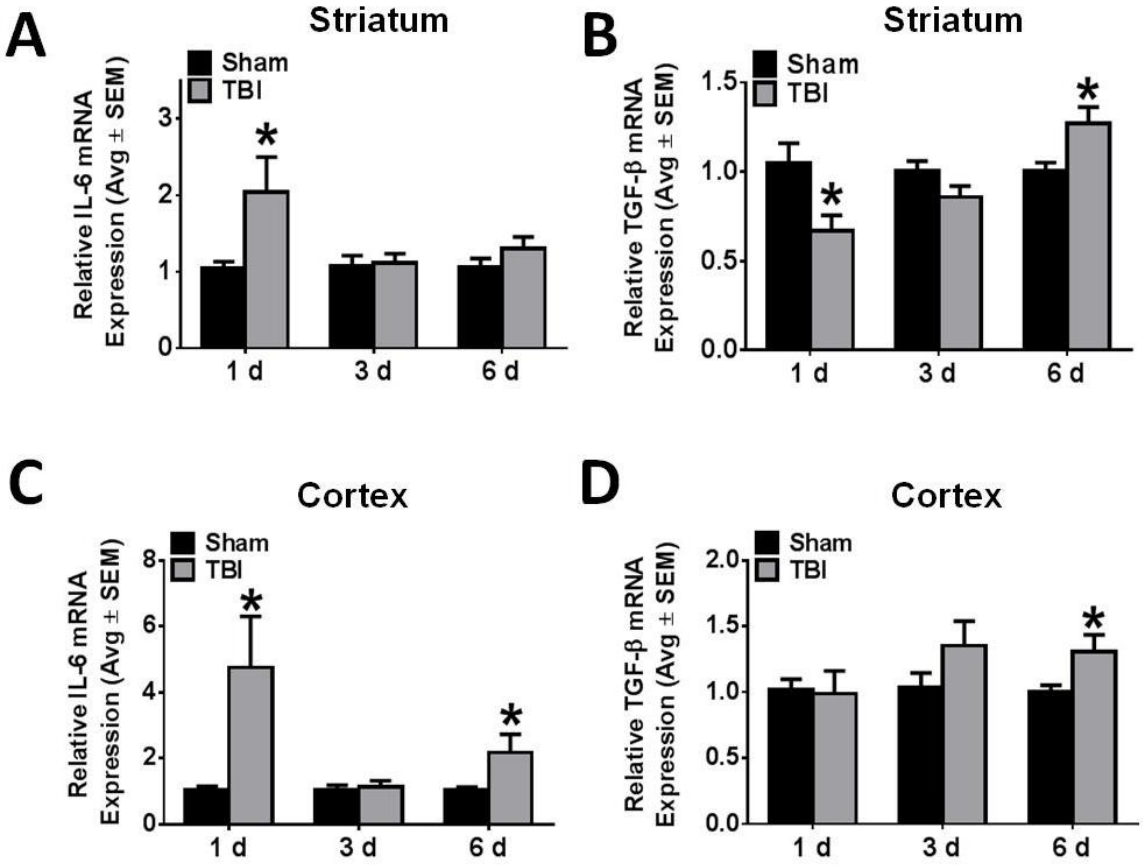

Fig. 4-8. CCI Induces Expression of Pro-Inflammatory and Anti-Inflammatory Cytokines. IL-6 and TGF-β mRNA expression were determined by RT-PCR using tubulin as a control gene. In ipsilateral striatum, IL-6 mRNA expression was measured at 1, 3 and 6 d post-injury (A) and TGF-β mRNA expression was measured at 1, 3 and 6 d post-injury (B). In ipsilateral cortex IL-6 mRNA expression was measured at 1, 3 and 6 d post-injury (C) and TGF-β mRNA expression was measured at 1, 3 and 6 d post-injury (D). Values reported as mean ± SEM. Student's T-test or Mann-Whitney U test was used to determine significance. *$p < 0.05$, n =4-16.

CCI Induces Expression of Mitochondria-Disrupting miRNAs in Ipsilateral Striatum and Cortex

We therefore hypothesized that miRNAs described in the literature to target SOD2 and PGC-1α mRNA would be increased in both striatum and cortex ipsilateral to injury. In the striatum, miR-21 was increased 2.5-fold over sham control rats at 3 d, but the increase in expression was completely attenuated by 6 d after CCI (Fig. 4-9A). miR-155 was induced earlier and with greater magnitude in the striatum, with a 2-fold increase at 1 d, a 5-fold increase at 3 d, and a return to 2-fold increased expression over sham control at 6 d post-injury (Fig. 4-9B). In ipsilateral cortex, miR-21 expression is unchanged at 1 d post-injury in CCI rats compared to sham controls but increases steadily at later time points with a respective ~4-fold and ~5.5-fold increase in expression at 3 and 6 d post-injury (Fig. 4-9C). Additionally, miR-155 is strongly induced in cortex following CCI, with 6-fold, 10-fold and 13-fold increases at 1, 3 and 6 days, respectively, in CCI rats compared to sham controls (Fig. 4-9D). These results are very interesting and indicate that increased miRNA expression may be one mechanism of mitochondrial suppression following CCI and represents a novel target to prevent mitochondria disruption in TBI.

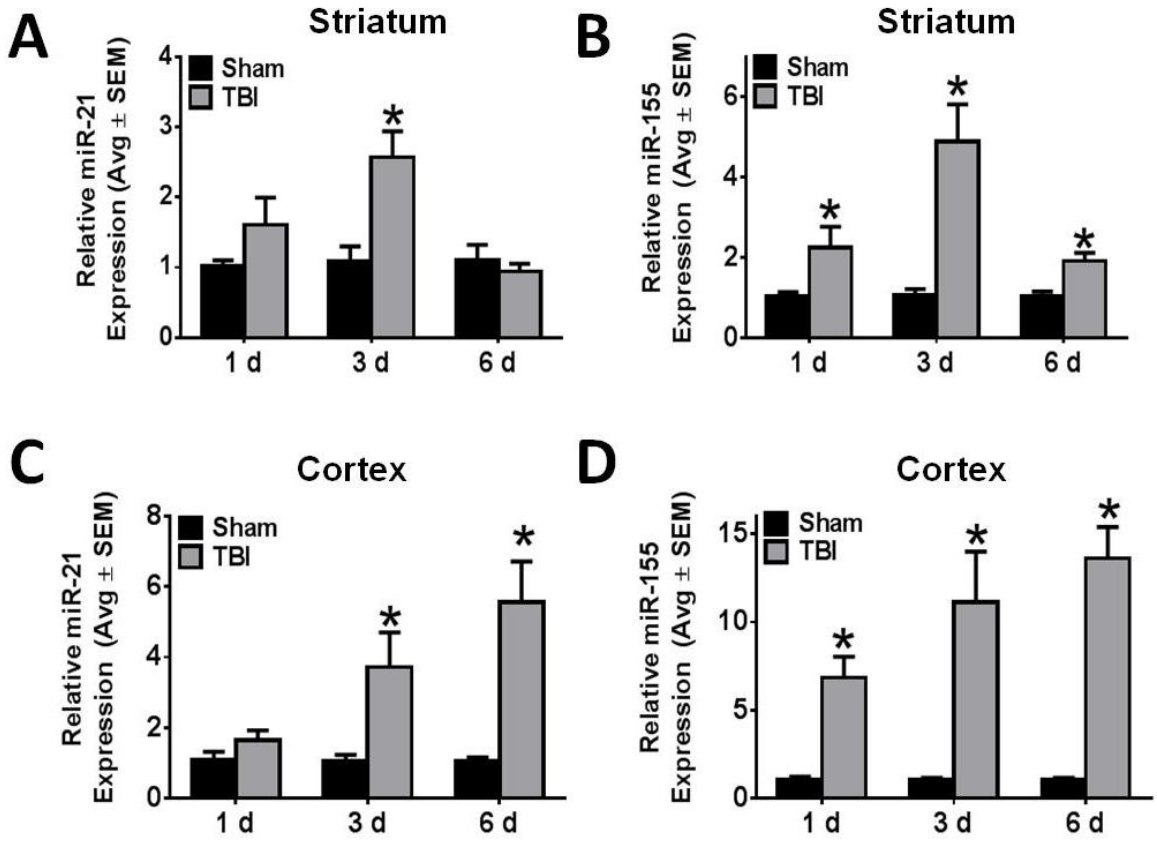

Fig. 4-9. CCI Induces Expression of Mitochondria-Disrupting miRNAs in Ipsilateral Striatum and Cortex. miR-21 and miR-155 mRNA expression were determined by RT-PCR using U6 as a control miRNA. In ipsilateral striatum, miR-21 expression was measured at 1, 3 and 6 d post-injury (A) and miR-155 expression was measured at 1, 3 and 6 d post-injury (B). In ipsilateral cortex miR-21 expression was measured at 1, 3 and 6 d post-injury (C) and miR-155 expression was measured at 1, 3 and 6 d post-injury (D). Values reported as mean ± SEM. Student's T-test or Mann-Whitney U test was used to determine significance. *$p < 0.05$, n =4-16.

CCI Causes Minimal Alterations in Contralateral Signaling Associated with Mitochondrial Dysregulation

Because we detected no changes in mitochondrial homeostasis in the contralateral striatum at 1, 3 or 6 days after initial insult, we wanted to examine the effect of acute injury on contralateral striatal signaling mechanisms we demonstrated to be altered in ipsilateral striatum post-CCI. We first measured SOD2 and UCP2 mRNA expression to determine if CCI caused increased oxidative stress in contralateral striatum. No change in SOD2 mRNA expression was detected at any time after injury, and UCP2 mRNA expression remained unchanged at days 1 and 3 but increased 1.5-fold 6 d post-injury (Fig. 4-10A and B). Neither IL-6 or TGF-β mRNA expression changed at any time post-injury, and expression of inflammation-associated miR-21 and miR-155 was unaltered at all time points examined after CCI (Fig. 4-10C-F). When considered with earlier findings that mitochondrial markers were unaltered in contralateral tissues, these results are consistent with studies that demonstrate deleterious effects of persistent oxidative stress on mitochondrial homeostasis. These findings further support a mechanistic link between inflammation and mitochondrial dysregulation through increased miRNA expression.

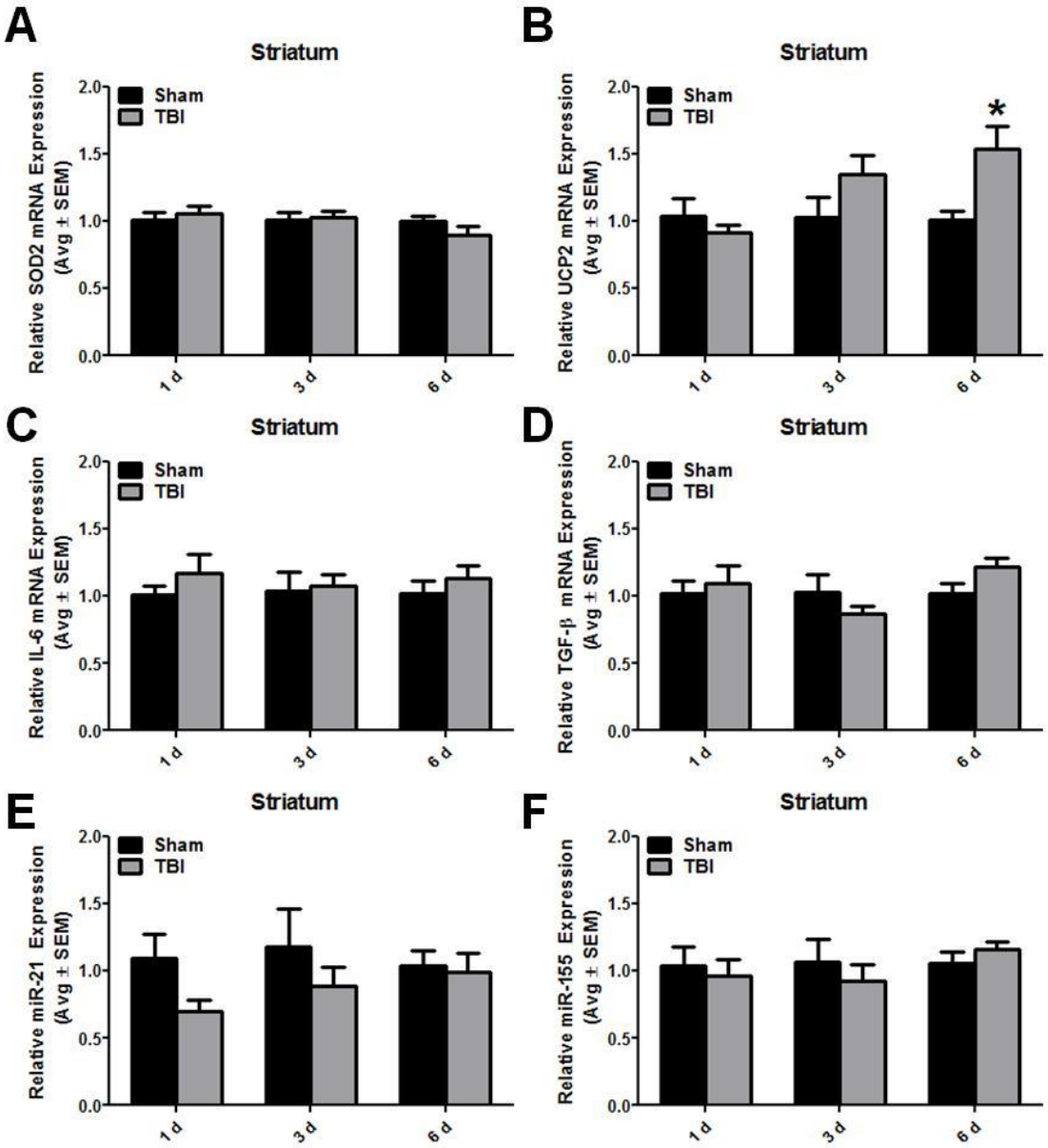

Fig. 4-10. CCI Causes Minimal Alterations in Contralateral Signaling Associated with Mitochondrial Dysregulation. SOD2, UCP2, IL-6 and TGF-β mRNA were measured using tubulin as a control gene. miR-21 and miR-155 expression were determined by RT-PCR using U6 as a control gene. At 1, 3 and 6 d post-injury, SOD2 (A), UCP2 (B), IL-6 (C), TGF-β (D), miR-21 (E) and miR-155 expression (F) were measured in contralateral striatum. Values reported as mean ± SEM. Student's T-test or Mann-Whitney U test was used to determine significance. *$p < 0.05$, n =4-16.

DISCUSSION

Traumatic brain injury is a multi-faceted acute injury state with a complex pathophysiology comprised of diverse pathways. The secondary phase of TBI is comprised of two prominent mechanisms: inflammatory signaling resulting from activation of resident microglia in the affected brain tissues and mitochondrial dysfunction consequent to increased oxidative stress and calcium flux caused by glutamate excitotoxicity. Although traditionally the effects of these pathways have been considered largely independent of each other, research increasingly suggests the existence of an axis between inflammatory and mitochondrial signaling (213, 240).

Current research has identified mitochondrial dysfunction as a target for pharmacological management of TBI; however, therapeutics that have been developed and tested in either animal models or TBI patients have uncovered several limitations in drug discovery for this disease, the most prominent of which is the narrow therapeutic window in which drug administration is effective (126-128, 135, 153, 172, 180, 181). Therefore, identification of a facet of mitochondrial dysfunction that peaks in the sub-acute phase of injury (days-weeks post-insult) and is subject to pharmacological modulation is paramount. Furthermore, little work has focused on characterizing the pattern and time course of mitochondrial dysregulation in separate structures after TBI, the determination of which would guide identification of an ideal therapeutic window for the treatment of post-TBI mitochondrial dysfunction. Finally, differential susceptibilities to mitochondrial injury in diverse tissues and cell types comprising the brain is an understudied area; for example, research into the effects of TBI on hippocampal function and subsequent memory deficit is extensive, whereas little is known about mitochondrial damage in the striatum after TBI, despite the fact that the striatum is increasingly implicated in emotional deficits that persist into the chronic phase of disease (241).

To first assess if differences in mitochondrial homeostasis are apparent in different brain structures and/or different time frames post-TBI, we performed CCI on naïve Long-Evans rats and collected ipsilateral and contralateral cortex, hippocampus and striatum at 1 d, 3 d and 6 d post-injury. Because the model we use delivers a unilateral impact to the sensorimotor cortex (SMC), we are able to assess motor deficits by comparing the percent error made by the affected forelimb during completion of a ladder task to that made by the unaffected forelimb and therefore verify that this injury causes severe deficits that are readily apparent as early as 1 d and persist through 6 d after insult.

To screen for differences in mitochondrial biogenesis and/or mitochondrial content, we measured PGC-1α mRNA and mtDNA copy number in all tissues at all time points collected. At 1 d post-injury, we detected no changes in PGC-1α mRNA or mtDNA copy number in ipsilateral or contralateral cortex, hippocampus or striatum. 3 d after injury, mtDNA was significantly decreased in ipsilateral hippocampus, and both PGC-1α mRNA and mtDNA copy number were significantly decreased in ipsilateral striatum. By 6 d after initial insult, mtDNA copy number had recovered to baseline levels in ipsilateral hippocampus, and PGC-1α mRNA was significantly decreased in both ipsilateral cortex and striatum. No differences in either of these measures were detected in contralateral tissues at 3 or 6 d post-injury.

In light of the decreased mitochondrial homeostasis markers observed in the striatum at 3 and 6 d and cortex at 6 d after impact, we decided to probe more markers of mitochondrial homeostasis in these tissues. We determined that expression of two mitochondrial-encoded components of the ETC was significantly decreased, while no difference was detected in nuclear-encoded NDUFS1 or TFAM mRNA.

Because TFAM was unchanged, the significant decrease in transcription of both mitochondrial-encoded genes is likely due to decreased mtDNA in the days previous to those sampled. In the case of the striatum, decreased mtDNA was detected at 3 d but had recovered by 6 d after injury; interestingly, the mitochondrial-encoded markers were at 60% of control at 3 d post-injury but had increased to 80% of control at 6 d after initial insult; these findings further support a hypothesis that decreased transcription of mitochondrial-encoded genes secondary to depletion of mtDNA is one mechanism by which mtDNA-encoded mRNA are decreased. However, the observation that ND1 and COXI mRNA transcription have not fully recovered when mtDNA has returned to control levels indicates that additional mechanisms for their depletion may also exist. Although a significant decrease in ipsilateral cortical mtDNA was not detected, one explanation is it significantly decreases and then recovers between days 3-6 and that the detected decreases in ND1 and COXI are artifacts of this change.

Since the cortex is the site of impact, it is very interesting that the ipsilateral striatum has significant decreases in mitochondrial homeostasis before any molecular deficits are detected in the cortex. Additionally, it is interesting that the decrease in PGC-1α mRNA in ipsilateral striatum is persistent through 6 d after injury and that the cortex first presents with a decrease in this same marker at this time point, suggesting a continued activation of damage-associated molecular pathways in both tissues.

Although we demonstrated that there were significant decreases in PGC-1α mRNA as well as mitochondrial-encoded gene expression in both the ipsilateral striatum and cortex at 6 d post-injury, no decreases in expression of corresponding proteins were detected. The sustained expression of PGC-1α protein is consistent with the unchanged expression of TFAM and NDUFS1 mRNA observed previously, since their transcription

is induced both directly and indirectly by the interaction of PGC-1α with transcription factors driving their expression. Additionally, these results may indicate that both the genes encoding PGC-1α as well as mitochondrial genes are excessively expressed, with a sensing mechanism that targets these mRNAs for degradation at baseline levels of physiological function (242).

After identifying a pattern of mitochondrial disruption in the sub-acute phase after TBI, we hypothesized that administering a mitochondrial biogenic agent to rats given CCI would reverse the observed deficits in mitochondrial homeostasis markers. Amoxapine, a 5-$HT_{2A/2C}$ antagonist that had previously been shown to induce MB and cross the BBB, was given daily to a group of CCI rats starting at 1 d post-injury, and mitochondrial homeostasis markers were measured 6 d after initial cortical impact. Decreases in mitochondrial homeostasis markers in CCI rats who were given diluent were comparable to those observed in previous experiments. However, contrary to expectations, treatment with amoxapine did not improve any of these mitochondrial measures. These results, paired with our earlier observations that deficits in mitochondrial homeostasis were persistent through the sub-acute phase of injury, suggest the ongoing activation of pathways that suppress several facets of mitochondrial health. It is possible that attempts to support mitochondrial function and biogenesis are not sufficient to overcome persistent activation of suppressive elements and that identifying these suppressive pathways will provide additional targets for pharmacological intervention to improve mitochondrial health and patient outcome after TBI.

Since acute oxidative stress has been demonstrated to contribute to depletion of mtDNA, we first assessed for persistent increases in oxidant markers (161). The antioxidant proteins SOD2 and UCP2 are activated in two phases. Release of ROS in

the early acute injury state activates a protein kinase D/NF-κB signaling pathway that results in the rapid induction of SOD2; after detoxification of acute ROS is complete, UCP2 is upregulated to sequester reactive lipid peroxides from the contents of the mitochondrial matrix by translocating them across the inner mitochondrial membrane and into the inner membrane space (155, 243). Both ipsilateral striatum and cortex have early increases in SOD2 mRNA, which returns to baseline as UCP2 mRNA increases. In the striatum, UCP2 mRNA decreases at 6 d compared to 3 d post-injury, but in the cortex, it continues to increase. These results indicate the persistent presence of reactive oxidative molecules in the mitochondria after TBI and provide a potential mechanism for the observed decrease in mtDNA copy number in the ipsilateral striatum. As previously discussed, it is possible that mtDNA copy number is decreased in the ipsilateral cortex between 3-6 d post-injury and that steadily increasing levels of UCP2 mRNA indicate an ongoing and strongly-induced compensatory mechanism to mitigate continued generation of reactive molecules.

Recent studies have demonstrated several pathways connecting inflammatory signaling and mitochondrial dysfunction (213, 240). Analysis of inflammation markers implicated by others in TBI pathophysiology demonstrated early increases in IL-6 mRNA and increased induction of TGF-β mRNA that became significantly increased at 6 d post-injury in both ipsilateral striatum and cortex. The ipsilateral cortex displayed in an interesting pattern of IL-6 mRNA expression, with a strong induction at 1 d, a return to baseline at 3 d and a significant increase again at 6 d post-injury. Since IL-6 is considered both a pro- and anti-inflammatory cytokine in TBI pathophysiology, one explanation for this pattern is that early increases in this cytokine indicate increased pro-inflammatory signaling while later induction may indicate a transition to an anti-inflammatory state; the concomitant increase in mRNA expression of TGF-β, another

anti-inflammatory mediator in TBI, suggests activation of an anti-inflammatory phase of signaling (140, 149).Finally, we suggest that increased inflammation in the sub-acute phase of TBI contributes to mitochondrial dysregulation through induction of miRNAs, non-coding RNAs that both promote degradation and block translation of their target mRNAs. MiR-21,which is induced by both IL-6 and TGF-β and has been demonstrated to decrease SOD2 mRNA, was increased significantly in both tissues; it returned to baseline in striatum at 6 d but continued to increase in ipsilateral cortex (244-246). These results are consistent with previous reports that miR-21 is increased after TBI in the cortex. Additionally, miR-155, which is induced by TGF-β and suppresses PGC-1α mRNA expression, was significantly increased at all time points in both tissues (244, 247).

Our results suggest that the striatum is highly susceptible to disruptions in mitochondrial homeostasis in the sub-acute phase after TBI and further indicate that these disruptions may be secondary to increased cytokine release and induction of miRNA expression after acute insult. Although our findings are consistent with other reports that the striatum is more sensitive to mitochondrial damage than either cortex or hippocampus, there are other potential explanations for our observations (194). Our model of CCI inflicts a severe cortical injury, and the most injured cells likely undergo rapid necrosis and are degraded by activated microglia; as a result, it is possible that our methods sample the cells that were functional enough to survive and that we are therefore not accurately capturing the effects of early mitochondrial dysfunction. Furthermore, it is possible these surviving cells contain mitochondria with sub-lethal injury and that the delayed disruption in mitochondrial homeostasis represents decompensation of those surviving mitochondria that do not sufficiently recover. The detection of continuing

mitochondrial disruptions in our latest point examined warrants further examination of mitochondrial dysfunction in the period of weeks to months post-injury.

It is also interesting that both miRNAs studied were much more strongly induced in the cortex than the striatum, despite the fact that the striatum seems to be more susceptible to mitochondrial disruptions at those same time points. It is possible that the cortex has a higher baseline expression of PGC-1α mRNA and that, therefore, miR-155 must be more highly induced to cause a decrease in its expression. Given reports that miRNA can exert effects on surrounding tissues via paracrine signaling, it is further possible that the miRNAs detected in the striatum are generated in the cortex and then released by dying cortical cells before signaling to adjacent striatal cells (248, 249).

Finally, the relationship between IL-6 and TGF-β and the disruption of mitochondrial homeostasis is contradictory, since both of these inflammatory mediators are considered pro-survival cytokines (140). It is possible that the observed mitochondrial deficits are due to completely separate mechanisms, with mtDNA copy number depleted by acute oxidative stress and downregulation of PGC-1α mRNA by pro-survival cytokines to decrease mitochondrial generation of ROS. The participation of NF-κB in both induction of SOD2 as well as in the regulation of PGC-1α indicates that inflammatory mediators may play a significant role in the modulation of acute oxidative stress through several coordinated pathways. Therefore, it is possible that disruption of mitochondrial homeostasis in the acute and sub-acute phase of TBI serves a protective role and that pharmacological intervention to increase mitochondrial content or function in this acute phase would have deleterious effects. However, it is further possible that these pathways are beneficial in the early phase, but become maladaptive after the first few days post-injury and that better understanding of the delicate balance between beneficial

and harmful mitochondrial dysregulation in TBI may lead to a better identification of an ideal therapy window and therefore improve mitochondrial therapeutic research. Furthermore, additional research to determine if a causative relationship exists between the identified putative pathways disrupting mitochondrial homeostasis may identify novel upstream targets to mitigate mitochondrial dysfunction following TBI.

CHAPTER 5

SUMMARY, CONTRIBUTION AND FUTURE DIRECTIONS

SUMMARY OF CURRENT LITERATURE

Pharmacological Induction of Mitochondrial Biogenesis for Treatment of AKI

Acute injury states in multiple organ systems share common pathophysiological features, including vascular disturbances, inflammation and mitochondrial dysfunction. Mitochondrial dysfunction is a critical mechanism of subcellular injury after ischemic injury in organs with high energy requirements, such as kidney and brain, and improvement of mitochondrial function through pharmacological induction of mitochondrial biogenesis (MB) or modulation of newly described mitochondrial suppressive pathways is a novel therapeutic option for treatment of acute organ injury that provides a variety of potential drug targets.

Previous work with our *in vitro* primary renal proximal tubule (RPTC) model demonstrated that oxidant stress induced mitochondrial dysfunction, as measured by decreased cellular respiration and ATP content, and that the return of these injury markers to baseline preceded recovery of cellular function (119). Furthermore, treatment of cells exposed to oxidant injury with pharmacological agents demonstrated to induce mitochondrial biogenesis increased markers of mitochondrial function, including mitochondrial protein expression, ATP content and cellular respiration (47, 48, 56).

Additional work in our laboratory has demonstrated that both acute ischemia/reperfusion acute kidney injury (I/R-AKI) and folic acid-induced AKI (FA-AKI) induced a suppression

of mitochondrial mRNA and protein expression that was detectable at 1 d post-injury and persisted through 6 d post-injury (122). Treatment of I/R-AKI mice with formoterol, a β_2-adrenergic receptor agonist previously demonstrated to induce MB in naïve mouse kidney, improved mitochondrial mRNA and protein expression and mitochondrial respiration and promoted recovery of renal function as demonstrated by decreased serum creatinine and reduced renal tubular necrosis in kidneys of formoterol-treated I/R-AKI mice (120). Finally, sildenafil, a PDE5 inhibitor and inducer of MB in both RPTC and naïve mouse kidney, increased mitochondrial mRNA expression and mtDNA copy number in FA-AKI mouse kidney and decreased kidney injury molecule-1 (KIM-1) expression (52).

These results suggest that pharmacological induction of MB is a viable strategy to reverse mitochondrial dysfunction following AKI and that reversal of mitochondrial dysfunction promotes recovery of renal function in multiple etiologies of AKI. Since acute injury in many organ systems share mitochondrial dysfunction as a common pathophysiological feature, these results may indicate that MB is a potential target for treatment of multiple acute organ injury states.

Further work in our laboratory identified the 5-HT_2 class of receptors as a potential target for the induction of MB. Treatment of primary RPTC with the 5-HT_2 non-selective agonist DOI increased cellular respiration and increased mitochondrial protein expression (56). Additionally, DOI promoted recovery of cellular respiration after TBHP-induced oxidant injury in RPTC, further indicating that the 5-HT_2 class of receptors is a viable target for pharmacological induction of MB and promotion of cellular recovery after AKI. However, the 5-HT_2 receptor class is composed of three diverse receptors, 5-$HT_{2A,}$

5-HT $_{2B}$ and 5-HT$_{2C}$, and more work was warranted to determine if an individual receptor in this class is responsible for the observed biogenic effects of DOI.

Mitochondrial Dysfunction in Traumatic Brain Injury

Like AKI, mitochondrial dysfunction as a result of acute oxidant stress is the primary mechanism of cell death following traumatic brain injury (TBI). In the immediate phase after initial insult, necrosis occurs secondary to total metabolic failure and ATP depletion. The secondary phase of injury is marked by subcellular injury that converges on mitochondrial dysfunction as a result of glutamate toxicity and ischemia, during which rapid increases in ROS generation cause propagation of oxidative stress through generation of reactive biomolecules such as lipid peroxides and protein carbonyls (135, 151, 152, 157, 158). These altered biomolecules disrupt the structure of the inner mitochondrial membrane (IMM), which distorts its structure and diminishes efficacy of the ETC components, further propagating ROS production at Complexes I and III (108, 152, 159).

Glutamate excitotoxicity causes an excessive influx of calcium, leading to an increased intracellular calcium concentration both in tissues directly affected by mechanical injury and those in the surrounding affected penumbra of injury (164). Excess intracellular calcium can lead to activation of calpains and other proteases, so mitochondrial sequestration of calcium through the mitochondrial calcium uniporter is important in the maintenance of cytosolic calcium homeostasis (135, 156, 158, 165). However, excessive calcium influx into the mitochondria can activate the mitochondrial permeability transition (MPT), in which the opening of mitochondrial permeability transition pores results in collapse of the mitochondrial proton gradient, ATP depletion and release of cytochrome c into the cytosol (152, 166, 167). Once translocated to the

cytosol, cytochrome c initiates cleavage of caspases 9 and 3, resulting in apoptosis (137, 166).

Both acute oxidative stress and calcium dysregulation cause a pattern of mitochondrial dysfunction with multiple potential targets for modulation of mitochondrial damage signaling after TBI. Current mitochondrial-directed therapies for TBI have focused on two main targets: reduction of oxidative injury using antioxidant therapy and electron transport chain bypass as well as inhibition of apoptotic signaling through inhibition of the MPT. However, these therapies have been severely limited by a number of factors, including narrow therapeutic windows for efficacy and a poor understanding of the time course of mitochondrial disruption and dysfunction after injury (126-128, 135, 153).

Although much research has implicated oxidative stress and apoptosis as the primary drug targets for mitigation of mitochondrial injury following TBI, little work has focused on the disruptions in mitochondrial biogenesis or alterations in mtDNA copy number and mitochondrial mRNA and protein expression. Furthermore, research in other ischemic organ injury states has demonstrated that mitochondrial homeostasis is persistently disrupted in days to weeks after insult, indicating that these patterns of mitochondrial disruption may have relatively large therapeutic windows in which support of mitochondrial function contributes to survival of cells that have been exposed to a sub-lethal injury (52, 120). Therefore, further exploration of the effects of TBI on mitochondrial homeostasis and function may reveal novel therapeutic targets to prevent cell death in the sub-acute phase of injury.

Finally, there is increasing evidence in other organ systems that cytokines released during inflammatory pathway activation after acute injury may suppress mitochondrial

biogenesis and homeostasis through direct interaction of inflammatory mediators with PGC-1α (213, 240). Additionally, current research has identified microRNAs (miRNAs) induced by both pro- and anti-inflammatory cytokines that are responsible for both direct and indirect down-regulation of several mitochondrial homeostasis targets, including SOD2 and PGC-1α (244-247, 250). Although the expression of two of these miRNAs, miR-21 and miR-155, have been demonstrated in the brain in acute injury states, no work to date has linked their expression to decreased mitochondrial homeostasis after TBI (246, 251).

CONTRIBUTIONS TO THE FIELD

The Role of the 5-HT_2 Receptor in Mitochondrial Biogenesis

Previous work in our laboratory demonstrated that the non-selective 5-HT_2 receptor agonist DOI was capable of inducing MB in primary RPTC. However, the 5-HT_2 receptor class is composed of three receptor subtypes, 5-$HT_{2A,}$5-HT_{2B} and 5-HT_{2C}, with different patterns of expression and physiological functions, and the dose of DOI used was non-specific for any of these receptor subtypes. Using a Seahorse Extracellular Flux assay to screen for biogenic molecules, we identified two potent inducers of MB: 5-HT_{2C} receptor agonist CP-809,101 and 5-HT_{2C} receptor antagonist SB-242,084, both of which increased FCCP-uncoupled oxygen consumption at nanomolar concentrations. Because 5-HT_{2C} receptor protein expression was previously described as being entirely localized to CNS tissue, we verified its expression in both whole mouse, rat and rabbit kidney as well as in RPTC, reporting for the first time expression of this receptor outside the CNS. After confirming that the receptor was expressed in our tissues of interest, we verified that both CP-809,101 and SB-242,084 increased PGC-1α mRNA expression, indicating that the observed increases in cellular respiration were due to increased MB. Therefore, we reported that both an agonist and antagonist paradoxically elicited the

same phenotypic response in the same cell type and sought to further characterize the biogenic potential of these compounds in renal tissue.

Both CP-809,101 and SB-242,084 induced increases in PGC-1α mRNA expression as well as expression of both nuclear- and mitochondrial-encoded mRNAs for components of the ETC in the kidney cortex of naïve C57BL/6 mice at 1 mg/kg doses, further indicating that these compounds were potent inducers of MB.

Because both an agonist and antagonist elicited the same biogenic effect, we proposed that these compounds might exert their biogenic effects through two different 5-HT_2 receptors via non-canonical signaling pathways. Because these compounds both considered specific ligands for the 5-HT_{2C} receptor, we administered both drugs to mice lacking this receptor. In the absence of this receptor, our markers of MB were more strongly induced, indicating that the action of these compounds at this receptor in the renal cortex is not responsible for their observed biogenic effect. Interestingly, however, we did observe a 20% decrease in PGC-1α mRNA expression in diluent-treated 5-HT_{2C} receptor KO mice compared to diluent-treated WT mice, suggesting that the 5-HT_{2C} receptor may play a novel role in the endogenous maintenance of mitochondrial homeostasis in the kidney.

After discovering that these compounds did not induce MB through the 5-HT_{2C} receptor, we next hypothesized that their biogenic actions were due to interaction with the 5-HT_{2A} receptor. We repeated our previous experiment using 129Sv mice lacking the 5-HT_{2A} receptor but failed to elicit increases in our biogenic markers in the WT mice. We posit that these observed differences in the ability of these compounds to induce MB may be due to the effect of strain differences in 5-HT_2 receptor biology, mitochondrial biogenesis

regulation or drug clearance capabilities. Given our inability to interpret the results of our 5-HT_{2A} receptor KO mouse experiment, we treated RTPC with siRNA directed toward either the 5-HT_{2A} or 5-HT_{2C} receptor for 72 h and then treated them with either the agonist CP-809,101 or antagonist SB-242,084. Both CP-809, 101 and SB-242,084 increased PGC-1α mRNA in RPTC treated with either negative control siRNA or 5-HT_{2C} receptor siRNA , but RPTC treated with 5-HT_{2A} receptor siRNA had no change in PGC-1α mRNA expression with either drug. These results strongly indicate that agonism of the 5-HT_{2A} receptor is mechanism by which 5-HT_2 receptor ligands induce mitochondrial biogenesis and that the 5-HT_{2A} receptor is a novel target for the induction of MB to treat mitochondrial dysfunction and promote cellular recovery in acute renal injury states.

Although both the agonist CP-809,101 and antagonist SB-242,084 induce MB in renal cortex, neither drug is a good candidate for further pharmaceutical development. In further testing to determine safety, CP-809,101 was determined to be genotoxic, while recent experiments with SB-242,084 in primates have suggested that it has a psychostimulant effect that may contribute to abuse potential (75, 252). Due to the risks inherent in developing a novel drug through Phase I clinical trials, we decided to screen compounds within our target classes that have already passed safety testing in humans. While no potent agonists of the 5-HT_{2A} receptor have undergone extensive pharmaceutical development, we identified a number of FDA-approved pharmaceuticals that act as potent antagonists at the 5-HT_{2A} receptor and found that the drug amoxapine, an SSRI/SNRI that also acts as a potent antagonist at both the 5-HT_{2A} and 5-HT_{2C} receptor, increased FCCP-uncoupled cellular respiration in a primary screening assay. Further investigation of this compound revealed that amoxapine increased PGC-1α mRNA in RPTC and that this observed increase was dependent on the 5-HT_{2A} receptor,

which supported our earlier observations that the 5-HT_{2A} receptor was responsible for the biogenic capacity of 5-HT_2 receptor ligands.

Amoxapine was demonstrated to increase PGC-1α mRNA expression in naïve mouse kidney but had no effect on PGC-1α mRNA expression in CNS tissues, including frontal cortex, hippocampus and striatum. These results indicate that this compound may induce biogenesis through a non-canonical signaling pathways downstream of the 5-HT_{2A} receptor, which has been demonstrated to couple pleiotropically to multiple G proteins (66). In addition to non-canonical coupling to the $G_{i/0}$ receptor, the 5-HT_{2A} receptor has been shown to couple to both phospholipase D (PLD) and phospholipase A_2 (PLA_2) (58, 65, 66). Finally, it has been demonstrated that some 5-HT_{2A} receptor ligands can signals independent of G protein coupled through β-arrestin-mediated activation of Akt/GSK3β (67, 69, 70).

Finally, amoxapine did not significantly increase mitochondrial biogenesis or homeostasis markers in renal cortex of FA-AKI mice and had no beneficial effect on either renal function or survival post-injury. Given that sildenafil, an inhibitor of intracellular PDE5, did recover some mitochondrial content and renal function after FA-AKI, these results were surprising. It is possible that this very severe injury induces dedifferentiation and migration cells in sub-lethally injured renal tubular cells, which causes downregulation of 5-HT_{2A} receptor at the cell membrane, or that disruption of the tubular cell membrane is sufficient to effect 5-HT_{2A} receptor expression at the cell surface. These results suggest that this class of receptors may not be a viable target for the treatment of severe acute renal tubular dysfunction, but that they may still have utility in treatment of chronic mitochondrial dysfunction that persists after the migrated tubular cells have proliferated and redifferentiation.

Disruptions in Mitochondrial Homeostasis Following TBI

Given the body of work that exists about mitochondrial dysfunction as a result of increased oxidative stress and calcium dysregulation following TBI, we hypothesized that mitochondrial homeostasis and biogenesis would be disrupted in the ipsilateral cortex and in the in the penumbra surrounding the initial injury site in the first week after insult in a controlled cortical impact (CCI) rat model of TBI. We found that the first detected decreases in mtDNA copy number expression of PGC-1α, ND1 and COXI mRNA were in the ipsilateral striatum; expression of the three mRNAs remained suppressed through 6 d post-injury but mtDNA copy number recovered at this time. The cortex had a delayed disruption in mitochondrial homeostasis but displayed a decrease in PGC-1α, ND1 and COXI mRNA at 6 d after injury. These results indicate persistent activation of pathways that contribute to mitochondrial dysregulation and may serve as therapeutic targets for treatment of TBI. However, the protein expression of all of these markers remained unchanged, indicating that there may be an early compensation mechanism to support MB as well as ETC function in the face of decreased mRNA expression.

We next hypothesized that treatment of CCI rats with a therapeutic that induces MB would be a beneficial strategy for management of mitochondrial disruption after TBI. We chose to use the previously described biogenic compound amoxapine because it was first designed as a neuroactive drug and is known to cross the BBB. However, amoxapine treatment did not significantly increase any of the suppressed markers of mitochondrial homeostasis at 6 d post-injury. Since this CCI model is considered a severe injury, it is possible that the cell damage is too harsh for effective recovery of

mitochondrial homeostasis or cellular function. It also provides further evidence of persistent activation of damage pathways that continue to actively suppress mitochondrial content regulation and/or function and suggests that attempts to induce MB may not be able to overcome these suppressive mechanisms.

We further hypothesized that these suppressive mechanisms may exert differential effects on markers of mitochondrial homeostasis. A detected increase in SOD2 mRNA at 1 d and UCP2 mRNA at 3 d and 6 d post injury in cortex and striatum provide evidence of both increased ROS production as well as propagation of oxidative stress by lipid peroxides and may represent a mechanism of mtDNA depletion, which has been demonstrated to occur as a result of acute oxidant injury (161). Time-dependent expression patterns of the cytokines IL-6 and TGF-β indicate the induction of both pro- and anti-inflammatory signaling and subsequent increases in their downstream targets miR-21 and miR-155 provide a putative link between the observed cytokine signaling and mitochondrial disruptions in both ipsilateral striatum and cortex. The absence of mitochondrial suppression as well as lack of antioxidant, cytokine or miRNA induction in contralateral tissues may lend further support to the existence of a link between these damage mediators and disruption of mitochondrial homeostasis.

FUTURE DIRECTIONS

The Role of the 5-HT_2 Receptor in Mitochondrial Biogenesis

Further research is warranted into the potential signaling pathways that lead to induction of MB through the 5-HT_{2A} receptor. One prominent question that is yet to be answered is if both agonists and antagonists elicit MB through an identical signaling pathway or through divergent signaling pathways. Given the body of research that indicates that the 5-HT_{2A} receptor can couple to a number of non-canonical signal pathways, it is likely that

these ligands signal through separate pathways to induce biogenesis. There are several techniques that can be employed to detect divergent signaling pathways that lead to induction of MB by these compounds. The first technique is to probe for release of signaling molecules that have been previously connected to biogenic signaling. There are commercially available ELISA kits that allow for detection of cAMP and cGMP after stimulation with a compounds; previous work in our laboratory has successfully reported the involvement of these mediators, particular cGMP, in the stimulation of MB in primary RPTC, making this signaling molecule a high-yield target for elucidation of 5-HT_{2A}-induced MB signaling (52). Additional work in our laboratory has successfully utilized pretreatment with a panel of inhibitors for common biogenic signaling pathways to probe for involvement of these pathways in biogenic signaling in response to physiological stimulus; pretreatment of RPTC with these inhibitors prior to administration of known 5-HT_{2A} receptor-targeted biogenic agents and subsequent measurement of PGC-1α mRNA expression may provide further information as to the signaling pathway(s) that mediate 5-HT_{2A} receptor-induced MB (38).

The final potential direction for this project is to use bioinformatics to examine chemical features within the 5-HT_{2A} receptor ligands that induce MB and to determine if our biogenic agonists and antagonists share chemical elements that could provide evidence of signaling through similar 5-HT_{2A} receptor-mediated mechanisms. Additionally, these comparisons of similarity can also identify a potential pharmacophore to design improved biogenic ligands for this receptor. Alternately, use of this approach to compare differences between 5-HT_{2A} receptor ligands that induce MB and those that do not may help identify the critical drug-receptor molecular interactions for successful induction of MB.

Disruptions in Mitochondrial Homeostasis Following TBI

There are many exciting avenues for further characterization of the patterns of mitochondrial disruption observed after acute CCI and for elucidation of potential suppressive pathways that inhibit recovery of mitochondrial function in naïve CCI rats or those treated with biogenic agents.

Because there is evidence that mitochondrial homeostasis is still disrupted at the latest time point we analyzed after TBI, it would be beneficial to follow patterns of disruption in the time period of weeks to months after CCI. The maintenance of mitochondrial protein expression, despite persistent decreases in PGC-1α, ND1 and COXI mRNA expression, indicates that there may be a compensatory mechanism to maintain mitochondrial function in the sub-acute phase of days after injury. It would be interesting to determine if such a compensatory mechanism exists and if eventually the mitochondria decompensate and begin to demonstrate decreases in mitochondrial protein expression.

Another exciting avenue of research is the elucidation of potential mitochondrial suppression pathways. Our current research has indicated that surrogate markers of persistent oxidative injury, inflammatory mediators and suppressive miRNAs downstream of these inflammatory mediators are all increased in ipsilateral striatum and cortex when a concomitant decrease in mitochondrial homeostasis markers is also observed. Neither these mediators nor mitochondrial dysregulation are detected in the contralateral tissues, which can be considered further evidence of the relationship between these damage pathways and mitochondrial disruption after TBI. Pharmacological agents that block these signaling pathways are commercially available. Pretreatment with antioxidants or with lipid peroxide scavengers can probe the relationship between persistent oxidative stress and depletion of mtDNA copy number.

Additionally, pretreatment with a monoclonal antibody directed toward IL-6 or with the TGF-β receptor antagonist GW 788388 could reveal a relationship between these agents and decreased mitochondrial homeostasis markers and would also indicate if expression of these cytokines leads to increased miR-21 and/or miR-155 expression. Finally, pretreatment with antagomirs that target and neutralize miR-21 and miR-155 would provide evidence of the relationship between these microRNAs and mitochondrial suppression following TBI. Although custom synthesis of these antagomirs would be prohibitively expensive, they are currently produced by Regulus Therapeutics, a company that has previously collaborated with academic researchers to answer questions such as ours. Although delivery of these antagomirs to the brain is another potential prohibitive factor, we may be able to harness the pathological increase in BBB permeability following TBI to increase delivery of these therapeutics. These exciting experiments could elucidate for the first time important signaling mechanisms leading to disruption of mitochondrial homeostasis and function after TBI. Elucidation of these pathways would also identify a number of novel drug targets for treatment of TBI and increase options for combination therapy to promote mitochondrial recovery after TBI.

A hypothesis proposed by other research groups is that differing cell populations in the striatum and cortex may contribute to their differential susceptibilities to mitochondrial injury (194). Therefore, immunohistological examinations of tissue sections collected following TBI could identify if antibody-labeled mitochondrial components are differentially altered in these different cell types.

Additional potential experiments include the development of a more clinically relevant model of TBI, since our current CCI model mimics severe injury. The majority of clinical cases are either mild or moderate in severity. The benefit of development of various

severity models is two-fold. First, it would allow us to determine if our observations about the striatum's increased susceptibility to mitochondrial disruption compared to that of the cortex is valid. It is possible that the severe model causes fulminant injury to the affected cortex, resulting in massive cell death, and that cells sampled in our experiments are those that are functioning well enough to survive; if this is the case, the decrease in PGC-1α mRNA expression observed a 6 d post-injury in this tissue may indicate persistent insult to mitochondrial structure and homeostasis and may represent decompensation of surviving sub-lethally injured mitochondria. To probe this hypothesis, a potential experiment would be to decrease the depth of penetration of the Impactor and determine whether cortical mitochondrial disruptions were detected at earlier time points. Finally, the current severity of our CCI model may limit our ability to effectively promote mitochondrial recovery, and mild to moderate TBI may be more responsive to treatment with MB agents.

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