Summer 2014

Effects of DFO-Induced Hypoxia on Key Signaling Mediators

Jennifer Candelora
jennifer.candelora@student.shu.edu

Effects of DFO-induced Hypoxia on Key Signaling Mediators

Jennifer Candelora

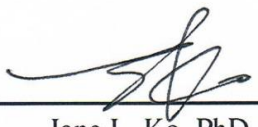
Submitted in partial fulfillment of the requirement for the

Degree of Master of Science in Biology from the

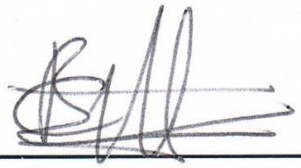
Department of Biological Sciences of Seton Hall University

August 2014

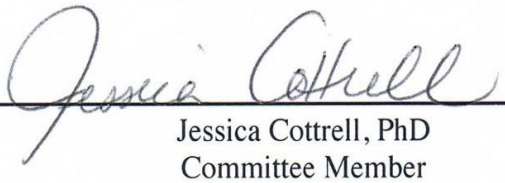
Approved by



Jane L. Ko, PhD
Mentor



Constantine Bitsaktsis, PhD
Committee Member



Jessica Cottrell, PhD
Committee Member

Jane L. Ko, PhD
Chair, Department of Biological Sciences

Acknowledgements

I could not be more grateful for the opportunity to complete my Master's Degree at Seton Hall University and would like to extend my deepest thanks and gratitude to all those who have made the journey, not only possible, but successful. I would like to thank Dr. Ko for her mentorship and guidance, as well as for giving me the opportunity to work with her in lab. I would also like to extend my thanks to my committee members, Dr. Constantine Bitsaktsis and Dr. Jessica Cottrell for their support and reassurance. Also, a special thanks to all the faculty, staff, and fellow students of the Biological Sciences Department at Seton Hall University.

My fellow lab mates also deserve full-hearted thanks for their hard work and companionship. Thanks to Natasha Pavlichko for her constant encouragement and Ashley Alexandre for always making me think on my feet. Also, another special thanks to Alberto Herrera for being dedicated to his undergraduate research.

Thanks are also extended to my friends, whose light-heartedness helped keep me grounded. Thanks also goes out to my family, to my parents, Betsy and Timothy Candelora, as well as to my sisters Faith and Victoria. And finally, I would also like to thank my fiancé, Matthew Babcock, for his selflessness, support and helpfulness in regards to my ambitions.

Table of Contents

List of Figures

Abstract

While diseases such as cancer and diabetes, or surgery and traumatic injury can cause hypoxia through a decrease in blood circulation to bodily regions or decrease cardiac output they can also associate with hypoxia-induced pain. Clinically, opioids, such as morphine, are used to modulate pain. The mu-opioid receptors (MORs) are one of three main types of opioid receptors and are key mediators in morphine-induced analgesia. Therefore, in this study, the effect of hypoxia on MOR gene expression was examined using human neuronal cells treated with DFO to create a hypoxic-mimic condition. We found that MOR expression was shown to decrease over increased exposure times to DFO. Recently, our laboratory reported that receptor associated C kinase (RACK-1), interacting with the transcription regulator Poly C Binding Protein (PCBP-1), can negatively regulate MOR gene expression using a yeast two-hybrid screening system. RT-PCR analysis revealed an increased in RACK-1 expression while a decrease in MOR expression under DFO treatment was observed. These results supported our recent finding that RACK-1 participates in the regulation of MOR expression. Neuronal cells surviving the DFO insult also displayed activation of the JAK/STAT pathway through Western blot analysis. Due to activation, the suppressors of cytokine signaling (SOCS) proteins, negative regulators of the JAK/STAT pathway, were investigated and detected via RT-PCR. These results suggest that there is an up-regulation of the SOCS proteins under hypoxia, and it may play a role in a form of neuroprotection by aiding in the decrease of inflammation associated with the JAK/STAT pathway.

Introduction

Disease is a term defined as a condition that affects a living organism, which impairs normal function, and is typically evident by distinguishing signs or symptoms. Diseases like diabetes affect 23.6 million people in the United States, according to the National Institute of Health. Patients with conditions such as physical trauma, cardiac arrest, pneumonia, stroke, diabetes, or cancer are considered to be enduring a disease, which can be identified by signs or symptoms that affect the associated bodily region. These diseases, and many others, share a common symptom: hypoxia. While diseases such as diabetes cause hypoxia through a decrease in blood circulation to the bodily regions, operation and post-operative events can also result in hypoxia by decreasing cardiac output due to the use of anesthesia. Since hypoxia is a symptom, as well as an outcome, within disease, it has become an important event to be investigated.

Hypoxia is a shortage in the supply of oxygen available for cellular consumption. The stress presented upon cells under hypoxia causes a reduction in the amount of ATP within the cell. For example, neurons under hypoxia are unable to maintain membrane potential and result in depolarization, calcium influx, and eventually cell death (Won et al, 2002). Hypoxia is a condition that is noxious to normal cells and cancer cells, however cancer cells have the ability to adapt to the low oxygen environment through the development of changes that allow them to survive as well as proliferate (Harris, 2002).

To survive a hypoxic assault, neurons enlist mechanisms that perpetuate survival and adaptation. One of the mechanisms that cells utilize in response to hypoxia involves a transcription factor known as hypoxia-inducible factor-1 (HIF-1) (Michiels, 2004; Vangeison et al, 2008). HIF-1 can up-regulate or down-regulate the expression of assorted genes against the hypoxic insult (Vangeison et al, 2008; Gao et al, 2013). This transcription factor is composed of two subunits,

HIF-1α and HIF-1β. Under normoxia, HIF-1α interacts with the Von Hippel-Lindau (VHL) tumor suppressor protein, a component of the E3 ubiquitin ligase complex (Warnecke et al, 2003). The oxygen sensation mechanism utilized for HIF-1α/VHL complex recognition is comprised of the oxygen dependent enzymatic hydroxylation of two conserved proline residues (Warnecke et al, 2003). The enzymes for these reactions are known as prolyl hydroxlases (PHDs) and are essential for the proteaosomal degradation of HIF-1α. This interaction causes ubiquitination of HIF-1α and leads to its labeling for proteaosomal degradation. Under hypoxia, HIF-1α accumulates and translocates into the nucleus where it forms a heterodimer with HIF-1β leading to the binding to hypoxia response elements (HREs). This binding increases activation of transcription and expression of several target genes, such as the glycolytic enzymes, vascular endothelial growth factor (VEGF) that leads to angiogenesis, as well as proapoptotic genes (Vangeison et al, 2008; Yokogami et al, 2013).

Mu-Opioid Receptor Regulation

Many compounds have been identified to create the hypoxic mimic condition; these hypoxic mimicking compounds include cobalt chloride ($CoCl_2$) (Wang and Semenza, 1993), nickel chloride ($NiCl_2$), and desferoxamine (DFO) (Wang and Semenza, 1993). DFO acts as a ferrous iron chelator and inhibits the prolyl hydroxylases that are essential for the proteaosomal degradation of HIF-1α (Harris, 2002). Increased DFO concentration has been shown to reduce neuronal NMB cell viability and increase cellular glutathione levels in the surviving cells (Figure 1A; Cook et al, 2010). The neurons that survived the DFO insult also do not exhibit any significant morphological changes in comparison to the non-treated neuronal cells. (Figure 1B; Cook et al,

2010). The surviving cells, however, did present with increased endogenous HIF-1 mRNA levels over extended exposure to DFO (Figure 1C; Cook et al, 2010). Another important feature was the decrease in mu-opioid receptor (MOR) mRNA levels within DFO treated cells. Opioid receptors are known to mediate pain sensation and they may also play a role in the survival. However, there are controversial results in MORs role in neuroprotection (Feng et al, 2012).

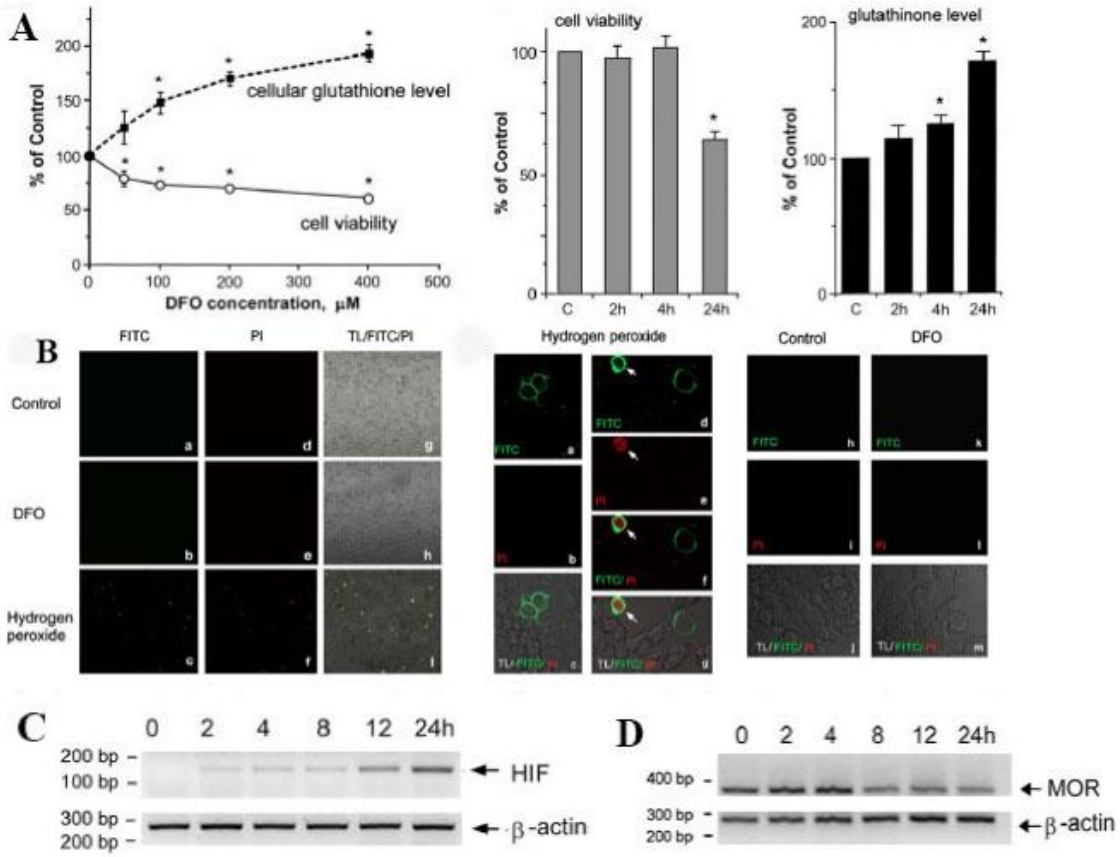


Figure 1: (A) Human NMB cells were treated with varying concentrations of DFO for 24 hours. Viability was measured (open circles) as the percentage of total cell number divided by the control (100%). Glutathione levels were measured similarly. Cell viability and glutathione levels were also measured in cells treated with 200 μM DFO for 2, 4, and 24 hours. (B) Effect of DFO exposure on cellular morphology of NMB cells. Cells surviving, control (no treatment), DFO, and hydrogen peroxide (positive control) exposure were stained using annexin-V-FLUOS (green color) and PI (red color). Images were obtained using confocal microscopy. (C) DFO treatment increases the expression of HIF-1 mRNA levels. Cells were treated with DFO for 2, 4, 8, 12, and 24 hours. RNA was extracted from cells without (control) and with DFO treatment. RT-PCR was performed using specific primers for HIF-1 and beta-actin (internal standard). (D) Effect of DFO on MOR mRNA levels. Cells were treated with DFO for 2, 4, 8, 12, and 24 hours. RNA was extracted from cells without (control) and with DFO treatment. RT-PCR was performed using specific primers for MOR and beta-actin (internal standard). (A-C) Adapted from Cook et al, 2010.

MOR expression and activation has been significantly studied, due to morphine's high affinity for the receptor, for inducing analgesia and tolerance (Le Merrer et al, 2009). These characteristics have made MOR the focus of pain management and addiction; and have led to the discovery of a reduction in functional MORs upon chronic opioid treatment (Christie et al, 1987). However, the exact mechanisms for the regulation of functional MORs under various conditions have yet to be completely uncovered (Le Merrer et al, 2009). One of the transcriptional regulators for the regulation of MOR gene expression was identified as poly C binding protein 1 (PCBP1), in both mouse (Ko and Loh, 2005) and human species (Cook et al, 2010; Nahar-Gohad et al, 2013). PCBP1 has been shown to participate in multiple levels of gene expression, from stimulating transcription, regulating RNA splicing and acting as a translational repressor (Choi et al, 2009). Recently, a new role for PCBP1 was discovered (Nahar-Gohad et al, 2013). It has been shown to directly interact with RACK-1, receptor activated C-kinase (Figure 2; Nahar-Gohad et al, 2013), and using human neuronal NMB cells RACK-1's interaction with PCBP-1 causes a decrease in the available PCBP-1, not allowing translocation of the transcriptional regulator into the nucleus to initiate transcription (Nahar-Gohad et al, 2013). Therefore, this study determined whether such a regulatory mechanism occurs under the hypoxia condition.

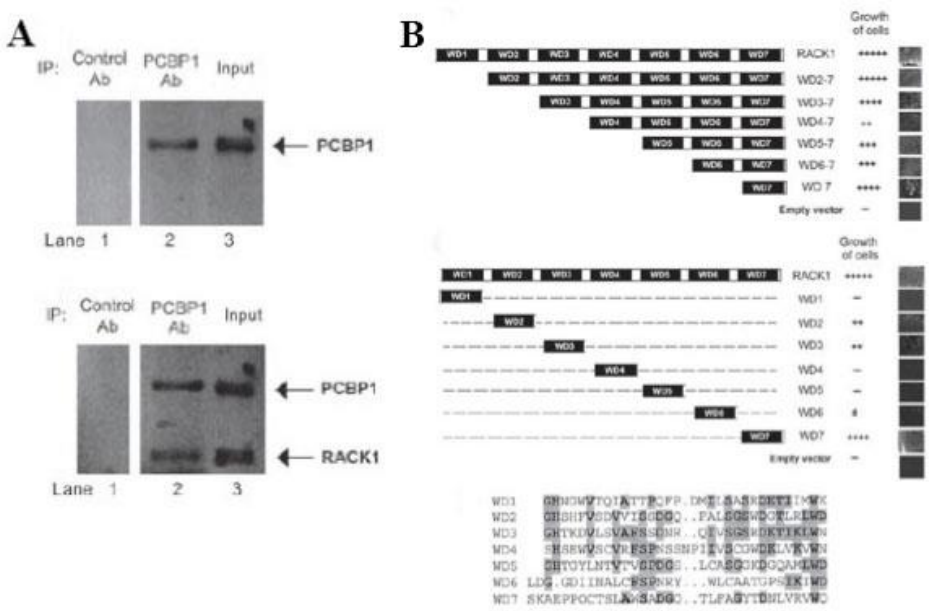


Figure 2: (A) Co-immunoprecipitation of PCBP1-RACK1. Using Western Blot analysis, whole NMB cell lysates were incubated with anti-PCBP1 or a non-specific IgG antibody (control). The same lysates were then probed with anti-RACK1 antibody. (B) Determination of RACK1-PCBP1 interaction domain using deletional analysis. Illustrates full length RACK1 and truncated RACK1 fragments. Each was cloned into pTRG vectors and each construct was co-transformed with full length PCBP1 using the two-hybrid system. Interaction between RACK1 and PCBP1 was determined by growth ability. Illustrates full-length RACK1 and individual domain constructs used to determine the interaction capability between RACK1 and PCBP1 using the two-hybrid system. Also present is the amino acid comparison of all RACK1 WD domains, with conserved amino acids highlighted. (A-B) Adapted from Nahar-Gohad et al, 2013.

JAK/STAT Activation and Suppression

Several signal transduction pathways are known to be involved in cell survival. Previous experimentation has shown the activation of the JAK/STAT pathway under DFO induced hypoxia (Rasmussen, 2012). The activation of the JAK/STAT pathway leads to many processes that stimulate cell proliferation, differentiation, migration, and apoptosis, all of which are required to support homeostasis (Rawlings et al, 2004).

Signaling through the JAK/STAT pathway can be initiated by a variety of ligands and their receptors. Ligands, such as cytokines (examples: interleukin (IL)-6, IL-11, and interferon (IFN)-alpha) bind to their receptors inducing dimerization of JAKs, which associate with the cytoplasmic domains of the receptor (Inagaki-Ohara et al, 2013; Yu et al, 2007). Dimerization leads to autophosphorylation of the JAK units at specific tyrosine residues, these residues further act as docking sites for transcription factors. Signal transduction and activation of transcription (STATs) factors are one of the main groups of transcription factors that interact with the activated JAK and become phosphorylated (p-STAT) at tyrosine residues. The p-STAT dimerizes for translocation into the nucleus where it will influence the initiation and transcription of multiple target genes. For example, STAT3, the protein of interest within these experiments, targets anti-apoptotic genes, pro-proliferation genes, and angiogenesis (Yu et al, 2009; Ogura et al, 2008; Yokogami et al, 2013). Regulation of the JAK/STAT pathway is mediated through a series of proteins known as suppressors of cytokine signaling (SOCS). These proteins produce a negative feedback loop to prevent excess activation of the JAK/STAT pathway (Qin et al, 2012).

This study therefore, examined the inhibition of pathways under the hypoxic response. The balance between the activation and the suppression of the JAK/STAT pathway is delicate and

important for normal homeostatic function (Rawlings et al, 2004). There are eight members of the SOCS family (SOCS1-7 and CIS), all of which are characterized by a homologous SH2 domain that allows their binding to tyrosine phosphorylated molecules and a C-terminal SOCS box that is essential for ubiquitination (Boyle et al, 2009; Babon et al, 2012). Two of the eight inhibitory proteins are considered potent suppressors, SOCS1 and SOCS3, which have an additional kinase inhibitory region (KIR) located near the N-terminal (Boyle et al, 2009). The KIR region allows for direct inhibition of JAK activity (Babon et al, 2012). It has been suggested that SOCS3 binds to both the JAKs and the receptor simultaneously, which leads to inhibition of specific cytokine produced signals (Babon et al, 2012). SOCS3 has been shown to be a non-competitive inhibitor of JAK, specifically inhibiting the ability to transfer a phosphate to the specific tyrosine of the adapting molecule (Inagaki-Ohara et al, 2013).

Irregularities in the activation of STAT3, such as overexpression or activation, can lead to increased expression of genes required for proliferation, survival, angiogenesis, and inflammation (Yu et al, 2009). The overexpression of these genes leads to tumorigenesis due to reduced expression of SOCS3, which is the main regulator of STAT3. For example, due to STAT3 activation and translocation, an increase in the growth factor VEGF can be seen (Yokogami et al, 2013). The increase is VEGF further activates the JAK/STAT3 pathway to create a mechanism that continuously ensures the presence of STAT3 in tumor cells (Yu et al, 2007). It has been shown that SOCS3 is essential for the regulation of cancer progression. In the pancreas, through the deletion of SOCS3, aberrant expression of STAT3 escalates the progression of pancreatic intraepithelial neoplasias and pancreatic ductal adenocarcinoma through the IL-6 activated STAT3/SOCS3 pathway (Lesina et al, 2011). SOCS3 has been suggested to function as a tumor suppressor that undergoes down regulation through hypermethylation of the SOCS3 promoter in

a variety of cancers (Inagaki-Ohara et al, 2013). However, SOCS3 is continuously expressed in melanoma cells, which may suggest a protective function (Fojtova et al, 2007). Previously, an increase in p-STAT3 has been seen within neuronal cells treated for extended periods of time with DFO (Figure 3; Rasmussen, 2012; *unpublished data*), which would lead to the assumption that SOCS3 levels would be low. Due to the inconsistency in the expression of SOCS3 between cancers, it is necessary to further understand the relationship between SOCS3 and other microenvironment conditions, such as hypoxia in this study.

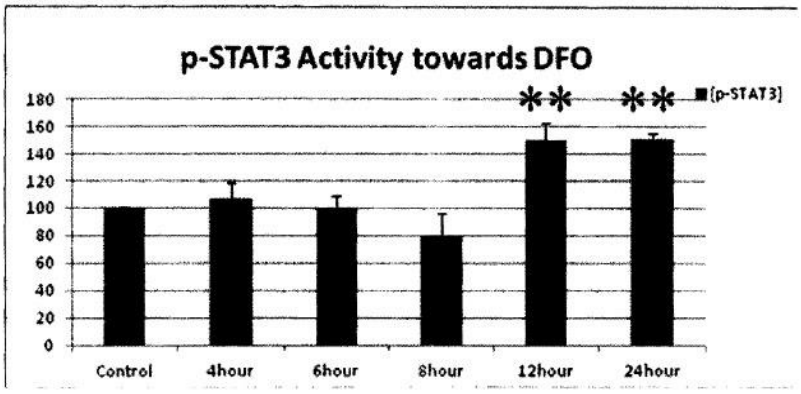


Figure 3: Phosphorylated STAT3 expression under extended exposure to DFO. NMB cells were exposed to DFO for 4, 6, 8, 12, and 24 hours. Western blot analysis revealed p-STAT3 levels. Quantification of p-STAT3 was done by normalization with beta-tubulin. Values are shown +/- SE. "**" Indicating a p value less than 0.005. (Rasmussen, 2012, *unpublished data*).

Materials and Methods

Cell Culture:

Human Neuroblastoma cells (NMB) were maintained in Roswell Park Memorial Institute Media (RPMI) (Invitrogen, CA) with 10% Fetal Bovine Serum infused with antibiotics, streptomycin and penicillin, in a 37 degrees Celsius and 5% CO_2 incubator. Cells were treated with single doses of 0.1 mM of desferoxamine (DFO) and incubated for varying exposure times. The DFO used is a product of Sigma-Aldrich (St. Louis, MO).

RNA Isolation:

Total RNA was extracted using Tri-reagent (Invitrogen, CA) and chloroform. The mixture was then centrifuged at 12000 RPM for 5 minutes at 4 degrees Celsius. The top phase, of the three phase separation, was removed as it contained the RNA, which was precipitated using isopropanol (molecular grade, Sigma-Aldrich). The mixture then underwent centrifugation for 5 minutes at 12000 RPM and 4 degrees Celsius. The RNA pellet was washed with 70% ethanol and allowed to dry. The pellet was resuspended in diethyl pyrocarbonate (DEPC) treated water. Concentration of the RNA was determined through spectrophotometry and calculated using absorbance at 260 nm.

RT-PCR:

Reverse transcription was performed using RNA for the synthesis of first strand cDNA and random hexamer primer (Qiagen, CA). The cDNA was synthesized for 50 minutes at 37 degrees Celsius and 15 minutes at 70 degrees Celsius. PCR amplification was performed under the settings

of 95 degrees Celsius for 1 minute, 68 degrees Celsius for 30 seconds annealing, and 72 degrees Celsius for 30 seconds for 32 cycles of the MOR gene. RACK1 gene amplification was performed under the settings of 95 degrees Celsius for 1 minute, 67 degrees Celsius for 30 seconds, and 72 degrees Celsius for 30 seconds over 21 cycles.

Agarose Gel Electrophoresis:

The products of PCR amplification were run on 2% aragose gels which contained ethidium bromide. Samples were allowed to separate for 30 minutes at 120 volts. Images of the gel and products were taken utilizing Alpha Imager with UV illumination. All products underwent quantification using Image Quant version 5.2.

Statistical Analysis

All acquired data was normalized and presented as +/- SEM. Statistical comparison between the groups, control and treatments, were preformed by use of the Student T-Test. All analysis was completed by use of InStat with statistical significance defined as $P<0.05$.

Results

Determination of MOR mRNA levels under hypoxia

The prevalence of hypoxia in many diseases as well as in operative and post-operative scenarios makes the mechanism by which MOR is regulated an important research objective. In addition, MOR is involved in the management of pain; therefore, the expression of MOR was investigated under the influence of DFO induced hypoxia in this study. Previous results have shown a decrease in MOR mRNA levels during various exposure periods of DFO treatment (Figure 1D). To determine if the decrease of MOR expression was maintained during a longer DFO exposure (48 hours), the level of MOR mRNA was examined. RNAs were extracted from NMB neuronal cells treated with DFO for 2, 4, 8, 12, 24, and 48 hours or with no treatment (control). RT-PCR was performed with specific MOR primers as well as beta-actin primers and PCR products were analyzed. Beta-actin was used as an internal standard for normalization. Figure 4 shows the gradual decrease in the mRNA levels of MOR over the time course. These results show that the mRNA levels of MOR gradually decreased significantly from 12 hours ($p<0.001$), 24 hours ($p<0.001$), and 48 hours ($p<0.001$) upon exposure to DFO.

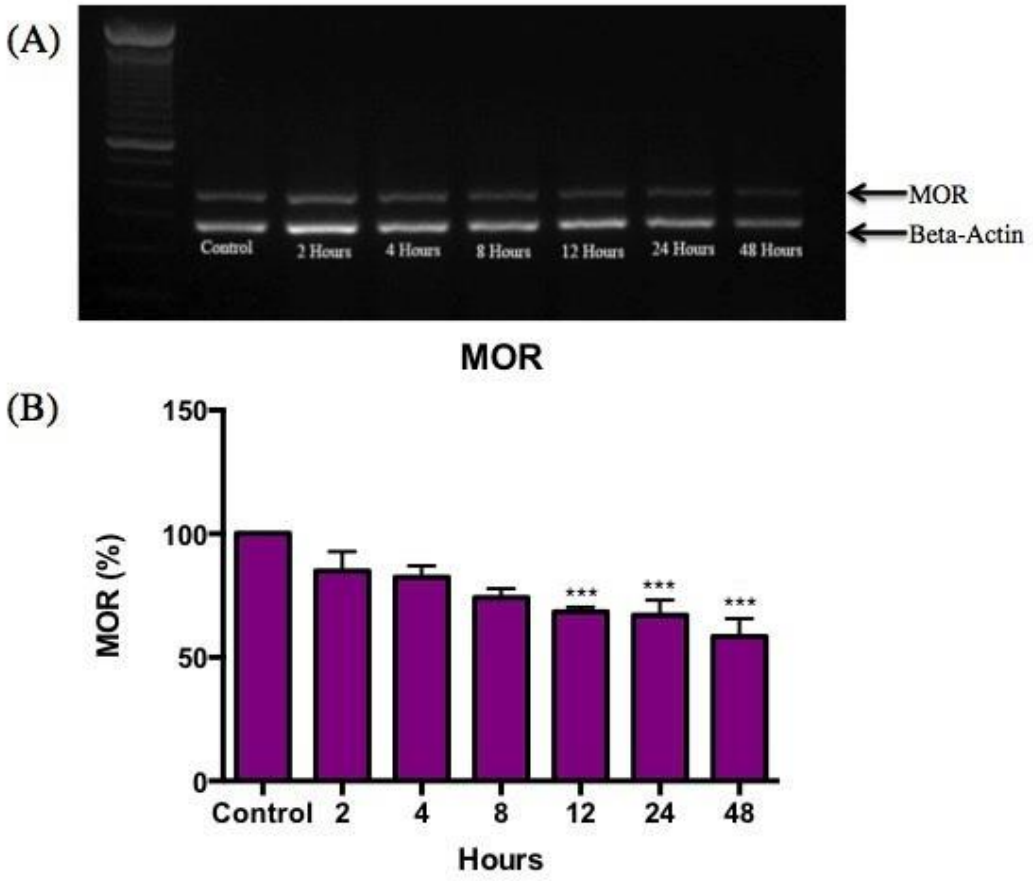


Figure 4: Expression of MOR mRNA in NMB cells under DFO exposure
(A) Results from RT-PCR analysis showing MOR gene expression in NMB cells treated with DFO with beta-actin as an internal control for normalization. Image shows the expression of MOR in treated and nontreated (control) samples of NMB cells plated (1 x 10^6), treated with 300 μM of DFO at 2, 4, 8, 12, 24, and 48 hours, and analyzed on a 2% agarose gel. (B) Normalized mRNA levels from control were defined as 100%. Quantitative analyses from five different experiments are presented as mean±SE, "***" indicating P<0.001.

Determination of RACK1 mRNA levels under hypoxia

Recently, our laboratory has demonstrated that RACK1 can negatively regulate MOR expression (Figure 2; Nahar-Gohad et al, 2013). RACK1 acts as a scaffolding protein and directly interacts with PCBP1, the MOR transcriptional regulator (Nahar-Gohad et al, 2013). Therefore, to examine if the RACK1 message was increased under DFO treatment, the expression of RACK1 under DFO-induced hypoxia was examined. RNAs were extracted from neuronal NMB cells treated without or with DFO for 2, 4, 8, 12, 24, and 48 hours. RT-PCR was performed with specific RACK1 primers as well as with beta-actin primers and the PCR products were analyzed. Beta-actin was used as an internal standard for normalization. Figure 5 shows the gradual increase in the mRNA levels of RACK1 overtime. The results show that the mRNA levels of RACK1 gradually increase significantly from 24 hours ($P<0.01$) to 48 hours ($P<0.001$) upon exposure to DFO. Taken together, Figure 4 and Figure 5 show an inversely proportional relationship between the mRNA expression of MOR and the mRNA expression of RACK1.

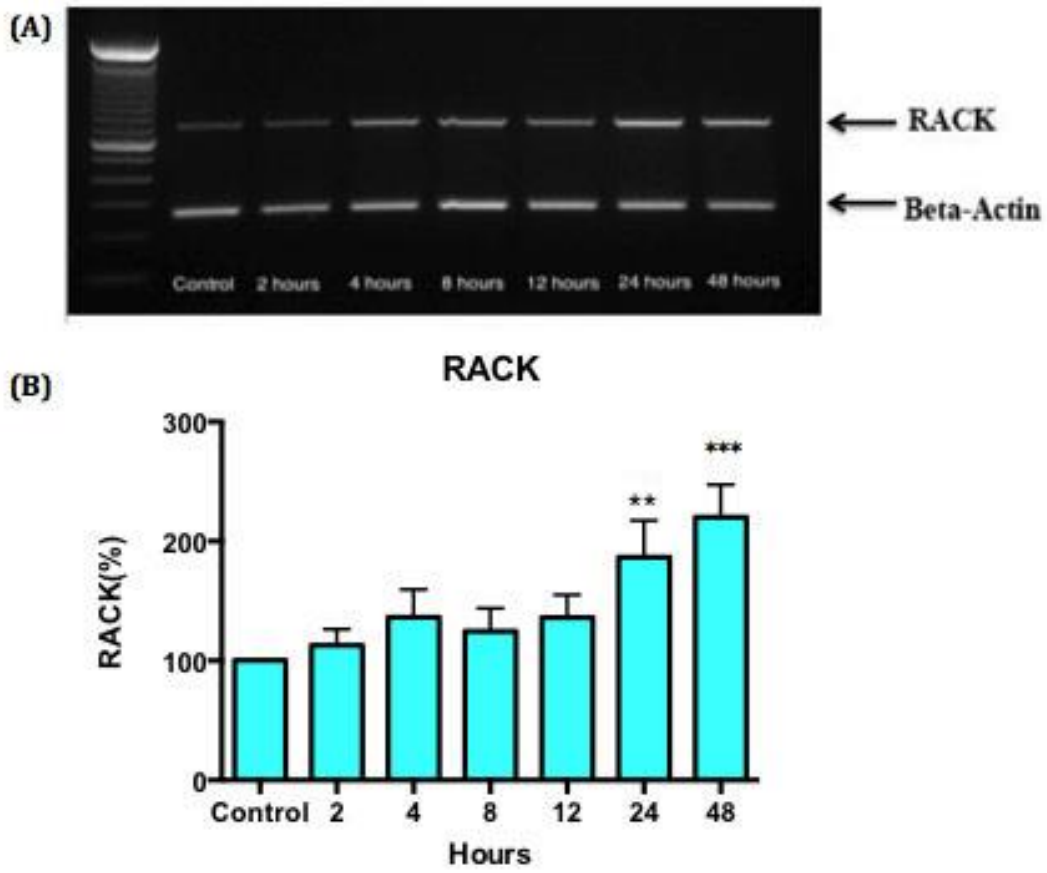


Figure 5: Expression of RACK1 mRNA in NMB under DFO exposure
(A) Results from RT-PCR analysis showing RACK1` gene expression in NMB cells treated with DFO with beta-actin as an internal control for normalization. Image shows the expression of RACK1 in treated and nontreated (control) samples of NMB cells plated (1 x 10^6), treated with 300 μM of DFO at 2, 4, 8, 12, 24, and 48 hours, and analyzed on a 2% agarose gel. (B) Normalized mRNA levels from control were defined as 100%. Quantitative analyses from nine different experiments are presented as mean±SE, "**" indicating P<0.01 and "***" indicating a P<0.001.

JAK/STAT Activation and Suppression

Determination of SOCS3 mRNA levels under hypoxia

Activation of the JAK/STAT pathway is crucial in the maintenance of homeostasis, and it can affect both proliferation and apoptosis by phosphorylation of STAT3; conditional on the initiation of specific target genes (Inagaki-Ohara et al, 2013). Formerly, Western Blot analyses showed that the level of p-STAT3 was activated within neuronal cells under DFO-induced hypoxia over a time course (Figure 3; Rasmussen, 2012; *unpublished data*). Results of these analyses show a significant increase in p-STAT3 levels at 12 and 24 hours ($p<0.005$).

The delicate balance between activation and suppression is key for normal homeostatic function. Therefore, the presence of an inhibitory mechanism to prevent excess JAK/STAT activation, such as the increase of SOCS3 levels, is essential. The levels of SOCS3 mRNA were then investigated. RNAs were extracted from neuronal NMB cells treated without or with DFO for 2, 4, 8, 12, 24, and 48 hours. RT-PCR was performed with specific primers for SOCS3 as well as with primers for beta-actin. PCR products were analyzed via gel electrophoresis. Beta-actin was used as the internal standard for normalization. Figure 6 showed the gradual increase in the mRNA levels of SOCS3 overtime. In summary, these results demonstrated that the mRNA levels of SOCS3 gradually increase with a significant increase seen at 24 hours ($P<0.05$) and48 hours ($P<0.005$) upon the exposure to DFO.

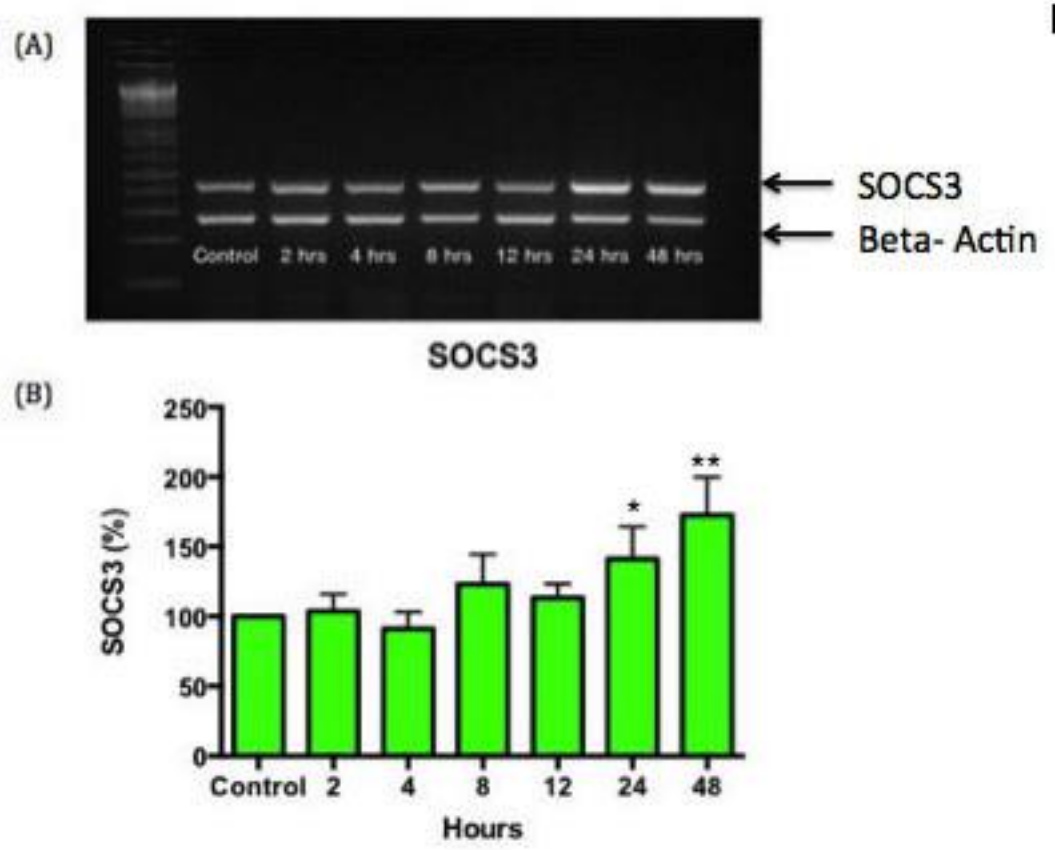


Figure 6: Expression of SOCS3 mRNA in NMB under DFO exposure
(A) Results from RT-PCR analysis showing SOCS3 gene expression in NMB cells treated with DFO with beta-actin as an internal control for normalization. Image shows the expression of SOCS3 in treated and nontreated (control) samples of NMB cells plated (1 x 10^6), treated with 300 μM of DFO at 2, 4, 8, 12, 24, and 48 hours, and analyzed on a 2% agarose gel. (B) Normalized mRNA levels from control were defined as 100%. Quantitative analyses from nine different experiments are presented as mean±SE, "*" indicating P<0.05 and "**" indicating P<0.005.

Discussion

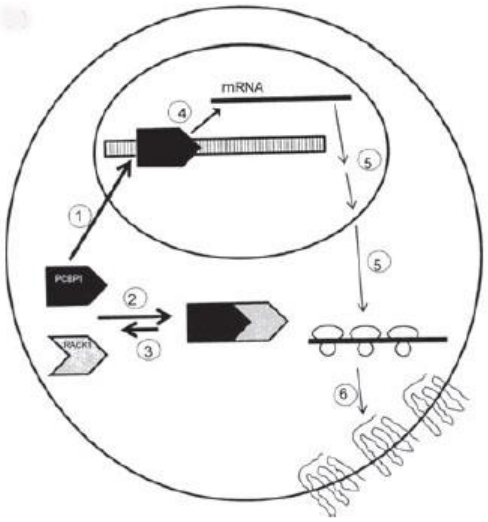
Cancer, disease, surgery, or traumatic injury can elicit the hypoxic condition and can further instigate pain sensation mediated through MORs. Therefore, it was important to investigate the mechanism of MOR expression under DFO induced hypoxia. In this study, we report that the data gathered under DFO induced hypoxia follows the theory that MOR mRNA expression reduction is a direct affect of the interaction between PCBP1, the MOR transcriptional regulator, and RACK1.

Under DFO induced hypoxia, NMB neuronal cells have shown a decrease in MOR mRNA levels over increased exposure times (Cook et al, 2010). In this study, the decrease is MOR mRNA levels under DFO induced hypoxia was verified and shows consistency with previous studies performed. In addition, MOR mRNA levels remained low under longer exposure to DFO (Figure 4). MOR expression has been shown to be regulated by PCBP1 at the level of transcription (Ko and Loh, 2005; Malik et al, 2006; Rivera-Gines et al, 2006; Cook et al, 2010) and has since been shown, though two-hybrid screening and co-immunoprecipitation, that there is a direct interaction between PCBP1 and RACK1 (Nahar-Gohad et al, 2013). Here, through mRNA analysis, MOR mRNA levels decreased over increased exposure times to DFO (Figure 4) while RACK1 mRNA levels increased over the same exposure times (Figure 5). These results agreed that DFO induced hypoxia influences a reduction in MOR expression via the same PCBP1-RACK1 interaction as shown in our lab's recent publication (Figure 7; Nahar-Gohad et al, 2013).

There is a long history of the use of opioid receptors as pain modulators. MOR has been extensively studied due to its actions in the mediation of morphine (Al-Hasani and Bruchas, 2011; Feng et al, 2012), which has lead to the inquiry of MORs potential affect on pain sensation due to

hypoxia. Under hypoxia, sensitization of high-threshold receptors allows an increase in pain with disease progression (Regan and Peng, 2000). A decrease in MOR mRNA expression has also been seen in the studies of many diseases such as fibromyalgia (Harris et al, 2007) and opiate addiction (Feng et al, 2012), as well as in studies of inflammatory pain (Aoki et al, 2014). The decrease in the availability of functional MORs lead to an increase in pain, as well as a decrease in the ability to treat pain with opiates (Harris et al, 2007; Aoki et al, 2014). With RACK1 playing a negative regulatory role in the regulation of MOR gene expression (Nahar-Gohad et al, 2013), this raises the question of what elevates RACK1 expression. This study, therefore, implicated that the change of RACK1 expression can be pathologically mediated; down-regulation or up-regulation of RACK1 seems to depend on the pathological location.

Alterations in RACK1 expression are associated with a variety of disorders; including brain development disorders, heart failure, renal failure, cancer, and others (Adams et al, 2011). Elevation in the expression of RACK-1 has been proposed to be a marker of prognosis in breast cancer (Cao et al, 2010) and elevation in expression has also been seen in various other cancers such as heptacellular carcinoma (Ruan et al, 2012), and melanoma (Lopez-Bergami et al, 2007). This data suggests that the increase in RACK1 expression reduces the ability to manage pain by decreasing the amount of MOR receptors available on the cells surface, which supports the notion that hypoxia can augment pain sensation due to the decrease in MOR expression (Regan and Peng, 2000). However, where the exact mechanism initiates MOR down-regulation and/or up-regulation of RACK1 will requires more comprehensive studies.



Adapted from Nahar-Gohad et al, 2013.

Figure 7: Simplified diagram showing the modularity of PCBP1 in MOR expression. PCBP1 (black) can be free to translocate into the nucleus to bind to DNA and initiate the transcription of the MOR mRNA that will then be translated into a functional receptor. When RACK1 (White) is available, PCBP1 (Black) with bind to RACK1, which will keep the transcriptional regulator from translocating into the nucleus to initiate transcription and leads to a decrease in the expression of MOR mRNA.

JAK/STAT Activation and Suppression

The balance between activation and inhibition of pathways is important to maintain homeostasis and adaptation for cell survival. Neuronal cells that survived the influence of DFO have shown evidence of JAK/STAT pathway activation by the presence of p-STAT3 observed by Western blot analysis. The detected signal for p-STAT3 was also observed to increase over exposure to DFO (Figure 3; Rasmussen, 2012; *unpublished data*). Together, these results lead to the investigation of the pathways main inhibitor, SOCS3, which is essential to maintain the balance between phosphorylated STAT3 and further activation of the pathway (Rawlings et al, 2004).

In this study, RT-PCR has shown an increase in mRNA levels of SOCS3 under increased exposure times to DFO (Figure 6), while the p-STAT3 signal also increased (Figure 3; Rasmussen, 2012; *unpublished data*). Together, these results indicated that the balance between the JAK/STAT pathway and SOCS3 may be important for neuroprotection under DFO-induced hypoxia challenge. Certainly, more studies will be required to determine the function and the related mechanisms.

SOCS3 inhibits through direct interaction with the JAK subunits and the related receptors simultaneously, blocking the phosphorylation sites that are required for further phosphorylation of the STAT3 factors and preventing advancement of signal (Babon et al, 2012; Kershaw et al, 2013). Over expression of SOCS3 has been seen in human neuroblastoma and is suggested to decrease the protective effect of STAT3 mediated insulin-like growth factor against TNF-α induced cell death (Baker et al, 2009). However, the increased presence of SOCS3 has been suggested to be correlated to the severity of inflammation. Inflammatory studies performed on a murine model of periodontal disease has shown a parallel increase is SOCS3 and p-STAT3 over the duration of inflammation and has led to the suggestion that the negative regulation that SOCS3 provides is

important for the regulation of expression of genes that are responsible for connective tissue degradation (Chaves de Souza et al, 2013). A similar elevation pattern in the expression of both STAT3 and SOCS3 has also been seen in glioblastoma and is suggested to contribute to its anti-apoptotic phenotype and malignancy (Baker et al, 2009). Together, these results reveal the controversy that plaques SOCS3's function and mechanism, as well as provides the reasoning for further exploration of its role in inflammation and neuroprotection.

Previously, Cook et al reported the increase in HIF-1 in neuronal NMB cells under DFO induced hypoxia at increasing exposure times, while the experiments presented here show an increase in SOCS3 under the same conditions (Figure 6). Interestingly, this parallel increase is similar to what has been seen in adipocytes under $CoCl_2$ induced hypoxia. Through ChIP assays, the SOCS3 gene was determined to possess two potential HREs and shown to be a direct target of HIF-1 (Jiang et al, 2013). These reported results suggest a more complex mechanism behind SOCS3 expression that goes beyond the concentration of p-STAT3 present within the activated pathway. More experimental analysis could lead to more mechanical similarities between the studies and may lead to a further understanding of the role SOCS3 plays in neuroprotection under DFO induced hypoxia.

References

Adams, D.R., Ron, D. and Kiely, P.A. (2011) RACK1, A multifaceted scaffolding protein: Structure and Function. *Cell Communication and Signaling*, 9(22).

Al-Hasani, R. and Bruchas, M.R. (2011) Molecular Mechanisms of Opioid Receptor-Dependent Signaling and Behavior. *Anesthesiology,* 115(6): 1363-1381.

Aoki, Y., Mizoguchi, H., Watanabe, C., Takeda, K., Sakurada, T. and Sakurada, S. (2014) Potential Involvement of μ- Opioid Receptor Dysregulation on the Reduced Antinociception of Morphine in the Inflammatory Pain State in Mice. *Journal of Pharmacological Sciences,* 124(2): 258-266.

Babon, J. and Nicola, N.A. (2012) The biology and mechanism of action of Suppressor of Cytokine Signaling 3 (SOCS3). *Growth Factors,* 30(4): 207- 219.

Babon, J., Kershaw, N.J., Murphy, J.M., Varghese, L.N., Laktyushin, A., Young, S.N., Lucet, I.S., Norton, R.S., and Nicola, N.A. (2012) Suppression of Cytokine Signaling by SOCS3: Characterization of the Mode of Inhibition and the Basis of its Specificity. *Immunity,* 36(2): 239-250.

Baker, B.J., Akhtar, L.N. and Benveniste, E.N. (2009) SOCS1 and SOCS3 in the Control of CNS Immunity. *Trends in Immunology*, 30(8): 392-400.

Boyle, K., Zhang, J.G., Nicholson, S.E., Trounson, E., Babon, J., McManus, E.J., Nicola, N.A., and Robb, L. (2009) Deletion of the SOCS box of suppressor of cytokine signaling 3 (SOCS3) in embryonic stem cells reveals SOCS box-dependent regulation of JAK but not STAT phosphorylation. *Cellular Signaling,* 21(3):394-404.

Cao, X.X., Xu, J.D., Xu, J.W., Liu, X.L., Cheng, Y.Y., Wang, W.J., Li, Q.Q., Chen, Q., Xu, Z.D., and Liu, X.P. (2010) RACK1 promotes breast carcinoma proliferation and invasion/metastasis in vitro and in vivo. *Breast Cancer Research Treatment.* 123(2):375-386.

Chaves de Souza, J.A., Nogueira, A.V., Chaves de Souza, P.P., Jung Kim, Y., Lobo, C.S., Lopes de Oliveira, G.J.P., Cirelli, J.A., Garlet, G.P. and Rossa Jr, C. (2013) SOCS3 Expression Correlates with Severity of Inflammation, Expression of Proinflammatory Cytokines, and Activation of STAT3 and p38 MAPK in LPS-Induced Inflammation *In Vivo*. *Mediators of Inflammation,* 2013.

Christie, M.J., Williams, J.T and North, R.A. (1987) Cellular Mechanisms of Opioid Tolerance: Studies in Single Brain Neurons. *Molecular Pharmacology.* 32(5):633-638.

Choi, H., Kim, C.S., Hwang, C.K., Song, K.Y., Law, P., Wei, L. and Loh, H.H. (2007) Novel function of the poly(c)-binding protein alpha-CP3 as a transcriptional repressor of the mu opioid receptor gene. *The FASEB Journal.* 21(14):3963-3973.

Choi, H., Hwang, C., Song, K., Law, P.Y., Wei, L.N. and Loh, H.H (2009) Poly (C)-binding Proteins as Transcriptional Regulators of Gene Expression. *Biochemical and Biophysical Research Communications.* 380(13): 431-436.

Cook, R., Karch, C., Nahar, P. and Ko, J. (2010) Effects of desferoxamine-induced hypoxia on neuronal human mu-opioid receptor gene expression. *Biochemical and Biophysical Research Communications.* 398(1): 56-61.

Feng, Y., He, X., Yang, Y., Chao, D., Lazarus, L. and Xia, Y. (2012) Current research on opioid receptor function. *Current Drug Targets*. 13(2): 230-246.

Fojtova, M., Boudny, V., Kovarik, A., Lauerova, L., Adamkova, L., Souckova, K., Jarkovsky, J., and Kovarik, J. (2007) Development of IFN-Gamma resistance is associated with attenuation of SOCS genes induction and constitutive expression of SOCS3 in melanoma cells. *British Journal of Cancer,* 97(2): 231-237.

Harris, Adrian L. (2002) Hypoxia – A Key Regulatory Factor in Tumor Growth. *Nature Reviews Cancer*. 2(1): 38-47.

Harris, R.E., Clauw, D.J., Scott, D.J., McLean, S.A., Gracely, R.H. and Zubieta, J.K. (2007) Decreased Central μ-Opioid Receptor Availability in Fibromyalgia. *The Journal of Neuroscience,* 27(37): 10000-10006.

Inagaki-Ohara, K., Kondo, T., Ito, M. and Yoshimura, A. (2013) SOCS, inflammation, and cancer. *JAKSTAT,* 2(3): ePublished.

Jiang, C., Kim, J.H., Li, F., Qu, A., Gavrilova, O., Shah, Y.M. and Gonzalez, F.J. (2013) Hypoxia-inducible Factor 1α Regulates a SOCS3-STAT3-Adiponectin Signal Transduction Pathway in Adipocytes. *The Journal of Biological Chemistry,* 288(6): 3844-3857.

Kershaw, N.J., Murphy, J.M., Liau, N.P.D., Varghese, L.N., Laktyushin, A., Whitlock, E.L., Lucet, I.S., Nicola, N.A. and Babon, J.J. (2013) SOCS3 binds specific receptor-JAK complexes to control cytokine signaling by direct kinase inhibition. *Nature Structural and Molecular Biology,* 20(4): 469-476.

Ko, J. L. and Loh, H. H. (2005) Poly C binding protein, a single-stranded DNA binding protein, regulates mouse mu-opioid receptor gene expression. *Journal of Neurochemistry.* 93(3): 749-761.

Le Merrer, J., Becker, A.J., Befort, K. and Kieffer, B. L. (2009) Reward Processing by the Opioid System in the Brain. *Physiological Reviews.* 89(4): 1379-1412.

Lesina, M., Kurkowski, M.U., Ludes, K., Rose-John, S., Treiber, M., Kloppel, G., Yoshimura, A., Reindl, W., Sipos, B., Akira, S., Schmid, R.M. and Algul, H. (2011) Stat3/Socs3 Activation by IL-6 Transsignaling Promotes Progression of Pancreatic Intraepithelial Neoplasia and Development of Pancreatic Cancer. *Cancer Cell*, 19(4): 456-469.

Lopez-Bergami, P., Huang, C., Goydos, J., Yip, D., Bar-Eli, M., Herlyn, M., Smalley, K.S.M., Mahale, A., Eroshkin, A., Aaronson, S. and Ronai, Z. (2007) Rewired Erk-jnk signaling pathways in melanoma. *Cancer Cell.* 11(5): 447-460.

Makeyev, A.V. and Liebhaber, S.A. (2002) The poly (c)-binding proteins: A multiplicity of functions and a search for mechanisms. *RNA,* 8(3): 265-278.

Malik, A.K., Flock, K.E., Godavarthi, C.L., Loh, H.H. and Ko, J.L. (2006) Molecular basis underlying poly-c binding protein 1 as a regulator of the proximal promoter of mouse mu-opioid receptor gene. *Brain Research,* 1112(1): 33-45.

Michiels, C. (2004) Physiological and Pathological Responses to Hypoxia. *American Journal of Pathology,* 164(6): 1875-1882.

Nahar-Gohad, P., Sultan, H., Esteban, Y., Stabile, A. and Ko, J. (2013) RACK1 identified as the PCBP1 interacting protein with a novel functional role in the regulation of human MOR gene expression. *Journal of Neurochemistry*. 124(4):466-477.

Ogura, H., Murakami, M., Okuyama, Y., Tsuruoka, M., Kitabayashi, C., Kanamoto, M., Nishihara, M., Iwakura, Y., and Hirano, T. (2008) Interleukin-17 Promotes Autoimmunity by Triggering a Positive-Feedback Loop via Interleukin-6 Induction. *Immunity,* 29(4):628-636.

Qin, H., Yeh, W.I., De Sarno, P., Holdbrooks, A.T., Liu, Y., Muldowney, M.T., Reynold, S.L., Yanagisawa, L.L., Fox III, T.H., Park, K., Harrington, L.E., Raman, C. and Benveniste, E.N. (2012) Signal transducer and activator of transcription-3/suppressor of cytokine signaling 3 (STAT3/SOCS3) axis in myeloid cells regulates neuroinflammation. *Proceedings of the National Academy of Sciences,* 109(13): 5004-5009.

Rasmussen, L. (2012) Alteration of Protein Kinases' Activities by Hypoxic Mimic Compounds. *Theses, Seton Hall University,* Paper 213.

Rawlings, J.S., Rosler, K.M. and Harrison, D.A. (2004) The JAK/STAT Signaling Pathway. *Journal of Cell Science,* 117(8):1281-1283.

Regan, J.M. and Peng, P. (2000) Neurophysiology of Cancer Pain. *Cancer Control,* 7(2): 111-119.

Rivera-Gines, A., Cook, R.J., Loh, H.H. and Ko, J.L. (2006) Interplay of Sps and poly(C) binding protein 1 on the mu-opioid receptor gene expression. *Biochemical & Biophysical Research Communications,* 345(1): 530-537.

Ruan, Y., Sun, L., Hao, Y., Wang, L., Xu, J., Zhang, W., Xie, J., Guo, L., Zhou, L., Yun, X., Zhu, H., Shen, A. and Gu, J. (2012) Ribosomal RACK1 promotes chemoresistance and growth in human hepatocellular carcinoma. *The Journal of Clinical Investigation.* 122(7): 2554-2566.

Vangeison, G., Carr, D., Federoff, H.J. and Rempe, D.A. (2008) The Good, the Bad, and the Cell Type-Specific Roles of Hypoxia Inducible Factor-1α in Neurons and Astrocytes. *The Journal of Neuroscience,* 28(8): 1988-1993.

Wang, G.L. and Semenza, G.L. (1993) Desferrioxamine induces erythropoietin gene expression and hypoxia-inducible factor 1 DNA-binding activity: implications for models of hypoxia signal transduction. *Blood Journal,* 82(12): 3610-3615.

Warnecke, C., Griethe, W., Weidemann, A., Jurgensen, J.S., William, C., Bachmann, S., Ivashchenko, Y., Wagner, I., Frei, U., Wiesner, M. and Eckardt, K. (2003) Activation of the hypoxia-inducinle factor-pathway and stimulation of angiogenesis by application of prolyl hydroxylase inhibitors. *FASEB Journal*. 17(9): 1186-1188.

Won, S.J., Kim, D.J. and Gwag, B.J. (2002) Cellular and molecular pathways of ischemic neuronal death. *Journal of Biochemistry and Molecular Biology* 35(1): 67-86.

Yokogami, K., Yamashita, S. and Takeshima, H. (2013) Hypoxia-induced decreases in SOCS3 increase STAT3 activation and upregulate VEGF gene expression. *Brain Tumor Pathology,* 30(3):135-143.

Yu, H., Kortylewski, M. and Pardoll, D. (2007) Crosstalk between cancer and immune cells: role of STAT3 in the tumor environment. *Nature.* 7(1): 41- 51.

Yu, H., Pardoll, D. and Jove, R. (2009) STATs in cancer inflammation and immunity: a leading role for STAT3. *Nature,* 9(11):798-809.