University of Nebraska Medical Center
BREAKTHROUGHS FOR LIFE

University of Nebraska Medical Center
DigitalCommons@UNMC

Theses & Dissertations

Graduate Studies

Fall 12-15-2023

The Role of Pre- and Perinatal Oxycodone Exposure on Postnatal Brain Development and Behavior: Assessing the Impact on Later Life Brain Injuries in Juvenile Rats

Austin M. Gowen
University of Nebraska Medical Center

Tell us how you used this information in this short survey.

Follow this and additional works at: https://digitalcommons.unmc.edu/etd

 Part of the Neuroscience and Neurobiology Commons

Recommended Citation

Gowen, Austin M., "The Role of Pre- and Perinatal Oxycodone Exposure on Postnatal Brain Development and Behavior: Assessing the Impact on Later Life Brain Injuries in Juvenile Rats" (2023). *Theses & Dissertations*. 773.
https://digitalcommons.unmc.edu/etd/773

This Dissertation is brought to you for free and open access by the Graduate Studies at DigitalCommons@UNMC. It has been accepted for inclusion in Theses & Dissertations by an authorized administrator of DigitalCommons@UNMC. For more information, please contact digitalcommons@unmc.edu.

The Role of Pre- and Perinatal Oxycodone Exposure on Postnatal Brain Development and Behavior: Assessing the Impact on Later Life Brain Injuries in Juvenile Rats

Austin M. Gowen

Presented to the Faculty of the University of Nebraska Graduate College in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

Graduate Program: Integrative Physiology and Molecular Medicine

Under the Mentorship of Professor Sowmya V. Yelamanchili

University of Nebraska Medical Center

Omaha, Nebraska

August 2023

Supervisory Committee:

Gurudutt Pendyala, Ph.D.

Chittibabu Guda, Ph.D.

Kelly Stauch, Ph.D.

Rebekah Gundry, Ph.D.

ACKNOWLEDGEMENTS

The research in this dissertation could not have happened without the time and investment of many people. First and foremost, among the individuals responsible for my ability to accomplish the presented works is my primary mentor Dr. Sowmya V. Yelamanchili. Without her support and scientific latitude, I would never have been able to undertake, let alone finish, this research. Thank you for allowing me to research and pursue my passion for health equity in brain injury; having ownership of my work and the ability to launch it from a discussion in a lab meeting all the way to published science was what enabled me to survive the long hours and roadblocks that happen so often in graduate school.

I would also like to offer my sincere thanks to my committee members, Drs. Gurudutt Pendyala, Rebekah Gundry, Chittibabu Guda, and Kelly Stauch for their thoughts and support throughout my journey. Whether it was behavioral work advice from Dr. Pendyala, mass spectrometry exploration from Dr. Gundry, encouragement to take a bioinformatics class from Dr. Guda, or teaching me synaptic mitochondrial assessment from Dr. Stauch my technical, professional, and personal growth was inspired by my committee. I have had ups and downs in my research, but the steady support from my committee has helped keep me on track throughout this tumultuous journey.

To those individuals who were in the trenches of graduate school with me I want simultaneously to say thank you and apologize for having to deal with me. Dr. Subhash Chand, thank you for being a steadfast supporter throughout my early learning curves. Specifically, thank you for approaching me as an equal and never making me feel less for where I was at in my education. To Dr. Katherine Odegaard, you paved the way as the first graduate student from Dr. Yelamanchili's lab and set a bar so high that I don't think anyone will ever meet it. To Dr. Farah Shahjin, you were an inspiration for accomplishing so much in the lab and at home, I don't know how you managed to do it all. To Mason Savine, thank you for being a steadfast source of joy in

the lab, and always bringing some pep into every day with your humor. To Minh-Nghi Nguyen and Adrian Flores, it was quite the journey when I moved over to Lied Transplant Center, but it was nice to have individuals to go through it all together with. I also want to thank each of the students who put their faith in me as a mentor: Luke Miles, Sanjay Srinivasan, Gisela Meza-Guzman, Yvette Incontro, Zoe Gaupp, Mark Bausch, and Jocelyn Oh. Each student I taught had their own unique set of gifts, but all of them were blessed with an intelligence that surpassed my own. The next generation is bright, and it was an honor to pass along what I have learned to them.

Further, the University of Nebraska Medical Center played an integral part in this research and my own growth. I would like to acknowledge Dr. Liu and the imaging core for the ^{1}H-MRI/S work they helped me with and the Center on NeuroDevelopment and Aging mini-grant that supported those works. Extend my gratitude to the mass-spectrometry core for their handling of my synaptosome samples. As well as express my gratitude to Dr. Stauch and Dr. Trease with the Seahorse core for training me and hosting me while I performed those long experiments. Further, I could not acknowledge my success without considering the incredible department that I called home throughout my graduate school. Thank you to the Department of Anesthesiology for being the best department a student could ask for from having exemplary office staff all the way to invested leadership. Drs. Steven Lisco and Karsten Bartels have gone above and beyond what I have seen in any other senior leadership in regard to involving themselves into the milieu of the people they lead. I hope if I ever reach their level of success that I can inspire the same degree of confidence and security that they radiate.

Taking a step backwards in time, before my graduate life, there are many people who were responsible for getting me to the doorstep of a Ph.D. Firstly, I would like to acknowledge Dr. Jill Blankenship for being the first person to really teach me science and all the positive and negative things that it entails; were it not for her letting me bungle experiments as a sophomore during my bachelors I would never have had the chance to fall in love with science. I also cannot avoid acknowledging all of the individuals who participated in my combat sports journey. As

anathema to science and healthcare as combat sports might be, the cultures of mixed martial arts, Brazilian jiu-jitsu, Muay Thai, boxing, and Tae-Kwon-Do gave me more than I could ever give back. I would also like to thank Jim and Melinda Winterscheid for being steadfast inspirations to continually grow throughout my life. You both helped me during critical periods of transition in my life and it pushed me have the confidence and courage to take each step forward that I needed to take.

I must also acknowledge my family, the unfortunate souls who have had to deal with me the longest. The work I present here might only be recognized in the ivory tower by scientific'y' ilk, but it is the product of blue-collar ethics, much of which was instilled in me by my uncles Jerry, Dan, and Paul. Jerry for teaching me how to take pride in the work I do and helping me with fabricating my, "brain hitter." Dan for teaching me how to K.I.S.S. (keep it simple stupid) as well as everything I know about tools and upkeeping a home. Paul for being a role model to me when I lived with him during the summers of my childhood, I will strive to be a father and educator as patient and present as you. I want to thank my mother and father in-law Nu and Vu Tran; thank you for accepting me into your family and letting me learn all the beautiful elements of both your family and Vietnamese culture. To my mother JoAnn, I thank you for the time and investment it took to educate a child with my energy. Enrolling me in martial arts might have been a last-ditch effort, but it was one of the greatest things that ever happened to me. There is no job that requires more of someone than being a parent. Having three children, each with a M.D. (in a few years), Ph.D., and J.D. while coming from a small farm is as positive an outcome as anyone could ask for. To my little sister Amber, I'm sorry that I'm leaving UNMC right as you start medical school, but I appreciate you being proud of me when I couldn't be proud of myself. To my older sister Annie, I can't write an acknowledgement that encompasses what is fair. I wouldn't have made it if it wasn't for you, and maybe that statement is enough to say all the things I can't put in writing. You are the smartest person I have ever met and surrounding myself with the geniuses that inhabit the world of science has only served to make that statement more accurate.

To the love of my life Alexandra. Acknowledging you for my Ph.D. seems comical because it is just one component of the life we have built together. While I wouldn't have this degree without you, I also wouldn't have anything I have now without you. Crediting you with just my Ph.D. would be an irredeemable shortchanging. The world could never know all you've done for me, but I do. Any good I bring to the world can be attributed to you, with every fiber of my being thank you.

ABSTRACT

The Role of Pre- and Perinatal Oxycodone Exposure on Postnatal Brain Development and Behavior: Assessing the Impact on Later Life Brain Injuries in Juvenile Rats

Austin M. Gowen, Ph.D.

University of Nebraska Medical Center, 2023

Supervisor: Sowmya V. Yelamanchili, Ph.D.

There has been a long history of research investigating the role of the gestational environment on later life health and disease. The progressive widespread illicit and prescribed use of opioids in the 21st century has seen opioids become one of the most significant gestational insults in the last century. The ability of opioids to pass both the placental barrier and blood-brain barrier makes them a particularly dangerous teratogen capable of altering the peripheral physiology, neurobiology, and behavior of affected children. The works herein investigate the impact of a widely prescribed opioid, oxycodone, on developmental neurobiology in the context of response to a later traumatic brain injury using a rat model of *in utero* opioid exposure.

Maternal opioid use poses a significant health concern not just to the expectant mother but also to the fetus. While epidemiological research has shown the heightened risk factors associated with *in utero* opioid exposure, little research has investigated what molecular mechanisms underly the vulnerabilities these children carry throughout development and into later life. To understand the implications of *in utero* opioid exposure on the developing brain, we sought to assess the response to one of the most common pediatric injuries: minor traumatic brain injury (mTBI). Using a rat model of *in utero* oxycodone (IUO) exposure and a low force weight drop model of mTBI, we show not only that neonatal opioid exposure significantly affects neuroinflammation, brain metabolites, synaptic proteome, mitochondrial function, and altered behavior in juvenile rats, but also, in conjunction with mTBI these aberrations are further

exacerbated. Specifically, we observed long term metabolic dysregulation, neuroinflammation, alterations in synaptic mitochondria, and impaired behavior were impacted severely by mTBI. Our research highlights the specific vulnerability caused by IUO exposure to a secondary stressor such as later life brain injury. In summary, we present a broad study to highlight the damaging effects of *in utero* opioid exposure in conjunction with mild brain injury on the developing brain.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADHD – attention deficit hyperactive disorder

ALA – alanine

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ASD – autism spectrum disorder

ASP – aspartate

ATP – adenosine triphosphate

BBB – blood brain barrier

BDNF – brain-derived neurotrophic factor

BSA – bovine serum albumin

cAMP – cyclic AMP

CBI/II – cannabinoid receptor 1/2

CDC – Centers for Disease Control and Prevention

CNS – central nervous system

CRE – creatine

DiffExpPro – differentially expressed protein

DG – dentate gyrus

DOHaD – developmental origins of health and disease

DOR – delta opioid receptor

DPI – days post injury

Dyn – dynorphin

ED – emergency department

ER – emergency room

ESC – eat, sleep, console

FA – formic acid

FC – fold change

FDR – false discovery rate

GABA – gamma-aminobutyric acid

GFAP – glial fibrillary acidic protein

GI - gastrointestinal

GLC – glucose

GLN – glutamine

GLU – glutamate

GLY – glycine

GO – gene ontology

GPCR – G-protein coupled receptor

HCD – high energy C-trap dissociation

Hcrt – hypocretin

HPA – hypothalamic-pituitary-adrenocortical axis

Iba1 – ionized calcium-binding adaptor molecule 1

IGF – insulin-like growth factor

IHC – immunohistochemistry

IL – interleukin

IM – isolation medium

IPA – ingenuity pathway analysis

IUO – *in utero* oxycodone exposure

KOR – kappa opioid receptor

LAC – lactate

LTD – long-term depression

LTP – long-term potentiation

LSD – lysergic acid diethylamide

MAS – mitochondrial assay solution

MDMA – methylenedioxy-methylamphetamine

METH – methamphetamine

MOR – mu opioid receptor

MRI/MRS – magnetic resonance imaging/spectroscopy

MS/MS – tandem mass spectrometry

mTBI – minor traumatic brain injury

MYO – myo-inositol

NAA – N-acetyl aspartate

NAc – nucleus accumbens

NAS – neonatal abstinence syndrome

NICU – neonatal intensive care unit

NMDA – N-methyl-D-aspartate

NMDG – N-methyl-D-glucamine

NOR – nociception opioid receptor

NOWS – neonatal opioid withdrawal syndrome

NSYPmito – non-synaptic mitochondria

OBD – opioid bowel disorder

OCR – oxygen consumption rate

OPRM1 – mu opioid receptor 1

OUD – opioid use disorder

Oxy – oxycodone

P – post-natal day

PBS – phospho-buffered saline

PC – phosphorylcholine

PCS – post-concussive syndrome

PFA – paraformaldehyde

PFC – prefrontal cortex

PK – pharmacokinetics

PKC – protein kinase C

PKD – pharmacodynamics

PTSD – post-traumatic stress disorder

PVN – paraventricular nucleus

qPCR – quantitative real-time polymerase chain reaction

RPM – rotations per minute

ROS – reactive oxygenation species

SUD – substance use disorder

SYP – synaptosome

SYPMito – synaptosomal mitochondria

TAU – taurine

TBS – tris-buffered saline

TBS-T – tris-buffered saline with Tween20

tCHO – total choline

TE – echo time

THC – Δ9-tetrahydrocannabinol

TL – tissue lysate

TLR4 – toll-like receptor four

TNF – tumor necrosis factor

TR – repetition time

TTR – time to right

UNMC – University of Nebraska Medical Center

US – United States of America

VOI – volume of interest

VTA – ventral tegmental area

ZOR – zeta opioid receptor

INTRODUCTION

Substances of Abuse and Addiction

Human history has a well-documented record of using psychoactive substances for recreational, religious, cognitive, and anesthesia related purposes [1]. Such substances, whether naturally occurring or synthetic, have shaped human history and culture for better and for worse. While modern science has elucidated these formerly mysterious substances and revealed much as to how and why they work, psychoactive drugs still play a complex role in human life. Many of these now illicit substances have been understood to be highly addictive and harmful. However, inherently each of these is just a pharmacological agent with the potential for both use and misuse. For instance, in the United States of America (US) a significant minority of people suffer from opioid addiction with major health and societal disbenefit, while on the other side a significant majority people consume caffeine regularly with great self-reported benefit. The spectrum of psychoactive substances in use today spans a wide array of utility, addiction, and danger. Broadly defined there are four clades of psychoactive drugs characterized by their physiological and mechanistic effects on the human body: stimulants, depressants, hallucinogens, and opioids.

It is important to understand that each of these classes of psychoactive substances can cause the disease of addiction. Addiction is a major challenge world-wide with an estimated annual death toll of 11.8 million with over 106,000 in the US alone [2, 3]. Many of the drugs with the greatest utility, are also highly addictive, for instance opioids constitute over 60% of overdose associated mortality in the US but are one of the most prescribed drugs [4]. It is critical then to distinguish between drug use, dependance, and addiction. An individual could be taking a prescribed dose responsibility for a medical purpose, thus being a drug user but not an abuser. At the same time, an individual could rapidly become dependent on their prescription; drug dependance is defined as the developed physical reliance on an exogenous substance in such a manner that functioning without the drug poses a significant challenge [5, 6]. Drug dependance

often leads to drug abuse and addiction. Drug abuse is defined as, “The use of a substance in a manner, amount, or situation such that the drug use causes problems or greatly increases the chance of problems occurring.” [6]. Recurrent use of a drug often leads to a diminished response upon taking the drug prompting the individual to take increasingly larger doses, quickly leading to drug abuse. This diminished potency, commonly referred to as sensitization, poses a significant threat to individuals. Such a sufferer might seek larger doses of a drug to achieve the same level of euphoria as well as to relieve the physiological crash one suffers as they rebound from the euphoric experience. Through continued use, this individual could rapidly transition from dependance to addiction. Drug addiction is separate from dependance as now not only is the sufferer physically reliant on the drug, but they are also behaviorally dependent on the drug. The classification of addiction as a disease in 1987 marked a change in our understanding of exactly how dangerous drugs of abuse are [7, 8]. Drugs of abuse can, over time, directly alter the brain, physiology, and behavior resulting in the disease of addiction.

Psychostimulants

Psychostimulants, whether illicit or legal, are one of the most widely abused class of drugs. The canonical reward pathway, or dopaminergic pathway, is conserved throughout most drugs of abuse, including psychostimulants, and is comprised of the activation of the dopaminergic neurons in the ventral tegmental area (VTA) and substantia nigra, which affects GABAergic neurons in the striatum [9]. The complex transition from reward stimulation to addiction also involves the activation of higher brain structures such as the limbic system and pre-frontal cortex (PFC) [9]. Psychostimulants, as their name suggests, stimulate the central nervous system (CNS) by enhancing or mimicking various catecholamine neurotransmitters such as dopamine [10, 11]. Some psychostimulants are also capable of acting on serotonin receptors to induce hallucinogenic and psychogenic effects [12]. The three most widely abused psychostimulants are methamphetamine (METH), cocaine, and nicotine [3]. Cocaine and methamphetamine both target

presynaptic monoamine receptors and are highly addictive drugs known for their severe withdrawal symptoms [13, 14]. The mechanism of addiction of these two substances likely lies in synaptic plasticity and memory pathways associated with dopaminergic stimulation [13, 14]. Nicotine, however, acts as an agonist to the nicotinic acetylcholine receptors residing on dopaminergic neurons to exert similar effects on the dopaminergic reward system [15]. Nicotine is one of the most widely consumed recreational addictive substances with known negative health implications due to its contingent legality in many countries.

Depressants

Depressants are so named for their ability to impair and depress both behavioral and mechanical functions typically through impacting γ-Aminobutyric acid (GABA) the brain's primary inhibitory signaling molecule. The most common depressants are alcohol, marijuana, and benzodiazepines; for some time, opioids were considered depressants, however the psychoactive effects of both exogenous and endogenous opioids have been understood to be unique. Alcohol, similar to nicotine in legality, is considered the most abused drugs in the world [16]. Δ9-tetrahydrocannabinol (THC), the primary psychoactive component in marijuana, has received significant attention recently due to its increasing social and legal acceptance worldwide [17]. While both substances are canonically described as depressants, their methods of action in the brain are very different. Alcohol use affects GABA, glutamate, NMDA, nicotinic acetylcholine receptors, and serotonin receptors through complex actions on both lipid bilayer properties and receptor function [18]. Acute alcohol use has been shown to increase extracellular dopamine release and alter synaptic plasticity. Alcohol, or ethanol, is the smallest molecule psychoactive drug known and as such typically requires a significantly higher blood concentration to generate psychoactive effects. THC, on the other hand, binds to the cannabinoid 1 (CB1) and 2 (CB2) receptors with CB1 being the predominant receptor on neurons [17]. THC operates as a retrograde neuromodulator inhibiting calcium channel activity of upstream neurons, as such the

effects of THC are largely dependent on the presynaptic neuron type [17]. If the presynaptic neuron is glutamatergic then the effect will be depressant and the opposite if the presynaptic neuron is GABAergic. Benzodiazepines, first developed in the mid-20th century are perhaps the most stereotypical depressant functionally as they enhance GABA directed inhibition in the brain as allosteric modulators of GABA receptors increasing channel opening frequency [19]. Benzodiazepines at one point, were the most prescribed drug worldwide for their potent anxiolytic function [20].

Hallucinogens

Hallucinogens, sometimes described as either being psychedelic or dissociative in nature, are powerful psychoactive drugs which typically result in dramatic somatosensory alterations. Hallucinogens operate primarily through serotonin receptor mediated functions, however, much about hallucinogens remains poorly understood [18]. The most common hallucinogens are Lysergic acid diethylamide (LSD), psilocybin, and 3,4-Methylene-dioxymethamphetamine (MDMA). LSD functions in the brain like most hallucinogens by impacting a wide variety of serotonin receptors, but exactly how it induces its psychedelic effects is not known though significant activation of the cerebral cortex has been shown during use [21]. Psilocybin is a naturally occurring dissociative and psychedelic hallucinogen found in many different species of fungi that strongly activates the 5-HT_{2A} serotonin receptor in the basal ganglia and cerebral cortex [22]. MDMA was initially developed in the early 1900s as an aid to psychotherapeutic treatment, but quickly become a recreational drug. MDMA is unique from other hallucinogens in that it not only binds to serotonin, but also affects norepinephrine and dopamine inhibiting the reuptake of these neurotransmitters causing them to remain active in the synaptic cleft [23]. Each of these drugs has undergone different shifts in public opinion, but all are now being considered for therapeutic efficacy in a variety of psychological disorders and diseases including post-traumatic stress disorder (PTSD), schizophrenia, as well as even treating addiction itself [23, 24].

Opioids

Opioids are both one of the oldest and newest drugs. Opioids, much like alcohol, psilocybin, and THC occur in nature in the opium poppy plant, however unlike alcohol, psilocybin, and THC, opioids have undergone pharmacologic advancement from the naturally occurring opium into a variety of prescribed and illicit drugs. The most common illicit opioids are opium and heroin, while the most common prescribed opioids are hydrocodone, oxycodone, morphine, and fentanyl [25]. Human intervention has turned opium derivatives into some of the most potent and lethal psychoactive drugs; raw opium can be consumed in gram quantities without fatality meanwhile only 30mg of heroin and less than 3mg of fentanyl can be fatal to an adult human [26]. Prescription opioids are typically given for pain management or used in combination with anesthetics, but they are also frequently abused and known for their addictive potential [27]. All opioids act as opioid receptor agonists which are widely dispersed in the brain and spinal cord, allowing for complex addiction pharmacodynamics. There are three principal opioid receptors μ-opioid receptor (MOR), κ-opioid receptor (KOR) and δ-opioid receptor (DOR) that act on GABAergic neurons as well as a variety of other neural and peripheral cell-types, each with a variety of sub-receptors [28]. In addition to the three principal opioid receptors there are also Z-opioid receptors (ZOR) and nociception receptors (NOR) [28]. On the surface, opioid addiction occurs in a remarkably similar network to psychostimulants following the mesolimbic reward pathway; however, the targets and substrates that mediate opioid addiction are very different. Part of what makes opioids such distinct psychoactive drugs is the complexity of the endogenous opioid system. Opioid receptors have multiple endogenous signaling molecules: enkephalins, endorphins, endomorphins, dynorphins, and nociceptin/orphanin [28]. Further, the ubiquitous nature of the opioid system being extant on neurons, neural immune cells, and throughout the periphery makes them one of the most complex and addictive psychoactive substances.

Analgesics and Anesthetics in Addiction

Pain management and addiction have had an intertwined history. Dating back to the emergence of the rational phase of medicine in the 16th century psychoactive drugs have been found in many therapies whether they were called laudanum, theriac, or ether [29]. Now, modern medicine uses pharmacologically developed compounds, often derived from plant biosimilars, to treat and manage pain. While the development of analgesics and anesthetics has revolutionized pain management, it has also come with major drawbacks. We now understand how and why many of the historical therapies work, or in many cases did not work, similarly we also have a better understand of the risks associated with these therapies. In fact fear of addiction is reported as one of the primary concerns by both patient and provider when discussing pain management and anesthesia [30]. Patients prescribed an opioid pain management therapy have displayed 4-fold greater likelihood of reporting addiction in the next 6 months [31]. Meanwhile, anesthesiologists prescribing these medications who are well educated on their harmful effects, but exposed to them nonetheless have a 7-fold greater likelihood of addiction as compared to other physicians [32].

While anesthesia and analgesia are well defined in the clinic, many people also report self-treating pain using psychoactive substances. Among individuals suffering from chronic pain, it has been reported that over half report using illicit substances including THC, cocaine, and heroin to help manage their pain [33]. Among this same cohort of individuals that reported abusing a prescription medicine 81% of these were specifically abusing an opioid, 69% doing so without a prescription [33]. Alcohol as a legal alternative in most places is also commonly abused among people attempting to manage pain [33, 34]. The danger and prevalence of pain management self-medication has been apparent throughout history but has come to a dangerous peak amid the opioid epidemic.

Pain management, specifically with prescribed medicines, is a double-edged sword. Patients with a prescription for a pain management, typically an opioid, often have a chronic noxious/painful stimulus justifying this prescription. However, these drugs also induce a pleasurable euphoria in addition to helping patients ameliorate their pain. As such these patients are more severely exposed to the harmful sequelae of stopping drug use. When attempting to quit using a drug that the body has become dependent on there is a rebound phase which presents itself as a physiological depressor often associated with depression, agitation, lack of appetite, impaired sleep, and psychosis. For the pain management patient this becomes even worse as their drug induced rebound is often coupled with a return of their previously managed pain. At this point addiction is not just positively reinforced through euphoria, but also negatively reinforced by both physiological rebound and return of previously managed pain. Of all the pain management prescriptions opioids are one of the most effective, most prescribed, and most addictive.

Opioid Use and Addiction

As previously stated, opiates and their derivatives have been used clinically and recreationally throughout human history. While opioids have been extant for millennia the development and mass production of the hypodermic syringe in 1853 alongside a series of successive wars in the latter half of the 1800s saw opioids, specifically morphine, surge in popularity for treatment of battlefield wounds, dysentery, and what is now diagnosed as PTSD [35]. To date, morphine is still widely used whether recreationally or clinically, but significantly more potent opioids are more often prescribed. However, the potent pain-relieving capability of these prescriptions is directly linked to their potential misuse, dependence, and addiction [36]. Currently the dramatic rate of prescription drug abuse of opioids has simultaneously seen an increase in the illicit use of opioids such as heroin [36]. This increase in use of prescription and non-prescription opioids has culminated in the public health crisis known as the opioid epidemic

in America [36]. In 2017 nearly 70% of controlled substance overdose related deaths were caused by opioid misuse alone and opioid-attributed overdose fatalities have increased three-fold since 2000 [37, 38]. Of the opioids used today, the most infamous are the prescription opioids such as oxycodone, hydrocodone, and morphine. For many individuals these prescribed drugs are the gateway into a more severe addiction. Perhaps the most abused and prescribed of these, and the focus of these works, is oxycodone.

Pharmacokinetics and Pharmacodynamics

Pharmacokinetics (PK) and pharmacodynamics (PKD) is largely dependent on the route of administration as chemical structure is highly conserved between opioids. Chemical structures for morphine (CID 5288826), hydrocodone (CID 5284569), and oxycodone (CID 5284603) illustrates how the new opioids share similar structure and relationship to their parent molecule morphine shown in Figure 1.

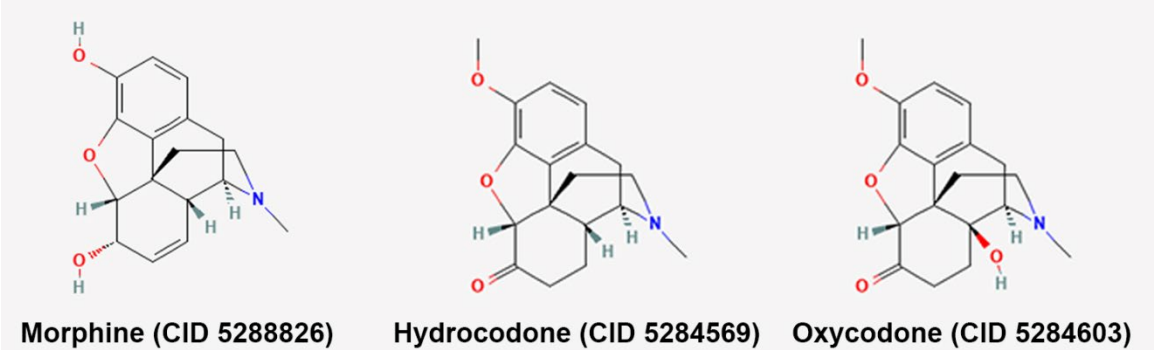


Figure 1) Chemical structures of three common opioids illustration their conserved structural identity. The prototypical structure of opioids is based on the morphine molecule, which is the key active compound in opium. Morphine, like other opioids, contains a core structure known as the morphinan skeleton, which is a fused ring system comprising three benzene rings (a structure known as a phenanthrene) and a fourth, piperidine ring attached. This core structure is critical for the interaction of opioids with their receptors.

While oxycodone can be given subcutaneously, intravenously, and orally the most commonly prescribed and misused form is oral tablet or pill. This also contributes heavily to their addictive potential as oral drugs have minimal adverse use experience interactions as compared specifically to injectables [39]. Oral administration directly affects PK/PKD largely due to first-pass metabolism by the gut and liver before the drug can reach broad circulation. In order to address this issue many opioids, such as oxycodone, are designed to mitigate first pass metabolism [40]. Oxycodone has a very low first-pass metabolism effect and significantly greater bioavailability as compared to its parent compound morphine [41]. Most oral oxycodone preparations have an onset within 30 minutes, and achieve peak analgesia in about 4 hours maintaining this throughout a duration up to 24 hours [40]. Intravenous administration generates nearly immediate pain relief, but these techniques are mainly utilized in perioperative situations. Intravenous opioids can be directly absorbed into the systemic circulation of the body negating much of the first pass effect, so this is mostly utilized with formulations like ketamine or morphine which are most effected by first past metabolism. Beyond this Oxycodone, and other opioids, primarily undergo phase 1 metabolism in the liver and subsequent excretion in the urine. The primary enzyme responsible for oxycodone metabolism is CYP3A4 which converts oxycodone into noroxycodone. Although a small percentage of oxycodone gets converted to oxymorphone by CYP2D6 in the liver and must then undergo phase 2 glucuronidation by UGT2B7. A full list of opioids and their metabolism is given in Table 1. In the normal patient oxycodone is about 45% protein bound and typically excreted as a conjugate. The average plasma half-life of oral oxycodone ranges between 2-3 hours and clearance half-life of 4-5 hours. While the PK of oxycodone is well understood, the PKD is largely dependent on the patient. While both individuals taking other drugs digested by CYP family enzymes as well as those with mutations in CYP genes have shown complications with oxycodone and other opioids these are rare cases that are outside the realm of this review. PKD principles which are most critical to oxycodone is the relative age and gestational status of the patient. Older patients have significantly decreased clearance of oxycodone while younger

patients and pregnant women display rapid clearance. An interesting note here is that oxycodone PK/PKD studies in pregnant women suggest that neonate exposure levels are comparable to the maternal plasma levels. However, what we know of neonates suggests that their clearance and metabolism of oxycodone is significantly different from the mother, potentially causing a tolerated dose in the mother to have different results in the neonate.

Opioid Drug	Primary Metabolic Pathway(s)
Morphine	Hepatic metabolism via glucuronidation, followed by renal elimination
Codeine	Hepatic metabolism via O-demethylation to morphine, which is then metabolized as above
Fentanyl	Hepatic metabolism via CYP3A4, with some renal elimination of unchanged drug
Oxycodone	Hepatic metabolism via CYP3A4 and glucuronidation, followed by renal elimination
Hydrocodone	Hepatic metabolism via CYP2D6 and CYP3A4, followed by renal elimination
Methadone	Hepatic metabolism via CYP3A4, with some renal elimination of unchanged drug
Buprenorphine	Hepatic metabolism via glucuronidation and CYP3A4, with some renal elimination of unchanged drug

Table 1) Table showing commonly used opioid drugs and their primary routes of metabolism. Hepatic metabolism via enzymes such as CYP3A4 and glucuronidation, as well as renal elimination, are common pathways for many of these drugs. However, individual patient characteristics may impact the metabolism and elimination of these drugs.

Oxycodone has particularly good bioavailability and its body distribution is very ubiquitous. In fact, oxycodone is capable of passing both of the principal stringent barriers in humans' the blood brain barrier (BBB) and the placental barrier. All opioids effect three main receptors as previously mentioned: MOR, KOR, and DOR however, oxycodone and most opioids primarily bind to MOR_1 and MOR_2 [42]. All three receptor types are widely dispersed throughout the body playing critical roles in most tissues [43]. All 3 opioid receptors have been found to be widely expressed in several peripheral tissues including the small intestine, large intestine, adrenal, kidney, lung, spleen, testis, ovary and uterus however for this work we will be focusing on their CNS impacts [44]. MOR have a wide distribution, but are most concentrated in the brain and spinal cord; specifically, MOR density is very high in the thalamus, nucleus accumbens (NAc), periaqueductal gray, medulla, amygdala, cortex, and dorsal horn of the spine [43]. MOR in the medulla and periaqueductal gray are principally responsible for the central nervous induction of anesthesia. These neurons then activate downstream inhibitory neurons which causes a cascade to reduce afferent nociceptive signaling going to the thalamic region. While mostly binding to MOR, oxycodone does have partial binding to KOR and DOR [42, 43, 45, 46]. KOR are most concentrated in the limbic-diencephalic regions, brain stem, and spinal cord; these receptors are thought to contribute to spinal analgesia and sedation affects however, some studies suggest that these also contribute strongly to opioid dependance [42, 47, 48]. DOR are widely distributed throughout the brain and spinal cord, but are over 80% internally located in dendrites as compared to MOR which are over 90% located on the plasma membrane of dendrites [49]. DOR are thought to play a leading role in the euphoric/dysphoric effect, inhibition generation, and some analgesic effects of oxycodone and other opioids [50]. Recent data has suggested the DOR induces analgesic effects by dimerization or interaction with MOR to potentiate their function [51, 52]. In fact, the primarily internalized DOR will bind to the cellular membrane upon opioid induced MOR activation among other stimuli such as high levels of pain [49]. Opioid receptor intracellular and

membrane bound dynamics have been shown to play a significant role on withdrawal, development, and synaptic plasticity [53-55].

All opioid receptors are G-protein coupled receptors (GPCR) and share similar downstream signaling pathways. Upon endogenous or exogenous ligand binding these receptors then activates a G-protein to phosphorylate its α-GDP subunit into α-GTP resulting in its dissociation of the β- and γ-complexed subunit intracellularly. Both the α-GTP and βγ complex then interact with target molecules to induce separate intracellular events. In classical opioid signaling this results in the phosphorylation induced decrease of cyclic AMP (cAMP) and interactions with ion channels resulting in the reduction of calcium ion inflow and increase of internal potassium ion inflow [43]. Opioid receptors on the presynaptic terminals of nociceptive fibers can indirectly inhibit voltage-dependent calcium channels, decreasing cAMP levels and blocking the release of downstream pain neurotransmitters, which results in analgesia. While these receptors typically induce analgesia via the activation of neurons synapsing to inhibitory neurons, they also have a variety of non-analgesic effects in the CNS which operate in differential manners beyond GABAergic signaling. These mechanisms include glutamatergic, serotonergic, and dopaminergic signaling to downstream neurons in the limbic reward system as well as in the prefrontal and mid cortical regions [56]. Opioids can also block NMDA receptors causing the activation of downstream serotonin and norepinephrine pathways; this dynamic is thought to be related to both idiopathic pain and tolerance associated with chronic opioid use and withdrawal [42].

Neonatal and Infant Opioid Pharmacokinetics

Drug metabolism in neonates and infants can differ significantly from that of adults due to a number of developmental factors. These differences can impact the pharmacokinetics and pharmacodynamics of medications and can result in increased risk of toxicity or reduced efficacy.

Understanding the mechanisms and clinical implications of neonatal and infant drug metabolism is therefore essential when investigating specifically IUO.

One key factor that can impact neonatal and infant drug metabolism is the immaturity of enzyme systems involved in drug metabolism. Many of the liver enzymes responsible for drug metabolism are not fully developed at birth, and may not reach adult levels of activity until several months or years of age [57]. For example, the activity of the liver enzyme CYP3A4, which is involved in the metabolism of many drugs, is lower in neonates and infants than in adults [58]. This can result in slower metabolism of drugs and increased risk of toxicity. Another factor that can impact neonatal and infant drug metabolism is the limited capacity for glucuronidation. Glucuronidation is a common pathway for the metabolism and elimination of opioids, but neonates and infants have a limited capacity for this process due to lower activity of UDP-glucuronosyltransferase enzymes [59]. This can result in slower metabolism of drugs that are primarily eliminated through glucuronidation, such as morphine and oxycodone. Renal function is also a key factor in neonatal and infant drug metabolism. Neonates and infants have lower renal blood flow and glomerular filtration rate than adults, which can impact the elimination of drugs that are primarily excreted through the kidneys [60]. This can result in slower elimination of drugs and increased risk of toxicity. Other factors that can impact neonatal and infant drug metabolism include variability in gastric pH and intestinal transit time, which can impact the absorption and bioavailability of orally administered drugs [57]. Additionally, neonatal and infant drug metabolism can change rapidly over the first few weeks and months of life as enzyme systems mature, and renal function improves making this a dynamic process.

Understanding these differences in drug metabolism is not only essential for safe and effective use of medications in neonates and infants, but also important when considering the long-term developmental changes caused by *in utero* exposure. Specifically, *in utero* opioid exposure can alter the development and function of dopamine signaling in the brain, leading to changes in reward processing and behavioral responses [9]. For example, *in utero* opioid exposure has been

shown to decrease dopamine receptor density and function in the striatum of rats, which can lead to decreased motivation and increased risk for substance use disorders [61, 62]. Further, as previously discussed, *in utero* opioid exposure can also impact the development and function of endogenous opioid peptides, such as enkephalins and endorphins, which are involved in the modulation of pain and reward [28]. Exposure to opioids *in utero* can lead to permanent epigenetic changes in the expression and function of endogenous opioid peptides, which can impact the development and function of reward pathways in the developing brain [63]. Altogether, while the plethora of data regarding adult opioid use and addiction can be considered neonatal and infant response to opioids needs to be evaluated independently.

Neonatal Opioid Withdrawal Syndrome Epidemic

Opioid misuse is widespread but is significantly elevated in populations who have been previously prescribed opioids. Prior to the early 20th century pregnant women were regularly prescribed opioids to aid with pregnancy related pain. Still today approximately 7% of pregnant women report using prescription opioid medication and these numbers have been consistently rising from 2010 - 2017 [64]. These numbers are even higher when analyzing Medicare reported data suggesting that, in some states, as high as 47% of women report receiving an opioid prescription during their pregnancy [65]. Furthermore, opioids are still regularly prescribed and often over-prescribed to women post-partum increasing the susceptibility of child-rearing population to developing an opioid dependance [66]. Despite these growing numbers increased data has come out illustrating the harm and complications associated with neonatal opioid exposure. When a neonate is exposed regularly to opioids, they can be born with neonatal opioid withdrawal syndrome (NOWS). This condition has rapidly become a secondary epidemic within the opioid epidemic resulting in nearly 30,000 children born suffering from NOWS in 2017 [64]. Understanding the unique PKD of pregnant mothers and how agonism of opioid receptors affects both mother and child is a complex and not fully investigated topic. Opioid receptor signaling is not only critical for

neurological function, but also their immune, heart, gut, and lung health. As such there are a variety of complications and considerations for the misuse of opioids by pregnant mothers.

Even prior to conception opioid use plays a role in a child's health as all three principle opioid receptors are extant in both the pre-implanted uterus and embryo. Extensive activation of these opioid receptors induce inhibited activity in calcium ion channels, a disruption known to lead to growth disability [67]. Morphine treated mice have shown a 40% reduction in embryo progression to blastocytes compared to saline treated counterparts [67]. Further, opioid use can negatively affect mothers' gastrointestinal function, renal function, liver function, and respiratory function. Opioid use is well known to induce bowel dysfunction mediated by opioid induced inhibition of water and electrolyte secretion into the lumen of the gut [68]. This is coupled with increased sympathetic nervous activity also causing a decrease in secretion and as a result stool remains in the gut for an extended period [68]. Similar fluid transposition issues can result in acute kidney injury and nephropathy [69, 70]. For pregnant mothers this has been indicated in increased hypertensive disorders of pregnancy and worsened outcomes for both the mother and neonate [71]. The liver is primarily responsible for metabolism of oxycodone and is likewise significantly impacted by its misuse. Opioid use is associated with and contraindicated in nonalcoholic fatty liver disease, cirrhosis, cholestasis, and hepatitis c associated hepatotoxicity [72, 73]. The liver's role in protecting and digesting compounds is critical to embryo survivability and development. Lastly respiratory depression and dyspnea caused by opioids can impact maternal and neonate outcomes due to pulmonary hypertension, embolism, and acid-base disturbances [74]. Pregnancy requires dynamic multiorgan adjustments in the maternal body, and the widespread impact of opioids and their receptors is capable of significantly effecting this shift to the detriment of both mother and neonate. Unfortunately, opioids have an even more complex impact on the physiology of the neonate as they develop.

The developing neonate relies heavily on proper opioid receptor function and endogenous opioid signaling. Disruption of this by exogenous opioids crossing the placental barrier have been

shown to cause significant developmental changes. The exact role of the opioid system during gestation is not fully understood, although it is most well characterized in the CNS. It is well known that endogenous opiates are critical in healthy development of the brain through neurite and dendrite outgrowth regulation as well as cell proliferation [75]. Broadly speaking exposure to exogenous opioids has a deleterious effect on these outcomes; works spanning the last three decades show that brain size, cell number, protein levels, and RNA levels are all decreased in animal models of gestational opioid exposure [76, 77]. These collective insults result in increased pre-term birth, physical defects, and behavioral disorders [78]. Our current understanding of exactly how complex and broad the effects of gestational opioid exposure and specifically neonatal opioid withdrawal syndrome (NOWS) is on children during development and into later life is currently extremely poor.

NOWS is a complex disorder and one that does not have a defined clinical therapeutic strategy currently [79]. While steps are established in the near-term immediately after birth, there is little to no tracking of children throughout development. Part of this is because, despite increased complication rates, impaired cognitive function, and altered behavior, the majority of NOWS children develop within tolerable bounds alongside their peers [80]. In 1995 Dr. David Barker first proposed the idea of developmental origins of health and disease (DOHaD) which suggests that many later life diseases and health complications are either caused or accelerated by early life insults [81]. This theory has been supported time and again in epidemiological studies for heart, brain, and gut development [82, 83]. The impact of NOWS in the lens of the DOHaD hypothesis proposes a particularly important and uninvestigated complication of the NOWS epidemic: do NOWS children have worsened responses to normally recoverable insults as compared to non-NOWS children? This question has been poorly investigated, but there exists data in both animal and human models suggesting that NOWS sufferers are not only acutely impacted by their opioid exposure but are also longitudinally susceptible to otherwise recoverable injuries in a normal population.

Developmental Effects of Opioid Exposure *in utero* and Future Vulnerability

Both animal and human data show behavioral, physiological, and brain-specific effects playing a major role throughout gestation and development for IUO exposed subjects. Our understanding behind the mechanisms responsible these widespread effects has improved significantly over the last two decades; however, significant gaps exist in our understanding of IUO associated vulnerabilities to common behavioral and physiological insults and diseases. Burgeoning research in the wake of the opioid epidemic of the late 20th century has shown that IUO exposed children have many developmental vulnerabilities and pathophysiological conditions [84]. The scope of these impacts extends to nervous system, peripheral physiology, and immunity. However, in many cases IUO exposed children can grow up alongside their unexposed peers [85]. Research has shown that while deficits exist across behavior, health, and academic performance in IUO exposed subjects these deficits are not significant enough many times to take them out of their age-cohort [85-87]. However, this does not address how IUO exposed individuals might be differentially vulnerable to otherwise minor or recoverable insults that occur during normal development. Considering the many behavioral and physiological domains that epidemiological data shows is affected by IUO and NOWS it is important to consider specific areas of vulnerability during development. For the purpose of this thesis study, we will consider the developmental vulnerabilities caused by IUO from three perspectives: neurological development, peripheral physiology, and behavior.

Neurodevelopmental Pathophysiology in IUO

NOWS and *in utero* opioid exposure can have significant impacts on a developing fetus's neuroanatomy and neurophysiology. It's important to note that the specifics can vary depending on the type of opioid, the timing and duration of exposure, and individual genetic factors. However, some consistent general findings have been reported. Most notably *in utero* opioid exposure has

been associated with alterations in the structure of the brain. Neuroimaging studies have shown changes in the volume and connectivity of various brain regions. For instance, there is evidence of smaller total brain volumes, altered white matter development, and changes in the basal ganglia, amygdala, and PFC [88]. These regions are involved in a range of functions, including emotional regulation, cognitive processing, and motor control. Specifically, the precentral gyrus of the motor cortex has been shown to be smaller and less myelinated in NOWS children [89]. It's thought that these structural changes might underpin some of the behavioral and cognitive difficulties seen in children with *in utero* opioid exposure. These neurological differences precipitate into multiple pathophysiological outcomes as the brain develops in these children. One of the most notable is that exposure to opioids *in utero* can influence the development of the HPA axis, the body's central stress response system [90]. Further, opioids act on the reward system of the brain, a network of structures including the VTA, NAc, and PFC [91]. Gestational opioid exposure can prime this reward network, leading to changes in how the brain processes rewarding stimuli, animal and epidemiological data suggests contributes to the increased risk of substance use disorders later in life [80].

Both the neural connectivity and anatomy are directly related to functional deficits in neurogenesis, myelination, and neurotransmitter production in opioid exposed neonates. Ultimately these pathways are impaired developmentally by gestational opioid exposure. Opioids can cross the placenta and directly interact with neural stem cells in the developing brain, affecting their proliferation and differentiation into neurons and glial cells [92-95]. This could lead to reduced neurogenesis and alterations in the balance of different cell types in the brain. Interestingly, it does not seem to induce a deficit in any specific cell-type but rather a disrupted balance [95]. These dysregulations are potentially responsible for the disrupted connectivity reported in NOWS children as they age; NOWS children have been reported to have autism-like neural networks with high-interconnectivity and potentially erroneous signaling pathways [96, 97]. Opioids can influence synaptic plasticity, the ability of synapses to strengthen or weaken over time. This

process is fundamental for learning, memory, and the formation of neural networks. Chronic opioid exposure can lead to changes in the expression of genes related to synaptic plasticity, potentially affecting synaptogenesis. Specifically, opioids are known to affect the production and function of growth factors and neurotransmitters that regulate neurogenesis and synaptogenesis, such as BDNF and GABA [98]. Changes in these factors could influence the survival, growth, and differentiation of neurons. BDNF supports the growth and maintenance of neurons by binding to its high-affinity receptor tropomyosin receptor kinase B and activating intracellular signaling pathways that promote neuron survival and growth [99, 100]. This is crucial in the early stages of brain development when neurons are forming and establishing connections with each other [101, 102]. Interestingly mixed research has shown upregulation of BDNF in specific areas such as the PFC and cortex whereas other research has shown downregulation of BDNF in the hippocampus after opioid exposure [98, 103, 104]. These dysregulations appear to be not only spatially dependent, but also temporally dependent during development and duration of opioid exposure to the neonate [105, 106]. Both BDNF and GABA are critical for the turnover of neural progenitor cells into functioning neurons during neurogenesis [106, 107]. Aside from neurons, the signaling pathways associated with multiple glia and resident immune cells are affected by *in utero* opioid exposure suggesting that neurological cellular function is heavily compromised [108].

Cellular biology in the brains' of *in utero* opioid exposed infants is significantly affected. Microglia, astrocytes, oligodendrocytes and neuronal composition of the brain is altered by *in utero* opioid exposure during development in large part due to the molecular pathways of progenitor cells being compromised [106, 108, 109]. Microglia are the primary immune cells of the brain and are involved in responding to injury and disease, as well as modulating synaptic activity [110]. Opioids have been shown to influence microglial activation, potentially leading to increased inflammatory signaling in the brain [108, 109]. This can induce a variety of intracellular signaling pathways, leading to the release of pro-inflammatory cytokines such as TNFα, IL-1β, and IL-6 [108, 109]. This neuroinflammation can disrupt normal brain development and

contributes to the neurodevelopmental problems associated with NOWS [86, 111]. Additionally, opioids can increase the expression of Toll-like receptor 4 (TLR4) on microglia, further amplifying the inflammatory response [109, 112]. Astrocytes express various types of opioid receptors (mu, kappa, and delta) [108]. When opioids bind to these receptors, they can modulate intracellular signaling pathways, potentially affecting astrocyte functions such as neurotransmitter uptake and release, ion homeostasis, and metabolic support to neurons [108, 113, 114]. For example, opioids can inhibit the release of glutamate from astrocytes, which could impact neuronal activity and synaptic plasticity [115]. Additionally, the microglial inflammatory signaling molecules TNFα and IL-1β can affect astrocyte health and function [116]. Oligodendrocytes are responsible for producing myelin, the insulating layer that surrounds axons and is crucial for efficient signal transmission [117]. How opioids affect oligodendrocyte function and while specific pathways are not fully understood, opioids may influence oligodendrocyte differentiation and myelin production via their effects on neurotransmitter systems and growth factors that regulate oligodendrocyte function [95, 118, 119]. For example, dopaminergic signaling is disrupted by opioid exposure and the dopaminergic pathway plays a role in regulating oligodendrocyte maturation and myelination [120]. Chronic opioid exposure can also induce oxidative stress in both adults and neonates, which may damage oligodendrocytes and disrupt myelin production [121, 122]. pathways involved in myelination. Many imaging studies in NOWS infants have found that decreased brain size, specifically in the cortex, is caused by thinned white-matter tracts suggesting that demyelination is not only occurring but also rampant in the motor cortex [89, 123, 124].

The molecular pathways underlying the cellular and morphological changes seen in the brain after *in utero* opioid exposure are very poorly understood. However, some pathways have been established if not fully elucidated. The endogenous opioid pathways are the most directly affected group by *in utero* opioid exposure. Overexposure to opioid, particularly during development, can cause opioid receptor internalization, receptor desensitization, and alterations in levels of endogenous opioid production [125-127]. This three-pronged insult is significant to all

cell types of the brain as opioid receptor and signaling occurs in neurons, microglia, and astrocytes [95]. Further, opioid receptors and their ligands often work in conjunction with other receptors. While this arena of research is still in its infancy, data has shown that TLR4 receptor mediated innate immunity receptors can be stimulated by opioid receptor ligands to induce an inflammatory cascade capable of causing microglial and neuronal cell population changes [109, 112]. Another well-established developmental pathway affected by gestational opioid exposure is the expression of BDNF and its receptor of TrkB [98, 103, 104]. This pathway is thought to be behind much of the differential neurogenesis and synaptogenesis seen in *in utero* opioid exposure and NOWS [128]. A last well documented pathway alteration are oxidative stress pathways which can affect cellular and morphological changes in the brain by mediating cell death or damage to all cell types in the brain [129].

Peripheral Physiology in IUO

Although CNS disorders are more focused in NOWS and *in utero* opioid research, many peripheral effects have also been noted. In some cases, these are thought to be downstream effects of CNS pathophysiology. For example, both autonomic function and HPA axis dysregulation have been shown affect chronic stress in infants which can result in fever-like symptoms, tachycardia, and excoriated skin [130]. However, opioid receptors are also found on many peripheral cell types playing integral roles in those systems. Some of the most impactful of these are the enteric nervous system, small and large intestine, lungs, reproductive organs, and immune cells. Exposure during the critical period of gestation can have lasting effects on the function of each of these systems.

The gastrointestinal (GI) tract and enteric nervous system which governs its autonomic function have been heavily implicated in opioid use for better or worse. Opioid misuse in adults is well known to induce GI motility dysfunction, typically manifesting as ileus, termed as opioid bowel disorders (OBD) [131]. Infant cases of ileus are particularly harmful due to their shortened GI tract,

non-solid diet, and high metabolic rate. Fortunately opioid antagonists such as methylnaltrexone have been shown to improve response to these conditions in pediatric populations [132]. However, it is important to understand any potential long-term effects, which to date have not been reported.

Chronic neonatal opioid exposure can significantly impair lung function and health [130, 133, 134]. Opioids exert their effects by binding to and activating opioid receptors, which are present in the brainstem's respiratory center and in the lung tissue itself, causing it to have a dual mechanism of impact [135]. Overstimulation of these receptors in both locations can depress respiration, ultimately leading to hypoventilation, hypoxia, and potential respiratory failure in severe cases [136]. Molecularly, this occurs through the inhibition of adenylyl cyclase and reduction in cAMP production, which diminishes the responsiveness of the respiratory center to carbon dioxide, leading to slower and less effective breathing [137]. In infant cases of withdrawal though, these symptoms can have oppositional and dangerous effects. Neonates experiencing NOWS often exhibit tachypnea, which can cause excessive loss of carbon dioxide and result in respiratory alkalosis [138, 139]. Moreover, persistent respiratory distress can lead to hypoxia, potentially resulting in pulmonary hypertension and affecting long-term lung development [139]. In the context of adult opioid misuse, withdrawal can similarly lead to dysregulated breathing patterns, albeit adults generally have more compensatory mechanisms to handle these changes. However, severe cases of opioid withdrawal in adults can still lead to significant respiratory distress and death [136]. NOWS poses considerable short-term and long-term risks to lung health and function, underlining the need for early identification and appropriate management.

Chronic neonatal opioid exposure and NOWS can significantly impact the function of immune cells and overall health. Opioids, acting through their receptors present on various immune cells including leukocytes, lymphocytes, and macrophages, can modulate immune responses [140]. The binding of opioids to these receptors triggers immunosuppression by inhibiting the activity of these cell types. This can result in diminished phagocytic activity, reduced

cytokine production, and overall suppression of both innate and adaptive immune responses during exposure [141]. Consequently, neonates with NOWS may have increased susceptibility to infections, posing significant health risks. Furthermore, these effects can have long-term implications, as early life immune disruption can potentially impact immune function later in life, making individuals more susceptible to various diseases [142]. When it comes to opioid withdrawal, it is known to cause a rebound hyperactivity of the immune system, which may result in excessive inflammation and tissue damage affecting the entire body [143]. Therefore, both neonatal opioid exposure and NOWS have considerable implications for immune cell function and health, highlighting the importance of effective interventions and long-term potential vulnerability of these individuals.

Health and Behavioral Pathophysiology in IUO

The implications of *in utero* opioid exposure and NOWS extend beyond immediate physical health consequences, with significant impacts on neurodevelopment, behavior, and long-term health. Emerging evidence has highlighted the profound effects of NOWS on multiple aspects of child development, including cognitive function, movement and pain perception, and social behavior, each of which are crucial to the individual's overall health, well-being, and quality of life [144, 145]. Further understanding how each specific pathophysiological network integrates into a whole health portrait for children exposed *in utero* to opioids gives a better understanding of the potential vulnerability these children might suffer compared to their unexposed peers.

Children with a history of NOWS are known to be at an increased risk of developing anxiety, mood, and memory disorders. The chronic *in utero* opioid exposure is believed to cause changes in the developing brain, particularly in areas like the amygdala and PFC that regulate emotion and cognition. Primarily this is thought to be from previously described disruptions in myelination and synaptogenesis. Disruption of normal brain development in these regions can lead to heightened anxiety and mood dysregulation. Some studies have suggested that a

heightened fear response is caused by the chronic activation of opioid receptors in the amygdala. Additionally, hippocampal size in NOWS children has been shown to be deceased, potentially leading to memory and learning deficits caused by decreased neurogenesis in the hippocampus [146]. This pattern of neurological impairment underscores the need for early intervention and continued mental health surveillance in this population. Particularly since disorders of anxiety, mood, and memory are the most readily targeted for behavioral intervention therapies. Animal enrichment studies have found that *in utero* opioid exposed subjects benefit more from environmental enrichment. Unfortunately, epidemiological data suggests that they are also more vulnerable to later life stressors.

NOWS also significantly affects motor development, muscle function, and pain perception. Opioid receptors are present in areas of the brain and spinal cord involved in motor control, and their altered signaling due to chronic opioid exposure may impair normal motor development, possibly leading to issues like hypotonia. Specifically, the precentral gyrus which is responsible for conscious motor control is significantly decreased in children diagnosed with NOWS. Motor function studies in children have found the NOWS children suffer both fine and gross motor control impairments [147, 148]. Furthermore, opioids have a significant impact on the perception of pain [108, 120, 149]. Chronic *in utero* opioid exposure could potentially alter the developing pain pathways, making these children more sensitive to pain or disrupting their ability to effectively cope with it [150]. Both issues lead towards significant physical environment interaction deficits. Motor control network learning utilizes both positive and negative reinforcement [151]. Having an impaired ability to execute motor control and potentially increased negative stimuli to failed activity could significantly affect these children. Indeed, epidemiological studies have found that children with a NOWS diagnosis are significantly more likely to be admitted into the ER. While many of these cases are related to infections, a significant portion of pediatric ER admittances are due to slips, falls, and other motor associated injuries [147].

Social development can also be significantly impacted in children with NOWS. Chronic opioid exposure has been associated with difficulties in social interaction, potentially due to alterations in brain regions involved in social cognition, such as the PFC and the amygdala. Some children may exhibit behaviors similar to those seen in autism spectrum disorder (ASD), including difficulties in communication and repetitive behaviors. However, it's important to note that while these behaviors may resemble those of ASD, the etiology and necessary interventions can differ significantly. However, children diagnosed with pre-natal opioid exposure and NOWS are at a significantly higher risk for attention deficit hyperactive disorders (ADHD) as well as ASD in later life [152]. Many neuroimaging studies on NOWS children have led to an understanding that no single brain region is targeted by *in utero* opioid exposure, but rather the connectome of the brain is altered. This means that each child could have an altered synaptic plasticity which induces such a disparate association of disorders that very well might depend on their environment [152]. As such, these children would benefit significantly from targeted social interventions and close monitoring of their social development throughout childhood.

Brain Injury as a Secondary Insult in NOWS

As previously discussed, significant advances have been made in establishing the negative molecular, cellular, and behavioral sequelae associated with IUO. Gestational insults have been well understood to induce later life vulnerability to injury and disease, further, many studies have observed these epidemiological effects in children suffering from NOWS [85, 151, 153, 154]. This makes IUO a marked risk factor for later life injury and disease and a potentially vulnerable population; in fact, falls are the most common non-fatal reason for ED visits in children and are the primary cause of pediatric mild-traumatic brain injury (mTBI) [155]. Given the well reported association of IUO/NOWS with motor-control deficits and motor cortex thinning persistent through peri-adolescence there is an urgent need to understand if this population is more vulnerable to one of the most prevalent pediatric injuries: mTBI [151]. mTBI is a widespread

injury with a 2020 report showing that 8.3% of children were diagnosed with at least one concussion before reaching 18 years old, and self-reported numbers being significantly higher [156]. The primary cause of mTBI in children is from slips, falls, and accidents during play [157]. Acute sequelae of mTBI overlap significantly with IUO associated pathophysiology such as increased inflammation, myelin disruption, and cognitive deficits [86, 111, 123, 158-160].

Closed-head brain injury, or mTBI, occurs when a sudden trauma or impact to the head causes injury to the brain without penetrating the skull. The primary injury, occurring at the moment of impact, induces shearing and stretching forces that lead to mechanical disruption of neuronal and vascular structures, causing immediate neuronal death, axonal damage, and in some cases hemorrhage [158]. This primary injury directly affects neurons, astrocytes, and blood vessels, leading to an immediate and localized injury site [158]. However, this primary damage triggers a cascade of secondary injury processes that can expand the damage over time and affect a wider area of the brain. Secondary injury involves processes such as neuroinflammation, oxidative stress, and excitotoxicity, which can further damage neurons and glial cells. It's characterized by increased intracranial pressure, cerebral ischemia, and edema [161, 162]. Notably, microglia, the brain's resident immune cells, become activated during this phase and can exacerbate the injury by releasing pro-inflammatory cytokines and contributing to oxidative stress [163]. While microglia are responsible for resolving the brain injury, hyperactive microglia and exacerbate long-term characteristics of brain injury, referred to as post-concussive syndrome (PCS) [164].

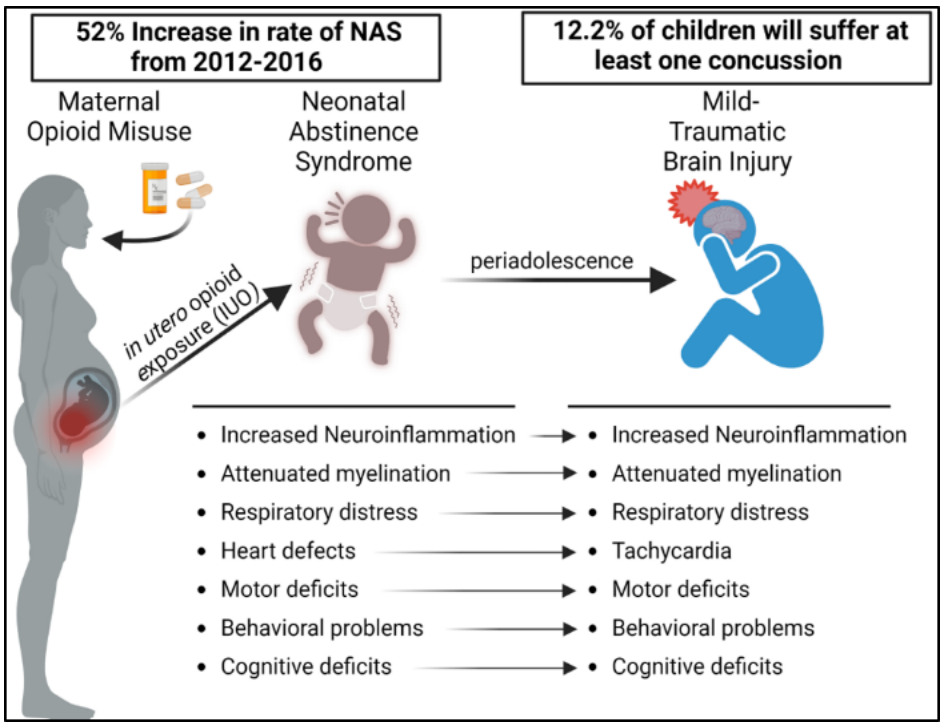


Figure 2) Graphical illustration of how *in utero* opioid exposure and minor traumatic brain injury have overlapping pathophysiology, and how both are widespread issues.

From a molecular perspective, chronic opioid exposure *in utero*, characteristic of NOWS, raises basal levels of inflammatory cytokines [86]. Similarly, in brain injury, the damage to neural tissue can trigger an inflammatory response, releasing pro-inflammatory cytokines which overlap with NOWS like interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), and various chemokines that contribute to secondary injury [158, 163]. This secondary injury is a product of dysregulated inflammatory response and results in including neuronal death and synaptic dysfunction [161, 162]. Now, consider a situation where a child with a history of NOWS suffers a brain injury. In a child with NOWS, the already dysregulated neuroimmune response might not adequately control this inflammatory reaction, leading to an overproduction of these cytokines. This cytokine storm could amplify the inflammatory response, contributing to more significant secondary neuronal injury, including neuronal death, synaptic dysfunction, and blood-brain barrier disruption (Figure 2). Further, it is well known that NOWS affects the endogenous opioid system through mechanisms such as receptor desensitization or down-regulation [125-127]. This could lead to an increased need for opioids including endogenous endorphins, enkephalins, endomorphins, and dynorphins to achieve the same analgesic effects. If a brain injury occurs in a child with NOWS, the already disrupted opioid system might not adequately buffer the pain and stress response. Both inflammation and the opioid system are critical for pain management and experiential pain; the dual disruption of this system in an opioid exposed individual suffering an mTBI could result in significant downstream effects both cellularly and in long-term behavior [149].

Understanding NOWS and mTBI associated inflammatory and molecular dysregulations we can also gain insight to the vulnerability of microglia, astrocytes, and neurons. Brain injury is typically resolved by the activation and recruitment of microglia to recycle and remove damaged cells. However, since *in utero* opioid exposure induces microgliosis and raises neuroinflammation the main mechanism of resolution of brain injury is impaired in this population [86, 111, 160]. Neuronal cascades post-mTBI can involve excitotoxicity, oxidative stress, mitochondrial dysfunction, and inflammation. Further, neurons which are stretched and deformed during primary

injury cascades release signals, primarily inflammatory cytokines, for the recruitment of microglia to attend to the site of injury [158]. However, hyperactive microglia can cause more harm than benefit in these cases, impairing the natural resolution of the injury. Further, this natural neuron loss and synaptic pruning post-mTBI may become rapidly pathophysiological in pediatric populations who suffered from NOWS, because *in utero* opioid exposure can hinder both neurogenesis and synaptogenesis [88]. These processes are necessary to allows neural networks to plasticly recover post-injury. What's more the excitotoxicity and inflammation associated with mTBI can also target astrocytes which are integral components of the synapse and BBB [163, 165]. In the short term, brain injury leads to the activation of astrocytes, a state referred to as astrogliosis. In this activated state, astrocytes undergo morphological and functional changes, including hypertrophy and increased expression of glial fibrillary acidic protein (GFAP) [166]. Reactive astrocytes can also release pro-inflammatory mediators that contribute to the inflammatory response [167]. Exacerbated astrogliosis has several negative effects including decreased ability to maintain a homeostatic environment in the brain and formation of glial scarring which impairs synaptic plasticity even further [166, 168]. Lastly, mTBI is heavily associated with short-term loss of myelin due to sheer-stress on neurons in the cortex caused by rotational or lateral head acceleration. Understanding that *in utero* opioid exposure causes impaired oligodendrocyte function resulting in already impaired white matter tracts, the inability to recover lost myelin can cause permanent signal transduction issues in neurons post-mTBI.

Behavioral issues in NOWS can stem from the molecular and cellular dysfunction described above, leading to cognitive impairments, emotional dysregulation, and social difficulties. Similarly, brain injury can lead to a range of cognitive and behavioral issues due to the localized or diffuse damage to brain structures. Notably, executive functions often suffer following brain injury, as these higher-order cognitive skills depend on multiple brain regions that can be affected by diffuse injury. If a child with NOWS experiences a brain injury, the pre-existing vulnerabilities in cognitive and emotional regulation could be exacerbated, leading to more severe

behavioral issues and potentially a longer or more difficult recovery process. Further, it has already been shown that NOWS primes children to be more emotionally reactive to their environment and experiences. A traumatic experience, such as an mTBI, could further impair their development and behavior. Considering that mTBI is also associated with development of post-traumatic stress disorders this dual hit could result in not only molecular crossover between mTBI and NOWS, but also behavioral and environmental cross over. Moreover, brain injury in children has been shown to affect scholastic performance, but specifically in the context of behavioral problems.

Overall, these potential effects suggest that NOWS and brain injury could interact in a 'double-hit' scenario, with each condition potentially exacerbating the impacts of the other. Extending from birth into early life these alterations have shown to have significant impacts longitudinally. Understanding NOWS and *in utero* opioid exposure as dynamic conditions that does not end after resolution of withdrawal and exposure related symptoms is critical for further progress and care of this growing population. However, the specific mechanisms and implications of the interaction between *in utero* opioid exposure and mTBI need further research for a comprehensive understanding.

Summary and Conclusion

The opioid epidemic has given rise to multiple generations of children suffering from NOWS and gestational opioid exposure. These children have been well documented to be able to function within or near their respective age cohort but suffer a multitude of pathophysiological conditions. As this vulnerable population continue to grow, much research has been undertaken to understanding opioid associated insults and injury. However, significant lack of understanding exists when regarding their vulnerability to common later life injuries. While growing epidemiological, behavioral, and imaging research in humans has expanded our understanding of the sequelae associated with gestational opioid exposure, animal models can serve to bridge

the gap in our understanding of the molecular and functional attributes that underlie these sequelae. We hypothesized that IUO exposure would result in a pathophysiological response to pediatric mTBI caused by IUO induced neuroinflammation and synaptic deficits that will have long term effects on behavior and recovery from injury. Specifically, do opioid exposed children experience differential outcomes to one of the most common pediatric injuries, mTBI, and what is the mechanism behind these differences. Utilizing an *in utero* oxycodone exposure paradigm in a rat model we investigated this question. Chapter One illustrates the acute response to mTBI in the cortex of juvenile rats using common biomarkers of inflammation, mTBI, and cellular function in the context of IUO. Chapter Two investigates how the synaptic environment, specifically the synaptic proteome and mitochondria, is altered by IUO exposure and mTBI, both separately and in conjunction. Chapter Three investigates the long-term consequences of IUO exposure and mTBI in the context of behavior and mitochondrial function. Each of these chapters contains data first published in Brain, Behavior, and Immunity: Health. These chapters are then followed by a conclusion highlighting the implications of these findings and directions to further not only research, but also advocacy and education for populations affected by gestational opioid exposure.

MATERIALS

Chemicals and Reagents

Name	Company
2-methylbutane	Sigma Aldrich (St. Louis, MO, USA)
Acetonitrile	Thermo Fisher Scientific (Waltham, MA, USA)
ADP	Sigma Aldrich (St. Louis, MO, USA)
Antimycin-A	Sigma Aldrich (St. Louis, MO, USA)
Ascorbate	Sigma Aldrich (St. Louis, MO, USA)
ATP-Mg	Thermo Fisher Scientific (Waltham, MA, USA)
Bicinchoninic acid assay	Thermo Fisher Scientific (Waltham, MA, USA)
Bis-Tris Gel	Thermo Fisher Scientific (Waltham, MA, USA)
Bovine Serum Albumin	Thermo Fisher Scientific (Waltham, MA, USA)
Bupivacaine HCl	Sigma Aldrich (St. Louis, MO, USA)
$CaCl_2$	Thermo Fisher Scientific (Waltham, MA, USA)
Cs-methanesolfonate	Thermo Fisher Scientific (Waltham, MA, USA)
Dirtect-Zol RNA Isolation	(Zymo Research, CA, USA)
EGTA	Thermo Fisher Scientific (Waltham, MA, USA)
FCCP	Sigma Aldrich (St. Louis, MO, USA)
Fetal Bovine Serum	Thermo Fisher Scientific (Waltham, MA, USA)
Formic Acid	Thermo Fisher Scientific (Waltham, MA, USA)

Glucose	Thermo Fisher Scientific (Waltham, MA, USA)
HEPES	Thermo Fisher Scientific (Waltham MA, USA)
Isoflurane	Sigma Aldrich (St. Louis, MO, USA)
KCl	Thermo Fisher Scientific (Waltham, MA, USA)
KH_2PO_4	Sigma Aldrich (St. Louis, MO, USA)
L-ascorbic acid	Thermo Fisher Scientific (Waltham, MA, USA)
Malate	Sigma Aldrich (St. Louis, MO, USA)
Mannitol	Roche Diagnostics (Basel, CH)
Mass spectrometry-grade trypsin	Thermo Fisher Scientific (Waltham, MA, USA)
$MgCl_2$	Sigma Aldrich (St. Louis, MO, USA)
MgSO4	Thermo Fisher Scientific (Waltham, MA, USA)
NaCl	Thermo Fisher Scientific (Waltham, MA, USA)
NaH2PO4	Thermo Fisher Scientific (Waltham, MA, USA)
NaHCO3	Thermo Fisher Scientific (Waltham, MA, USA)
NA-pyruvate	Thermo Fisher Scientific (Waltham, MA, USA)
NMDG	Thermo Fisher Scientific (Waltham, MA, USA)
Nonfat Milk	Thermo Fisher Scientific (Waltham, MA, USA)
Oligomycin	Sigma Aldrich (St. Louis, MO, USA)
Optimal Cutting	Sakura Finetek (Torrance, CA, USA)

Temperature compound	
Oxycodone hydrochloride	Sigma Aldrich (St. Louis, MO, USA)
Oxygen Gas	Matheson Gas (Omaha, NE, USA)
Paraformaldehyde	Sigma Aldrich (St. Louis, MO, USA)
PepClean C18 spin columns	Thermo Fisher Scientific (Waltham, MA, USA)
Percoll	Sigma Aldrich (St. Louis, MO, USA)
Ponceau S stain	Thermo Fisher Scientific (Waltham, MA, USA)
Protease Inhibitor	MilliporeSigma (Burlington, MA, USA)
Pyruvate	Sigma Aldrich (St. Louis, MO, USA)
sodium dodecyl sulfate–polyacrylamide	Roche Diagnostics (Basel, CH)
Succinate	Sigma Aldrich (St. Louis, MO, USA)
Sucrose	Thermo Fisher Scientific (Waltham, MA, USA)
Superblock	Thermo Fisher Scientific (Waltham, MA, USA)
SuperSignal West Pico Chemiluminescent Substrate	Thermo Fisher Scientific (Waltham, MA, USA)
Tetramethylphenylenediamine (TMPD)	Sigma Aldrich (St. Louis, MO, USA)
Tris-buffered Saline (TBS)	Thermo Fisher Scientific (Waltham, MA, USA)
Triton	Thermo Fisher Scientific (Waltham, MA, USA)
Tween20	Thermo Fisher Scientific (Waltham, MA, USA)

Antibodies

Primary Antibody	Catalog #	Company	Concentration
Gfap	G-3895	Sigma-Aldritch	1:1000
Iba1	019-19741	WAKO	1:1000
Total Oxphos	Ab110413	Abcam	1:1000
Sod2	PA5-30604	Invitrogen	1:1000
Fumarase	PA5-82899	Invitrogen	1:1000
Tfam	PA5-80107	Invitrogen	1:1000

Secondary Antibody	Catalog #	Company	Concentration
Anti-mouse IgG	31430	Thermo Fisher Scientific	1:5000
Anti-rabbit IgG	31460	Thermo Fisher Scientific	1:5000
Anti-Mouse Alexa Fluor 594	A-11005	Thermo Fisher Scientific	1:500
Anti-Rabbit Alexa Fluor 488	A-11008	Thermo Fisher Scientific	1:500

Quantitative Real-Time PCR Probes

Gene Name	Catalog #
18s rRNA	Hs99999901_s1
Gapdh	Rn01775763_g1
Gfap	Rn01460869_g1
Aif1	Rn00567906_g1
Tnf	Rn01525859_g1
Il1b	Rn00580432_m1
Il6	Rn01410330_m1
Cxcl1	Rn00578225_m1
Cxcl2	Rn00586403_m1
Il10	Rn99999012_m1
Mapt	Rn00691532_m1
Bdnf	Rn02531967_s1
Apoe	Rn00593680_m1
S100b	Rn04219408_m1
Mbp	Rn01399619_m1
Nefl	Rn00582365_m1

Buffers and Solutions

Name	Composition
Antibody Solution (Western Blots)	*0.75 g BSA* *50 mL TBS-T*
Synaptosome Isolation Medium	*225 mM Sucrose* *75 mM mannitol* *1 mM EGTA* *5 mM HEPES* *Protease inhibitor Tablet* *(Adjusted to pH 7.4)*
Mass Spectrometry Solvent B	*0.1% Formic Acid in 80% Acetonitrile*
Mitochondrial Assay Solution (MAS)	*70 mM sucrose* *220 mM mannitol* *10 mM KH_2PO_4* *5 mM $MgCl_2$* *2 mM HEPES* *1 mM EGTA* *2 mM malate* *4μM FCCP* *2% w/w BSA* *(Adjusted to pH 7.4)*
Synaptosome Homogenization Buffer	*320 mM Sucrose* *4 mM HEPES*

	Protease Inhibitor Tablet
	900 mL deionized water 100 mL 10X
	TBS
TBS-T	*1 mL Tween20*

METHODS

Animals

Male and female Long Evans 006 rats were obtained from Charles River Laboratories Inc. (Wilmington, MA, USA) and condition housed in a 12 h light–dark cycle and fed ad libitum. Equal ratio of sex distribution (n = 3) was used for all molecular experiments to accurately represent the population distribution. Sex differences were statistically assessed in all experiments, however n = 3 provides insufficient power to ascertain sex differences. For all experiments 1 male and 1 female animal from N = 3 biological dams were used. Pups from 14 saline gavaged dams and 16 oxycodone gavaged dams were used in these works. Animals were assigned randomly to various experimental groups within litters by Excel random number generation blocking. Power analysis was performed using previously published works as a baseline reference to determine sample sizes [169-171]. All procedures and protocols were approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

In utero oxycodone treatment

The development of the IUO treatment paradigm was adapted from previously published studies [169-171]. Nulliparous female (70-80 days of age) Long Evans rats were treated with oxycodone HCl (Sigma Aldrich, St. Louis, MO, USA) dissolved in saline via oral gavage, a graphical timeline is included in the supplement (Figure 3). An ascending dosing procedure was used wherein doses of 10 mg/kg/day oxy were orally gavaged for 5 days. After five days a final dose of 15 mg/kg/day was administered, after which females were mated with proven male breeders, and the treatment regimen continued throughout mating, gestation, and parturition until weaning at post-natal day (P) 21. Pups are removed at P21 and allowed to recover from opioid exposure for 7 days. Because the half-life of oxy is relatively short and drug distribution can be

altered by pregnancy, dams were monitored daily for signs of opiate withdrawal, such as weight loss, diarrhea, and irritability throughout gestation.

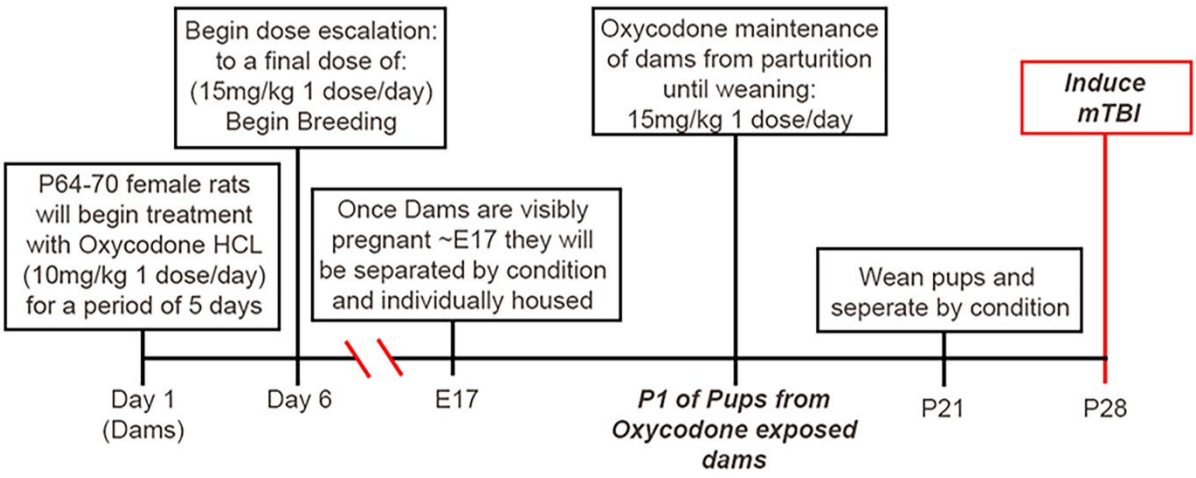


Figure 3) A timeline of maternal oxycodone dosing and pup handling procedures leading up to mTBI induction.

Phenotypic measurements and mTBI

Body weight and head size diameter, as measured from the front of each ear, were obtained from saline and IUO conditions at post-natal day 28 prior to injury. After injury righting reflex, or time to right (TTR) was recorded as the time from impact until a supinated animal placed pronates and mobilizes all four limbs supporting its own weight. For all animals time was kept ensuring that no more than 5 seconds passed from removal from anesthesia to mTBI induction.

mTBI Induction

This procedure was adapted from Mychasiuk et al., 2014 [172]. At P28 rats were placed in a pre-primed isoflurane chamber with 5% isoflurane in 100% oxygen for 45 seconds to induce anesthesia. Animals were removed from the chamber and subjected to a toe-pinch to assess sufficient plane of anesthesia. Animals were then placed, pronated, on a scored tin-foil platform 15 cm above a foam landing pad. A guide tube was centered above the mid-line of the subjects' head. A 100-mg weight was locked at the 500-cm height with a 505-cm line attached; the weight was released and allowed to free-fall and impact the subject (Figure 4). Subjects broke through the scored tin-foil base and were allowed to freely rotate post-impact to land supinated on the foam landing pad. Animals were subcutaneously administered 1mg/kg of bupivacaine to the site of impact and placed, supinated, in a cage and observed until normal movement was observed. No mortality or signs of complication were observed in any subjects post-mTBI. Sham conditions were identically anesthetized, placed on the tin-foil platform, and given 1mg/kg bupivacaine, however, no mTBI or rotational movement was suffered. The four conditions seen in the study are *in utero* saline exposure with sham (Sal+Sham), *in utero* saline exposure with mTBI (Sal+mTBI), *in utero* oxycodone exposure with sham (IUO+Sham), and *in utero* oxycodone exposure with mTBI (IUO+mTBI).

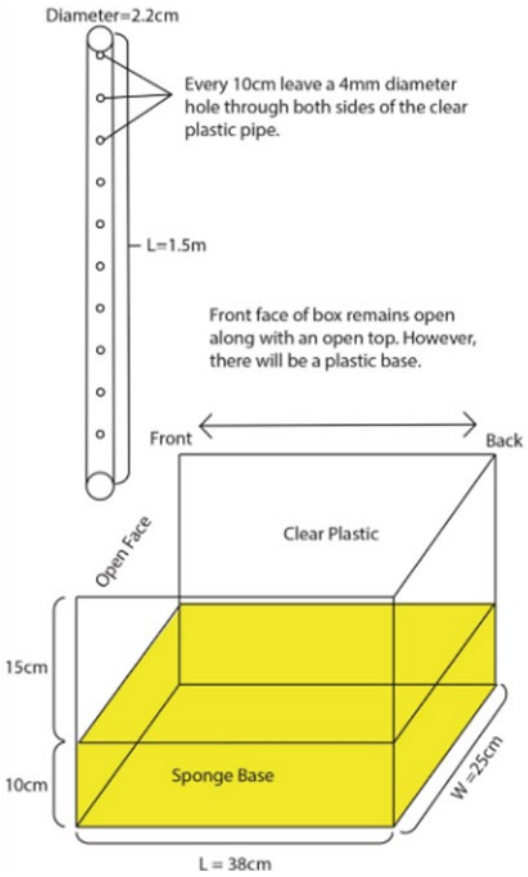


Figure 4) Design and dimensions of weight drop mTBI induction platform and guide tube.

RNA isolation and qPCR

Total RNA from cortex tissue was isolated from the randomly selected male or female pups of each treatment condition at DPI 2, 6, and 10 using the Direct-Zol RNA kit (Zymo Research, CA, USA). A custom TaqMan probe plate was purchased from Applied Biosystems designed for mTBI and inflammation associated genes (Materials). Analysis by real-time PCR (rt-PCR) was performed, and the delta-delta Ct method was used to calculate fold change and statistical significance as previously described [169].

Immunohistochemical (IHC) Analysis

Rat brains were collected after perfusion with sterile 1X PBS followed by 4% PFA. Brains were placed in cooled 4% PFA overnight at 4 °C. Following this, brains were cryoprotected by resting in a solution of 20% sucrose for 24 hours followed by a 30% sucrose solution for 24 hours at 4 °C. Following sucrose preparation tissues were snap frozen in Optical Cutting Temperature Compound (OCT) molds via immersion in a pre-cooled -80 °C 2-methylbutane bath. Frozen tissues were stored at -80 °C until cryosectioning. Sections were cut at -20 °C in 10μm thick sections at interaural 6.48mm and bregma -2.52 axes as described by Paxinos and Watson's rat brain atlas to include both the site of direct injury as well as the primary and secondary motor cortex. Tissues were warmed to 37 °C for 10 minutes then washed in 1X PBS three times before being permeabilized for 15 minutes in 0.3% Triton. Permeabilized tissue was blocked using 3% normal goat serum and 3% FBS before incubation at 4 °C overnight in primary antibody (Materials). Sections were then washed in PBS three times before 1-hour incubation with appropriate secondary antibody and DAPI for nuclear staining. Sections were mounted using Prolong Gold Antifade reagent. Sections were imaged using EVOS M5000 (Thermo Fisher) microscope with DAPI (357/447 nm), GFP (470/525 nm), and Texas Red (585/524) fluorescent light cubes. Microglia were labeled using primary Iba1/Aif1 and secondary Alexa Fluor 488 secondary (Materials). Quantification images were captured at the apical cortex using 10x

magnification, and representative images were taken using a 40x magnification. Cell quantification was performed using FIJI [173] by converting 10x IHC captured images to 8-bit then threshold gating using Sal+Sham controls to assess number of cells and relative size of microglia for all conditions.

^{1}H-MRI/MRS Acquisition

P30 pups were used for *in vivo* localized ^{1}H-MRS imaging of the cortex similarly to previously published works in the hippocampus [170]. Animals were anesthetized using 1–1.5% isoflurane in 100% oxygen and maintained 40–80 breaths/minute. MRI and ^{1}H-MRS data were obtained using a Bruker® Biospin 7 Tesla/21 cm small animal scanner (Bruker, Billerica, MA), operating at 300.41 MHz, using a laboratory-built 22 mm diameter quadrature birdcage volume coil. All first- and second-order shim terms were first automatically adjusted in the volume-of-interest (VOI) using MAPSHIM® (Bruker, Billerica, MA), with a final shim performed manually to achieve a water line width of 10–15 Hz. The water signal was suppressed by variable power radiofrequency pulses with optimized relaxation delays (VAPOR). MR images were acquired for anatomical reference using a multi-slice rapid acquisition with relaxation enhancement (RARE) sequence (Effective echo time (TE) = 36 ms, Rare Factor = 8, repetition time (TR) = 4,200 ms, Number of Averages (NA) = 2, Scan Time = 3 m 21 s; FOV = 20 × 20 mm2, Matrix Size = 256 × 256, Spatial Resolution = 0.078125 × 0.078125 mm2, Number of Slices = 29, Slice Thickness = 0.5 mm). ^{1}H-MRS data sets were obtained using semiLASER localization with timing parameters (TE/TR = 40/4,000 ms, 576 averages, 2,048 points) from a 2 × 5.187 × 1.557 mm3 (16.15 μL) VOI located in the mid-cortex. Pulse types and specifications: Excitation: hermite 90, duration = 0.7 ms, bandwidth = 5,400 Hz; 1st and 2nd Refocusing: hyperbolic secant, duration = 4 ms, bandwidth = 9484.5 Hz. The acquisition time was 38:24 min per data set. All pulses were applied with a frequency offset of −600 Hz to center the pulse bandwidth between Creatine (CRE) and N-Acetyl Aspartate (NAA). For the water suppression module, the spoiler strength matrix was

calculated automatically. Spoiler strength was 35%; spoiler duration was 1.5 ms. For each experiment, one data set was acquired without water suppression to be used as the water concentration reference during quantitation. Unsuppressed water spectra were obtained with identical metabolite spectra parameters except for the following: TR = 10,000 ms, NA = 1, and Receiver Gain = 64. One 64 average (for quality assessment) plus four 128-average data sets were acquired for metabolite measurements using a combination of VAPOR scheme for water suppression. Model parameters and constraints for quantification were generated using spectra from phantoms (n = 14) for the following metabolites: Alanine (ALA), Aspartate (ASP), Gamma-Aminobutyric acid (GABA), Glucose (GLC), Glutamine (GLN), Glutamate (GLU), Glycine (GLY), Lactate (LAC), Myo-inositol (MYO), Phosphorylcholine (PC), Taurine (TAU), total choline (tCHO), CRE, and NAA. Phantoms of each metabolite were prepared in pH 7.5 phosphate buffer (100 mM) and contained 3-(trimethylsilyl)-1-propane-sulfonic acid and sodium formate as chemical shift and phasing references. Spectra for each metabolite at known concentrations were acquired using semiLASER sequences at 40 ms TE, maintaining the phantom at 38 °C with a circulating water jacket during spectral acquisition. The set of metabolite spectra formed a metabolite basis set, which was used as prior knowledge during quantification. In all conditions, n = 6 except for IUO+Sham (n = 5) where one female animal was excluded for imaging complications.

Purified Synaptosome Isolation

To investigate the effects of *in utero* and postnatal oxy exposure on synaptic transmission, we isolated purified synaptosomes (SYP) following the protocol designed by Ahmed, Holt, Riedel, and Jahn [13] with minor modifications. 500mg of cortical tissue from each condition (n = 6) was homogenized in 9 mL of ice-cold homogenization buffer (320 mM sucrose, 4 mM HEPES, protease inhibitor tablet) using a Wheaton Overhead Stirrer with ten strokes at 2k–3k RPM. The homogenized mixture was centrifuged at 1000 × *g* for 10 min at 4 °C, and the pellet (P1; cell fragments and nuclei) was discarded. The supernatant was centrifuged at 12,000 × *g* for 20 min

at 4 °C. The pellet (P2; crude synaptosomes) was washed carefully in 1 mL of homogenization buffer. Suspended crude synaptosomes were layered on top of a sucrose gradient (1.20M, 0.80M, and 0.60M) and spun in a SW41 Ti Rotor (Beckman Coulter, Brea, CA, USA) at 145,000 × *g* for 40 minutes at 4 °C. The synaptosomal band was collected at the 0.80M and 1.20M interface. The collected band was resuspended with 9mL of homogenization buffer and spun again using the SW41 Ti Rotor at 145,000 × *g* for 40 minutes at 4 °C. This pellet was resuspended in 200-300 µL of PBS + protease inhibitor (Roche Diagnostics) depending on the size of the pellet garnering our purified synaptosome samples.

Mass Spectrometry

Mass spectrometry was performed using previously published methodologies [174]. Briefly, 100 µg of protein per purified SYP sample from six biological replicates per condition was taken, and detergent was removed by chloroform/methanol extraction. With 100 mM ammonium bicarbonate, the protein pellet was resuspended followed by digestion with MS-grade trypsin (Thermo Fisher) at 37 °C overnight. PepClean C18 spin columns (Thermo Scientific) were used to clean peptides. Cleaned peptides were re-suspended in 2% acetonitrile (ACN) and 0.1% formic acid (FA), and 500 ng of each sample was loaded onto trap column Acclaim PepMap100, 75 µm × 2 cm C18 LC Columns (Thermo Scientific) at a flow rate of 4 µL/min. Samples were then separated with a Thermo RSLC Ultimate 3000 (Thermo Scientific) on a Thermo Easy-Spray PepMap RSLC C18, 75 µm × 50 cm C-18 2 µm column (Thermo Scientific) with a step gradient of 4–25% solvent B (0.1% FA in 80% ACN) from 10–130 min and 25–45% solvent B for 130–145 min at 300 nL/min and 50 °C with a 180-min total run time. Following separation, the Thermo Orbitrap Fusion Lumos Tribrid (Thermo Scientific) mass spectrometer, in a data-dependent acquisition mode, was used to analyze the eluted peptides. All samples were batched and randomized for all runs. An MS scan (from m/z 350–1800) was acquired in the Orbitrap with a resolution of 120,000. The AGC target for MS1 was set as 4 × 105 and ion filling time set as 100

ms. To isolate the most intense ions a charge state of 2–6, a 3 s cycle was performed followed by fragmentation using HCD fragmentation with 40% normalized collision energy and detected at a mass resolution of 30,000 at 200 m/z. The AGC target for MS/MS was set as 5 × 104 and ion filling time set at 60 ms; dynamic exclusion was set for 30 s with a 10-ppm mass window. For identification of proteins a search was conducted where the MS/MS data were viewed against the Swiss–Prot *Rattus norvegicus* protein database downloaded in May 2019, using the in-house mascot 2.6.2 (Matrix Science, Canton, MA, USA) search engine. The search criteria consisted of full tryptic peptides with a maximum of two missed cleavage sites. The variable modifications included acetylation of protein N-terminus and oxidized methionine. The fixed modification was carbamidomethylation of cysteine. Additional parameters set include the precursor mass tolerance threshold, which was set at 10 ppm, and the maximum fragment mass error which was set at 0.02 Da. A false discovery rate (FDR) of ≤1% was used to determine the significant threshold of the ion score. Progenesis QI proteomics 4.1 (Nonlinear Dynamics, Milford, MA, USA) was then utilized to perform qualitative analysis.

Bioinformatics

Identified proteins were pruned for those with 2 or more unique peptides. Proteins were considered differentially expressed if the FDR-corrected p-value was ≤0.05 and displayed a fold change ≥ 1.5. Further exclusion criteria were placed to reduce the number of significant upregulated hits in the IUO+Sham and IUO+mTBI conditions by only including proteins with a fold change ≥ 1.5 as compared to ACTB (Table 2). These cut offs were determined by the variability of protein quantification values as compared to known standard proteins ACTB and GAPDH. Venn diagram was generated using bioinformatics.psb.ugent.be/webtools/Venn/ online resource. Heatmap was generated using the top expressed mitochondrial proteins shared between IUO+Sham and IUO+mTBI conditions in Graph Pad Prism software (La Jolla, CA, USA). Cytoscape plugin ClueGO was used to perform gene ontology (GO) analysis of biological

processes on significantly differentially expressed proteins [175]; criteria placed were: *Rattus norvegicus*, all experimental, GO term fusion, and pV ≤ .05. Further, enriched disease-associated pathways were identified using the Ingenuity Pathway Analysis (IPA) software (Ingenuity® Systems, Redwood City, CA, USA). Canonical pathway analysis in IPA was performed by comparing the differentially expressed proteins against known canonical pathways (signaling and metabolic) within the IPA database.

	Sal+Sham vs Sal+mTBI	Sal+Sham vs IUO+Sham	Sal+Sham vs IUO+mTBI	Sal+mTBI vs IUO+mTBI	IUO+Sham vs IUO+mTBI
Total Mapped Proteins	**4579**	**4260**	**4315**	**4327**	**4120**
Total DEPs	**142**	**1388**	**1704**	**1672**	**131**
Total DEPs (Normalized to ACTB)	**142**	**322**	**465**	**385**	**131**
Upregulated DEPs (Normalized to ACTB)	**24**	**184**	**359**	**248**	**95**
Downregulated DEPs (Normalized to ACTB)	**118**	**138**	**106**	**137**	**36**

Table 2) List of total mapped proteins and numbers after normalization to ACTB. DiffExpPro considered if $p < 0.05$, unique peptides > 2, and ± 1.5 fold change.

Immunoblotting

15ug of protein as determined by Pierce bicinchoninic acid assay (Thermo Fisher Scientific, Waltham, MA, USA) from each animal were loaded into 4–12% Bis-Tris wells (Invitrogen, Waltham, MA, USA) under reducing conditions, followed by transfer to a nitrocellulose membrane using iBlot2 (Invitrogen). Ponceau S stain (Thermo Fisher Scientific) was used on the nitrocellulose membrane for equal protein loading detection and quantification. SuperBlock was used to block nonspecific antibody binding (Thermo Fisher Scientific, Waltham, MA, USA). After blocking, membranes were incubated overnight at 4 °C with a primary antibody (Supplementary Table 2). Primary and secondary antibody dilutions were done according to the manufacturer's suggestion and are shown in Supplementary Table 2. Blots were developed using Azure cSeries Imager (Azure Biosystems, Dublin, CA, USA) with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

Isolation of Synaptic and Non-Synaptic Mitochondria

Synaptic mitochondria were isolated from P30 rats using modifications to a method described previously [56]. Following decapitation, brains were rapidly removed, micro-dissected and placed in ice-cold isolation medium (IM) containing 225 mM sucrose, 75 mM mannitol, 1 mM EGTA, 5 mM HEPES, and cOmplete Mini, EDTA-free protease inhibitor cocktail (Roche Diagnostics) adjusted to pH 7.4. All homogenization and centrifugation steps were carried out on ice and at 4°C, respectively. Brains were minced and homogenized with 35 strokes in a Dounce homogenizer. The homogenate was then centrifuged at 1,300 x g for 3 min. Supernatant was collected and the pellet was resuspended in IM and centrifuged again at 1,300 x g for 3 min. The pooled supernatants were centrifuged at 21,000 x g for 10 min. This pellet was then resuspended in 15% Percoll and layered on top of a 24% and 40% Percoll gradient (prepared from 100% Percoll solution containing 225 mM sucrose, 75 mM mannitol, 1 mM EGTA, and 5 mM HEPES adjusted to pH 7.4 with HCl). Following centrifugation for 8 min at 30,700 x g the banding near the interface

of the upper two layers of the gradient, containing mainly synaptosomes, was collected and diluted in IM. This synaptosomal fraction was then transferred to a nitrogen cavitation vessel (Parr Instrument Company) where the pressure was equilibrated to 1000 psi for 15 min followed by depressurization to ATM pressure, which released synaptic mitochondria. This suspension was then added to the top of 24% Percoll and centrifuged for an additional 10 min at 30,700 x g. The pellet containing the synaptic mitochondria was resuspended in IM and centrifuged at 16,700 x g for 10 min. Finally, the pellet was resuspended in IM with fatty acid free BSA followed by centrifugation at 6,900 x g for 10 min. This final pellet containing (w/v) SDS and 0.1M DTT adjusted to pH 7.6. Lysates were incubated at 95°C for 5 min then briefly sonicated. Protein concentrations were determined using a Pierce 660 nm Protein Assay.

Seahorse Electron Flow Analysis of Synaptic and Non-Synaptic Mitochondria

The Seahorse XF24 Flux Analyzer (Seahorse) was equilibrated to 37°C overnight and a modified protocol was used based on previous work in mouse liver mitochondria [176]. The isolated synaptic mitochondria and non-synaptic (10 µg) were plated in V7-PS XF24 cell culture microplates in a volume of 50 µl mitochondrial assay solution (MAS) containing 70 mM sucrose, 220 mM mannitol, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 2 mM HEPES, 1 mM EGTA and 0.2% fatty-acid free BSA with 10 mM pyruvate, 2 mM malate, and 4 µM FCCP. After centrifugation for 15 min at 2,000 x g to attach mitochondria, 450 µl of MAS (containing substrate) was added to each well, and the plate was incubated at 37°C for 8 min to equilibrate temperature. The final concentrations of additions to the wells were 4 mM ADP, 2.5 µg/ml oligomycin, 4 µM FCCP, and 4 µM antimycin A for the coupling assay and 2 µM rotenone, 10 mM succinate, 4 µM antimycin A, and 10 mM ascorbate with 100 µM TMPD for the electron flow assay. The electron flow assays were run in 4-6 technical replicate wells for each independent biological replicate (electron flow assay, n = 6 for saline conditions and n = 3 for IUO conditions). XF24 data was calculated using the algorithm

previously described and used by the Seahorse software package [62]. Statistical analysis was conducted in PRISM (GraphPad Software) using one-way ANOVA and post-test with Tukey's multiple comparison tests.

Rotarod

P61-64 animals were assessed for motor function and balance using the Rotarod (Panlab, Model LE8305 76-0771; Barcelona, Spain). A central rod with a diameter of 60mm made of Perspex and knurled to provide adequate grip is divided into 5 lanes. Animals were placed on the rod within a lane 215mm above a trip box at a starting speed of 4 rotations per minute (RPM). The start of time recording began an acceleration protocol by which the rod would increase its RPM by .12 rotations per second to a maximum of 40 rotations over a 300 second period. Animals underwent 6 independent trials with 20 minutes between each trial. The first three trials were considered training trials, while the last three trials were considered performance trials. Rats were placed on the rotarod for 10s at 4 RPM, and then latency to fall was measured for each animal after acceleration protocol began. Rats were considered to fall if they fell from the rod, turned around, or clung to the rod for two full rotations. All assessments were conducted within the first 6 hours of the animal facility light-cycle.

Marble Burying

P61-64 animals were assessed for activity and anxiety using marble burying assessment as described in previously published works [169]. A rat cage (929 cm^2, 43.18 × 21.59 × 20.32 cm) containing an even 5 cm layer of ¼-inch corncob bedding (Envigo #7097), and 20 standard glass marbles (15 mm diameter, 5.2 g) were lightly placed in a 5 × 4 arrangement on top of the bedding. The subject was placed into the cage, and the cage was covered for 30 min. The animal was removed, and the marbles were imaged and scored by a scorer blinded to the conditions. A marble was considered buried if more than 70% of the marble was under the bedding. All assessments were conducted within the first 6 hours of the animal facility light-cycle.

Social Preference and Social Novelty

Both social novelty and social preference were carried out in P65 animals using an in-house built chamber as described in previously published works [169]. Briefly, a 90 × 40 × 40 cm acrylic chamber was divided into three 30 × 40 × 40 cm compartments. The left and right compartments contained 15 × 15 × 40 cm perforated isolation cubes. Perforations were spaced 1 cm apart along the entirety of both isolation cubes to allow for scenting and touching but not entrance.

To evaluate social novelty, a naive animal of the same sex and of similar age and size was placed into the left isolation cube. A cagemate of the test animal's housing cage was placed into the right isolation cube. The test animal was placed into the central chamber. For assessing social preference, a new naive rat (not used in social novelty) was placed into the left isolation cube, a rubber toy in the right isolation cube, and the test animal into the central chamber. After 5 min of acclimation, the two doors were lifted and the test animal was allowed to freely explore the entirety of the social chamber for 15 min. Animals were then returned to their housing cages, and the social chamber was cleaned and sterilized.

Scoring for both social tests consisted of the time the animal spent in each chamber, the number of entries into each chamber, and the number of active contacts toward one of the isolation cubes. Entry into a chamber was scored if an animal's head and all four paws were within the compartment. An active contact was defined as any attempt to sniff, paw, scratch, touch, or stretch toward any of the isolation cubes when inside the compartment containing an isolation cube. Testing was recorded, and recordings were scored manually by scorers blinded to the conditions. All assessments were conducted within the first 6 hours of the animal facility light-cycle.

Hot Plate Nociception Testing

An animal was placed on an Incremental Hot Plate set at a starting temperature of 25 °C (IITC Life Science, Irving, CA, U.S.A.). Temperature increased by 5°C per minute with maximum temperature of 50°C. During testing animals were constantly monitored by an attendant. When the animal licked its back paw, a standardized behavior to avoid heat, the test was stopped. Duration and intensity of heat was recorded using software provided by IITC Life Science. The testing plate was sanitized between each run to prevent eliminate scent-related confounds. All assessments were conducted within the first 6 hours of the animal facility light-cycle.

Maternal Pup Retrieval

A total of n = 6 dams with litters between 9-12 pups per condition were assessed for maternal behavior at 3 days post-birth. Dams were taken to the behavioral room at the beginning of their light cycle. A 1-hour acclimation period was allowed. The dam was allowed to voluntarily end nursing and was then removed from the home chamber temporarily. Three randomly selected pups were moved to three corners of the cage furthest from the central nursing nest. Dam was returned to the center of the home cage and recording begins. Time till first retrieval, second retrieval, third retrieval, and crouched nursing was recorded over a duration of 10 minutes. If dams failed to initiate nursing within 10 minutes their recording was noted at 600 seconds. Home cage was returned to animal room and left undisturbed. Pups in litters used for pup retrieval were not used in any further behavioral assessment.

Statistical Analyses

Data were analyzed for outliers using standard outlier tests in GraphPad Prism (version 9.3.1) finding that all data was normally distributed. Significance was determined using appropriate statistical testing outlined within figure legends for specific comparisons made followed by Tukey’s or Dunnett’s multiple comparison correct where appropriate with a minimum

criterion for significance of $p \leq .05$. All data are presented as mean ± SEM with individual data points. Figure 5a-b, 7, and 23 data were analyzed using Welch's unpaired t-test to compare between two conditions to compare populations with unequal sample size. In figures 5c, 9a-c, 11, 14a-c, 15a-c, 19, 21a-c, and 22 when comparing between four conditions Sal+Sham, Sal+mTBI, IUO+Sham, and IUO+mTBI one-way ANOVA was performed with Tukey's multiple comparison correction if the same control animals were used between comparisons. In figures 8a-c, 9c-d, 10, 17, 18a-b, 20 a-b, and 21d two way ANOVA was performed when comparing multiple variables between the four conditions with Tukey's multiple comparison correction when the same control animals were used between comparisons. Results were visualized using GraphPad Prism (version 9.3.1).

CHAPTER 1

Characterization of *in utero* Oxycodone Exposure's Effect on Response to Mild Traumatic Brain Injury

Abstract

The rising opioid crisis has also seen a growing number of children born suffering neonatal opioid withdrawal syndrome (NOWS). These opioid exposed children are known to have altered physiology, cognition, and behavior. However, to date no published works have explored the potential vulnerability of these children to later life insults. Using a preclinical rodent model that mimics oxycodone (oxy) exposure *in utero* (IUO) we sought to characterize the response to pediatric minor traumatic brain injury (mTBI) in unexposed subjects versus opioid exposed subjects. We employed an integrative approach investigating the righting reflex, physiological measurements, qPCR, and immunohistochemistry (IHC) to elucidate the differential vulnerability to mTBI between saline and IUO conditions. We found significant deficits in righting reflex in our IUO+mTBI conditions that coincided with increased neuroinflammation revealed in both IHC and qPCR studies. Further we found significantly increased biomarkers of neurodegeneration and mTBI in both IUO+Sham and IUO+mTBI conditions. Additionally, in IUO+mTBI condition and exacerbated metabolic stress disorder is revealed by ^{1}H-MRS showing multiple key metabolites being dysregulated. Given the rising population of NOWS children these data are critical for understanding the molecular and physiological vulnerability of IUO exposed children to one of the most common childhood injuries: mTBI.

Introduction

The ongoing opioid epidemic has seen record numbers of children born suffering from neonatal opioid withdrawal syndrome (NOWS) caused by *in utero* opioid exposure [177]. The ability for opioids to pass both the placental barrier and blood-brain barrier (BBB) makes them particularly impactful neurological teratogens [78, 153, 178]. First line therapeutic interventions for NOWS relies on a methadone weaning protocol to avoid withdrawal related complications, further extending the duration children are exposed to opioids [179]. *In utero* opioid exposure causes many neuropathophysiological outcomes including cortical thinning, increased basal neuroinflammation, attenuated myelination, motor deficits, behavioral issues, and cognitive impairment [89, 111, 151, 153, 178]. Significant advances have been made in establishing the negative molecular and physiological sequelae associated with *in utero* opioid exposure, however, to date there has been few reports understanding how these changes would impact later life development. Broadly, gestational insults as a whole have been well understood to induce later life vulnerability to injury and disease, and many studies have also observed these epidemiological effects in children suffering from NOWS [85, 151, 153, 154]. Interestingly, recent data shows that children who suffered from NOWS have a significantly increased rate of emergency department (ED) visits throughout childhood [85]. This makes *in utero* opioid exposure a marked risk factor for later life injury and disease in a potentially vulnerable population; in fact, falls are the most common non-fatal injury for ED admittance in children and are the primary cause of pediatric mild-traumatic brain injury (mTBI) [155]. Given the well reported association of NOWS with motor-control deficits and motor cortex thinning persistent through peri-adolescence [89, 153] there is an urgent need to understand if this population is more vulnerable to one of the most prevalent pediatric injuries: mTBI [151].

mTBI is one of the most common injuries with a 2020 report showing that 8.3% of children were diagnosed with at least one concussion before reaching 18 years old, and self-reported

numbers being significantly higher [156]. The primary cause of mTBI in children is from slips, falls, and accidents during play [157]. The acute sequelae of mTBI overlap significantly with *in utero* opioid exposure associated pathophysiology such as increased inflammation, myelin disruption, and cognitive deficits. However, mTBI associated patient-perceived symptoms can be recoverable in as short of a time frame as 15 minutes to 2 weeks in most cases [180]. Despite this a significant minority of mTBI sufferers have longitudinal symptoms that impose serious deficits including accelerated neurodegenerative disease progression [159, 181-185]. What is poorly understood is why some individuals develop these negative symptoms post-mTBI while others recover normally. Currently, the dominant theory is that individuals with poor outcomes have an innate or induced susceptibility to neurotrauma due to genetic or environmental risk factors, yet what these risk factors are remains largely unknown [186-189].

In the current study, we utilized a previously established regimen to mimic *in utero* and post-natal oxycodone exposure (IUO) in a rat model, combined with a low-force closed-head weight drop model of mTBI to mimic a recoverable brain injury that could be caused by a slip or fall which make up the majority of juvenile mTBI [155]. Utilizing a closed head weight drop model is ideal for investigating head injuries similar to those seen in the pediatric population because it mimics both the contusion injury at the site of injury as well as the rotational acceleration associated injury [190]. Assessing physiological and molecular assessments commonly used in the clinic and field, for the first time, we demonstrate that IUO exposure creates a susceptible environment in the brain, that when challenged with a mild stressor such as mTBI, results in exacerbated negative outcomes. These data shed light on novel mechanisms that warrants for increased patient and provider consideration of brain trauma in this vulnerable subset of the population who are gestationally exposed to opioids.

Results

IUO Induces Disparities to Development and Righting Reflex Post-mTBI:

IUO exposure has been reported to affect physiological development in rodent and rat models, including weight, head size, and BMI [88, 145, 169, 191-194]. To assess the effect of the IUO exposure on physiological development, we measured the weight and head circumference as previously described [195]. IUO and saline exposed conditions were measured at post-natal day (P) 28, an age that mimics early childhood in humans and corresponds not only to a highly active stage for neurological development but also a vulnerable age for mTBI [196]. At P28, IUO exposed conditions did not show significant alterations in weights as compared to the saline condition (Figure 5a), however, displayed significantly decreased head diameter (Figure 5b). Next, the saline and IUO rats underwent mTBI procedure and righting reflexes or time-to-right (TTR) was calculated to inform on the severity of injury as a measure of the rats' ability to regain normal motor function. TTR as defined by time it takes a supinated rat to awaken from anesthesia and mobilize all four limbs supporting its own weight after mTBI or sham induction. Within the saline conditions, a significant increase ($p \leq 0.004$) in TTR was observed in animals that underwent mTBI (Sal+mTBI) when compared to animals that underwent the sham procedure (Sal+Sham) (Figure 5c). Within the IUO conditions, animals that underwent mTBI (IUO+mTBI) displayed significant increase in TTR when compared to IUO+Sham ($p \leq 0.0001$) but also when compared across other saline conditions, Sal+Sham ($p \leq 0.0001$) and Sal+mTBI ($p \leq 0.0001$). On the other hand, no significant differences were seen between the saline and IUO conditions that underwent just the sham procedure. Further, the data also suggests that the combination of IUO+mTBI has a greater impact on TTR when compared to IUO exposure or mTBI alone with a 3.31-fold and 2.35-fold increase in TTR, respectively. To ensure that our model was consistently targeting the same bilateral neuroanatomical region and not resulting in major intracranial bleeding or skull fractures some animals were immediately sacrificed post-mTBI to assess for these presentations (Figure 6). Our results show that we can consistently target the medial apical cortex of P28 rats

and that our damage is not resulting in intracranial hematoma or fracturing of the skull suggesting that our model is anatomically representative of a mTBI that could be caused by a slip or fall. Interestingly the liver and heart were also assessed in these animals since significant cardiac and renal effects have been observed in NOWS children. While no significant difference in liver size or anatomical characteristics of were noticed, a significant decrease in heart size was noticed between saline and IUO exposed pups across three different time points. (Figure 7)

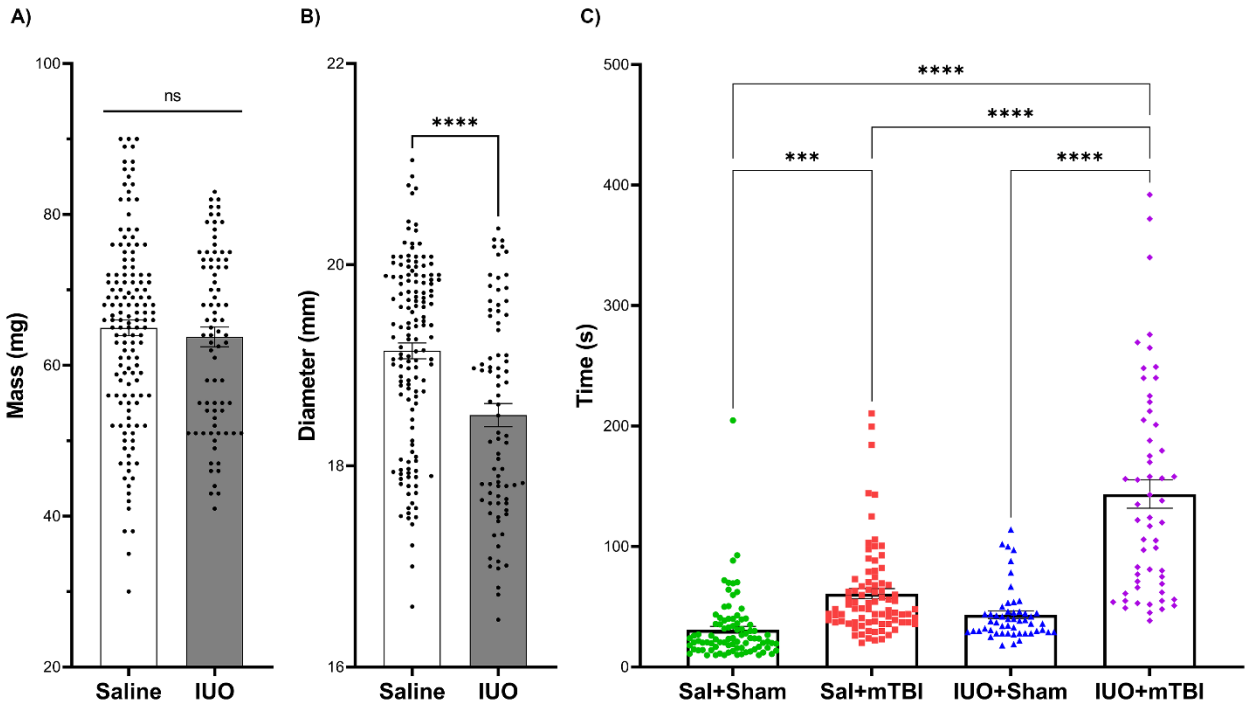


Figure 5) IUO exposure causes pathophysiological head diameter and righting reflex in response to mTBI. At post-natal day 28 *in utero* saline (Saline) and oxycodone (IUO) exposed animals were subjected to Sham or mTBI procedures and measured for weight, head diameter, and righting reflex. **(A)** IUO exposure does not significantly affect animal weight at P28 ($p \geq 0.48$) but significantly decreases **(B)** head diameter. Data is represented as Mean ± SEM, n = 141 for Saline and n = 80 for IUO; ****$p < 0.0001$. Welch's unpaired t-test was performed. **(C)** Post-anesthesia either Sham or mTBI procedure animals were assessed for their time-to-right (TTR). Sal+mTBI groups showed a significantly increased TTR when compared to Sal+Sham conditions ($p \leq 0.004$); IUO+mTBI conditions had a significantly increased TTR as compared to Sal+Sham ($p \leq 0.0001$), Sal+mTBI ($p \leq 0.0001$), and IUO+Sham ($p \leq 0.0001$). Data are represented as Mean ± SEM; n=86 for Sal+Sham, n=82 for Sal+mTBI, n=52 for IUO+Sham and n = 56 for IUO+mTBI; *$p \leq 0.05$, **$p \leq 0.001$, ***$p \leq 0.005$, ****$p \leq 0.0001$. One-way ANOVA with Tukey's multiple comparison test was performed.

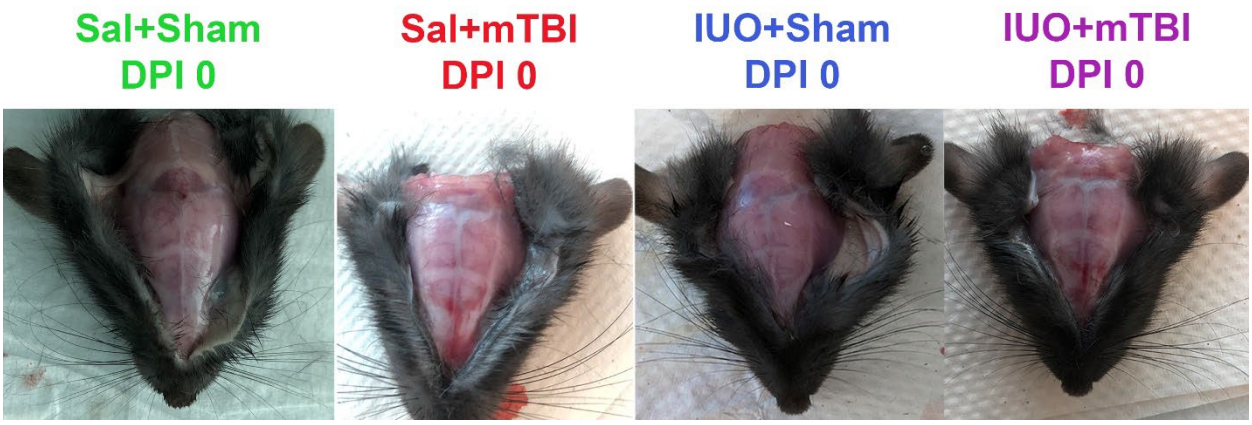


Figure 6 Representative photos of rat skulls post-mTBI. P28 animals post-sham or -mTBI induction. Photos taken within 30 minutes post-sham or -mTBI induction.

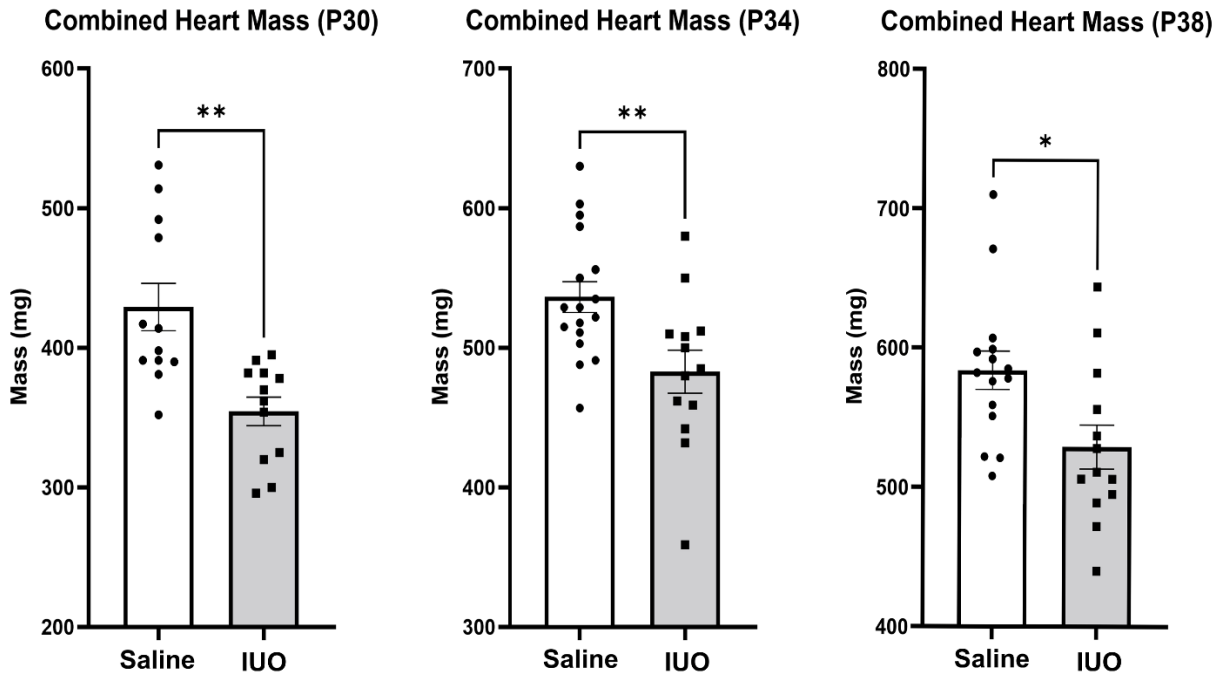


Figure 7) Mass of whole heart compared between Sal+Sham and IUO+Sham conditions. No significant differences we found to be seen by mTBI when comparing to their respective uninjured condition. Data is represented as Mean ± SEM; *p ≤ 0.05, **p ≤ 0.001, ***p ≤ 0.005, ****p ≤ 0.0001. Welch's unpaired t-test was performed.

Assessment of Cortical Neuroinflammatory and Classical Biomarkers Post-mTBI:

To understand the effects of IUO in the brain post-TBI, we assessed the expression of several well reported marker genes associated with mTBI induced inflammation across days post injury (DPI) 2 and 10 [197]. At DPI 2, qPCR analysis revealed a significant increase in both the inflammatory (*Aif1, Il1β, Il6, and Tnfα*, Figure 8a) as well in classical mTBI biomarkers (*Bdnf, Mapt, Mbp, Nefl, and S100β*, Figure 8b) in the Sal+mTBI conditions when compared to Sham controls [197]. Further, no significant upregulation in the expression of inflammatory genes was observed at DPI 10 in Sal+mTBI conditions, apart from one gene, *Nefl,* that showed increased expression (Figure 8b). On the other hand, the IUO conditions (IUO+Sham and IUO+mTBI) displayed significant upregulation of inflammatory genes: *Cxcl1, Il6, and Tnfα*, across all DPI timepoints (Figure 8c & 8e). Additionally, classical mTBI biomarker genes (*Bdnf, Mapt, Nefl, and S100β*) were significantly upregulated across all DPI timepoints (Figure 8d & 8f). However, no significant differences were seen between IUO+mTBI conditions when compared to IUO+Sham. Next, we performed immunohistochemistry (IHC) to investigate inflammatory cells of the brain, microglia, at the superior site of the cortex closest to the weight drop impact. IHC results show no significant changes in microglia count in the Sal+mTBI conditions as compared to the Sal+Sham but a trending increase at DPI 2 and 10 (Figure 9). However, in the IUO+mTBI we see significant increase in microglia numbers and size at DPI 2 and 10 suggesting an exacerbated response after acute injury with an impaired ability to return to baseline post 10 days mTBI (Figure 9). The increased total size of the microglia suggests an increased proportion of ramified M1 type microglia responding to the injury.

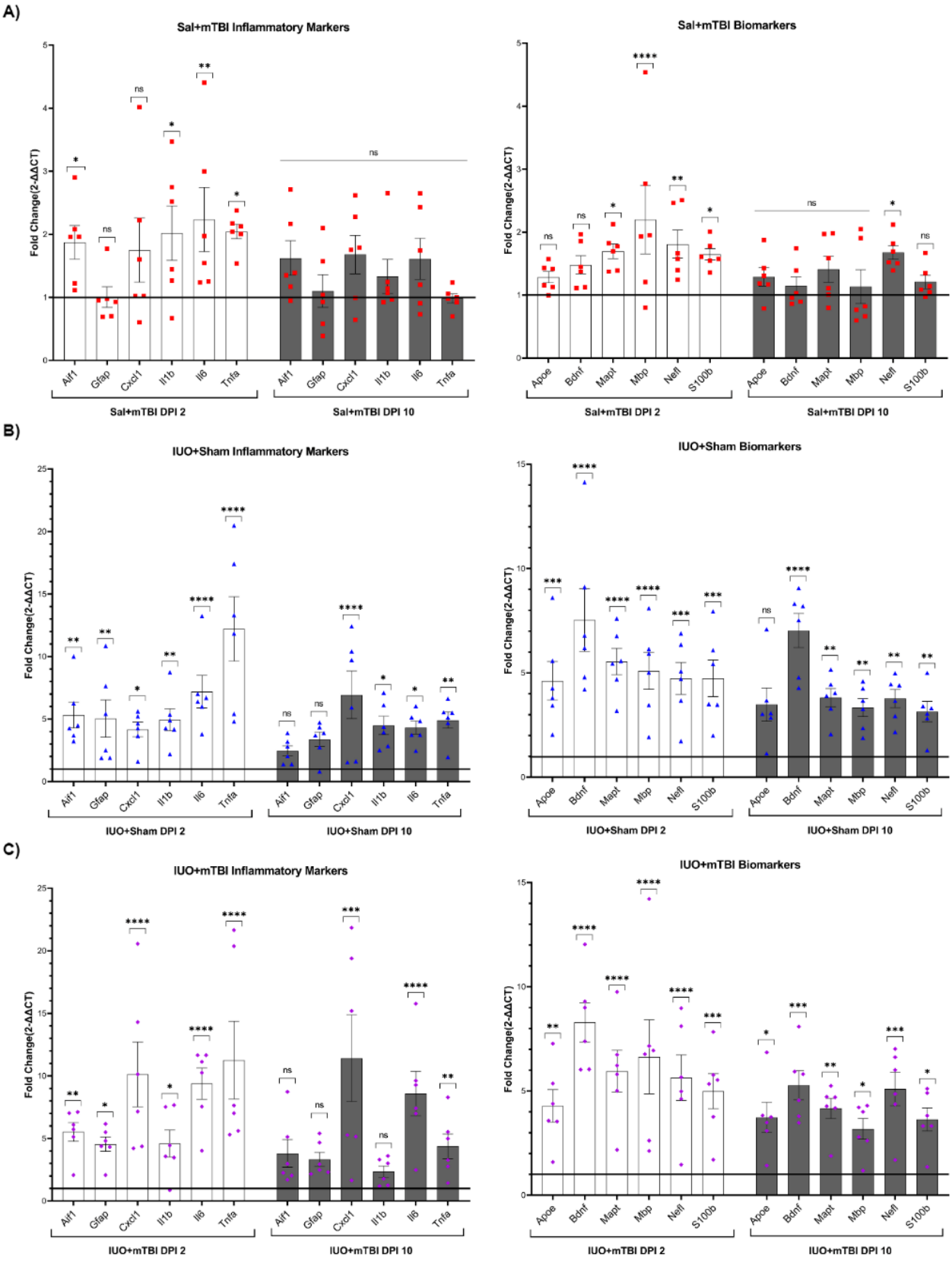

A)
Sal+mTBI Inflammatory Markers
Fold Change(2-ΔΔCT)
Alf1
Gfap
Cxcl1
Il1b
Il6
Tnfa
Sal+mTBI DPI 2
Sal+mTBI DPI 10
Sal+mTBI Biomarkers
Apoe
Bdnf
Mapt
Mbp
Nefl
S100b
B)
IUO+Sham Inflammatory Markers
IUO+Sham DPI 2
IUO+Sham DPI 10
IUO+Sham Biomarkers
C)
IUO+mTBI Inflammatory Markers
IUO+mTBI DPI 2
IUO+mTBI DPI 10
IUO+mTBI Biomarkers

Figure 8) Common gene markers of inflammation and brain injury are increased in mTBI and IUO exposure. Cortical brain tissue was assessed for mRNA levels of key biomarkers of inflammation and brain injury at 2- and 10- days post mTBI/Sham procedures using quantitative real-time PCR analysis. **(A)** Sal+mTBI conditions showed a significant increase of inflammatory markers Aif1, Il1b, Il6, and Tnfα as well as increased markers of brain injury Bdnf, Mapt, Nefl, and S100β. However, these effects were largely ameliorated at DPI 10. Nefl was the only significantly increased marker at DPI 10. **(B)** IUO+Sham conditions showed a significant increase in all biomarkers of inflammation and injury at DPI 2. At DPI 10 once again all markers were significantly increased. No significant gender differences were found within groups. **(C)** For the IUO+mTBI group all markers were significantly increased at DPI 2. While at day 10 Cxcl1, Il6, Tnfα, Apoe, Bdnf, Mapt, Mbp, Nefl, and S100β were increased. No significant gender differences were noted within groups. Additionally, when comparing IUO+Sham to IUO+mTBI no significant differences were found. Data are displayed as Mean ± SEM; for all conditions equal number of sexes were used n = 6 ; *p ≤ 0.05, **p ≤ 0.001, ***p ≤ 0.005, ****p ≤ 0.0001. Analysis was performed by two-way ANOVA with Fisher's LSD.

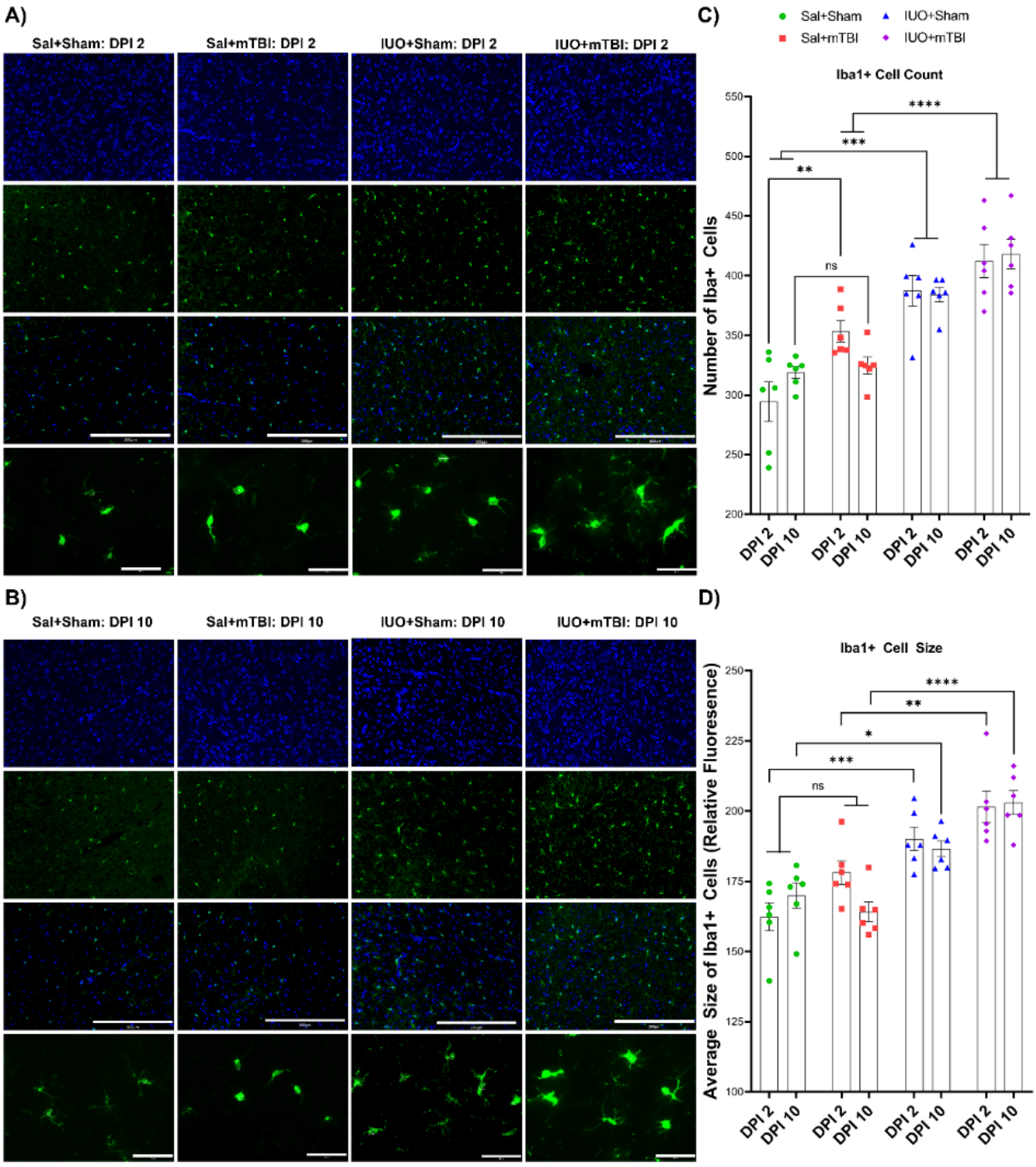

A)
Sal+Sham: DPI 2
Sal+mTBI: DPI 2
IUO+Sham: DPI 2
IUO+mTBI: DPI 2
B)
Sal+Sham: DPI 10
Sal+mTBI: DPI 10
IUO+Sham: DPI 10
IUO+mTBI: DPI 10
C)
Sal+Sham
Sal+mTBI
IUO+Sham
IUO+mTBI
Iba1+ Cell Count
Number of Iba+ Cells
550
500
450
400
350
300
250
200
****
***
**
ns
DPI 2
DPI 10
D)
Iba1+ Cell Size
Average Size of Iba1+ Cells (Relative Fluoresence)
250
225
200
175
150
125
100
****
**
*
***
ns

Figure 9) Immunohistochemical analysis of cortical macrophages show that IUO exposure increases macrophage number and cell size. Representative Iba1^{+} stained sections at **(A)** DPI 2 and **(B)** DPI 10 from apical cortex for all four conditions shows increased number and size of Iba1+ cells in IUO conditions. **(C)** Quantification of total number of cells shows a significant increase in Sal+mTBI as compared to Sal+Sham at DPI 2 ($p \leq 0.0028$), but not at DPI 10 ($p > 0.98$). At DPI 2 and 10 both IUO+Sham and IUO+mTBI show increased number of Iba1+ cells when compared to Sal+Sham ($p \leq 0.001$), this significance is also observed in Sal+mTBI at DPI 10 for IUO+Sham ($p \leq 0.0026$) and IUO+mTBI ($p < 0.0001$). However, only IUO+mTBI was significantly increased at DPI 2 as compared to Sal+mTBI ($p \leq 0.0029$). **(D)** Quantification of the relative size of counted cells in Figure 9c shows a trending but not significant increase in Iba1+ cell size in Sal+mTBI conditions as compared to Sal+Sham conditions at DPI 2 ($p > 0.0636$). IUO+Sham showed a significant increase in cell size when compared to Sal+Sham at DPI 2 ($p \leq 0.0003$) and DPI 10 ($p \leq 0.0461$), there was also a significant increase for IUO+mTBI at DPI 2 and DPI 10 ($p \leq 0.0001$). Further, IUO+mTBI showed a significant increase in Iba1+ cell size as compared to Sal+mTBI at DPI 2 ($p \leq 0.0023$) and DPI 10 ($p \leq 0.0001$). At DPI 10 IUO+mTBI Iba1+ cells were also significantly increased in size as compared to IUO+Sham conditions ($p \leq 0.0475$). For all conditions equal sexes were used ($n = 6$), and no significant gender differences were noted within groups. Data are displayed as Mean $\pm$ SEM (*$p \leq 0.05$; **$p \leq 0.001$; ***$p \leq 0.005$, ****$p \leq 0.0001$). Analysis was performed by two-way ANOVA with Tukey multiple comparison correction.

Discussion

It is well-known that gestational drug exposure poses significant threat to the developing fetus, affecting overall brain development and consequently leading to maladaptive behaviors [169, 170, 198]. Moreover, additional risk factors such as age of parents, education, and other stressors such as mild brain injury caused by slips and falls in infants can exacerbate these neurodevelopmental deficits. In this study, we utilized mTBI as a model to recapitulate these events in juvenile rats showing for the first time that *in utero* oxycodone exposure can induce vulnerability to one of the most common childhood injuries. Specifically characterizing IUO induced anatomical pathophysiology which overlap with predictors for worsened outcomes post-mTBI. Additionally, neuroinflammatory markers, microglial recruitment, and microglial morphology suggest that IUO induces molecular markers of vulnerability with similar characteristics of brain injury.

Our first observations show that IUO exposed animals suffered decreased head diameter (Figure 5), a condition often associated with cortical thinning and developmental deficits in NOWS children [89]. Prescription opioid exposure has shown significant decrease in cortical volume of both left and right precentral gyrus, a region of the brain critical for motor function [89]. This deficiency in the precentral gyrus has also been implicated in significant motor deficiencies in children diagnosed with NOWS, particularly, prevalent in children from the ages of 4.5 – 6 years old [151]. An additional consideration is the behavior of the dams while on oxy as rearing behavior is known to impact brain and behavior [199, 200]. Next, we induced mTBI at P28 after 7 days without any oxycodone exposure through maternal breastmilk, which translates to a neurodevelopmental human age of a ~5-year-old [201, 202]. At 5 years old not only are NOWS children still suffering from motor deficits, but this is also one of the most vulnerable ages for juvenile mTBI due to slips, falls, and accidents during play [151, 155, 157]. To measure some of the subtle motor changes immediately post-mTBI, we quantitated righting reflex or time to right

(the time each rat took to right itself from a supine to prone position after anesthesia) in the juvenile rats (Figure 5c). Righting reflex has long been used as a measure of outcomes post-mTBI and directly suggests severity of injury in regards to both cellular and behavioral measures [203]. Our IUO+mTBI condition had revealed a significant impairment in TTR as compared to their Sal+mTBI counterparts, suggesting that IUO exposure rendered a significant vulnerability to brain injury (Figure 5c).

Increased TTR latency can be representative of multiple potential issues in the brain such as inflammatory response, synaptic depolarization, and mitochondrial dysfunction but all are suggestive that injury was more severe in IUO exposed animals [203-206]. TTR is a critical measure of neurological dysfunction post-mTBI, however it can also have causes outside of the CNS. One key factor with peripheral origins that impacts TTR is reperfusion of the brain driven by a specialized cranial anastomose called the circle of Willis [207]. Cerebral reperfusion is critical for post-mTBI energetics to restore proper function; interestingly we noticed significantly decreased heart mass in rats at P30, P34, and P38 [208, 209]. While we did measure cerebral blood flow this is an important consideration of future works. To date few studies have analyzed cardiac defects in NOWS models despite these infants having significantly increased risk factor for cardiac events [210]. Considering the strong metabolic disruption caused by mTBI that relies on fresh oxygenated blood flow to properly restore function, if there is disruption to this system it could be behind some of the etiology associated with worsened outcomes post-mTBI.

Next, we sought to validate potential molecular mechanisms underlying this differential response to injury by investigating gene expression changes of common markers for inflammation and mTBI. Interestingly, our results showed a stark overlap of increased expression of genes associated with brain injury in IUO conditions. While our Sal+mTBI group did show an increased expression in some genes such as *Il1b*, *Il6*, *Tnfα, Mapt, Mbp, Nefl, and S100b,* these markers returned to baseline by DPI 10 apart from *Nefl*. This is not surprising as in healthy pediatric

populations only 2.3% of patients experience longitudinal symptoms after an mTBI [211]. Further, in both human data and rodent models of mTBI significant improvement of proinflammatory cytokines have been shown to recover significantly by DPI 6 [212-214]. Intriguingly, the only marker of mTBI significantly increased at DPI 10 in Sal+mTBI conditions was *Nefl,* which has been reported as a long-term indicator of axonal injury in mTBI patients [215]. However, in both IUO+Sham and IUO+mTBI conditions increased levels almost all markers persisted through to DPI 10 (Figure 8). This sustained increase only in the IUO conditions suggests that IUO exposure not only induces chronic inflammation but also, exacerbates inflammatory responses to subsequent mild brain injury. Previous works have shown that neuroinflammation is not only associated with opioid dependence but also with IUO exposure, and furthermore found that gestational opioid exposure induces functional brain injury [86, 216]. Further, an increase in expression of *Apoe* and *Mbp* in IUO conditions are in support of previous research works that also reported elevated levels during gestational opioid exposure [217, 218]. Next, we also found an increased microglial recruitment as well size in the cortex of IUO exposed animals that remained consistent through DPI 10 when compared to Sal animals (Figure 9). These Iba1+ cells could also be from invading macrophages, particularly since mTBI is known to disrupt the BBB and allow for increased peripheral macrophage activity leading to increased inflammation [219]. Our data clearly illustrates that a mild brain injury, which under normal circumstances is completely recoverable, could lead to a series of damaging molecular events in IUO exposed individuals. IUO exposure elicits an increased basal neuroinflammatory response, which has been previously reported, but also that this inflammation is increased in post-mTBI suggesting that, potentially, these IUO exposed subjects cannot respond appropriately to a brain injury. Additionally, the increased recruitment of microglia is associated with the onset of neurodegenerative pathogenesis seen in Alzheimer's Disease [220]. Interestingly we also see significantly increased *Mapt* expression in our qPCR assay in IUO exposed conditions. Mapt is a protein whose pathological expression is directly involved in Alzheimer's Disease pathogenesis

[221]. Together, this data suggests that IUO exposed brain is not only more vulnerable to injury but is also displaying early markers of neurodegenerative disease progression. It has long been established that brain injury leads to increased risk for neurodegenerative and dementia related diseases [222]. This comorbidity could explain the immune overlap in our IUO model with markers of both brain injury and Alzheimer's Disease. In summary, our data strongly suggests that IUO exposure creates a vulnerable environment in the brain, which, when challenged with another stressful event during development, such as mTBI, could potentially exacerbate neurological complications in later life. While these works focus on acute and longitudinal sequelae ranging between comparative rodent ages of childhood and young adulthood, future studies should address NOWS models in the context of the aging brain as it is possible that NOWS children are not only vulnerable to mTBI, but also more vulnerable to developing mTBI induced onset of neurodegeneration and dementia.

CHAPTER 2

Rats Exposed to Oxycodone *in utero* Display altered cortical metabolites and synaptic protein profiles

Abstract

In recent years much work has gone into investigating the repercussions of the opioid crisis in the United States of America and across the globes. However, there is still a paucity of research assessing the pediatric with neonatal opioid exposure and in severe cases neonatal opioid withdrawal syndrome (NOWS). Epidemiological data has found significant differences in health related outcomes for children with *in utero* opioid exposure, but the etiology behind these worsened outcomes is still poorly understood. This gap of knowledge is critical to understanding the vulnerability of this growing pediatric population as well as progressing towards therapeutic interventions. Recent work has found that both peripherally and in the brain there is metabolic disruption associated with animal models of NOWS. Considering the most metabolically active unit of the brain is at the synapse we sought to investigate if neonatal opioid exposure induced deficits in synaptic mitochondria, and further if these subjects could appropriately respond to an acute metabolically associated injury in the brain. Using an established rodent model of *in utero* oxycodone exposure (IUO) we used quantitative mass spectrometry-based proteomics to investigate protein profiles of synaptosomes in IUO subjects. To model an acute neurological stressor we utilized a low-force mild traumatic brain injury (mTBI) on these subjects to investigate if a normally recoverable injury resulted in worsened outcomes. We found that both synaptic and whole brain tissue displayed significantly altered profiles in response to IUO and mTBI. These results culminated in revealing significant functional deficits in oxygen consumption in specifically synaptic mitochondria. Together these results show that neonatal opioid exposure is a disease with metabolic origins that severely impact not only key metabolites and proteins, but also mitochondrial function. Moreover, this metabolic dysregulation causes a normally recoverable

mTBI to be further exacerbated indicating that pediatric populations exposed to opioids *in utero* are more vulnerable than is currently acknowledged.

Introduction

The opioid epidemic has given rise to a concerning increase in the number of children born with Neonatal Opioid Withdrawal Syndrome (NOWS) as a result of *in utero* opioid exposure. However, there remains a notable gap in understanding the developmental challenges associated with neonatal opioid exposure and NOWS. Marked differences in health outcomes have been observed in children exposed to opioids *in utero*, yet the underlying mechanisms responsible for these adverse effects remain unclear [85, 151, 153, 154]. Recent investigations have uncovered metabolic disruptions in both peripheral and brain tissues associated with animal models of NOWS [88, 170, 223-225]. However, to date, no data investigating mitochondrial function or specific units of the brain have been performed. Given that the synapse represents the most metabolically active unit in the brain, it is essential to explore whether neonatal opioid exposure induces deficits in synaptic mitochondria and whether these subjects can adequately respond to acute metabolically associated brain injuries. Filling this knowledge gap is crucial for comprehending the vulnerability of this growing pediatric population and advancing therapeutic interventions.

In this study, an established rodent model of *in utero* oxycodone exposure (IUO) was utilized, employing quantitative mass spectrometry-based proteomics to examine the protein profiles of synaptosomes in IUO subjects. To simulate an acute neurological stressor, a low-force mild traumatic brain injury (mTBI) was induced in these subjects to determine whether a typically recoverable injury would yield exacerbated outcomes. The findings revealed significant alterations in the profiles of both synaptic and whole brain tissue proteins in response to IUO and mTBI. Moreover, the study unveiled substantial functional deficits in both cortical metabolite profiles and mitochondrial oxygen consumption specifically within synaptic mitochondria. These

results collectively indicate that neonatal opioid exposure gives rise to metabolic dysfunction severely impacting cortical metabolites, proteins, and mitochondrial function. Furthermore, the observed metabolic dysregulation exacerbates the effects of an otherwise recoverable mTBI, underscoring the heightened vulnerability of pediatric populations exposed to opioids *in utero* beyond current recognition.

Mild traumatic brain injury (mTBI), commonly known as a concussion, is characterized by transient functional disturbances in brain cells, including neurons, astrocytes, and microglia. These disturbances can result in immediate and delayed consequences. Acutely, mTBI can lead to neuroinflammation, increased oxidative stress, and excitotoxicity, disrupting synaptic connectivity and neurotransmitter signaling. This disruption can manifest as cognitive impairments, memory deficits, and mood disturbances. Furthermore, mTBI can impact the integrity of the blood-brain barrier, leading to increased permeability and potential infiltration of inflammatory cells and cytokines into the brain parenchyma. In the context of NOWS, the combination of neonatal opioid exposure and mTBI may have synergistic effects on brain health. Neonatal opioid exposure already disrupts metabolic processes, and when coupled with mTBI, the impaired metabolic function may exacerbate otherwise recoverable outcomes of mTBI. The altered protein profiles and impaired mitochondrial function observed in this work suggests that the synaptosomes of IUO subjects may render the brain less capable of responding to the metabolic demands imposed by the mTBI. Consequently, this synergistic effect can intensify neuroinflammation, oxidative stress, and excitotoxicity, exacerbating neuronal damage and compromising recovery.

Understanding the complex interplay between neonatal opioid exposure, mTBI, and their effects on metabolic and cellular processes is critical for developing targeted interventions. By identifying the molecular mechanisms underlying these interactions, future works can uncover potential therapeutic targets to mitigate the long-term consequences of both NOWS and mTBI in

affected pediatric populations. Ultimately, this knowledge may pave the way for novel therapeutic strategies and patient education aimed at improving the health outcomes and quality of life for children exposed to opioids *in utero* and those who experience subsequent brain injuries.

Results

IUO Exposure Alters Metabolite Signatures in the Cortex

Metabolites in the brain are regulated in a spatiotemporal manner during critical neurodevelopmental periods to meet energy demands placed on the brain. We previously reported that rats which were exposed *in utero* to oxy showed distinct alterations in brain metabolites [193]. However, it is unknown how IUO exposed subjects would respond to a neurological insult such as mTBI which places acute energy demand on the brain. Accordingly, P30 animals at DPI 2 were assessed for levels of cortical metabolites by ^{1}H-MRS. Metabolites assessed were N-Acetyl-Aspartate (NAA), Alanine, Choline, Creatine, GABA, Glucose, Glutamate, Glutamine, Glycine, Lactate, Myoinositol, and Taurine. Out of these, NAA, myoinositol, lactate, and glucose showed significant differences in the IUO+mTBI condition as compared to Sal+Sham (Figure 10). Both NAA and Myoinositol are clinically relevant biomarkers of patient outcomes across multiple neurological injuries and diseases [226-229]. Interestingly, only NAA, which originates from the mitochondria of neurons, was significantly decreased in both IUO+Sham (mean = 66.91 $\pm$ 9.656 $p \leq 0.0032$) and IUO+mTBI (mean = 67.48 $\pm$ 10.373 $p \leq 0.0036$) as compared to both saline conditions (Figure 10). On the other hand, myoinositol which is predominantly located in glial cells, showed increased expression in the IUO+mTBI animals when compared to the Sal+Sham condition. A concomitant decrease in glucose, the precursor of myoinositol, was seen along with an increase in lactate expression. Intriguingly, increase in lactate and corresponding decrease in glucose is a characteristic hallmark of brain injury [230]. Hence, the data suggests a significant metabolic stress in the IUO+mTBI condition as compared to the

Sal+mTBI condition (Figure 10). Total metabolite signal was not significantly affected between all conditions (Figure 11) suggesting no technical errors during the acquisition of the data.

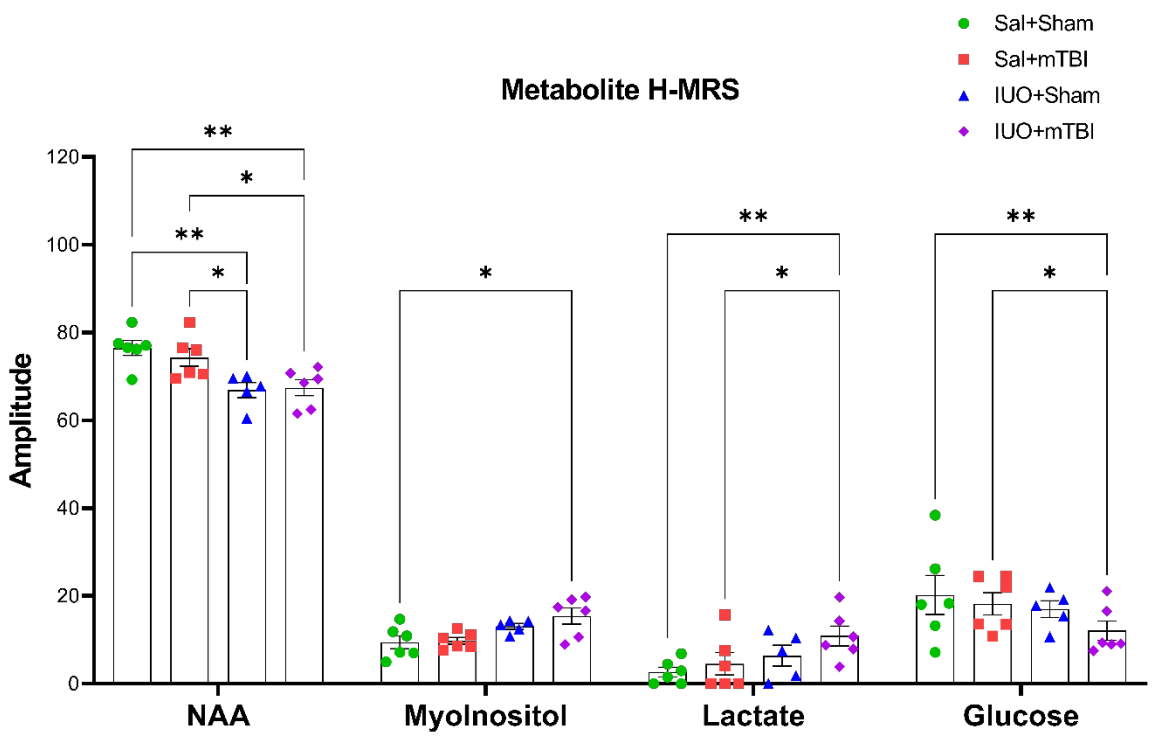


Figure 10) ^{1}H-MRS to identify significantly altered metabolites in IUO+Sham and IUO+mTBI conditions. Metabolite signal is significantly affected in mitochondrially derived NAA for both IUO+Sham ($p \leq 0.003$) and IUO+mTBI conditions ($p \leq 0.0036$) as compared to Sal+Sham conditions. IUO+Sham ($p \leq 0.0217$) and IUO+mTBI ($p \leq 0.0259$) conditions also show decreased NAA as compared to Sal+mTBI conditions. Myoinositol ($p \leq 0.0499$), lactate ($p \leq 0.0076$), and glucose ($p \leq 0.0085$) are only significantly affected in IUO+mTBI conditions as compared to Sal+Sham. When comparing IUO+mTBI to Sal+mTBI only lactate ($p \leq 0.0383$) and glucose ($p \leq 0.0466$) are significantly altered. For Sal+Sham, Sal+mTBI, and IUO+mTBI, all numbers were balanced for sexes (n = 6, 3 males and 3 females) and for IUO+Sham, n = 3 males and n = 2 females were used. Data are displayed as Mean ± SEM; *$p \leq 0.05$; **$p \leq 0.001$; ***$p \leq 0.005$, ****$p \leq 0.0001$. Analysis performed by two-way ANOVA with Fisher's LSD.

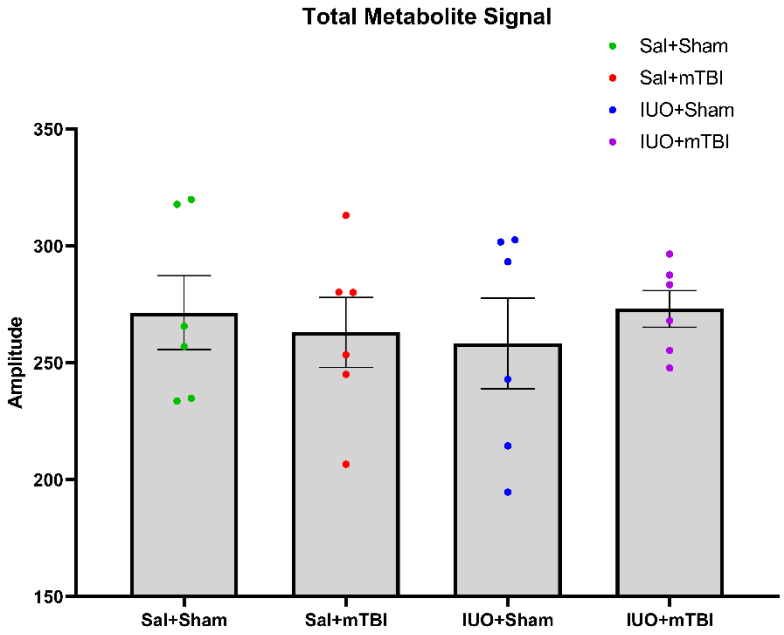


Figure 11) Total Metabolite Signal. For Sal+Sham, Sal+mTBI, and IUO+mTBI, all numbers were balanced for sexes (n = 6, 3 males and 3 females) and for IUO+Sham, n = 3 males and n = 2 females were used. Data are displayed as Mean ± SEM ; *p ≤ 0.05; **p ≤ 0.001; ***p ≤ 0.005, ****p ≤ 0.0001. Analysis performed by one-way ANOVA with Tukey's multiple comparison correction.

Cortical Synaptosomes Display Unique Protein Signatures

Cortical metabolomic data suggests that IUO+mTBI significantly alters metabolite expression in the brain. It is well known that synaptic structures are the most active metabolic areas of the neuron. In order to further understand whether changes in brain metabolism affects synaptic function, we first evaluated the proteomic profile of cortical synaptosomes (SYP), pinched off nerve terminals that represent an important component in neurotransmission and synaptic plasticity [231-233]. We purified cortical SYP from animals at DPI 2 and performed high-throughput quantitative mass spectrometry to characterize the synaptic proteome. A consistent number of total proteins were identified for all comparisons (Supplementary Table 3) suggesting controlled analysis for all comparisons. By further employing a criterion of ≥ 2 unique peptides and a $p \leq 0.05$ we were able to identify differentially expressed proteins (DiffExpPro) (Table 2). Only proteins which were observed in all technical replicates for each sample were considered. DiffExpPro between Sal+mTBI, IUO+Sham, and IUO+mTBI as compared to Sal+Sham shows some overlapping, but mostly distinct DiffExpPro profiles for each condition (Figure 12a). Only 5 proteins were found to be shared between the three conditions as compared to Sal+Sham: CYP51A1, HEXB, MGLL, TMEM256, and ZC2HC1A. Sal+mTBI conditions had 8 proteins which overlapped with IUO+mTBI conditions: SCRN1, ATXN10, SEC13, PIK3R4, PSMB4, SLC22A17, and DNM1L. No significant pathways were discovered in ClueGO or IPA analysis for these shared proteins. However, between IUO+Sham and IUO+mTBI conditions, 88 proteins were shared (Figure 12a). Since this is the largest number of overlapping proteins, we further performed functional analysis using ClueGO and heatmap. Results revealed that top hits (10 proteins), all of which were upregulated, were related to mitochondrial function (Figure 12b). ClueGO analysis of the 88 shared proteins between the IUO conditions also revealed that they are involved in mitochondrial function and oxidative stress pathways (Figure 12c). Interestingly, of the 88 shared proteins, 23 were directly related to mitochondrial function according to the MitoCarta3.0 database [234]. Further, Ingenuity Pathway Analysis (IPA) identified a strong association between IUO

exposure and mitochondrial dysfunction showing it as the most significantly associated pathway in IUO+Sham and the fifth most significant in IUO+mTBI conditions (Figure 13b, c). Further, we analyzed the shared upstream regulators for the IUO conditions. Results indicated that 5 of the top 10 regulators are known to be directly related to mitochondrial function or disease in the brain: HTT, RGS6, TFAM, NFU1, and KIT [235-239] (Figure 13d).

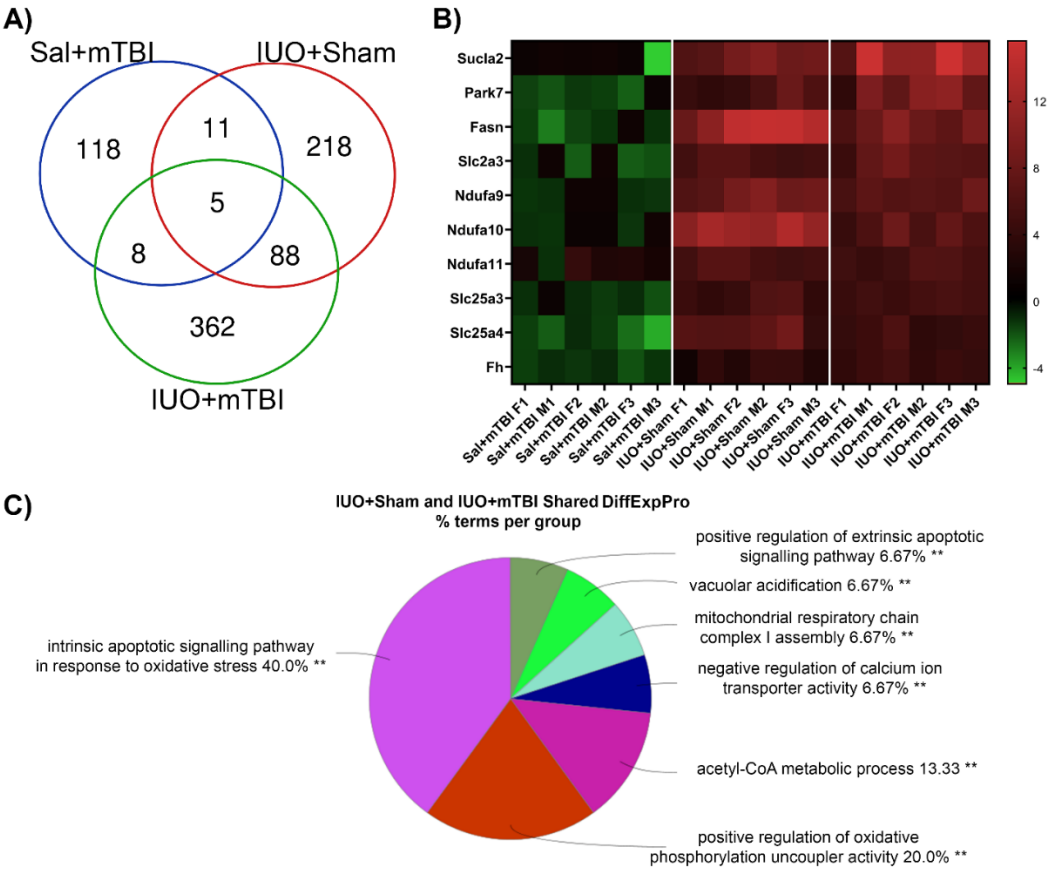
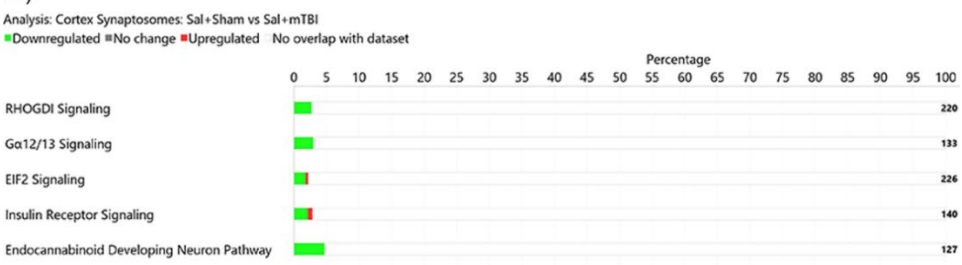
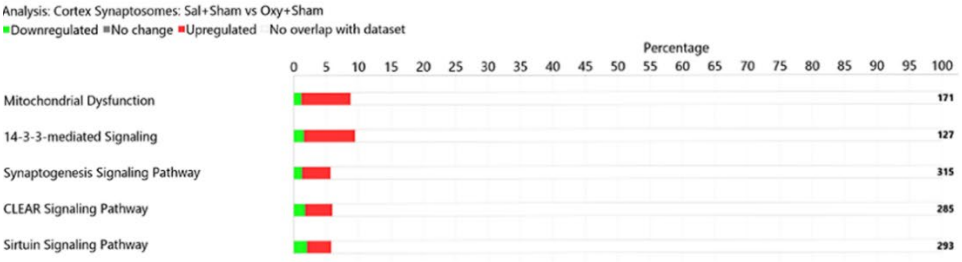


Figure 12) Synaptic proteome identified by quantitative mass spectroscopy were associated with mitochondrial dysfunction in IUO conditions. **(A)** 88 overlapping DiffExpPro between IUO+Sham and IUO+mTBI conditions were found while only 8 were shared between Sal+mTBI and IUO+mTBI. Between all three groups, only five total DiffExpPro were shared as compared to Sal+Sham. **(B)** A heatmap of the top ten mitochondria associated DiffExpPro shows the significant overlap between IUO+Sham and IUO+mTBI in mitochondria proteins. **(C)** Clue-GO analysis of the 88 shared DiffExpPro between IUO+Sham and IUO+mTBI show a strong relationship to pathways associated with oxidative stress associated apoptosis, oxidative uncoupler activity, and respiratory complex I assembly. For all conditions, animals were balanced for sex (n = 6; 3 males and 3 females). (Clue-GO group term category *p ≤ 0.05; **p ≤ 0.001).

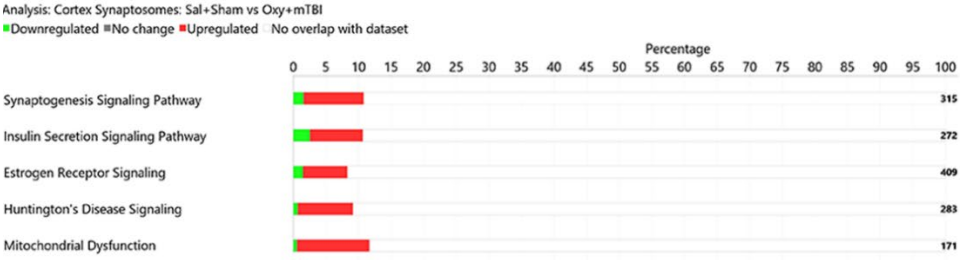
A)



B)



C)



D)

Upstream Regulator	Expr Fold Change	Molecule Type	Activation z-score	p-value of overlap
HTT	5.176	transcription regulator	1.506	7.43E-08
RICTOR	4.475	other	-3.154	0.000186
RIC8A	3.686	other		0.025
AP2B1	2.982	transporter		0.025
RGS6	2.528	enzyme		0.0494
HBA1/HBA2	2.436	transporter	1	0.0222
CAB39L	1.771	kinase	1.342	0.00244
TFAM	1.753	transcription regulator		0.0129
NFU1	1.725	other		0.0222
KIT	-23.827	transmembrane receptor		0.0325

Figure 13) Ingenuity pathway analysis. **(A)** Analysis of Sal+mTBI DiffExpPro shows small effects on pathways, mostly downregulated. The most significant effect is a 5% protein overlap with the endocannabinoid neuron development pathway. **(B)** IPA analysis of IUO+Sham DiffExpPro show the most affected correlation is mitochondrial dysfunction with a 9% overlap alongside other inflammatory and synaptic pathway deficits. **(C)** IPA analysis of IUO+Sham DiffExpPro show the most affected correlation is the synaptogenesis signaling pathway followed by insulin secretion pathway. Mitochondrial dysfunction also has a strong correlation with a 12% DiffExpPro overlap. **(D)** IPA analysis of upstream regulators found matching our dataset identified top ten regulatory proteins: HTT, RICTOR, RIC8A, AP2B1, RGS6, HBA1/2, CAB39L, TFAM, NFU1, and KIT. For all conditions equal sexes were used (n = 6).

Identification of Unique Mitochondrial Protein Dysregulation in the Cortical Synapse

To further investigate mitochondrial proteins, we isolated both cortical tissue lysate (TL) and SYP protein from DPI 2, DPI 6 and DPI 10 animals and immunoblotted for all five primary mitochondrial complexes: NDUFB8 (CI), SDHB (CII), UQCRC2 (CIII), MTCO1 (CIV), and ATP5A (CV). These complex proteins were also indicated in our SYP proteomics data (Figure 16) Results indicated that that at DPI 2 TL and SYP displayed unique profiles for each of the conditions (Figure 14a & 15a). In TL Sal+mTBI was only significantly increased for CII protein SDHB as compared to Sal+Sham. Interestingly, TL from IUO+mTBI conditions displayed significantly increased levels of CIII and CV proteins as compared to both Sal+Sham, while in IUO+Sham conditions there was a significant increase in all five complexes (Figure 14a). Further, at DPI 6, we see a significant return to Sal+Sham levels for both Sal+mTBI and IUO+Sham conditions (Figure 14b). In IUO+mTBI conditions though a significant increase in CIII and CV protein levels was maintained through DPI 6. At DPI 10 in TL, we see non-significant differences for all complex in all conditions (Figure 14c). On the other hand, SYP isolated from IUO+Sham conditions at DPI 2 displayed increased expression in mitochondrial complexes I-IV but not in complex V, while SYP isolated from IUO+mTBI conditions showed significantly increased levels for all five complexes when compared to Sal+Sham (Figure 15a). Further, IUO+mTBI conditions also showed increased levels of CIII marker when compared to IUO+Sham (Figure 15a). Similar to TL, in SYP at DPI 6 we see no significant differences between Sal+mTBI and IUO+Sham as compared to Sal+Sham conditions in any mitochondrial complex proteins. However, in IUO+mTBI conditions, we observed a significant upregulation of CII, CIII, and CV protein markers as compared to Sal+Sham. Interestingly, at DPI 10 in SYP, we see a significant decrease of complex protein levels in the IUO+mTBI conditions for CI, CII, CIV, and CV (Figure 15c). In summary, we see a dynamic increase in mitochondria complex channel proteins in both IUO+Sham and IUO+mTBI conditions at DPI 2, but in the IUO+mTBI conditions these increases shifted to downregulation over the 10-day recovery window after mTBI. This data suggests that there is a dynamic and

exacerbated response by synaptic mitochondria to mTBI in the IUO exposed subjects that rebounds during a 10-day recovery period. While equal number of genders were used, we did not find a significant difference in any gender comparisons except for at DPI 6 in the Sal+mTBI condition as compared to Sal+Sham (Figure 17).

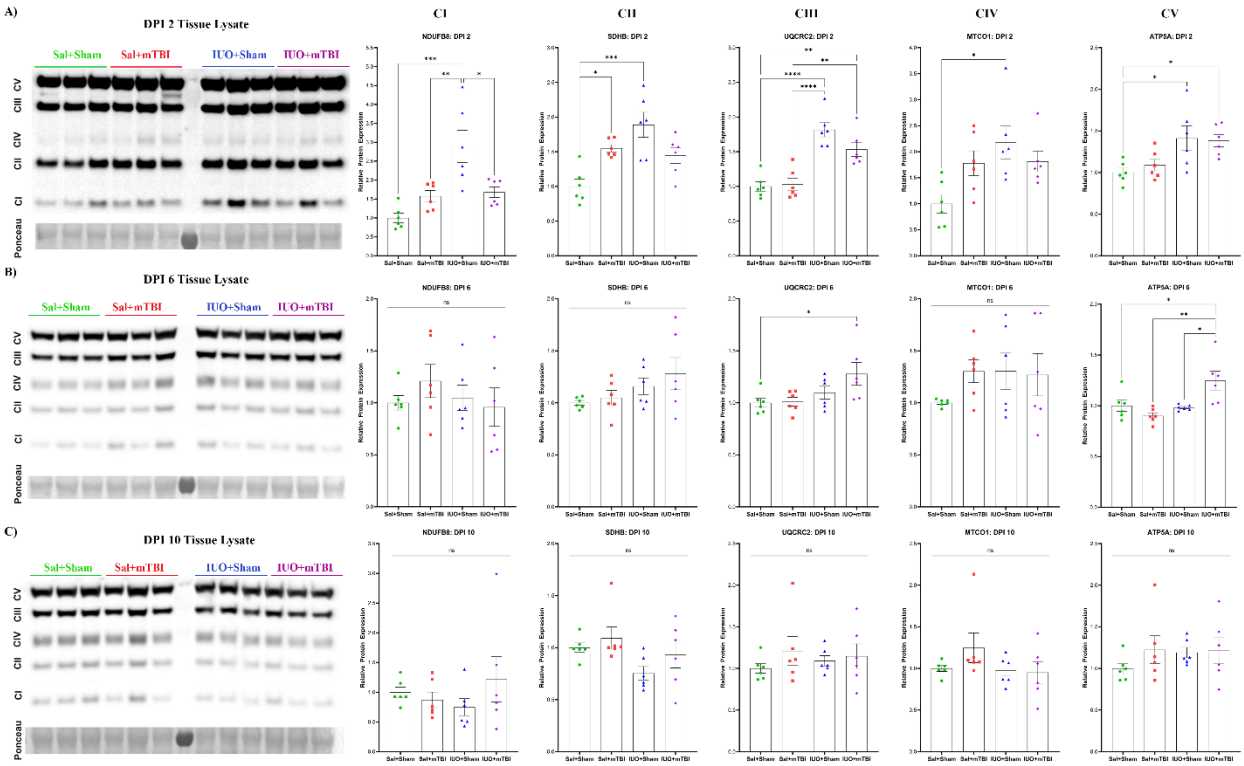


Figure 14) Western blotting conducted on cortical tissue lysates found acute but not longitudinal alterations in the expression of oxidative phosphorylation complex proteins. **(A)** At DPI 2 significant increase in complex proteins were noted in TL for IUO+Sham conditions in all five complex proteins. Significant increase in complex proteins in TL for IUO+mTBI conditions in CI, CIII, and CV. **(B)** At DPI 6 in TL a significant increase in complex proteins was only noted in IUO+mTBI conditions for CIII and CV as compared to Sal+Sham. **(C)** At DPI 10 no changes in complex proteins could be seen in TL. Spectral protein values from cortical tissue lysates were normalized to ponceau staining and then data was compared to Sal+Sham conditions. For all conditions equal sexes were used (n = 6). Data are displayed as Mean ± SEM ; *p ≤ 0.05; **p ≤ 0.001; ***p ≤ 0.005, ****p ≤ 0.0001. Analysis performed by one-way ANOVA with Tukey multiple comparison correction.

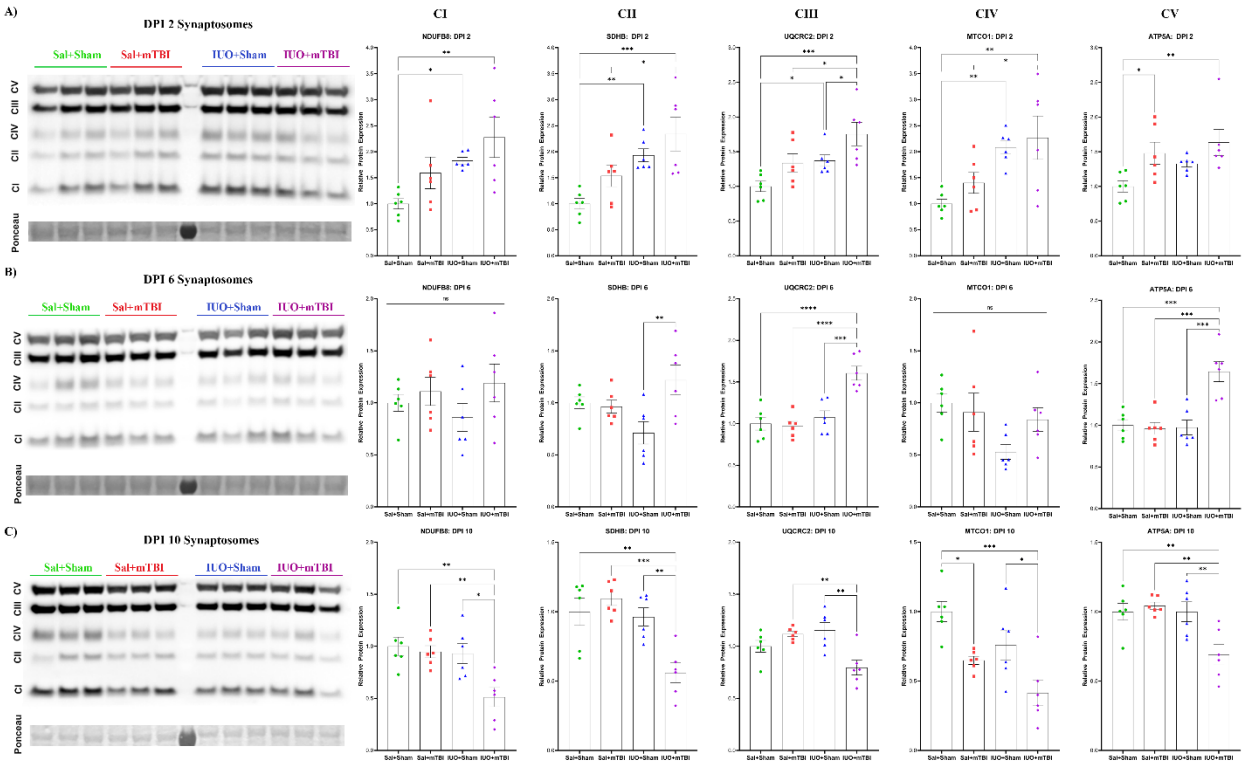


Figure 15) Western blotting conducted on cortical synaptosomes found time specific alterations in the expression of oxidative phosphorylation complex proteins. **(A)** At DPI 2 significant increase in complex proteins were noted in SYP for IUO+Sham conditions in complexes I, II, III, and IV proteins as compared to Sal+Sham conditions. Significant increase in complex proteins in SYP for IUO+mTBI conditions in all five complex proteins as compared to Sal+Sham. Further, in complexes II, III, and IV IUO+mTBI conditions were also significantly increased as compared to Sal+mTBI. **(B)** At DPI 6 in SYP a significant increase in complex proteins was only noted in IUO+mTBI conditions for CIII and CV as compared to all other conditions. Interestingly, in complex II protein SDHB IUO+mTBI was significantly increased as compared to IUO+Sham only. **(C)** At DPI 10 a significant decrease in complex IV protein MTCO1 was noted in Sal+mTBI conditions as compared to Sal+Sham conditions. Further, lower levels could be seen in IUO+mTBI conditions as compared to all other conditions for Complexes I, II, and V. Additionally, IUO+mTBI conditions had lower levels of complex III and IV proteins as compared to IUO+Sham conditions. Spectral protein values from cortical synaptosomes were normalized to ponceau staining and then data was compared to Sal+Sham conditions. For all conditions equal sexes were used (n = 6). Data are displayed as Mean ± SEM; *p ≤ 0.05; **p ≤ 0.001; ***p ≤ 0.005, ****p ≤ 0.0001. Analysis performed by one-way ANOVA with Tukey multiple comparison correction.

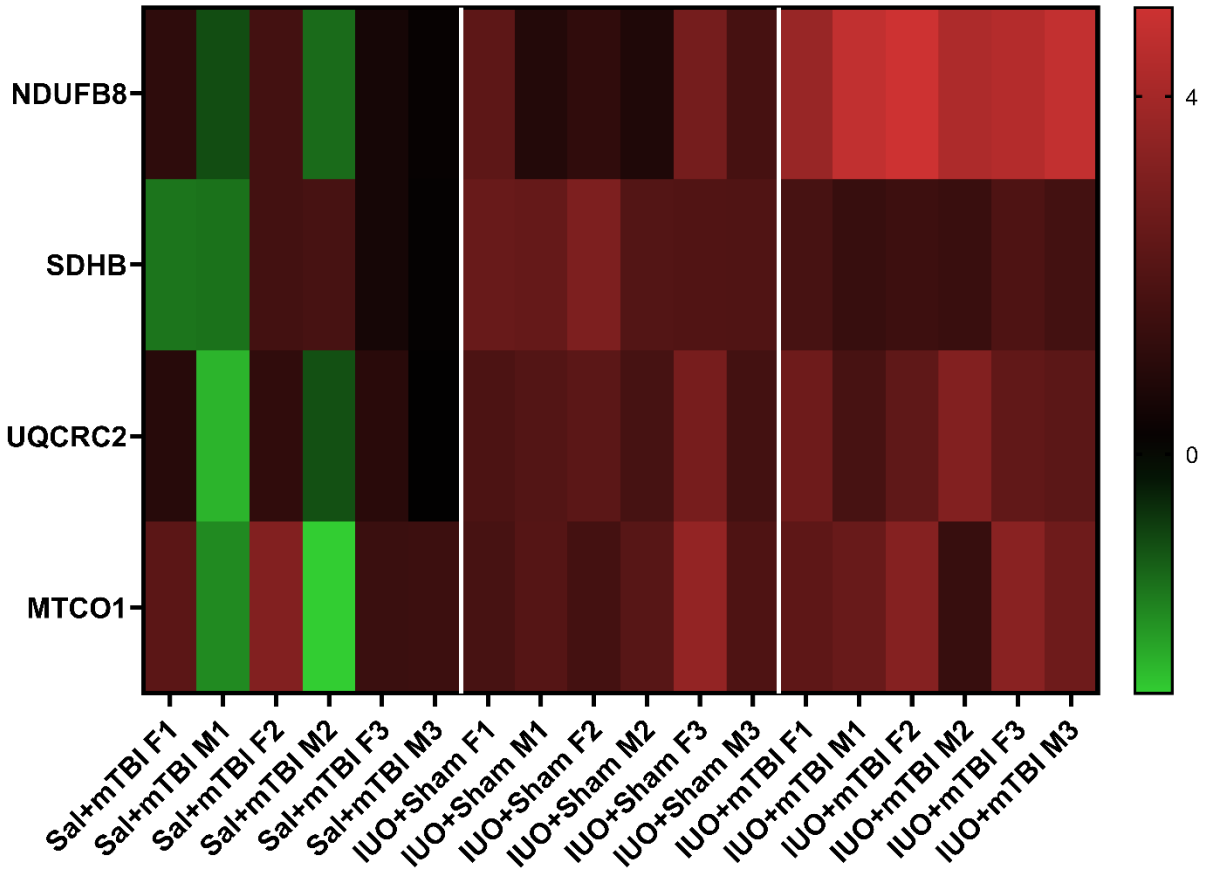


Figure 16) A heatmap of four mitochondria complex proteins identified as DiffExpPro in our SYP proteomics at DPI 2. Complex I (NDUFB8), Complex II (SDHB), Complex III (UQCRC2), and Complex IV (MTCO1). Levels of change are comparable to results found in western blotting validation shown in Figure 15. ATP5A was not identified as a DiffExpPro in our proteomics results.

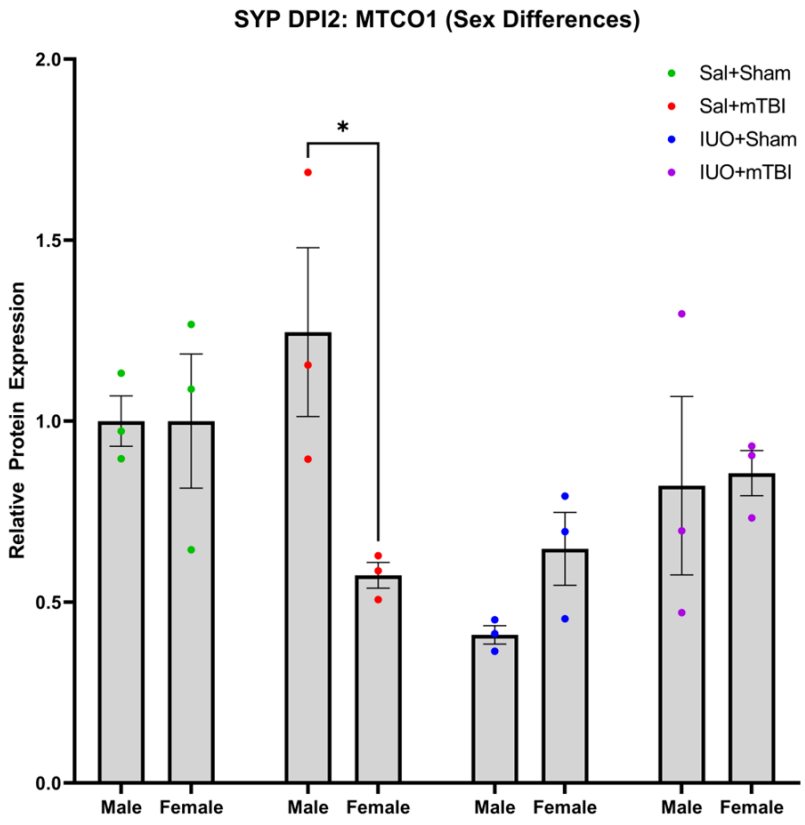


Figure 17) Gender comparisons for MTCO1 levels in cortical SYP at DPI 6. For all conditions equal sexes were used (n = 3). Data are displayed as Mean ± SEM ; *p ≤ 0.05; **p ≤ 0.001; ***p ≤ 0.005, ****p ≤ 0.0001. Analysis performed by one-way ANOVA with Tukey multiple comparison correction

Seahorse Analysis of Non-Synaptic and Synaptic Mitochondrial Respiration

To further investigate the role of mitochondria, we performed mitochondrial functional analysis using Seahorse Extracellular Flux Analyzer. First, mitochondria were isolated from SYP (SYPmito) and non-synaptic (NSYPmito) fractions from cortical brain tissue extracted from animals at DPI 2 and oxygen consumption rate (OCR) was measured in the presence of carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), an uncoupling agent known to disrupt ATP synthesis, while using pyruvate+ malate to drive complex I function. Both SYPmito and NSYPmito isolated from Sal conditions showed no significant changes in both FCCP blocked basal respiration and pyruvate+malate driven maximal respiration (Figure 18a, b). Results revealed that in response to mTBI, the NSYPmito from both the IUO+Sham and IUO+mTBI conditions had significantly increased basal and maximal OCR (Figure 18a). However, the SYPmito in both IUO conditions found to have significantly increased maximal respiration but no difference at basal respiration (Figure 18b). When calculating the individual complex OCR (subtracting background OCR of rotenone inhibited Complex I from basal respiration and Antimycin-A inhibited Complex III from succinate driven respiration), we identified complex specific effects. Both SYPmito and NSYPmito showed no differences in OCR among the Sal+mTBI conditions from Sal+Sham (Figure 18a, b). In NSYPmito, only the IUO+Sham condition had significantly increased OCR in complex II and IV as compared to Sal+Sham, however, in the SYPmito, the IUO+mTBI conditions had significantly increased OCR in Complex II and IV but no differences in Complex I when compared to both Sal+Sham and Sal+mTBI conditions (Figure 18a, b). While SYPmito isolated from IUO+Sham showed significantly increased Complex IV activity when compared to both Sal+Sham and Sal+mTBI conditions (Figure 18b). Together, these data suggest to us that IUO+Sham exposure results in overactive synaptic mitochondrial respiration in whole tissue mitochondrial samples when compared to Sal+Sham, while IUO+mTBI and Sal+mTBI are non-significantly lower than their uninjured controls. Further, IUO+mTBI causes specifically synaptic mitochondria to have significantly increased respiration as compared to Sal+Sham and Sal+mTBI.

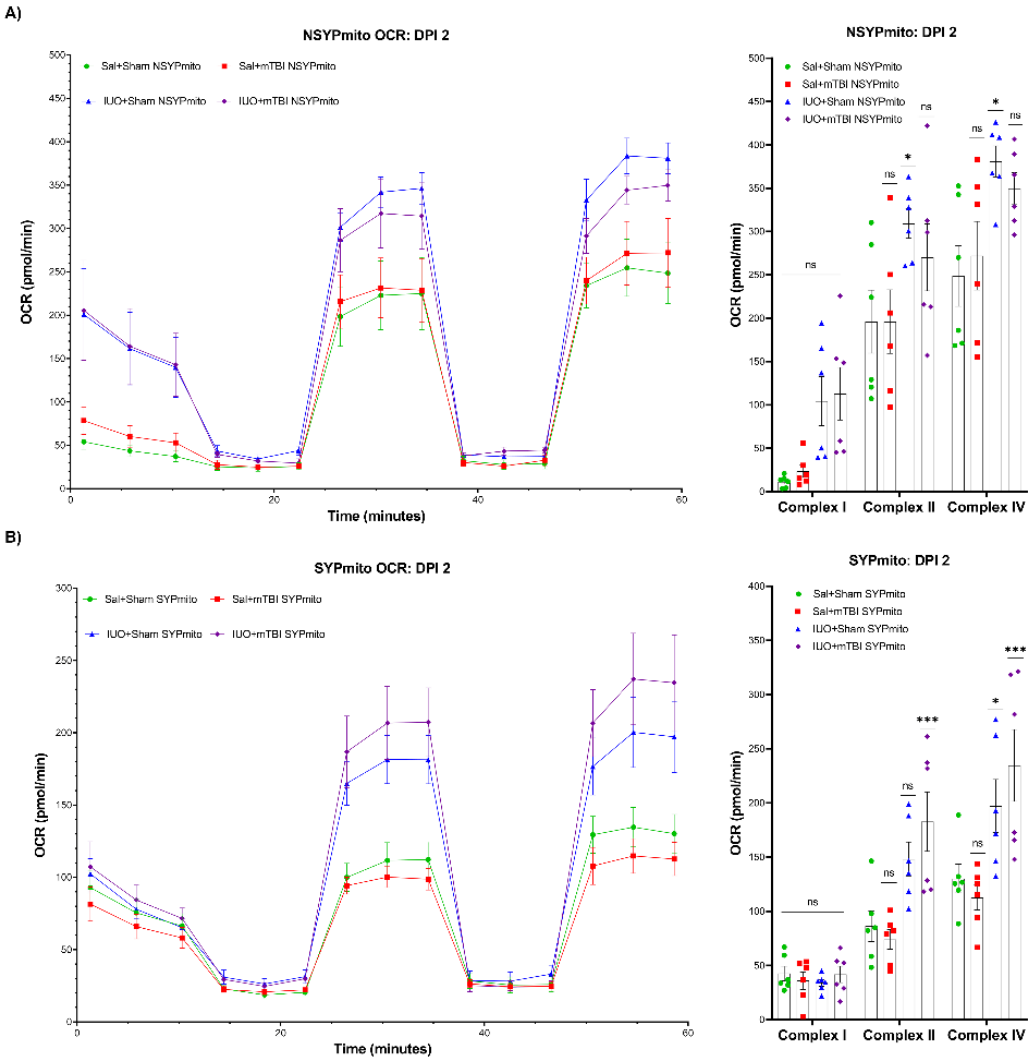


Figure 18) Oxygen consumption rate of NSYPmito and SYPmito showed acutely increased respiration in SYPmito for IUO conditions. **(A)** At DPI 2, NSYPmito displayed significantly increase basal and maximal respiration in both IUO conditions. However, only IUO+Sham showed an increased Complex II and Complex IV respiration. **(B)** At DPI 2 SYPmito displayed significantly increase maximal respiration but no change in basal respiration. IUO+Sham showed increased Complex IV associated respiration while IUO+mTBI conditions showed increased Complex II and Complex IV associated respiration. For all conditions equal sexes were used (n = 6) and one Sal+Sham animal was run in each plate. Data are displayed as Mean ± SEM ; *p ≤ 0.05; **p ≤ 0.001; ***p ≤ 0.005, ****p ≤ 0.0001. Analysis performed by two-way ANOVA with Tukey multiple comparison correction.

Discussion

The impact of neonatal opioid withdrawal syndrome (NOWS) on mitochondrial function is a critical area of investigation, given its potential implications for both development and response to injury. The opioid crisis, characterized by the widespread misuse and addiction to opioids, has led to a significant rise in NOWS cases, placing a growing population of children at risk. Mitochondria play a vital role in energy production, synaptic function, and cellular homeostasis, making their dysfunction a key contributor to the pathophysiology of various neurological disorders. Understanding the specific effects of NOWS on mitochondrial function is essential for elucidating the mechanisms underlying the long-term consequences observed in affected children and developing targeted therapeutic interventions. Our findings show significant decrease in the mitochondrial derived metabolite NAA in IUO exposed animals (Figure 10). Decreased NAA is directly associated with decreased levels of ATP and metabolic imbalance after brain injury. Seeing similar NAA profiles in IUO exposure alone suggests that gestational oxy exposure disrupts mitochondrial and metabolic function in brain [240-242]. Further, increased myoinositol levels in IUO+mTBI conditions is intriguing as myoinositol is a clinical biomarker of outcomes post-mTBI in human patients (Figure 10) [226]. Myoinositol serves as a biomolecular intermediary in many neurotransmitters and hormones, a decrease would therefore suggest a dysfunction in neuronal signaling post-injury. Further, myoinositol is derived from glucose which also shows significant decrease alongside with a concomitant increase in lactate in only IUO+mTBI conditions. The dysregulation of both, predominantly, glial derived myoinositol and neuron derived NAA suggests that metabolic distress is not necessarily cell-type specific, but rather whole-brain. Interestingly, the level of glucose decrease has been previously reported to be relative to the degree of injury [243, 244]. Even more alarming is that pediatric populations are particularly vulnerable to mTBI related glucose crisis as glucose metabolism peaks at approximately 5 years old to over 200% of adult glucose metabolism levels [245]. Lactate has long been identified as an alternative to glucose, acting as a reserve fuel source in brain injury [246]. Increased lactate use

by neurons has been shown to promote reactive oxygen species (ROS) production and subsequent oxidative stress [247-249]. Metabolically induced oxidative stress in response to mTBI among IUO exposed subjects could be responsible for both the inflammatory response and impaired righting reflex. In summary, data so far strongly suggests that the IUO exposed brain is particularly incapable to meet energy demands post-injury and hence led us to investigate the most metabolically active unit of the brain, the synapse.

Quantitative proteomics of purified cortical synaptosomes revealed a strong relationship between IUO exposure and mitochondrial dysfunction. Bioinformatic analysis using ClueGO tool revealed a total of 88 shared DiffExpPro between IUO+Sham and IUO+mTBI identifying pathways for apoptotic signaling in response oxidative stress, regulation of oxidative stress, and acetyl-CoA metabolism (Figure 12c). Intriguingly, acetyl-CoA is the immediate precursor to NAA and many pathophysiological abnormalities associated with lower NAA are thought to be caused by loss of acetyl-CoA [250]. The observed relationship between molecular pathways associated with acetyl-CoA metabolism further validates the significance of the decreased NAA levels in both IUO exposure conditions. Further with IPA analysis, we found that metabolic and mitochondrial functional pathways are perturbed specifically in IUO+mTBI conditions. This includes signaling pathways associated with Huntington's Disease where regulators TFAM and HTT were found to be upregulated in IUO+mTBI conditions (Figure 13c, d). The nuclear-encoded regulatory protein TFAM which was strongly identified in our proteomics data is critical in regulation of mtDNA replication, transcription, and packaging [235]. In chronic neurological disease states, such as late-stage Huntington's Disease or Parkinson's Disease, TFAM levels are typically lower and thought to be caused by increased oxidative damage, however our proteomics data show TFAM levels are significantly elevated in the IUO+mTBI SYP. Increased TFAM levels have been shown to be neuroprotective in multiple studies investigating metabolic diseases of the brain in both human and rat models [251-254]. An increase (~2 fold) in TFAM could explain a possible

compensatory mechanism for the increased energy demand in response to both IUO exposure and a brain injury. Chronic TFAM driven respiratory compensation in response to injury may become rapidly pathophysiologic during aging [235, 255]. Future studies investigating mtDNA regulation, specifically TFAM in aged subjects who were exposed to opioids *in utero*, might shed light into new mechanisms and pathways to investigate dementia related diseases. IPA analysis also revealed increase in proteins relating to insulin secretion signaling pathways, mitochondrial dysfunction, and synaptic signaling pathways (Figure 13). Insulin resistance and other metabolic disorders have been heavily implicated in adult opioid misuse disorders, but have not been investigated sufficiently in neonatal exposure [250]. However, insulin resistance is a known marker for mortality in cases of brain injury and is known to be exacerbated in response to brain injury [256, 257]. Interestingly, dysregulation of both synaptic signaling and insulin secretion pathways can be directly connected to mitochondrial dysfunction. Further, mitochondria play a significant role in inflammatory signaling and is a key regulator for many of the inflammatory genes noted in our previous qPCR data: *Cxcl1*, *Il6*, *Il1β*, *Tnfα* (Figure 8). In order to assess the role of the mitochondria in IUO exposure, we first analyzed the proteins in the mitochondrial oxidative phosphorylation complex (Figure 14, 15). We see that immediate post-injury there is a dramatic increase in all mitochondrial complex proteins in both whole tissue lysate (TL) and synaptosomes (SYP), however, the levels came back to baseline in TL, but a significant decrease was observed in SYP (Figure 14, 15). Our data clearly suggests an increase in synaptic mitochondria function to keep up with the energy demands immediate post-injury and hence a significant change in mitochondrial function was seen at DPI 2 (Figure 18). The synaptosome represents a critical microenvironment of the brain which contains key neurotransmitters managed by synaptic vesicle (SV) release, pre- and post-synaptic membrane proteins, and a significant proportion of neural mitochondria [258]. As such the synaptosome provides a window of insight into the key functional unit of the brain which has been implicated in several neurological vulnerabilities [259]. Traditional whole brain investigations might drown out the impacts given to this vulnerable microenvironment,

and our data indicates that the synapse is specifically affected by IUO exposure. It is well known that the mitochondria at the synapse, both neural and astrocytic, are under extreme stress post-mTBI [259, 260]. Furthermore, IUO exposure exacerbates the effects of the injury as clearly demonstrated in the decreased levels of the mitochondrial proteins in SYP (Figure 15c).

In order to further validate that IUO exposure causes significant mitochondrial stress, we assessed mitochondrial function. We observed no significant differences in oxygen consumption in either NSYPmito or SYPmito in the Sal+mTBI conditions suggesting that mitochondrial function is not significantly altered in mTBI alone (Figure 18b, c). However, we did see a significant increase in complex II and IV oxygen consumption in NSYPmito at DPI 2 for IUO+Sham groups along with a trending increase in IUO+mTBI (Figure 18a). This data dovetails with our previous observations in tissue lysate complex proteins at DPI 2, where we saw an increase in all complex proteins in IUO+Sham, however for IUO+mTBI we only identify significant changes in complexes III, IV, and V (Figure 14a). Moreover, in IUO+mTBI conditions at DPI 2, SYPmito have significantly increased OCR in Complex II and IV while IUO+Sham conditions have a significantly increased OCR for only Complex IV (Figure 18b). Again, this correlates with our western blot data on complex proteins in SYP where, in all complexes, IUO+mTBI showed greater average levels of expression at DPI 2 (Figure 15a).

The investigation of metabolic complexities and their interplay with mTBI has shed light on the potential role of *in utero* opioid exposure as an additional metabolic risk factor that can exacerbate TBI outcomes [261]. The convergence of IUO exposure and TBI pathophysiology highlights the significance of mitochondrial dysfunction, which has been implicated in disease progression and metabolic disorders. Emerging evidence suggests that IUO exposure leads to mitochondrial hyperactivity, which can become pathophysiological following TBI. These findings emphasize that IUO-induced mitochondrial stress contributes to functional and molecular impairments, which are further exacerbated in response to injury. By unraveling the intricate

relationship between IUO exposure, mitochondrial function, and TBI, we gain valuable insights into the underlying mechanisms driving the worsened outcomes observed in this vulnerable population.

CHAPTER 3

Longitudinal analyses of neuroinflammatory, mitochondrial, and behavioral profiles associated with mTBI in the *utero* oxycodone exposed animals

Abstract

Understanding how neonatal opioid exposure and neonatal opioid withdrawal syndrome (NOWS) negatively effects the pediatric population has been a growing field. While epidemiological data strongly supports some worsened outcomes throughout life for this population, the etiology behind these worsened outcomes remains a significant gap of knowledge in the field. Currently NOWS children often develop within their peer cohort and while rigorously attended shortly after birth, their gestational environment is not widely considered in the clinic as they age despite known impacts to their behavior and motor function. Further, our previous works have shown in rats that neonatally opioid exposed offspring have an impaired ability to respond to a common insult: minor traumatic brain injury (mTBI). Using an established rodent model of *in utero* oxycodone exposure (IUO) we used qPCR, Seahorse Extracellular Flux Analyzer, and relevant behavioral assessments to assess how a pediatric mTBI in neonatally opioid exposed offspring affects outcomes in early adulthood. We found that while many acutely observed molecular effects were no longer observed in later life, both mitochondrial function and behavior were significantly altered in IUO+Sham and IUO+mTBI conditions. Our results suggest that IUO+mTBI causes long term impairment to synaptic mitochondria and deficits in social, motor, and anxiety-like behaviors. Together these results show that neonatal opioid exposure can have long-term developmental effects in the brain on behavior and metabolism.

Introduction

Understanding the impact of neonatal opioid exposure and neonatal opioid withdrawal syndrome (NOWS) on the pediatric population has emerged as a critical field of research. While

epidemiological data strongly supports the presence of worsened outcomes throughout the lives of children affected by NOWS, the underlying etiology responsible for these adverse effects remains a significant knowledge gap in the field. Presently, while NOWS children receive intensive care after birth, as they age, they are often treated with the same consideration as compared to their peer cohort. However, many diseases and health disparities are known to have their roots in developmental exposure and environment [81]. Consideration of *in utero* exposure to opioids as a longitudinal risk factor is something that needs to be addressed.

Notably, recent research indicates that children who experienced NOWS have a significantly higher frequency of emergency department (ED) visits throughout childhood [85]. This highlights the substantial risk posed by *in utero* opioid exposure for later-life injuries and diseases, particularly considering that falls constitute the leading cause of non-fatal injuries leading to ED admissions in children and are also the primary contributor to pediatric mTBI [155]. Considering the well-established association between NOWS and persistent motor control deficits, as well as motor cortex thinning that persists into peri-adolescence [89, 153], it is crucial to determine whether this population exhibits longitudinal deficits to recovery from one of the most prevalent pediatric injuries: mTBI [6].

mTBI is a common pediatric injury, with children under the age of six being one of the most at risk demographics. However, typically mTBI are recoverable with symptoms relieving between 15 minutes to 2 weeks in the majority of cases. Despite this, a small minority of individuals who suffer a mTBI go on to experience longitudinal, or even lifelong, symptoms which are collectively known as post-concussive syndrome (PCS). It is not fully understood why a small minority of people suffer PCS, but a prevailing hypothesis is that vulnerability caused genetics, environment, or a combination of both is responsible. Given our previous works have shown that in the short term, two – ten days, rats exposed to opioid *in utero* have worsened outcomes after suffering a pediatric mTBI we sought to investigate these effects longitudinally.

In this study, we employed a well-established rodent model of *in utero* oxycodone exposure (IUO) and utilized a range of techniques including quantitative polymerase chain reaction (qPCR), Seahorse Extracellular Flux Analyzer, and relevant behavioral assessments to explore the impact of pediatric mTBI outcomes in early adulthood for IUO offspring. Our aim was to assess how the combination of IUO and mTBI influences molecular, metabolic, and behavioral outcomes with a significant recovery period with no insult administered. While some acutely observed molecular effects dissipated with age, our findings revealed persistent alterations in both mitochondrial function and behavior in the IUO+Sham and IUO+mTBI conditions. These results highlight that IUO combined with mTBI leads to long-term impairment of synaptic mitochondria and the emergence of deficits in social behavior, motor function, and anxiety-like behaviors. Collectively, these findings demonstrate that neonatal opioid exposure can have enduring developmental effects on brain function, behavior, and metabolism.

By investigating the consequences of neonatal opioid exposure and its interaction with pediatric mTBI, this study contributes to our understanding of the long-term implications of early-life opioid exposure on neurodevelopment. The identification of altered mitochondrial function and behavior underscores the significance of considering the impact of gestational opioid exposure on subsequent developmental outcomes. These insights have important clinical implications, emphasizing the need for comprehensive and integrated care for children exposed to opioids during the neonatal period. By recognizing and addressing the long-term developmental effects of neonatal opioid exposure greater strides can be made towards establishing health equity for this vulnerable population.

Results

DPI 38-40 qPCR

Our previous works demonstrated acute disruptions to key genes associated with neuroinflammation and biomarkers of minor traumatic brain injury (mTBI) across days post injury

(DPI) 2 and 10 [197]. We wanted to understand if there were any longitudinal disruptions that were associated with IUO+Sham and IUO+mTBI conditions so we reassessed these genes at DPI 38-40 which correlates to approximately young adulthood [262]. Utilizing a quantitative real-time PCR technique, we assessed expression differences of *Gfap*, *Aif1*, *Tnfα*, *Il1β*, *Il6*, *Cxcl1*, *Mapt*, *Bdnf*, *Apoe*, *S100β*, *Mbp*, and *Nefl* in cortical brain tissue (Figure 19). We found that among the biomarkers of injury *Bdnf* was the only marker increased at DPI38-40 in both IUO+Sham (FC average = 5.06; $p \leq .0001$) and IUO+mTBI (FC average = 3.18; $p \leq .0005$) as compared to Sal+Sham conditions. Further, when assessing inflammatory markers, the only gene which was altered in both IUO+Sham (FC average = 2.52; $p \leq .0008$) and IUO+mTBI (FC average = 2.06; $p \leq .0265$) as compared to Sal+Sham was microglial marker *Aif1*.

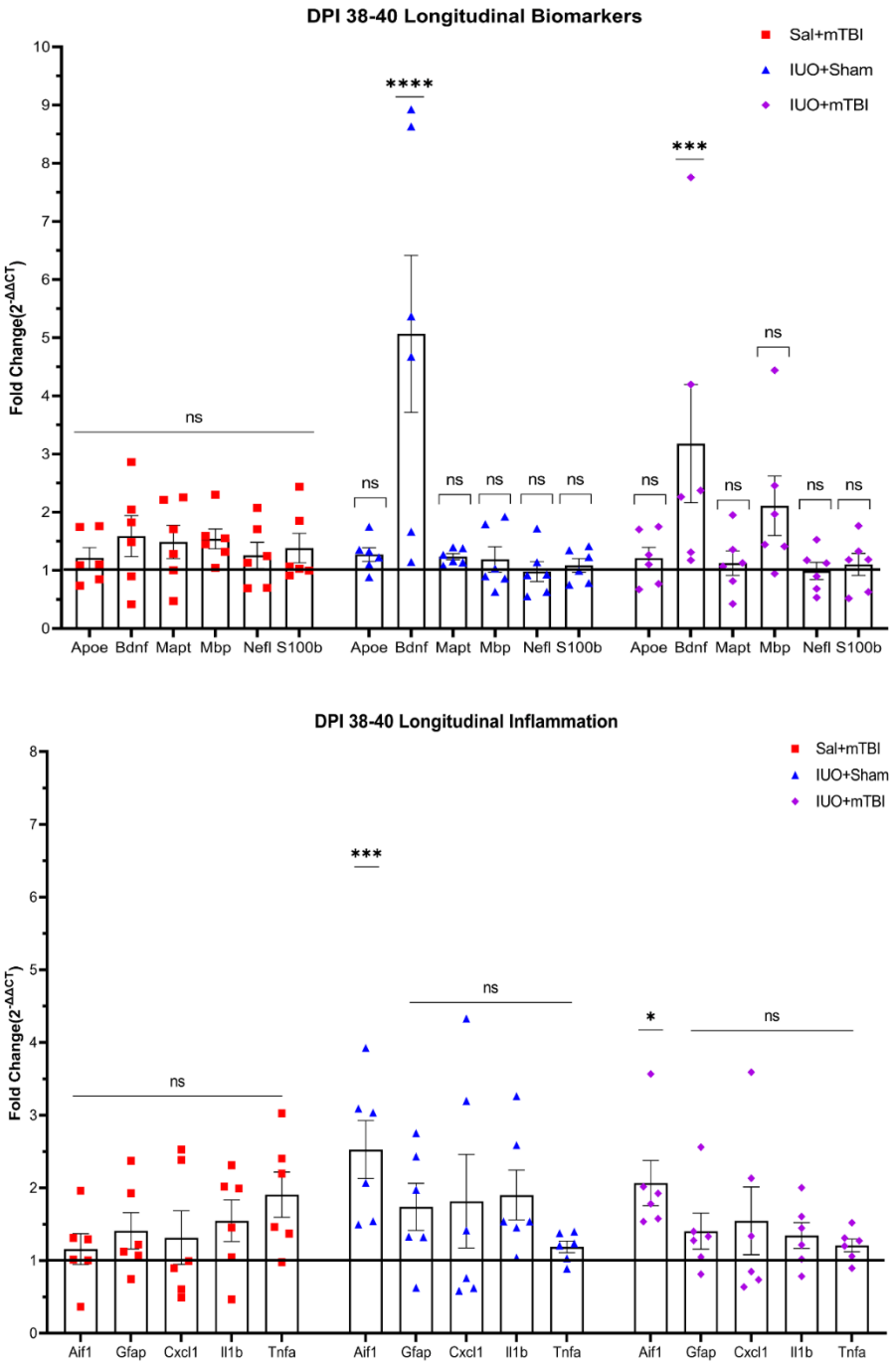


Figure 19) Common gene markers of inflammation and brain injury. Cortical brain tissue was assessed for mRNA levels of key biomarkers of inflammation and brain injury at 38-40 days post mTBI/Sham procedures using quantitative real-time PCR analysis. IUO+Sham and IUO+mTBI conditions display significantly increased Bdnf and Aif1 gene markers as compared to Sal+Sham. No significant differences were observed in the Sal+mTBI condition. No significant gender differences were found within groups. Data are displayed as Mean ± SEM; for all conditions equal number of sexes were used n = 6 ; *p ≤ 0.05, **p ≤ 0.001, ***p ≤ 0.005, ****p ≤ 0.0001. Analysis was performed by two-way ANOVA with Dunnett's Correction.

DPI 38-40 Seahorse Analysis of Non-Synaptic and Synaptic Mitochondria

To further investigate the role of mitochondria longitudinally, we performed mitochondrial functional analysis using Seahorse Extracellular Flux Analyzer in these samples at DPI 38-40. First, mitochondria were isolated from SYP (SYPmito) and non-synaptic (NSYPmito) fractions from cortical brain tissue extracted from animals at DPI 2 and oxygen consumption rate (OCR) was measured in the presence of carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), an uncoupling agent known to disrupt ATP synthesis, while using pyruvate+ malate to drive complex I function. At DPI 38-40 there were no significant differences discovered in NSYPmito OCR (Figure 20c), however, in SYPmito OCR was significantly decreased when comparing IUO+Sham and IUO+mTBI conditions to Sal+Sham controls (Figure 20d), showing an inverse effect as compared to our previous results at DPI 2. Both succinate and ASC/TMPD supported maximal respiration as well as basal respiration was significantly impaired in both IUO conditions as compared to Sal+Sham conditions suggesting an impairment of mitochondrial function in these animals (Figure 20d). While investigating complex specific OCR, IUO+mTBI conditions showed significantly decreased SYPmito OCR in all three complexes (Figure 20d). Further, a significant decrease was noted in the IUO+Sham conditions in Complex I (Figure 20d). Together, these data suggest to us that while we found IUO exposure results in overactive synaptic mitochondrial respiration during childhood, by adulthood mitochondrial respiratory capacity is significantly impaired. Specifically, that these outcomes are worsened when comparing Sal+mTBI and IUO+mTBI indicating differential mitochondrial response to injury between oxy exposed vs unexposed offspring.

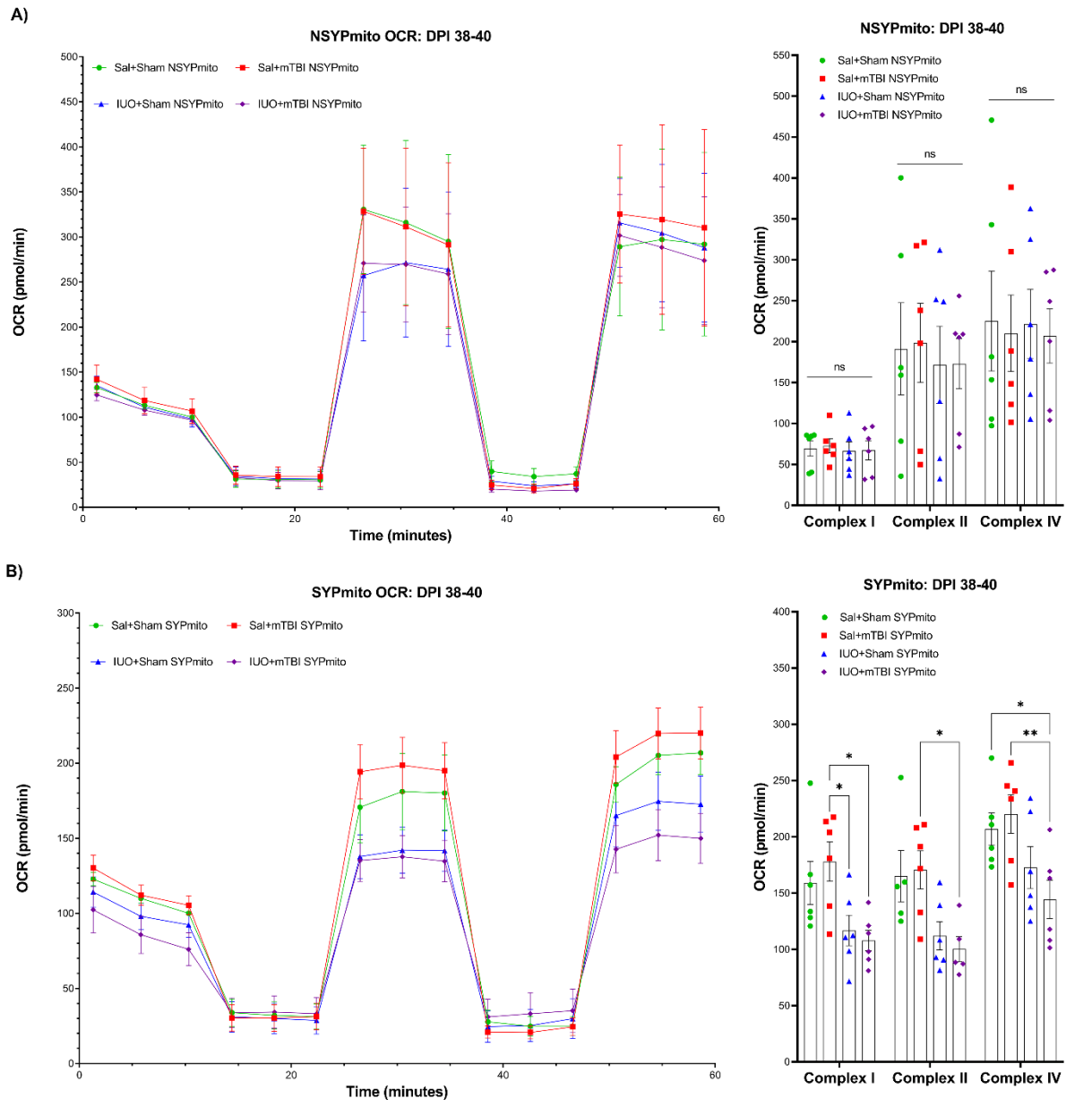


Figure 20) Oxygen consumption rate of NSYPmito and SYPmito showed decreased oxygen consumption in IUO+mTBI at DPI 38-40. **A)** At DPI 38-40, no significant changes were seen in basal, maximal, or complex specific respiration among all conditions. **(B)** At DPI 38-40, a significant decrease in maximal respiration was seen in both IUO conditions, however, only IUO+mTBI displayed a significant decrease in basal respiration as compared to both saline groups. IUO+Sham and IUO+mTBI showed a significant decrease in Complex I respiration as compared to Sal+mTBI condition. IUO+mTBI further showed a decreased respiration as compared to Sal+mTBI for Complex II associated OCR. IUO+mTBI also showed a decreased respiration as compared to both saline conditions for Complex IV associated respiration. For all conditions equal sexes were used (n = 6) and one Sal+Sham animal was run in each plate. Data are displayed as Mean ± SEM ; *p ≤ 0.05; **p ≤ 0.001; ***p ≤ 0.005, ****p ≤ 0.0001. Analysis performed by two-way ANOVA with Tukey multiple comparison correction.

Behavior Analysis

Our previous research found that IUO exposure showed minimal impact on social and anxiety-like behaviors in F1 generation but significant social impairments in the F2 generations [169]. Here, using the same methodology we sought to understand if IUO exposure and a later life injury such as mTBI would precipitate into behavioral deficits. To assess this, we performed marble burying, social novelty, and social preference experiments on all conditions between DPI 33-37 (Figure 21a, b). We found that IUO conditions were significantly less likely to spend time in the compartment with a novel animal as opposed to a cagemate when compared to Sal+Sham animals (Figure 21a). Interestingly though, despite the decreased time spent near the naïve animal in the IUO+mTBI conditions there was a significant increase in the number of entries into the naïve compartment but an unchanged number of touches by the IUO+mTBI condition (Figure 21a). To further understand this relationship, we assessed the number of touches per entry by all conditions and found that for IUO+mTBI conditions there were significantly lower number of touches per entry for the naïve animal, but not for the cagemate (Figure 21a). We also performed social preference assessment comparing the desire to interact with a toy versus a novel animal (Figure 21b). We found that in both IUO conditions the rats were again significantly less likely to spend time in the naïve animal's compartment and had decreased number of touches per entry (Figure 21b). However, the IUO+Sham conditions were also significantly more likely to spend time near the toy and had greater number of touches despite having no difference in the number of attempted touches per entry with the toy; further, the IUO+Sham condition was also more likely to attempt to touch the toy (Figure 21b). Beyond social deficits, NOWS children are also significantly more likely to suffer motor deficits and anxiety related behaviors [169]. Our social data suggests that IUO animals, despite having fewer interactions per entry, were highly active during the behavioral assessment which indicates anxiety-like motor patterns. To further understand this anxiety-like behavior we performed marble burying assessment for compulsive

motor behavior and found that IUO+mTBI conditions were significantly more likely to perform marble burying than both Sal+Sham and Sal+mTBI conditions (Figure 21c). To investigate the role of IUO and mTBI on motor function, we performed Rotarod assessment across six trials consisting of three learning trials and 3 test trials (Figure 21d). We noted significant and consistent decrease in motor function in both IUO+Sham and IUO+mTBI conditions in trials 4, 5, and 6. Hot plate nociception tests were also performed at DPI 32 to investigate any nociceptive differences between groups prior to behavioral tests, however no significant differences were observed (Figure 22). An additional consideration is the behavior of the dams while on oxy, as rearing behavior is known to impact brain and behavior of pups [199, 200]. While other studies in rats have found impairments in specific maternal care behaviors such as nursing and vocalization, we found no significant differences in pup-retrieval behavior between oxy dosed dams vs saline (Figure 23) [263]. However human caretaking and nurturing patterns are much more intricate than multiparous rodent models, which makes maternal behavior a potentially confounding factor when translating behavioral data [264].

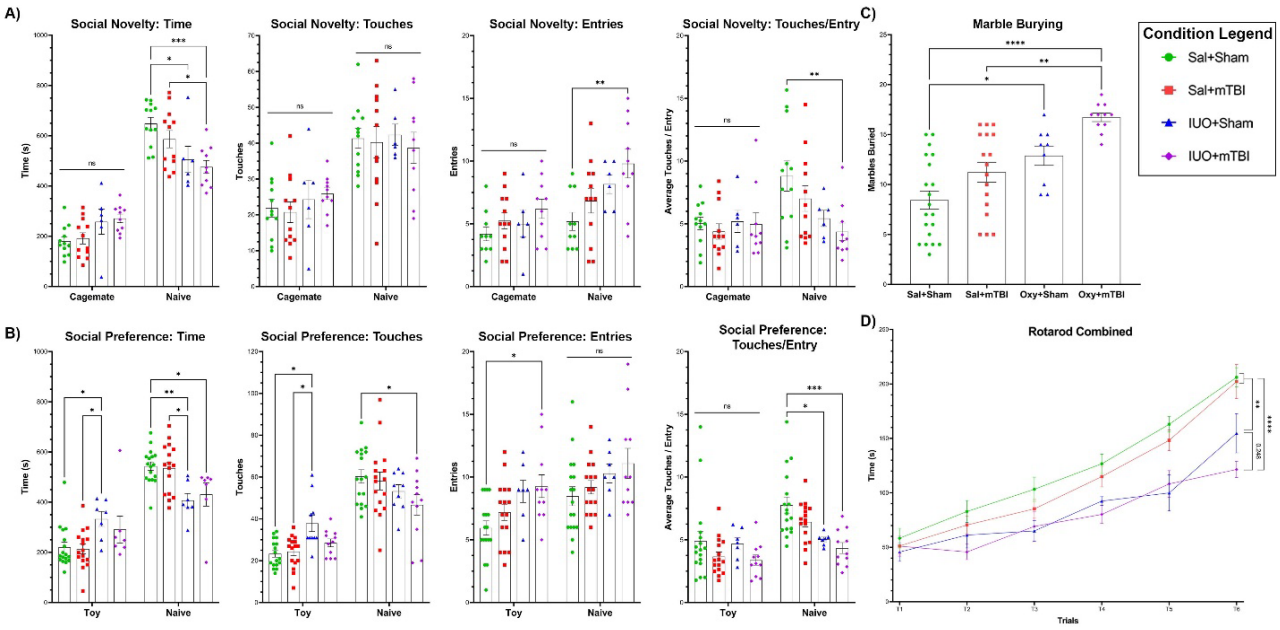


Figure 21) Behavioral analysis found impairments in social, anxiety, and motor behaviors in IUO+mTBI conditions. **(A)** Social novelty testing showed no changes in sociality for Sal+mTBI conditions as compared to Sal+Sham. No changes were observed in cagemate interactions in all conditions. Naïve animal time spent was significantly decreased in both IUO conditions as compared to Sal+Sham. IUO+mTBI animals displayed significantly increased number of naïve animal compartment entries, but a significantly decreased number of touches per entry. **(B)** Social preference testing showed no changes in sociality for Sal+mTBI conditions as compared to Sal+Sham. IUO+Sham animals spent more time in the toy compartment and attempted more touches while also spending significantly less time in the Naïve animal compartment and attempting less naïve touches per entry. IUO+mTBI condition spent significantly less time in the Naïve animal compartment and attempting less naïve touches per entry. **(C)** Marble burying assessment of anxiety and compulsive like behavior showed the IUO+Sham animals buried more marbles than Sal+Sham conditions but a non-significantly different amount as compared to Sal+mTBI. IUO+mTBI buried significantly more marbles as compared to both Sal+Sham and Sal+mTBI. **(D)** Rotarod assessment showed a significant decrease in latency to fall in both IUO conditions in trials 5 and 6 as compared to both Sal+Sham and Sal+mTBI. No significant differences were observed in trials 1, 2, and 3. Data are displayed as Mean ± SEM; *p ≤ 0.05; **p ≤ 0.001; ***p ≤ 0.005, ****p ≤ 0.0001. Analysis performed by one-way ANOVA with Tukey multiple comparison correction for 9a, 9b, and 9c. 9d analysis performed by two-way mixed-model ANOVA with Tukey multiple comparison correction.

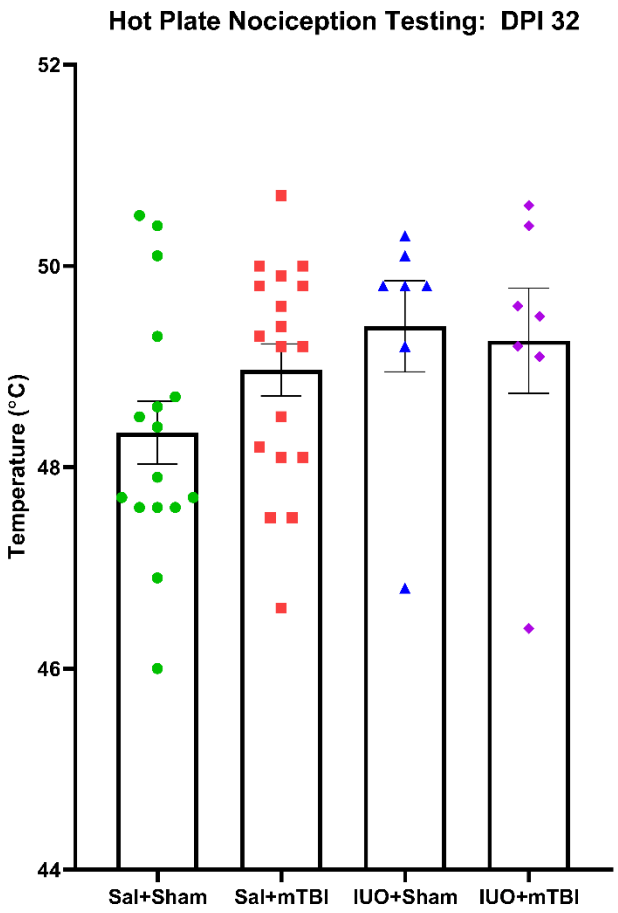


Figure 22) Hot plate nociception testing found no differences between conditions. For all conditions equal sexes were used (n = 3). Data are displayed as Mean ± SEM ; *p ≤ 0.05; **p ≤ 0.001; ***p ≤ 0.005, ****p ≤ 0.0001. Analysis performed by one-way ANOVA with Tukey multiple comparison correction.

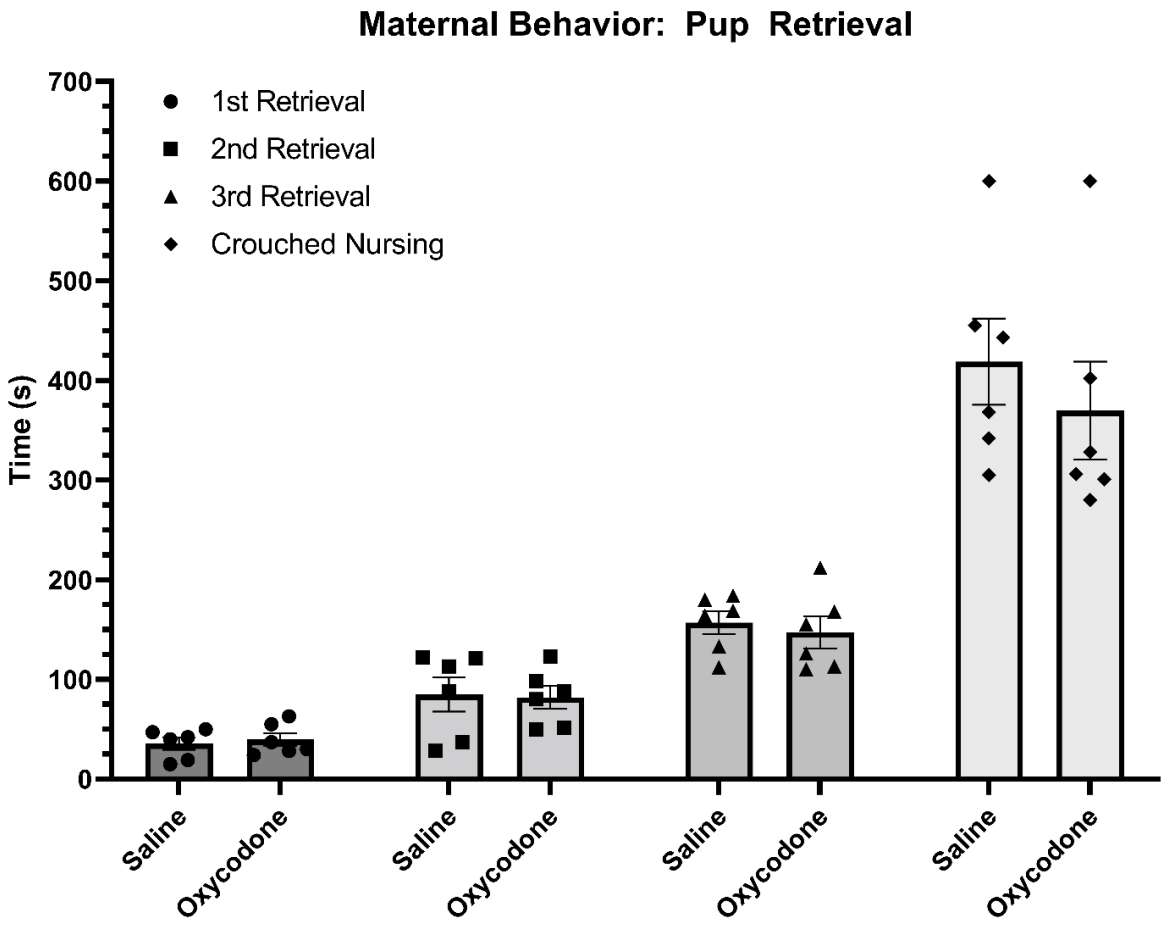


Figure 23) Maternal caretaking behaviors were assessed using pup retrieval. For both saline and oxycodone dose dams n = 6 was use and no significant changes were observed. Data are displayed as Mean ± SEM ; *p ≤ 0.05; **p ≤ 0.001; ***p ≤ 0.005, ****p ≤ 0.0001. Analysis performed by one-way ANOVA with Tukey multiple comparison correction.

Discussion

The impact of critical developmental periods on longitudinal health and vulnerability has been well established across multiple organs, diseases, and outcomes. However, to date in response to rising number of children born suffering *in utero* opioid exposure and NOWS there is insufficient work assessing longitudinal health and outcomes in this vulnerable population. Population and epidemiological studies have shown definitive differences in the pediatric population suffering from NOWS, but health equity for these individuals as they age is still far behind due to a gap of knowledge in the molecular systems impacted longitudinally by IUO exposure. Further, while children who suffer NOWS typically develop with the rest of their cohort, data has shown that up to 8 years of age they have significantly increased hospital costs associated with later life ED, outpatient, and inpatient admittance as compared to their unexposed peers. As such while, we treat NOWS and *in utero* opioid exposure in neonates, they bear longitudinal disadvantages that need to be considered during childhood and beyond.

Firstly, our previous works as well as those of other groups has shown increased basal inflammation in the brains of *in utero* opioid exposed neonates but has not taken this data out into young adulthood. While no inflammatory signaling molecules (*Tnfα*, *Il1β*, *Il6*, and *Cxcl1)* were observed to be altered, a significantly higher amount of *Aif1* mRNA was observed at DPI 38-40 (Figure 19). This observation coincides with our acute findings which showed both increased *Aif1*+ cells and *Aif1* mRNA at DPI 2 and 10 in the cortex of IUO rats. Seeing longitudinally increased levels of a key microglial marker in both IUO+Sham and IUO+mTBI conditions at DPI 38-40 indicates that, potentially, even into adulthood the brains of these subjects are primed for exacerbated inflammatory response mediated by microglia. Further, when observing biomarkers associated with brain injury and neurological disorders (*Mapt*, *Bdnf*, *Apoe*, *S100β*, *Mbp*, and *Nefl*) we found that the only longitudinally changed marker was *Bdnf* which was significantly increased in both IUO+Sham and IUO+mTBI conditions as compared to Sal+Sham (Figure 19). *Bdnf* has

been researched in animal models of both brain injury and *in utero* opioid exposure. The majority of published literature has found a decrease of BDNF in the hippocampus and plasma or opioid exposed rats. Our finding of increased *Bdnf* mRNA levels at early adulthood, well removed from any opioid exposure, is interesting as typically increased BDNF levels are associated with improved outcomes in both neurodegenerative diseases and cognitive performance. However, human studies have found that aberrantly increased *Bdnf* levels are associated with increased risk factors for epilepsy and is thought to be associated with BDNF/TrkB autism pathways [265, 266].

Next, finding the majority of acute inflammatory markers were recovered in adulthood, we sought to discover if our previous findings of increased mitochondrial OCR at DPI 2 was maintained into adulthood. Interestingly, we found an oppositional result in our early adult subjects in SYPmito (Figure 20). For NSYPmito there were no significantly observed differences between any conditions or complexes at DPI 38-40. However, in the SYPmito we found complex I associated OCR to be decreased significantly in both IUO+Sham and IUO+mTBI conditions as compared to Sal+mTBI conditions, but not Sal+Sham interestingly. However, while a strong trend in decreased OCR was noted for complexes II and IV in the IUO+Sham condition as compared to both the Sal+Sham and Sal+mTBI conditions, this finding was not significant. In IUO+mTBI conditions though both complex II and IV displayed significantly decreased OCR as compared to Sal+mTBI conditions. When comparing IUO+mTBI to Sal+Sham conditions the only complex which had significantly downregulated OCR was complex IV. However, area under the curve analysis for reads after injections of both succinate and ascorbate+TMPD, which push for maximal respiration, show that maximal respiration is significantly decreased in only IUO+mTBI conditions as compared to Sal+Sham. Considering the history of associating both brain injury and neurological diseases with impaired mitochondrial function, it is possible that gestational opioid exposure should be considered as a metabolic risk factor [261].

Lastly, we wanted to see if our findings of longitudinal molecular and mitochondrial deficits associated with IUO+Sham and IUO+mTBI conditions translated into behavioral deficits in early adulthood. Furthermore, it has been extensively established that mitochondrial dysfunction and chronic inflammation are factors in known behavioral deficits associated NOWS such as autism, anxiety, social impairment, and motor deficiencies [169, 267-271]. Interestingly, anxiety, social impairment, and motor dysfunction are also concomitant in pediatric brain injury [182, 272-274]. We sought to understand if mTBI would result in exacerbated NOWS associated behavioral changes in IUO exposed subjects at DPI 33-37. Many behavioral and functional deficits have been noted in children who had suffered from NOWS. Specifically, these children are significantly more likely to suffer social impediments including up to an 80% increased likelihood to develop autism by age 8 as well as a 68% increased chance to have developmental motor disorders [61]. Our data showed that IUO exposed rats have impaired anxiety-like behavior, social preference behavior, and motor function (Figure 21b, c, d). This data supports current epidemiological data in humans that show similar deficits in NOWS children [61]. What is more fascinating is that a heightened effect is seen in the IUO+mTBI conditions. For example, in the IUO+mTBI conditions we see not only impaired preference to spend time with a naïve rat, but also an increased number of entries towards the naïve rats resulting in significantly impaired touches per entry for a naïve rat (Figure 21a). This is again shown in the social preference experiment for both IUO+Sham and IUO+mTBI conditions (Figure 21b). This suggests that animals exposed to both stressors, although exhibiting a curiosity to explore, failed to participate in social interaction indicating autistic-like behavioral patterns, where social interaction isn't unwanted just atypical [275]. Our marble burying data, which represents more generalized anxiety, also suggests that IUO exposure induces anxiety like behaviors which become exacerbated in IUO+mTBI conditions (Figure 21c). Notably, no significant differences are seen in any behavioral measure in our Sal+mTBI conditions. Yet again our data supports that the damage caused by an mTBI, while normally fully recoverable, in IUO exposed subjects results in behavioral deficits. Specifically,

these behavioral measures related to NOWS associated behavioral deficits are exacerbated in IUO exposed rats who suffer a juvenile mTBI. Furthermore, significantly impaired motor function seen in IUO conditions suggests that NOWS children are not only likely to be vulnerable to the sequelae of mTBI at the molecular level but may also be more likely to suffer an mTBI as young adults (Figure 21d). This further supports human data which has found deficits in the size of the motor cortex of NOWS children and epidemiological data of greater ER admittance for injuries [85, 151]. Epidemiological data shows that NOWS children are significantly more likely to be admitted to the ED for injury than non-opioid exposed children [276]. Considering that mTBI caused by slips, falls, and accidents during play, is one of the most common admittance reasons for children, the impaired motor control caused by IUO, could be considered as a contributing factor. In summary, our data shows that *in utero* opioid exposure not only renders the brain vulnerable to pediatric brain injury but also at the molecular level, causes significant neuroinflammation, alterations in brain metabolites, and synaptic mitochondria dysfunction that further precipitates into behavioral deficits observed in NOWS children.

DISCUSSION AND FUTURE DIRECTIONS

Discussion

The cumulative findings reported in this thesis are both novel and beneficial to the future of the field of *in utero* opioid exposure and NOWS. Utilizing a rodent model to mimic oxy exposure *in utero* alongside a weight drop model of mTBI we have elucidated multiple gaps of knowledge in the field. Utilizing microdissection techniques, we have characterized alterations in the cortex for IUO exposure, mTBI, and IUO+mTBI. Specifically, we have shown in great detail that IUO exposure causes significant metabolic dysfunction in the cortex. Further, we have shown that behavioral and molecular response to mTBI in IUO exposed rats is pathophysiological as compared to unexposed controls. Lastly, we have characterized significant differences in the synaptic microenvironment and revealed it to be a site of significant mitochondrial dysfunction in both NOWS and mTBI. The results described in these works serves to describe each of these *in utero* opioid exposure associated patterns for the first time. A sufficient molecular, cellular, and behavioral baseline has been established for future works to target a newly revealed etiology as a potential therapeutic target for NOWS sufferers.

Our group and others have characterized IUO associated phenotypical deficits. Our model of IUO has been established in multiple studies looking at the phenotypic measurement deficits associated with *in utero* oxy exposure in a rodent model [63, 169-171]. Our findings have previously found decreased body weights and shorter body lengths at P28 [169]. Our results corroborated decreased interaural head diameter; however, we did not show a significant decrease in bodyweight for animals at P28. While this does disagree with our previous findings it is possible that this is a confound for differences in litter size between the two groups. It has been well established in multiparous animals that litter size is inversely correlated with average aggregate weight of the offspring [277, 278]. Since opioids are a known impactful teratogen, it is possible that our oxy paradigm decreases litter size and thus inversely affecting the weight of IUO

pups. While statistically we did not find a significant variability in the size of the litters between saline and oxy dosed dams, there was an absolute difference in the mean number of pups born. Future studies with appropriate power to assess this difference should be performed in our model. Some studies on oxy and buprenorphine combinatorial administration found a significant decrease in viable embryos and an increase in resorbed embryos for oxy and buprenorphine dosed dams [263]. Further, we did not find significant differences in infant mortality postpartum or significant differences in pup-retrieval, a key maternal caretaking measure (Figure 23). While other studies have found maternal caretaking differences in opioid dosed dams, these are typically response times to ultrasonic pup vocalization [263]. Another significant phenotypic measurement noted previously is a decreased head diameter. Our study further corroborated this, once again showing significantly decreased head diameter in IUO rats at P28. This coincides with reports in humans of decreased white mater and head circumference observed in human children suffering NOWS [123, 124]. While this study did not specifically assess for white matter degeneration, future studies should consider this element, particularly with coincident mTBI. A singular brain injury is known to cause white matter degeneration year after the insult [159]. Altogether, our model represents *in utero* opioid exposure while not resulting in high infant mortality or abnormal behaviors that could confound experimental interpretation.

mTBI has a significant amount of overlapping pathophysiology with IUO exposure and NOWS. Given this overlap in sequelae and the prevalence of mTBI in pediatric populations, it makes mTBI a relevant and potentially precipitous stressor in this already vulnerable population. Our closed-head weight drop model of mTBI allows for the most accurate representation of the common etiologies behind pediatric mTBI: slips/falls, head-object collisions, and motor vehicle accidents. Most cases of mTBI can be fully recovered in terms of patient perceived and observable symptoms in minutes to days after injury [180]. We found we were able to replicate similar symptomology and recoverability in our rat model of mTBI. Sal+mTBI conditions had

significantly altered righting reflex, increased neuroinflammation, and increased markers of mTBI (Figures 5, 8). However, these markers of injury and inflammation were recovered in the Sal+mTBI groups by day 6 (Figure 8). Further, our IHC results found an acutely increased number of Iba1+ cells in the cortex of Sal+mTBI rats at DPI 2, but not an increased Iba1+ cell size (Figure 9). Both effects were non-significantly different from Sal+Sham conditions by DPI 10 (Figure 9). Further no significant differences were noted in our cortical ^{1}H-MRS assessment of brain metabolites or in our functional seahorse analysis (Figures 10, 18). Beyond DPI 2 the only mitochondrial oxidative phosphorylation complex protein that was significantly affected was the decrease of MTCO1 in Sal+mTBI conditions as compared to Sal+Sham (Figure 15). Interestingly, we also found a between sex difference at DPI 6 in the Sal+mTBI condition where females showed significantly lower levels of MTCO1 as compared to both female Sal+mTBI and female Sal+Sham animals (Figure 17). This suggests that, potentially, the mitochondrially encoded MTCO1 is significantly impacted by even a low-force phenotypically recoverable pediatric mTBI. A study in mice utilizing low-intensity blast induce brain injury also found decreased MTCO1 post-injury, however these decreased levels were statistically recovered by DPI 7 albeit trending decreased [279]. Another study utilizing adult rats and a controlled cortical contusion (CCI) model of mTBI found decreased MTCO1 in their proteomic analysis that was recovered by thyroxine treatment [280]. Together these two studies connect MTCO1 and molecular metabolism in mTBI. Interestingly both found diminished duration and magnitude of MTCO1 decrease despite using more severe models of mTBI. Two possible explanations of this could be our use of a pediatric model and the use of a model which allows for rapid acceleration and deceleration after impact. Further we also identified key synaptic proteins altered by pediatric mTBI as compared to Sal+Sham conditions; while this was not the specific focus of these works, at the date of writing this manuscript, ours is the first works to assess specifically cortical synaptosomal proteomics in the context of pediatric mTBI. We identified 142 DiffExpPro in the Sal+mTBI condition as compared to Sal+Sham, a brief GO analysis of these DiffExpPro has been included in the

appendices (Appendix – A). Altogether we have shown the ability to recapitulate a mild and recoverable brain injury that is mechanistically representative of the common sources of mTBI in children.

Elucidating the role of IUO exposure on impacting later life brain vulnerability was a key focus of these works. As such the IUO+Sham condition reveals the deficits specifically associated with NOWS. We found significant and overlapping pathophysiological biology that has been seen repeatedly in NOWS and other *in utero* models of opioid exposure. We found significant increase in markers of inflammation and brain injury in our uninjured IUO+Sham condition. While inflammation has been widely established in *in utero* models of opioid exposure markers of neurodegeneration and brain injury have not been commonly looked at [86, 111]. While biomarkers of NOWS and gestational opioid exposure for diagnosis is not a critical issue, seeing the overlap in gene expression with stroke, brain injury, and neurodegenerative disease is a significant finding (Figures 8, 19). Much of this effect is thought to be mediated by toll-like receptor four (TLR4) and myeloid differentiation protein 2 (MD-2), both which opioids act as agonists for [160]. This profound inflammatory reaction is what has caused experts in the opioid field to describe gestational opioid exposure as a neuroinflammatory disease [86]. Further, Iba1+ cell count and size was seen to increase in cortical tissue for IUO+Sham conditions as compared to Sal+Sham. Interestingly, when investigating cortical brain metabolites NAA was found to be significantly decreased at P30 (Figure 10). In our previous investigation using the same model we found a significant increase in NAA in the hippocampus at P17 [170]. This dichotomy is interesting, but ultimately the time-point and region differences make it impossible to correlate these results. However, seeing NAA, a key mitochondrial metabolite originating from neurons, significantly altered indicates that metabolic dysregulation is component of IUO exposure. To further support this our cortical synaptosome proteomic results found significant alterations in key metabolic pathways in IUO+Sham conditions (Figures 12, 13). Together this data points towards

mitochondrial pathophysiology as a possible etiology for IUO associated deficits seen in both these works and our groups previous works [63, 169-171].

As previously mentioned, IUO and mTBI have significantly overlapping pathophysiology and the mitochondrial impacts found in IUO conditions are known hallmarks of brain injury. However, when assessing IUO+mTBI condition for inflammatory markers and biomarkers of brain injury we did not find any significant differences from the IUO+Sham condition at any of the three timepoints (Figures 8, 19). Despite seeing significant differences as compared to both Sal+Sham and Sal+mTBI for most of these markers at all time points. Whether this is because IUO exposure itself induces a maximal response in these pathways, or because the insult of mTBI is not being compensated for remains is challenging to determine. Further, in our IHC data no significant differences in Iba1+ cell size or number was observed in cortical tissue despite trending increased when comparing IUO+mTBI to IUO+Sham conditions (Figure 9). When assessing cortical metabolites in the IUO+mTBI condition we found significant differences in Lactate and Glucose as compared to both Sal+Sham and Sal+mTBI but not IUO+Sham conditions (Figure 10). Again, however, there was a trending difference in both lactate and glucose in the IUO+Sham condition as compared to Sal+Sham that matched the significant difference found in IUO+mTBI animals. This suggests that while IUO alone is not enough to generate enough stress to significantly alter cortical metabolites, the combination of IUO+mTBI causes a signficant pathophysiological change. Considering the non-significant changes in inflammatory markers and Iba1+ cell count or size between IUO+Sham and IUO+mTBI conditions, the mitochondrial and oxidative stress differences found in our synaptosomal proteomics data was a primary culprit for driving these observed metabolic deficits associated with IUO+mTBI. What is interesting is that when we investigated shared DiffExpPro in both IUO+Sham and IUO+mTBI conditions to determine connecting proteins between the two conditions we found oxidative stress and metabolic processes to be the principle pathways affected (Figure 12). Our western blotting validation of

identified oxidative phosphorylation complex proteins revealed significant upregulation at DPI 2 in IUO+Sham, and IUO+mTBI conditions in both tissue lysate and synaptosomes (Figures 14, 15). However, by DPI 10 no significant effects were observed in tissue lysate for either conditions, but in synaptosomes IUO+mTBI had a significantly decreased expression of NDUFB8, SDHB, UQCRC2, and ATP5A as compared to both Sal+mTBI and IUO+Sham (Figure 15). This suggests that while at DPI 2 complex expression is similar for IUO+Sham and IUO+mTBI, at DPI 10, specifically in the synapse, there is significant downregulation for only IUO+mTBI conditions. While literature investigating NOWS and *in utero* opioid exposure as a metabolic disorder are not currently extant in the field, these works suggest that this could be a principle etiology behind much of the disease sequelae.

When attempting to interrogate functional deficits associated with both IUO and IUO+mTBI we first characterized mitochondrial function at DPI 2, or P30. Our findings were consistent with our western blotting data in part. For NSYPmito, IUO+Sham conditions showed a significantly increased OCR in complexes II and IV as well as greater maximal respiration when compared to both Sal+Sham and Sal+mTBI conditions. However, while trending, no significant differences were noticed in our IUO+mTBI condition at DPI 2 when compared to any other groups. In SYPmito though, IUO+mTBI was very significantly increased for complexes II and IV as compared to both Sal+Sham and Sal+mTBI conditions, while IUO+Sham was only increased in complex IV. While the observed acute alterations in mitochondrial function at the synapse suggest a differential response to mTBI in IUO exposed subjects, it is critical to understand if these effect cause functional and behavioral deficits longitudinally. DPI 38-40, or P66-68, which translates to young adulthood is a sufficient time frame for complete recovery from a mTBI; at this time point after injury and after the majority of rat neurogenesis and synaptogenesis is finished we repeated our assessment of oxygen consumption in synaptic and non-synaptic mitochondria. Our findings again seemed to dovetail with our western blot differences at DPI 2 and DPI 10, where a

significant increase in OCR was observed at DPI 2, however, at DPI 38-40 OCR was significantly decreased (Figures 18, 20). Pathophysiologically overactive mitochondria are associated with exacerbated disease and injury response and are known to cause mitochondrial damage primarily caused reactive oxidation species (ROS) production [248, 281]. This temporal shift in OCR for IUO+mTBI conditions suggests that a hyperactive metabolic response to brain injury in IUO exposed subjects eventually leads to mitochondrial dysfunction. The mitochondria is a central regulator of inflammation in cells, and if IUO exposure induces mitochondrial hyperreactivity during early development ROS damage could be responsible for IUO associated inflammation [271]. It is well established that ROS damage can cause oxidative damage to mitchondrial proteins, lipids, and DNA impairing mitochondrial function over time and potentially leading to apoptosis [282]. Further, it is known that impaired mitochondrial function is associated with an array of psychiatric disorders primarily related to the allosteric stress response [283]. These behaviors are often associated with stress, anxiety, and impaired sociality [267, 270, 283-285]. In our data we show significant social impairment and increased anxiety like behavior in the IUO+mTBI groups (Figure 21). Throughout all behavioral experiments no significant difference was observed between IUO+Sham and IUO+mTBI, however, we see exacerbated effects in the IUO+mTBI condition across social, marble burying, and rotarod performance as compared to Sal+Sham and Sal+mTBI (Figure 21). The most stark contrasts can be seein in social novelty and marble burying data, both of which, suggest that while IUO+Sham conditions are impacted, IUO+mTBI conditions show an even further exacerbated effect on behavior. This data suggests that while IUO+Sham and IUO+mTBI many times are not significantly different, the combinatorial effect of both IUO exposure and mTBI causes significant deficits that are not observed in either insult alone. It is alos possible that our behavior data is not sufficiently powered to detect the differences between IUO+Sham and IUO+mTBI, because when comparisons are made between just those two groups and not correcting for multiple comparisons, we see significant differences between IUO+Sham and IUO+mTBI occur in two key behavioral measures marble burying and

motor learning (Appendix - B). Taken together this data suggests very strongly that gestational environment, and specifically gestational exposure to oxy, plays a significant role in worsening longitudinal outcomes both in terms of synaptic mitochondria and behavior.

Future Directions

The presented research does much to advocate for health equity of those exposed *in utero* to not only opioids, but also other teratogens and it should incite healthcare providers to consider a child's gestational history during treatment and diagnosis. The works here also provide a strong baseline for future therapeutic investigations; whether pharmaceutical or behavioral the principal etiologies behind IUO induced vulnerability revealed in these works can be targeted. Indeed, there are some epidemiological data and case studies to support some of the findings of this work that targeting metabolic stress could be a potential avenue of therapy for gestational opioid exposure. The most promising future directions will be divided into two categories based on the time of intervention. First, interventions which target the pregnant mother to improve offspring outcome. Second, interventions for the neonate to improve outcomes.

Maternal treatment to improve infant outcome of NOWS is a field with little conclusive data. However, there are data which suggest that maternal education and breastfeeding, even while on opioid substitution therapy, improves outcomes in children [286-288]. As such one of the first adjunctive therapies that helps the neonate is supplementing the mother with opioid substitution therapy. Indeed while gestational methadone exposure is not without complications the outcomes are much improved as compared to other illicit or prescription opioids [288]. Beyond this data for opioid substitution therapy there is a gap in the field for therapies that target the mother to improve neonate outcomes, however there is promising epidemiological and case study data. Opioid use disorder is known to correlate heavily with metabolic syndromes, and is also associated with the modification of reward circuitry for both food and drug seeking behaviors [289, 290]. Further chronic opioid consumption, as is seen in mothers with children who go on to suffer

NOWS, increases sign of insulin resistance and diabetes [290, 291]. This data then suggested to clinical researchers that diabetes could be a comorbid risk factor for NOWS children, however the opposite was revealed. A population study found that after controlling for variables, children born to mothers with diabetes had a 6.8% ($p \leq .0382$) lower rate of displays NOWS phenotypes [292]. The authors conclude that further interrogation into the role of NOWS and glucose metabolism needs to be performed. Given the strong association in our model with, not only cortical glucose consumption but also significant biphasic disruption of cortical synaptic mitochondria after brain injury suggests that opioid induced metabolic syndrome could be passed from mother to child. Future animal studies could assess using a diabetic dam model to understand how this provides protective effects in the case of NOWS. Infants born to diabetic mothers are exposed to greater amounts of insulin and are more likely to develop subsequent hypoglycemia [293]. Together with the data presented in this dissertation it is apparent that one potential pathway to protecting children exposed to opioids *in utero* could lie in targeting maternal metabolic syndromes.

Much more commonly therapeutic strategies involve treating neonates to ameliorate the immediate effects of NOWS and *in utero* opioid exposure. As with the mother, the best therapeutic strategy is to wean the neonate off opioids in a safe manner [179]. Most pharmacological interventions are targeted towards finding better opioid drugs for opioid substitution therapy, currently those commonly used are methadone and buprenorphine [294]. The most promising non-opioid therapeutic intervention is currently clonidine which is used to ameliorate the tachycardia, hypertension, and hyperhidrosis seen in opioid withdrawal [87, 294]. Aside from dealing with the principal issue of withdrawal symptoms there have been various interventions targeting decreasing time spent in the neonatal intensive care unit (NICU). One such recent multi-site interventional study utilizing the east, sleep, console (ESC) care approach was able to reduce NICU stay time in a cohort of 837 neonates by an average of 6.7 days and these neonates were 63% less likely to require drug therapy interventions [295]. A two year evaluation of outcomes in

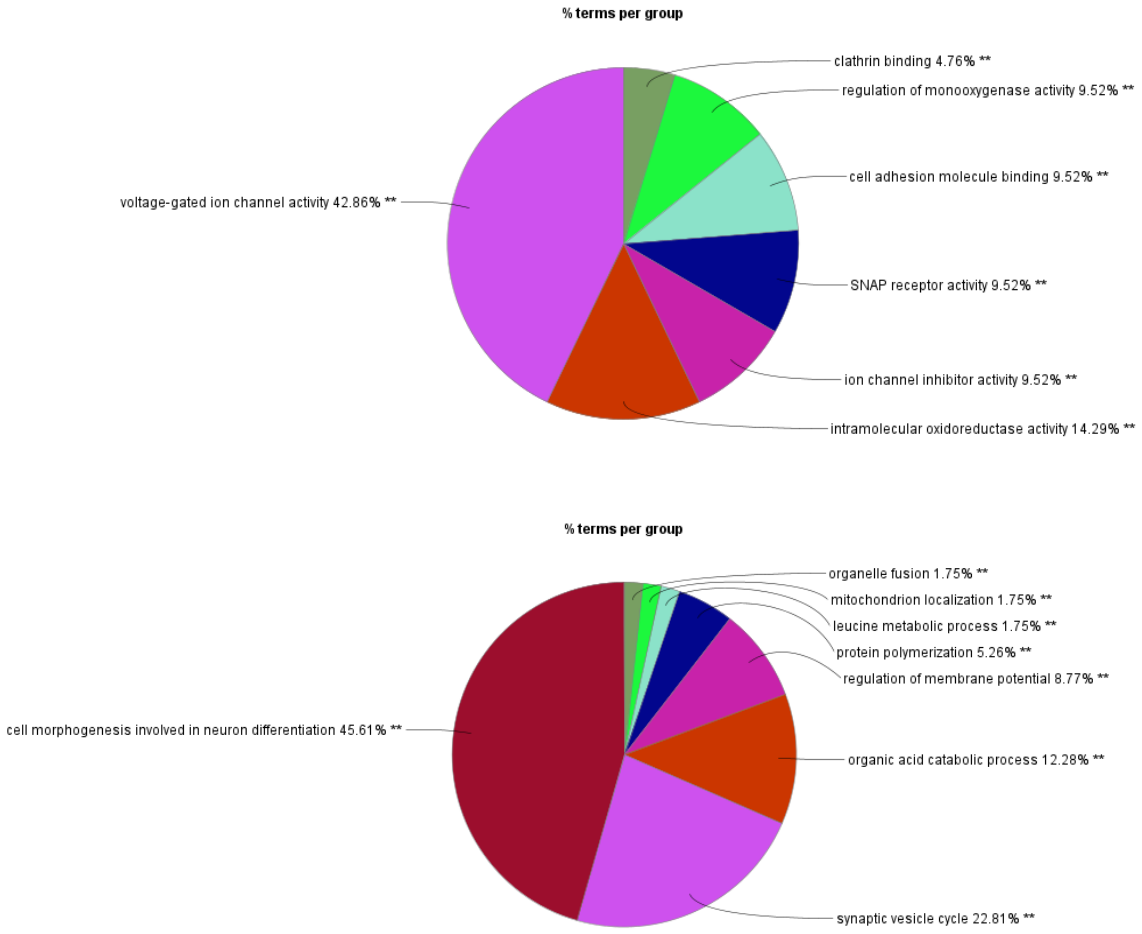
this population is currently under investigation as of the time of writing. These future works should seek to investigate if the ESC approach can improve key negative outcomes such as inpatient hospitalization, outpatient encounters, ED visits, white matter tract degeneration, and decreased brain region size that has been observed with NOWS [85, 89, 123]. Future works could target multiple interventions for these children based on the works here and in other preclinical animal models. A great amount clinical studies have gone into addressing metabolic disorders in both mother and infant through dietary supplementation. Current and past clinical trials have investigated caloric supplementation, non-lactose milk, and various formula nutrition modifications most with minimal effect or no published results (NCT04419857, NCT02178189, NCT03549936). A highly investigated supplement for pre-term and underweight children, both very common in NOWS, is poly-unsaturated fatty acids (PUFAs) [296]. Dietary supplementation with PUFAs to serve as antioxidants is a ubiquitously researched approach across multiple diseases spanning both body and brain [297]. While no published works specifically address oxidative stress as a component of *in utero* opioid exposure prior to these works, other works have shown benefits of antioxidant therapy to ameliorate alcohol induced oxidative stress in neonates [298]. Further, previous work by our group and others has identified significant perturbations in the hypocretin and orexin system associated with both adult and neonatal opioid exposure [169, 299]. The co-localization of opioid and orexin receptors on neurons makes this a promising target for future therapeutics, and indeed preclinical data as shown that targeting with a dual orexin inhibitor ameliorates opioid associated addiction and relapse in adults [299]. An additional strategy strongly supported in clinical research could be to target pathological inflammation by blocking TLR4. TLR4 is now known to be agonized by opioids and a strong component of the inflammatory cascade progression [112, 300]. A widely investigated molecule for the therapeutic treatment of opioid addiction is a phosphodiesterase inhibitor known as Ibudilast (IBUD) which has off-target effects as a TLR4 antagonist [301, 302]. IBUD has had both preclinical and clinical success in ameliorating opioid addiction related inflammation, as well as

inflammation associated with heart, kidney, and brain diseases [302-308]. While safety profiles haven't been established in the US, IBUD has shown good tolerability in Japan among pediatric populations. Future preclinical work could investigate its suitability in neonates to potentially push this strong candidate forward for resolving *in utero* opioid associated neuroinflammation [309-311].

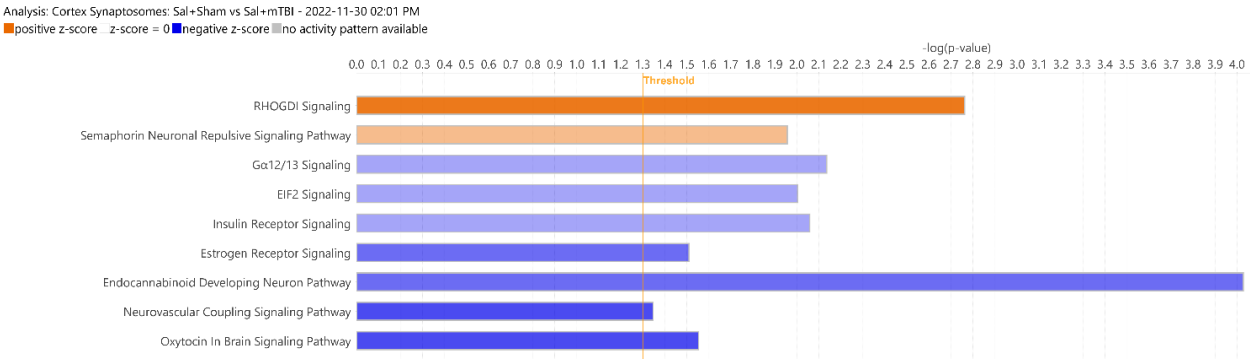
Taken together the works of this manuscript provide a solid bedrock for future investigation of NOWS and *in utero* opioid exposure as not only an inflammatory disease, but also a metabolic disease. Previous works have characterized size and white matter discrepancies in the cortex of NOWS sufferers; our works have shown not only specific cortical molecular perturbations, but also significant vulnerability to a normally recoverable brain injury which most impacts the cortices. Children born suffering from opioid exposure have a significant neurological vulnerability that needs to be addressed, both in the clinic through patient and provider education, and in clinical trials to develop strategies for ameliorating negative outcomes. Beyond the scope of our model utilizing oxy, it is the hope that these collective works put into perspective the long reaching developmental effects of insults during gestational development. It is perhaps best said by the much cited paper Killing Me Softly: The Fetal Origins Hypothesis, which posits that the effects of fetal conditions are not only persistent, but also that they can remain latent for many years until age or another potentiation event reveals the so-called cracks in the foundation [312]. Our work provides a novel insight into exactly these vulnerabilities are within the cortical and synaptic microenvironment, and a novel path forward to help mitigate the lifelong negative effects of *in utero* opioid exposure.

APPENDICES

Appendix A –

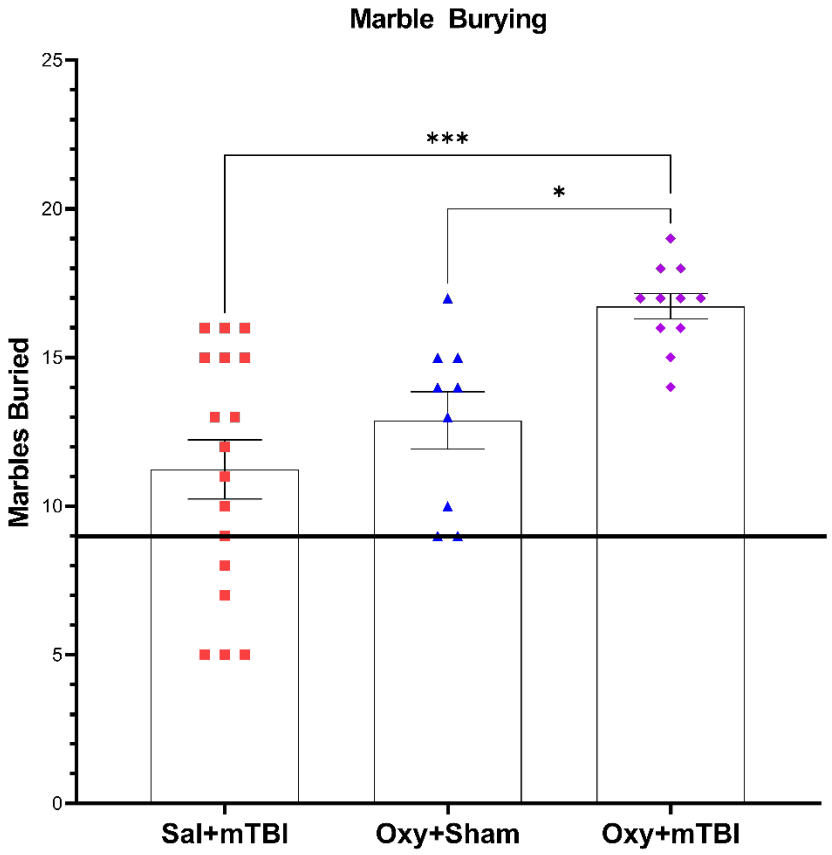


ClueGO analysis of Sal+mTBI synaptosomal proteins as compared to Sal+Sham conditions. Top is pathways related to molecular function and bottom is for pathways related to biological function. We see significant overlap in voltage-gated ion channel activity, SNAP receptor activity, cell adhesion molecule binding, and synaptic vesicle cycle which are all intrinsically linked and associated with mTBI induced excitotoxicity.

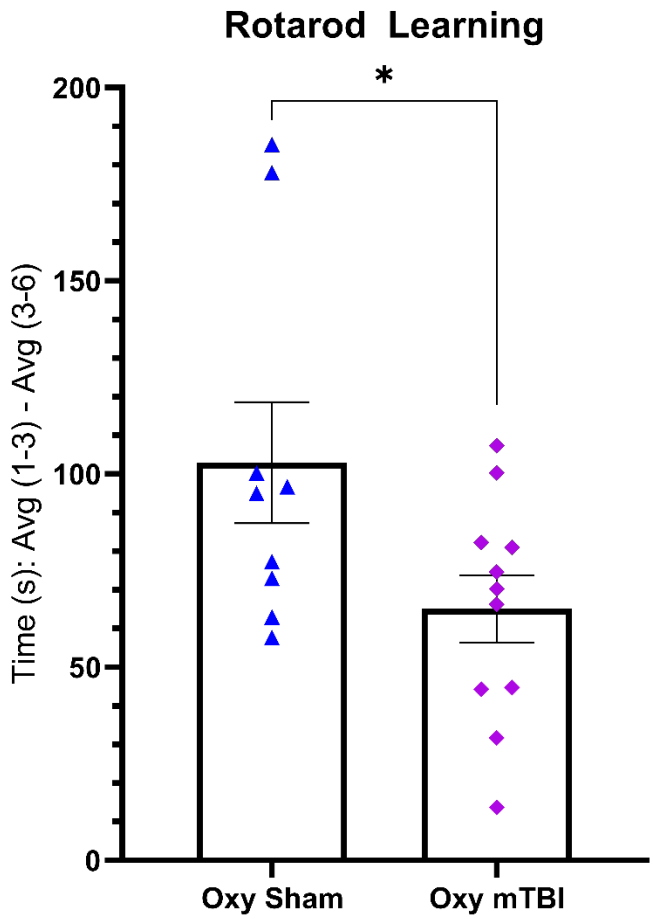


IPA analysis of Sal+mTBI synaptosomal proteins as compared to Sal+Sham conditions. Significant perturbations are noticed in insulin signaling and ion channel activity. The most significant pathways are the upregulation of RHOGDI signaling and downregulation of the endocannabinoid developing neuron pathway.

Appendix B –



Marble burying assessment of anxiety and compulsive like behavior showed the IUO+Sham animals buried more marbles than Sal+Sham conditions but a non-significantly different amount as compared to Sal+mTBI. IUO+mTBI buried significantly more marbles as compared to both Sal+Sham and Sal+mTBI. Data are displayed as Mean ± SEM; *p ≤ 0.05; **p ≤ 0.001; ***p ≤ 0.005, ****p ≤ 0.0001.



Rotarod assessment showed significantly motor learning impairment in IUO+mTBI conditions as measure by improvement in latency to fall from trials 1-3 as compared to trials 4-6. Data are displayed as Mean ± SEM; *p ≤ 0.05; **p ≤ 0.001; ***p ≤ 0.005, ****p ≤ 0.0001.

BIBLIOGRAPHY

1. Davenport-Hines, R., *The pursuit of oblivion: A social history of drugs*. 2012: Hachette UK.
2. Cano, M., *Racial/ethnic differences in US drug overdose mortality, 2017–2018.* Addictive Behaviors, 2021. **112**: p. 106625.
3. Nations, U., *World drug report.* United Nations publication, 2020.
4. Mattson, C.L., et al., *Trends and geographic patterns in drug and synthetic opioid overdose deaths—United States, 2013–2019.* Morbidity and Mortality Weekly Report, 2021. **70**(6): p. 202.
5. Wikler, A., *Present status of the concept of drug dependence.* Psychological medicine, 1971. **1**(5): p. 377-380.
6. Ray, O., *Drugs, Society, and Human Behavior.* 1978.
7. Volkow, N.D., G.F. Koob, and A.T. McLellan, *Neurobiologic advances from the brain disease model of addiction.* New England Journal of Medicine, 2016. **374**(4): p. 363-371.
8. Landry, M., *Addiction diagnostic update: DSM-III-R psychoactive substance use disorders.* Journal of psychoactive drugs, 1987. **19**(4): p. 379-381.
9. Volkow, N.D. and M. Morales, *The brain on drugs: from reward to addiction.* Cell, 2015. **162**(4): p. 712-725.
10. Treatment, C.f.S.A., *Treatment for stimulant use disorders.* 1999.
11. Wise, R.A., *Catecholamine theories of reward: a critical review.* Brain research, 1978. **152**(2): p. 215-247.
12. Müller, C.P. and J.R. Homberg, *The role of serotonin in drug use and addiction.* Behavioural brain research, 2015. **277**: p. 146-192.
13. Marshall, J.F., et al., *Methamphetamine-induced neural and cognitive changes in rodents.* Addiction, 2007. **102**: p. 61-69.

14. Nestler, E.J., *The neurobiology of cocaine addiction.* Science & practice perspectives, 2005. **3**(1): p. 4.

15. Mansvelder, H.D. and D.S. McGehee, *Cellular and synaptic mechanisms of nicotine addiction.* Journal of neurobiology, 2002. **53**(4): p. 606-617.

16. Rajendram, R., G. Lewison, and V. Preedy, *Worldwide alcohol-related research and the disease burden.* Alcohol and Alcoholism, 2006. **41**(1): p. 99-106.

17. Costa, B., *On the pharmacological properties of Δ9-tetrahydrocannabinol (THC).* Chemistry & biodiversity, 2007. **4**(8): p. 1664-1677.

18. Morgenstern, J. and R. Longabaugh, *Cognitive–behavioral treatment for alcohol dependence: A review of evidence for its hypothesized mechanisms of action.* Addiction, 2000. **95**(10): p. 1475-1490.

19. Griffin, C.E., et al., *Benzodiazepine pharmacology and central nervous system–mediated effects.* Ochsner Journal, 2013. **13**(2): p. 214-223.

20. Schmitz, A., *Benzodiazepine use, misuse, and abuse: a review.* Mental Health Clinician, 2016. **6**(3): p. 120-126.

21. Liechti, M.E., *Modern clinical research on LSD.* Neuropsychopharmacology, 2017. **42**(11): p. 2114-2127.

22. Passie, T., et al., *The pharmacology of psilocybin.* Addiction biology, 2002. **7**(4): p. 357-364.

23. Wagner, M.T., et al., *Therapeutic effect of increased openness: Investigating mechanism of action in MDMA-assisted psychotherapy.* Journal of Psychopharmacology, 2017. **31**(8): p. 967-974.

24. Nigam, K.B. and A.K. Pandurangi, *Do Hallucinogens Have a Role in the Treatment of Addictions? A Review of the Current Literature.* SN Comprehensive Clinical Medicine, 2021. **3**: p. 1385-1395.

25. Hawk, K., et al., *Past-year prescription drug monitoring program opioid prescriptions and self-reported opioid use in an emergency department population with opioid use disorder.* Academic Emergency Medicine, 2018. **25**(5): p. 508-516.

26. Florence, C., et al., *The economic burden of prescription opioid overdose, abuse and dependence in the United States, 2013.* Medical care, 2016. **54**(10): p. 901.

27. Kolodny, A., et al., *The prescription opioid and heroin crisis: a public health approach to an epidemic of addiction.* Annual review of public health, 2015. **36**: p. 559-574.

28. Kosten, T.R. and T.P. George, *The neurobiology of opioid dependence: implications for treatment.* Science & practice perspectives, 2002. **1**(1): p. 13.

29. Zimmermann, M., *[History of pain treatment from 1500 to 1900].* Schmerz, 2007. **21**(4): p. 297-306.

30. Goebel, J.R., et al., *Addressing Patients' Concerns about Pain Management and Addiction Risks.* Pain Management Nursing, 2010. **11**(2): p. 92-98.

31. Zedler, B., et al., *Risk factors for serious prescription opioid-related toxicity or overdose among Veterans Health Administration patients.* Pain Med, 2014. **15**(11): p. 1911-29.

32. Bryson, E.O. and J.H. Silverstein, *Addiction and substance abuse in anesthesiology.* Anesthesiology, 2008. **109**(5): p. 905-17.

33. Alford, D.P., et al., *Primary Care Patients with Drug Use Report Chronic Pain and Self-Medicate with Alcohol and Other Drugs.* Journal of General Internal Medicine, 2016. **31**(5): p. 486-491.

34. Riley III, J.L. and C. King, *Self-report of alcohol use for pain in a multi-ethnic community sample.* The Journal of Pain, 2009. **10**(9): p. 944-952.

35. Hart, C.L., C. Ksir, and O.S. Ray, *Drugs, society & human behavior*. 2013: McGraw-Hill New York.

36. Manchikanti, L., et al., *Opioid epidemic in the United States.* Pain physician, 2012. **15**(3S): p. ES9.

37. Scholl, L., et al., *Drug and opioid-involved overdose deaths—United States, 2013–2017.* Morbidity and mortality weekly report, 2019. **67**(51-52): p. 1419.

38. Nelson, L.S., D.N. Juurlink, and J. Perrone, *Addressing the opioid epidemic.* Jama, 2015. **314**(14): p. 1453-1454.

39. Comerford, D., *Techniques of opioid administration.* Anaesthesia & Intensive Care Medicine, 2008. **9**(1): p. 21-26.

40. Kaiko, R., *Pharmacokinetics and pharmacodynamics of controlled‐release opioids.* Acta anaesthesiologica scandinavica, 1997. **41**(1): p. 166-174.

41. Sindt, J.E. and R.H. Jenkinson, *18 - Nonintravenous Opioids*, in *Pharmacology and Physiology for Anesthesia (Second Edition)*, H.C. Hemmings and T.D. Egan, Editors. 2019, Elsevier: Philadelphia. p. 354-368.

42. Trescot, A.M., et al., *Opioid pharmacology.* Pain Physician, 2008. **11**(2 Suppl): p. S133-53.

43. Pathan, H. and J. Williams, *Basic opioid pharmacology: an update.* Br J Pain, 2012. **6**(1): p. 11-6.

44. Wittert, G., P. Hope, and D. Pyle, *Tissue Distribution of Opioid Receptor Gene Expression in the Rat.* Biochemical and Biophysical Research Communications, 1996. **218**(3): p. 877-881.

45. Ross, F.B. and M.T. Smith, *The intrinsic antinociceptive effects of oxycodone appear to be κ-opioid receptor mediated.* Pain, 1997. **73**(2): p. 151-157.

46. Yang, P.-P., et al., *Activation of delta-opioid receptor contributes to the antinociceptive effect of oxycodone in mice.* Pharmacological Research, 2016. **111**: p. 867-876.

47. Karkhanis, A., K.M. Holleran, and S.R. Jones, *Dynorphin/kappa opioid receptor signaling in preclinical models of alcohol, drug, and food addiction.* International review of neurobiology, 2017. **136**: p. 53-88.

48. Lalanne, L., et al., *The kappa opioid receptor: from addiction to depression, and back.* Frontiers in psychiatry, 2014: p. 170.

49. Wang, Y., E.J. Van Bockstaele, and L.-Y. Liu-Chen, *In vivo trafficking of endogenous opioid receptors.* Life Sciences, 2008. **83**(21): p. 693-699.

50. Befort, K., et al., *Effects of delta opioid receptors activation on a response inhibition task in rats.* Psychopharmacology (Berl), 2011. **214**(4): p. 967-76.

51. Fan, T., et al., *A Role for the Distal Carboxyl Tails in Generating the Novel Pharmacology and G Protein Activation Profile of μ and δ Opioid Receptor Hetero-oligomers *.* Journal of Biological Chemistry, 2005. **280**(46): p. 38478-38488.

52. Wang, H.-B., et al., *Coexpression of δ-and μ-opioid receptors in nociceptive sensory neurons.* Proceedings of the National Academy of Sciences, 2010. **107**(29): p. 13117-13122.

53. Miura, M., et al., *Compartment-specific modulation of GABAergic synaptic transmission by μ-opioid receptor in the mouse striatum with green fluorescent protein-expressing dopamine islands.* Journal of Neuroscience, 2007. **27**(36): p. 9721-9728.

54. Tejeda, H.A. and A. Bonci, *Dynorphin/kappa-opioid receptor control of dopamine dynamics: Implications for negative affective states and psychiatric disorders.* Brain Research, 2019. **1713**: p. 91-101.

55. Liao, D., et al., *Distinct effects of individual opioids on the morphology of spines depend upon the internalization of mu opioid receptors.* Molecular and Cellular Neuroscience, 2007. **35**(3): p. 456-469.

56. Fields, H.L. and E.B. Margolis, *Understanding opioid reward.* Trends in Neurosciences, 2015. **38**(4): p. 217-225.

57. Thigpen, J.C., B.L. Odle, and S. Harirforoosh, *Opioids: A Review of Pharmacokinetics and Pharmacodynamics in Neonates, Infants, and Children.* European Journal of Drug Metabolism and Pharmacokinetics, 2019. **44**(5): p. 591-609.

58. Tréluyer, J.M., et al., *Evidence of impaired cisapride metabolism in neonates.* British journal of clinical pharmacology, 2001. **52**(4): p. 419-425.

59. Neumann, E., et al., *Age-dependent hepatic UDP-glucuronosyltransferase gene expression and activity in children.* Frontiers in Pharmacology, 2016. **7**: p. 437.

60. Iacobelli, S. and J.-P. Guignard, *Maturation of glomerular filtration rate in neonates and infants: an overview.* Pediatric Nephrology, 2021. **36**(6): p. 1439-1446.

61. Weller, A.E., et al., *Neonatal Opioid Withdrawal Syndrome (NOWS): A Transgenerational Echo of the Opioid Crisis.* Cold Spring Harb Perspect Med, 2021. **11**(3).

62. Byrnes, E.M. and F.M. Vassoler, *Modeling prenatal opioid exposure in animals: current findings and future directions.* Frontiers in neuroendocrinology, 2018. **51**: p. 1-13.

63. Odegaard, K.E., G. Pendyala, and S.V. Yelamanchili *Generational Effects of Opioid Exposure*. Encyclopedia, 2021. **1**, 99-114 DOI: 10.3390/encyclopedia1010012.

64. Hirai, A.H., et al., *Neonatal Abstinence Syndrome and Maternal Opioid-Related Diagnoses in the US, 2010-2017.* JAMA, 2021. **325**(2): p. 146-155.

65. Desai, R.J., et al., *Increase in prescription opioid use during pregnancy among Medicaid-enrolled women.* Obstet Gynecol, 2014. **123**(5): p. 997-1002.

66. Osmundson, S.S., J.Y. Min, and C.G. Grijalva, *Opioid prescribing after childbirth: overprescribing and chronic use.* Curr Opin Obstet Gynecol, 2019. **31**(2): p. 83-89.

67. Chen, Y., et al., *Preimplantation Mouse Embryo Is a Target for Opioid Ligand-Receptor Signaling1.* Biology of Reproduction, 2014. **91**(1).

68. Leppert, W., *The impact of opioid analgesics on the gastrointestinal tract function and the current management possibilities.* Contemp Oncol (Pozn), 2012. **16**(2): p. 125-31.

69. Golosova, D., et al., *Role of opioid signaling in kidney damage during the development of salt-induced hypertension.* Life Science Alliance, 2020. **3**(12): p. e202000853.

70. Mallappallil, M., et al., *What Do We Know about Opioids and the Kidney?* Int J Mol Sci, 2017. **18**(1).

71. Greiner, K.S., et al., *Opioid Use and Pregnancy Outcomes in Women With a Hypertensive Disorder of Pregnancy [31C].* Obstetrics & Gynecology, 2019. **133**: p. 39S.

72. Moon, A.M., et al., *Opioid Use Is More Common in Nonalcoholic Fatty Liver Disease Patients with Cirrhosis, Higher BMI, and Psychiatric Disease.* Digestive Diseases, 2021. **39**(3): p. 247-257.

73. Verna, E.C., A. Schluger, and R.S. Brown, *Opioid epidemic and liver disease.* JHEP Reports, 2019. **1**(3): p. 240-255.

74. Yamanaka, T. and R.T. Sadikot, *Opioid effect on lungs.* Respirology, 2013. **18**(2): p. 255-62.

75. Seatriz, J.V. and R.P. Hammer, *Effects of opiates on neuronal development in the rat cerebral cortex.* Brain Research Bulletin, 1993. **30**(5): p. 523-527.

76. Yanai, J., et al., *Alterations in septohippocampal cholinergic innervations and related behaviors after early exposure to heroin and phencyclidine.* Developmental Brain Research, 1992. **69**(2): p. 207-214.

77. Smith, C.J., et al., *Prenatal opioid exposure inhibits microglial sculpting of the dopamine system selectively in adolescent male offspring.* Neuropsychopharmacology, 2022. **47**(10): p. 1755-1763.

78. Yazdy, M.M., R.J. Desai, and S.B. Brogly, *Prescription Opioids in Pregnancy and Birth Outcomes: A Review of the Literature.* J Pediatr Genet, 2015. **4**(2): p. 56-70.

79. Raffaeli, G., et al., *Neonatal abstinence syndrome: update on diagnostic and therapeutic strategies.* Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy, 2017. **37**(7): p. 814-823.

80. Conradt, E., et al., *Annual Research Review: Prenatal opioid exposure – a two-generation approach to conceptualizing neurodevelopmental outcomes.* Journal of Child Psychology and Psychiatry, 2023. **64**(4): p. 566-578.
81. Barker, D.J., *The origins of the developmental origins theory.* Journal of internal medicine, 2007. **261**(5): p. 412-417.
82. Suzuki, K., *The developing world of DOHaD.* Journal of developmental origins of health and disease, 2018. **9**(3): p. 266-269.
83. Fleming, T., M. Velazquez, and J. Eckert, *Embryos, DOHaD and david barker.* Journal of developmental origins of health and disease, 2015. **6**(5): p. 377-383.
84. Mactier, H. and R. Hamilton, *Prenatal opioid exposure – Increasing evidence of harm.* Early Human Development, 2020. **150**: p. 105188.
85. Liu, G., et al., *A Longitudinal Healthcare Use Profile of Children with a History of Neonatal Abstinence Syndrome.* J Pediatr, 2019. **204**: p. 111-117.e1.
86. Jantzie, L.L., et al., *Prenatal opioid exposure: The next neonatal neuroinflammatory disease.* Brain, Behavior, and Immunity, 2020. **84**: p. 45-58.
87. Bader, M.Y., et al., *A Retrospective Review Following the Addition of Clonidine to a Neonatal Abstinence Syndrome Treatment Algorithm.* Front Pediatr, 2021. **9**: p. 632836.
88. Merhar, S.L., et al., *Prenatal opioid exposure is associated with smaller brain volumes in multiple regions.* Pediatric research, 2021. **90**(2): p. 397-402.
89. Hartwell, M.L., et al., *Association of Prenatal Opioid Exposure With Precentral Gyrus Volume in Children.* JAMA Pediatrics, 2020. **174**(9): p. 893-896.
90. Conradt, E., S.E. Crowell, and B.M. Lester, *Early life stress and environmental influences on the neurodevelopment of children with prenatal opioid exposure.* Neurobiology of stress, 2018. **9**: p. 48-54.
91. Darcq, E. and B.L. Kieffer, *Opioid receptors: drivers to addiction?* Nature Reviews Neuroscience, 2018. **19**(8): p. 499-514.

92. Xu, C., et al., *Kappa opioid receptor controls neural stem cell differentiation via a miR-7a/Pax6 dependent pathway.* Stem Cells, 2021. **39**(5): p. 600-616.

93. Fauser, A.-M., et al., *Role of microRNA-132 in Opioid Addiction through Modification of Neural Stem Cell Differentiation*. 2022, MDPI. p. 1800.

94. Narita, M., et al., *Role of δ-opioid receptor function in neurogenesis and neuroprotection.* Journal of neurochemistry, 2006. **97**(5): p. 1494-1505.

95. Stiene-Martin, A., et al., *Opioid system diversity in developing neurons, astroglia, and oligodendroglia in the subventricular zone and striatum: impact on gliogenesis in vivo.* Glia, 2001. **36**(1): p. 78-88.

96. Vishnubhotla, R.V., et al., *Brain structural connectome in neonates with prenatal opioid exposure.* Frontiers in Neuroscience, 2022. **16**: p. 952322.

97. Rees, P., et al., *Childhood health and educational outcomes after neonatal abstinence syndrome: a systematic review and meta-analysis.* The Journal of Pediatrics, 2020. **226**: p. 149-156. e16.

98. Lum, J.S., et al., *Prenatal methadone exposure impairs adolescent cognition and GABAergic neurodevelopment in a novel rat model of maternal methadone treatment.* Progress in Neuro-Psychopharmacology and Biological Psychiatry, 2021. **110**: p. 110281.

99. Yoshii, A. and M. Constantine-Paton, *Postsynaptic BDNF-TrkB signaling in synapse maturation, plasticity, and disease.* Developmental neurobiology, 2010. **70**(5): p. 304-322.

100. Nagappan, G. and B. Lu, *Activity-dependent modulation of the BDNF receptor TrkB: mechanisms and implications.* Trends in neurosciences, 2005. **28**(9): p. 464-471.

101. Daskalakis, N.P., et al., *Early life stress effects on glucocorticoid—BDNF interplay in the hippocampus.* Frontiers in molecular neuroscience, 2015. **8**: p. 68.

102. Kundakovic, M., et al., *DNA methylation of BDNF as a biomarker of early-life adversity.* Proceedings of the National Academy of Sciences, 2015. **112**(22): p. 6807-6813.

103. Wan, L., et al., *RACK1 affects morphine reward via BDNF.* Brain Research, 2011. **1416**: p. 26-34.

104. Meng, M., et al., *Region-specific expression of brain-derived neurotrophic factor splice variants in morphine conditioned place preference in mice.* Brain Research, 2013. **1519**: p. 53-62.

105. Colgan, L.A., et al., *PKCα integrates spatiotemporally distinct Ca2+ and autocrine BDNF signaling to facilitate synaptic plasticity.* Nature neuroscience, 2018. **21**(8): p. 1027-1037.

106. Maisonpierre, P.C., et al., *NT-3, BDNF, and NGF in the developing rat nervous system: parallel as well as reciprocal patterns of expression.* Neuron, 1990. **5**(4): p. 501-509.

107. Mattis, V.B., et al., *HD iPSC-derived neural progenitors accumulate in culture and are susceptible to BDNF withdrawal due to glutamate toxicity.* Human molecular genetics, 2015. **24**(11): p. 3257-3271.

108. Machelska, H. and M.Ö. Celik, *Opioid receptors in immune and glial cells—implications for pain control.* Frontiers in immunology, 2020. **11**: p. 300.

109. Watkins, L.R., et al., *The "toll" of opioid-induced glial activation: improving the clinical efficacy of opioids by targeting glia.* Trends Pharmacol Sci, 2009. **30**(11): p. 581-91.

110. Graeber, M.B. and W.J. Streit, *Microglia: biology and pathology.* Acta neuropathologica, 2010. **119**: p. 89-105.

111. Vasan, V., et al., *Neonatal opioid exposure: public health crisis and novel neuroinflammatory disease.* Neural Regen Res, 2021. **16**(3): p. 430-432.

112. Zhang, P., et al., *Toll-Like Receptor 4 (TLR4)/Opioid Receptor Pathway Crosstalk and Impact on Opioid Analgesia, Immune Function, and Gastrointestinal Motility.* Front Immunol, 2020. **11**: p. 1455.

113. Corkrum, M., et al., *Opioid-mediated astrocyte–neuron signaling in the nucleus accumbens.* Cells, 2019. **8**(6): p. 586.

114. Belcheva, M.M., et al., *μ and κ opioid receptors activate ERK/MAPK via different protein kinase C isoforms and secondary messengers in astrocytes.* Journal of Biological Chemistry, 2005. **280**(30): p. 27662-27669.

115. Nam, M.-H., et al., *Signaling mechanisms of μ-opioid receptor (MOR) in the hippocampus: disinhibition versus astrocytic glutamate regulation.* Cellular and Molecular Life Sciences, 2021. **78**: p. 415-426.

116. Grace, P.M., S.F. Maier, and L.R. Watkins, *Opioid-induced central immune signaling: implications for opioid analgesia.* Headache: The Journal of Head and Face Pain, 2015. **55**(4): p. 475-489.

117. Bradl, M. and H. Lassmann, *Oligodendrocytes: biology and pathology.* Acta neuropathologica, 2010. **119**: p. 37-53.

118. Vestal-Laborde, A.A., et al., *The opioid system and brain development: effects of methadone on the oligodendrocyte lineage and the early stages of myelination.* Developmental neuroscience, 2014. **36**(5): p. 409-421.

119. Velasco, B., E. Mohamed, and C. Sato-Bigbee, *Endogenous and exogenous opioid effects on oligodendrocyte biology and developmental brain myelination.* Neurotoxicology and teratology, 2021. **86**: p. 107002.

120. Ramos, K., et al., *Pro-inflammatory signaling in endothelial cells and oligodendrocytes: new players in chronic pain and dysregulation of opioid efficacy.* The Journal of Pain, 2010. **11**(4): p. S26.

121. Mohamed, H.M. and A.M. Mahmoud, *Chronic exposure to the opioid tramadol induces oxidative damage, inflammation and apoptosis, and alters cerebral monoamine neurotransmitters in rats.* Biomedicine & pharmacotherapy, 2019. **110**: p. 239-247.

122. Ward, P., et al., *N-acetylcysteine mitigates acute opioid withdrawal behaviors and CNS oxidative stress in neonatal rats.* Pediatric research, 2020. **88**(1): p. 77-84.

123. Merhar, S.L., et al., *White Matter Injury and Structural Anomalies in Infants with Prenatal Opioid Exposure.* AJNR Am J Neuroradiol, 2019. **40**(12): p. 2161-2165.

124. Towers, C.V., et al., *Neonatal Head Circumference in Newborns With Neonatal Abstinence Syndrome.* Pediatrics, 2019. **143**(1).

125. Koch, T. and V. Höllt, *Role of receptor internalization in opioid tolerance and dependence.* Pharmacology & therapeutics, 2008. **117**(2): p. 199-206.

126. Williams, J.T., et al., *Regulation of μ-opioid receptors: desensitization, phosphorylation, internalization, and tolerance.* Pharmacological reviews, 2013. **65**(1): p. 223-254.

127. Suresh, S., K. Anand, and F. FCCM, *Opioid tolerance in neonates: a state-of-the-art.* Paediatric Anaesthesia, 2001. **11**(5): p. 511-521.

128. Subedi, L., et al., *Plasma brain-derived neurotrophic factor levels in newborn infants with neonatal abstinence syndrome.* Frontiers in pediatrics, 2017. **5**: p. 238.

129. Jenner, P., *Oxidative damage in neurodegenerative disease.* The Lancet, 1994. **344**(8925): p. 796-798.

130. Ordean, A. and B.C. Chisamore, *Clinical presentation and management of neonatal abstinence syndrome: an update.* Research and Reports in Neonatology, 2014: p. 75-86.

131. Holzer, P., *Opioid receptors in the gastrointestinal tract.* Regulatory Peptides, 2009. **155**(1): p. 11-17.

132. Garten, L., P. Degenhardt, and C. Bührer, *Resolution of opioid-induced postoperative ileus in a newborn infant after methylnaltrexone.* Journal of Pediatric Surgery, 2011. **46**(3): p. e13-e15.

133. Russ, J.B., R. Simmons, and H.C. Glass, *Neonatal encephalopathy: beyond hypoxic-ischemic encephalopathy.* Neoreviews, 2021. **22**(3): p. e148-e162.

134. Johnson, K., C. Gerada, and A. Greenough, *Treatment of neonatal abstinence syndrome.* Archives of Disease in Childhood-Fetal and Neonatal Edition, 2003. **88**(1): p. F2-F5.

135. Etches, R.C., A.N. Sandler, and M.D. Daley, *Respiratory depression and spinal opioids.* Canadian Journal of Anaesthesia, 1989. **36**(2): p. 165-185.

136. Dahan, A., L. Aarts, and Terry W. Smith, *Incidence, Reversal, and Prevention of Opioid-induced Respiratory Depression.* Anesthesiology, 2010. **112**(1): p. 226-238.

137. Dahan, A., et al., *Averting Opioid-induced Respiratory Depression without Affecting Analgesia.* Anesthesiology, 2018. **128**(5): p. 1027-1037.

138. Osborne, A., et al., *Cardiorespiratory anomalies and increased brainstem microglia in a rat model of neonatal opioid withdrawal syndrome.* Respiratory Physiology & Neurobiology, 2022. **296**: p. 103800.

139. Bloch-Salisbury, E., et al., *Physiologic dysregulation in newborns with prenatal opioid exposure: Cardiac, respiratory and movement activity.* Neurotoxicology and Teratology, 2022. **92**: p. 107105.

140. Miller, N.W., et al., *The impact of opioid exposure during pregnancy on the human neonatal immune profile.* Pediatric Research, 2022. **92**(6): p. 1566-1574.

141. Haroutounian, S., *Postoperative opioids, endocrine changes, and immunosuppression.* Pain Rep, 2018. **3**(2): p. e640.

142. Newville, J., et al., *Perinatal Opioid Exposure Primes the Peripheral Immune System Toward Hyperreactivity.* Frontiers in pediatrics, 2020. **8**: p. 272-272.

143. Roy, S., et al., *Opioid Drug Abuse and Modulation of Immune Function: Consequences in the Susceptibility to Opportunistic Infections.* Journal of Neuroimmune Pharmacology, 2011. **6**(4): p. 442-465.

144. Balalian, A.A., et al., *Prenatal exposure to opioids and neurodevelopment in infancy and childhood: A systematic review.* Front Pediatr, 2023. **11**: p. 1071889.

145. Boggess, T. and W.C. Risher, *Clinical and basic research investigations into the long-term effects of prenatal opioid exposure on brain development.* Journal of neuroscience research, 2022. **100**(1): p. 396-409.

146. Riggins, T., et al., *Memory ability and hippocampal volume in adolescents with prenatal drug exposure.* Neurotoxicology and teratology, 2012. **34**(4): p. 434-441.

147. Maguire, D.J., et al., *Long-Term Outcomes of Infants with Neonatal Abstinence Syndrome.* Neonatal Network, (5): p. 277-286.

148. Robey, A., et al., *Relations among prospective memory, cognitive abilities, and brain structure in adolescents who vary in prenatal drug exposure.* Journal of Experimental Child Psychology, 2014. **127**: p. 144-162.

149. Morales-Medina, J.C., et al., *Evidence for endogenous opioid dependence related to latent sensitization in a rat model of chronic inflammatory pain.* International Journal of Molecular Sciences, 2023. **24**(3): p. 2812.

150. Cleary, B.J., et al., *Methadone dose and neonatal abstinence syndrome—systematic review and meta-analysis.* Addiction, 2010. **105**(12): p. 2071-2084.

151. Yeoh, S.L., et al., *Cognitive and Motor Outcomes of Children With Prenatal Opioid Exposure: A Systematic Review and Meta-analysis.* JAMA Network Open, 2019. **2**(7): p. e197025-e197025.

152. Vishnubhotla, R.V., et al., *Brain structural connectome in neonates with prenatal opioid exposure.* Front Neurosci, 2022. **16**: p. 952322.

153. Larson, J.J., et al., *Cognitive and Behavioral Impact on Children Exposed to Opioids During Pregnancy.* Pediatrics, 2019. **144**(2).

154. Oei, J.L., et al., *Neonatal Abstinence Syndrome and High School Performance.* Pediatrics, 2017. **139**(2).

155. Taylor, C.A., et al., *Traumatic brain injury–related emergency department visits, hospitalizations, and deaths—United States, 2007 and 2013.* MMWR Surveillance Summaries, 2017. **66**(9): p. 1.

156. *Concussions and Brain Injuries in Children: United States, 2020*, S. National Center for Health, Editor. 2021, https://dx.doi.org/10.15620/cdc:111174: Hyattsville, MD.

157. Araki, T., H. Yokota, and A. Morita, *Pediatric Traumatic Brain Injury: Characteristic Features, Diagnosis, and Management.* Neurol Med Chir (Tokyo), 2017. **57**(2): p. 82-93.

158. Kaur, P. and S. Sharma, *Recent Advances in Pathophysiology of Traumatic Brain Injury.* Curr Neuropharmacol, 2018. **16**(8): p. 1224-1238.

159. Johnson, V.E., et al., *Inflammation and white matter degeneration persist for years after a single traumatic brain injury.* Brain, 2013. **136**(Pt 1): p. 28-42.

160. Yen, E., et al., *A review of the genomics of neonatal abstinence syndrome.* Frontiers in Genetics, 2023. **14**: p. 1140400.

161. Chesnut, R.M., et al., *The role of secondary brain injury in determining outcome from severe head injury*, in *50 Landmark Papers every Trauma Surgeon Should Know*. 2019, CRC Press. p. 63-66.

162. Hinson, H.E., S. Rowell, and M. Schreiber, *Clinical evidence of inflammation driving secondary brain injury: a systematic review.* The journal of trauma and acute care surgery, 2015. **78**(1): p. 184.

163. Karve, I.P., J.M. Taylor, and P.J. Crack, *The contribution of astrocytes and microglia to traumatic brain injury.* British journal of pharmacology, 2016. **173**(4): p. 692-702.

164. Eyolfson, E., et al., *Microglia dynamics in adolescent traumatic brain injury.* Journal of neuroinflammation, 2020. **17**(1): p. 1-19.

165. Yi, J.-H. and A.S. Hazell, *Excitotoxic mechanisms and the role of astrocytic glutamate transporters in traumatic brain injury.* Neurochemistry international, 2006. **48**(5): p. 394-403.

166. Escartin, C., et al., *Reactive astrocyte nomenclature, definitions, and future directions.* Nature neuroscience, 2021. **24**(3): p. 312-325.

167. Sofroniew, M.V., *Multiple roles for astrocytes as effectors of cytokines and inflammatory mediators.* The Neuroscientist, 2014. **20**(2): p. 160-172.

168. Zhuo, J., et al., *Diffusion kurtosis as an in vivo imaging marker for reactive astrogliosis in traumatic brain injury.* Neuroimage, 2012. **59**(1): p. 467-477.

169. Odegaard, K.E., et al., *Characterization of the intergenerational impact of in utero and postnatal oxycodone exposure.* Translational psychiatry, 2020. **10**(1): p. 1-11.

170. Odegaard, K.E., et al., *A Holistic Systems Approach to Characterize the Impact of Pre- and Post-natal Oxycodone Exposure on Neurodevelopment and Behavior.* Front Cell Dev Biol, 2020. **8**: p. 619199.

171. Shahjin, F., et al., *Brain-derived extracellular vesicle microRNA signatures associated with in utero and postnatal oxycodone exposure.* Cells, 2019. **9**(1): p. 21.

172. Mychasiuk, R., et al., *A novel model of mild traumatic brain injury for juvenile rats.* J Vis Exp, 2014(94).

173. Schindelin, J., et al., *Fiji: an open-source platform for biological-image analysis.* Nature Methods, 2012. **9**(7): p. 676-682.

174. Nguyen, N.M., et al., *Decoding the Synaptic Proteome with Long-Term Exposure to Midazolam during Early Development.* Int J Mol Sci, 2022. **23**(8).

175. Shannon, P., et al., *Cytoscape: a software environment for integrated models of biomolecular interaction networks.* Genome Res, 2003. **13**(11): p. 2498-504.

176. Rogers, G.W., et al., *High throughput microplate respiratory measurements using minimal quantities of isolated mitochondria.* PLoS One, 2011. **6**(7): p. e21746.

177. Kuehn, B., *Opioid Use Disorder During Pregnancy.* JAMA, 2018. **320**(12): p. 1232-1232.

178. Little, B., et al., *Teratogenic effects of maternal drug abuse on developing brain and underlying neurotransmitter mechanisms.* Neurotoxicology, 2021. **86**: p. 172-179.

179. Hall, E.S., J. Meinzen-Derr, and S.L. Wexelblatt, *Cohort Analysis of a Pharmacokinetic-Modeled Methadone Weaning Optimization for Neonatal Abstinence Syndrome.* J Pediatr, 2015. **167**(6): p. 1221-5.e1.

180. Carroll, L., et al., *Prognosis for mild traumatic brain injury: results of the WHO Collaborating Centre Task Force on Mild Traumatic Brain Injury.* Journal of rehabilitation medicine, 2004. **36**(0): p. 84-105.

181. Li, W., et al., *Traumatic brain injury and age at onset of cognitive impairment in older adults.* Journal of Neurology, 2016. **263**(7): p. 1280-1285.

182. Perry, D.C., et al., *Association of traumatic brain injury with subsequent neurological and psychiatric disease: a meta-analysis.* J Neurosurg, 2016. **124**(2): p. 511-26.

183. Tweedie, D., et al., *Time-dependent cytokine and chemokine changes in mouse cerebral cortex following a mild traumatic brain injury.* Elife, 2020. **9**.

184. Arciniegas, D.B. and H.S. Wortzel, *Emotional and behavioral dyscontrol after traumatic brain injury.* Psychiatric Clinics, 2014. **37**(1): p. 31-53.

185. Calvillo, M. and A. Irimia, *Neuroimaging and Psychometric Assessment of Mild Cognitive Impairment After Traumatic Brain Injury.* Front Psychol, 2020. **11**: p. 1423.

186. Cherry, J.D., et al., *Variation in TMEM106B in chronic traumatic encephalopathy.* Acta Neuropathologica Communications, 2018. **6**(1): p. 115.

187. Asken, B.M., et al., *Factors influencing clinical correlates of chronic traumatic encephalopathy (CTE): a review.* Neuropsychology review, 2016. **26**(4): p. 340-363.

188. Rickards, T.A., C.C. Cranston, and J. McWhorter, *Persistent post-concussive symptoms: A model of predisposing, precipitating, and perpetuating factors.* Applied Neuropsychology: Adult, 2022. **29**(2): p. 284-294.

189. Caplain, S., et al., *Early Detection of Poor Outcome after Mild Traumatic Brain Injury: Predictive Factors Using a Multidimensional Approach a Pilot Study.* Front Neurol, 2017. **8**: p. 666.

190. Shultz, S.R., et al., *The potential for animal models to provide insight into mild traumatic brain injury: translational challenges and strategies.* Neuroscience & Biobehavioral Reviews, 2017. **76**: p. 396-414.

191. Jantzie, L.L., et al., *Prenatal opioid exposure: The next neonatal neuroinflammatory disease.* Brain Behav Immun, 2020. **84**: p. 45-58.

192. Newville, J., et al., *Perinatal opioid exposure primes the peripheral immune system toward hyperreactivity.* Frontiers in Pediatrics, 2020. **8**: p. 272.

193. Odegaard, K.E., et al., *A holistic systems approach to characterize the impact of pre-and post-natal oxycodone exposure on neurodevelopment and behavior.* Frontiers in Cell and Developmental Biology, 2021. **8**: p. 619199.

194. Bailey, B.A., et al., *Impact of in utero opioid exposure on newborn outcomes: beyond neonatal opioid withdrawal syndrome.* The Journal of Maternal-Fetal & Neonatal Medicine, 2022. **35**(25): p. 9383-9390.

195. Odegaard, K.E., et al., *Characterization of the intergenerational impact of in utero and postnatal oxycodone exposure.* Translational psychiatry, 2020. **10**(1): p. 329-329.

196. Andersen, S.L., *Trajectories of brain development: point of vulnerability or window of opportunity?* Neuroscience & Biobehavioral Reviews, 2003. **27**(1-2): p. 3-18.

197. McDonald, S.J., S.R. Shultz, and D.V. Agoston, *The known unknowns: an overview of the state of blood-based protein biomarkers of mild traumatic brain injury.* Journal of Neurotrauma, 2021. **38**(19): p. 2652-2666.

198. Ross, E.J., et al., *Developmental consequences of fetal exposure to drugs: what we know and what we still must learn.* Neuropsychopharmacology, 2015. **40**(1): p. 61-87.

199. Labella, M.H., et al., *Adapting an Evidence-Based Home Visiting Intervention for Mothers With Opioid Dependence: Modified Attachment and Biobehavioral Catch-up.* Front Psychol, 2021. **12**: p. 675866.

200. Wallin, C.M., S.E. Bowen, and S. Brummelte, *Opioid use during pregnancy can impair maternal behavior and the Maternal Brain Network: A literature review.* Neurotoxicology and Teratology, 2021. **86**: p. 106976.

201. Semple, B.D., et al., *Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species.* Prog Neurobiol, 2013. **106-107**: p. 1-16.

202. Tsujimoto, S., *The prefrontal cortex: functional neural development during early childhood.* Neuroscientist, 2008. **14**(4): p. 345-58.

203. Grin'kina, N.M., et al., *Righting Reflex Predicts Long-Term Histological and Behavioral Outcomes in a Closed Head Model of Traumatic Brain Injury.* PLoS One, 2016. **11**(9): p. e0161053.

204. Rowe, R.K., J.B. Ortiz, and T.C. Thomas, *Mild and Moderate Traumatic Brain Injury and Repeated Stress Affect Corticosterone in the Rat.* Neurotrauma Rep, 2020. **1**(1): p. 113-124.

205. Creeley, C.E., et al., *Multiple episodes of mild traumatic brain injury result in impaired cognitive performance in mice.* Acad Emerg Med, 2004. **11**(8): p. 809-19.

206. Mashour, G.A., et al., *Recovery of consciousness and cognition after general anesthesia in humans.* eLife, 2021. **10**: p. e59525.

207. Cipolla, M.J., *The cerebral circulation.* 2016.

208. Grossman, E., et al., *Cognitive impairment in mild traumatic brain injury: a longitudinal diffusional kurtosis and perfusion imaging study.* American Journal of Neuroradiology, 2013. **34**(5): p. 951-957.

209. Kinoshita, K., *Traumatic brain injury: pathophysiology for neurocritical care.* Journal of intensive care, 2016. **4**: p. 1-10.

210. Ramphul, K., S.G. Mejias, and J. Joynauth, *In-utero exposure to opioid increases the risk of congenital heart defects.* EXCLI journal, 2020. **19**: p. 239.

211. Barlow, K.M., et al., *Epidemiology of postconcussion syndrome in pediatric mild traumatic brain injury.* Pediatrics, 2010. **126**(2): p. e374-e381.

212. Helmy, A., et al., *The cytokine response to human traumatic brain injury: temporal profiles and evidence for cerebral parenchymal production.* Journal of Cerebral Blood Flow & Metabolism, 2011. **31**(2): p. 658-670.

213. Helmy, A., et al., *Principal Component Analysis of the Cytokine and Chemokine Response to Human Traumatic Brain Injury.* PLOS ONE, 2012. **7**(6): p. e39677.

214. Shojo, H., et al., *Genetic and histologic evidence implicates role of inflammation in traumatic brain injury-induced apoptosis in the rat cerebral cortex following moderate fluid percussion injury.* Neuroscience, 2010. **171**(4): p. 1273-1282.

215. Graham, N.S.N., et al., *Axonal marker neurofilament light predicts long-term outcomes and progressive neurodegeneration after traumatic brain injury.* Science Translational Medicine, 2021. **13**(613): p. eabg9922.

216. Zhang, Y., et al., *Alterations of expression of inflammation/immune-related genes in the dorsal and ventral striatum of adult C57BL/6J mice following chronic oxycodone self-administration: a RNA sequencing study.* Psychopharmacology, 2017. **234**: p. 2259-2275.

217. Sanchez, E.S., et al., *Opioid addiction and pregnancy: Perinatal exposure to buprenorphine affects myelination in the developing brain.* Glia, 2008. **56**(9): p. 1017-1027.

218. Pappas, A., et al., *Transcriptomics of Maternal and Fetal Membranes Can Discriminate between Gestational-Age Matched Preterm Neonates with and without Cognitive Impairment Diagnosed at 18–24 Months.* PLOS ONE, 2015. **10**(3): p. e0118573.

219. Gadani, S.P., et al., *Dealing with danger in the CNS: the response of the immune system to injury.* Neuron, 2015. **87**(1): p. 47-62.

220. Hansen, D.V., J.E. Hanson, and M. Sheng, *Microglia in Alzheimer's disease.* J Cell Biol, 2018. **217**(2): p. 459-472.

221. Dubois, B., et al., *Preclinical Alzheimer's disease: Definition, natural history, and diagnostic criteria.* Alzheimers Dement, 2016. **12**(3): p. 292-323.

222. Graham, N.S. and D.J. Sharp, *Understanding neurodegeneration after traumatic brain injury: from mechanisms to clinical trials in dementia.* Journal of Neurology, Neurosurgery & Psychiatry, 2019. **90**(11): p. 1221-1233.

223. Calarco, C.A., et al., *Mitochondria-related nuclear gene expression in the nucleus accumbens and blood mitochondrial copy number after developmental fentanyl exposure in adolescent male and female C57BL/6 mice.* Frontiers in psychiatry, 2021: p. 1910.

224. Kasala, S., et al., *Exposure to morphine and caffeine induces apoptosis and mitochondrial dysfunction in a neonatal rat brain.* Frontiers in Pediatrics, 2020. **8**: p. 593.

225. Dunn, A.D., et al., *Molecular and long-term behavioral consequences of neonatal opioid exposure and withdrawal in mice.* Frontiers in Behavioral Neuroscience, 2023. **17**: p. 1202099.

226. Voevodskaya, O., et al., *Brain myoinositol as a potential marker of amyloid-related pathology: A longitudinal study.* Neurology, 2019. **92**(5): p. e395-e405.

227. Ng, T.S., et al., *Neuroimaging in repetitive brain trauma.* Alzheimers Res Ther, 2014. **6**(1): p. 10.

228. Tumati, S., S. Martens, and A. Aleman, *Magnetic resonance spectroscopy in mild cognitive impairment: systematic review and meta-analysis.* Neuroscience & Biobehavioral Reviews, 2013. **37**(10): p. 2571-2586.

229. Kantarci, K., et al., *1H magnetic resonance spectroscopy, cognitive function, and apolipoprotein E genotype in normal aging, mild cognitive impairment and Alzheimer's disease.* Journal of the International Neuropsychological Society, 2002. **8**(7): p. 934-942.

230. Patet, C., et al., *Cerebral Lactate Metabolism After Traumatic Brain Injury.* Current Neurology and Neuroscience Reports, 2016. **16**(4): p. 31.

231. Evans, G.J., *The synaptosome as a model system for studying synaptic physiology.* Cold Spring Harbor protocols, 2015. **2015**(5): p. pdb. top074450.

232. Whittaker, V., I. Michaelson, and R.J.A. Kirkland, *The separation of synaptic vesicles from nerve-ending particles (synaptosomes').* Biochemical Journal, 1964. **90**(2): p. 293.

233. Bai, F. and F.A. Witzmann, *Synaptosome proteomics.* Subcellular Proteomics: From Cell Deconstruction to System Reconstruction, 2007: p. 77-98.

234. Rath, S., et al., *MitoCarta3.0: an updated mitochondrial proteome now with sub-organelle localization and pathway annotations.* Nucleic Acids Res, 2021. **49**(D1): p. D1541-d1547.

235. Kang, I., C.T. Chu, and B.A. Kaufman, *The mitochondrial transcription factor TFAM in neurodegeneration: emerging evidence and mechanisms.* FEBS Lett, 2018. **592**(5): p. 793-811.

236. Itoh, K., et al., *Mitochondrial dynamics in neurodegeneration.* Trends in cell biology, 2013. **23**(2): p. 64-71.

237. Bifsha, P., et al., *Rgs6 is required for adult maintenance of dopaminergic neurons in the ventral substantia nigra.* PLoS genetics, 2014. **10**(12): p. e1004863.

238. Invernizzi, F., et al., *Cavitating leukoencephalopathy with multiple mitochondrial dysfunction syndrome and NFU1 mutations.* Frontiers in genetics, 2014. **5**: p. 412.

239. Huang, Z., et al., *The stem cell factor/Kit signalling pathway regulates mitochondrial function and energy expenditure.* Nature Communications, 2014. **5**(1): p. 4282.

240. Tavazzi, B., et al., *Cerebral Oxidative Stress and Depression of Energy Metabolism Correlate with Severity of Diffuse Brain Injury in Rats.* Neurosurgery, 2005. **56**(3).

241. Signoretti, S., et al., *Biochemical and neurochemical sequelae following mild traumatic brain injury: summary of experimental data and clinical implications.* Neurosurgical Focus FOC, 2010. **29**(5): p. E1.

242. *Metabolic Acetate Therapy for the Treatment of Traumatic Brain Injury.* Journal of Neurotrauma, 2010. **27**(1): p. 293-298.

243. Yoshino, A., et al., *Dynamic changes in local cerebral glucose utilization following cerebral concussion in rats: evidence of a hyper-and subsequent hypometabolic state.* Brain research, 1991. **561**(1): p. 106-119.

244. Bergsneider, M., et al., *Cerebral hyperglycolysis following severe traumatic brain injury in humans: a positron emission tomography study.* Journal of neurosurgery, 1997. **86**(2): p. 241-251.

245. Prins, M.L., *Glucose metabolism in pediatric traumatic brain injury.* Child's Nervous System, 2017. **33**(10): p. 1711-1718.

246. Glenn, T.C., et al., *Lactate: brain fuel in human traumatic brain injury: a comparison with normal healthy control subjects.* J Neurotrauma, 2015. **32**(11): p. 820-32.

247. Jia, L., et al., *Rheb-regulated mitochondrial pyruvate metabolism of Schwann cells linked to axon stability.* Developmental cell, 2021. **56**(21): p. 2980-2994. e6.

248. Yang, S. and G. Lian, *ROS and diseases: Role in metabolism and energy supply.* Molecular and cellular biochemistry, 2020. **467**: p. 1-12.

249. Corkey, B.E. and J.T. Deeney, *The redox communication network as a regulator of metabolism.* Frontiers in Physiology, 2020. **11**: p. 567796.

250. Vagnozzi, R., et al., *Hypothesis of the Postconcussive Vulnerable Brain: Experimental Evidence of Its Metabolic Occurrence.* Neurosurgery, 2005. **57**(1).

251. Zhong, Y., et al., *Mitochondrial transcription factor A overexpression and base excision repair deficiency in the inner ear of rats with d-galactose-induced aging.* The FEBS Journal, 2011. **278**(14): p. 2500-2510.

252. Hayashi, Y., et al., *Reverse of age-dependent memory impairment and mitochondrial DNA damage in microglia by an overexpression of human mitochondrial transcription factor a in mice.* Journal of Neuroscience, 2008. **28**(34): p. 8624-8634.

253. Morimoto, N., et al., *Effect of mitochondrial transcription factor a overexpression on motor neurons in amyotrophic lateral sclerosis model mice.* Journal of neuroscience research, 2012. **90**(6): p. 1200-1208.

254. Oka, S., et al., *Human mitochondrial transcriptional factor A breaks the mitochondria-mediated vicious cycle in Alzheimer's disease.* Scientific reports, 2016. **6**(1): p. 37889.

255. Tuppen, H.A., et al., *Mitochondrial DNA mutations and human disease.* Biochimica et Biophysica Acta (BBA)-Bioenergetics, 2010. **1797**(2): p. 113-128.

256. Sekar, S., et al., *Concussion/mild traumatic brain injury (TBI) induces brain insulin resistance: a positron emission tomography (PET) scanning study.* International journal of molecular sciences, 2021. **22**(16): p. 9005.

257. Mowery, N.T., et al., *Stress insulin resistance is a marker for mortality in traumatic brain injury.* Journal of Trauma and Acute Care Surgery, 2009. **66**(1): p. 145-153.

258. Whittaker, V., *Thirty years of synaptosome research.* Journal of neurocytology, 1993. **22**(9): p. 735-742.

259. Jamjoom, A.A.B., et al., *The synapse in traumatic brain injury.* Brain, 2021. **144**(1): p. 18-31.

260. Jamjoom, A.A.B., et al., *The synapse in traumatic brain injury.* Brain, 2020. **144**(1): p. 18-31.

261. Lai, J.-q., et al., *Metabolic disorders on cognitive dysfunction after traumatic brain injury.* Trends in Endocrinology & Metabolism, 2022. **33**(7): p. 451-462.

262. Sengupta, P., *The Laboratory Rat: Relating Its Age With Human's.* Int J Prev Med, 2013. **4**(6): p. 624-30.

263. Schlagal, C.R., et al., *Maternal Opioid Exposure Culminates in Perturbed Murine Neurodevelopment and Hyperactive Phenotype in Adolescence.* Neuroscience, 2021. **463**: p. 272-287.

264. Velez, M.L., C. Jordan, and L.M. Jansson, *Reconceptualizing non-pharmacologic approaches to Neonatal Abstinence Syndrome (NAS) and Neonatal Opioid Withdrawal Syndrome (NOWS): A theoretical and evidence–based approach. Part II: The clinical application of nonpharmacologic care for NAS/NOWS.* Neurotoxicology and teratology, 2021. **88**: p. 107032.

265. Martínez-Levy, G., et al., *Increased expression of BDNF transcript with exon VI in hippocampi of patients with pharmaco-resistant temporal lobe epilepsy.* Neuroscience, 2016. **314**: p. 12-21.

266. Correia, C., et al., *Increased BDNF levels and NTRK2 gene association suggest a disruption of BDNF/TrkB signaling in autism.* Genes, Brain and behavior, 2010. **9**(7): p. 841-848.

267. Ülgen, D.H., S.R. Ruigrok, and C. Sandi, *Powering the social brain: Mitochondria in social behaviour.* Current Opinion in Neurobiology, 2023. **79**: p. 102675.

268. Rossignol, D.A. and R.E. Frye, *Evidence linking oxidative stress, mitochondrial dysfunction, and inflammation in the brain of individuals with autism.* Frontiers in physiology, 2014. **5**: p. 150.

269. Misiewicz, Z., et al., *Multi-omics analysis identifies mitochondrial pathways associated with anxiety-related behavior.* PLoS genetics, 2019. **15**(9): p. e1008358.

270. Hollis, F., et al., *Mitochondrial function in the brain links anxiety with social subordination.* Proceedings of the National Academy of Sciences, 2015. **112**(50): p. 15486-15491.

271. Picca, A., et al., *Mitochondrial dysfunction, oxidative stress, and neuroinflammation: Intertwined roads to neurodegeneration.* Antioxidants, 2020. **9**(8): p. 647.

272. Zamani, A., R. Mychasiuk, and B.D. Semple, *Determinants of social behavior deficits and recovery after pediatric traumatic brain injury.* Experimental neurology, 2019. **314**: p. 34-45.

273. Bigler, E.D., et al., *Neuroimaging and social behavior in children after traumatic brain injury: Findings from the Social Outcomes of Brain Injury in Kids (SOBIK) study.* NeuroRehabilitation, 2013. **32**(4): p. 707-720.

274. Ryan, N.P., et al., *Executive function mediates the prospective association between neurostructural differences within the central executive network and anti‐social behavior after childhood traumatic brain injury.* Journal of child psychology and psychiatry, 2021. **62**(9): p. 1150-1161.

275. Umagami, K., et al., *Loneliness in autistic adults: A systematic review.* Autism, 2022. **26**(8): p. 2117-2135.

276. Cunha-Oliveira, T., A.C. Rego, and C.R. Oliveira, *Cellular and molecular mechanisms involved in the neurotoxicity of opioid and psychostimulant drugs.* Brain Research Reviews, 2008. **58**(1): p. 192-208.

277. Zindove, T.J., et al., *Relationships between litter size, sex ratio and within-litter birth weight variation in a sow herd and consequences on weaning performance.* Translational Animal Science, 2021. **5**(3).

278. Epstein, H.T., *The Effect of Litter Size on Weight Gain in Mice.* The Journal of Nutrition, 1978. **108**(1): p. 120-123.

279. Song, H., et al., *Proteomic Analysis and Biochemical Correlates of Mitochondrial Dysfunction after Low-Intensity Primary Blast Exposure.* J Neurotrauma, 2019. **36**(10): p. 1591-1605.

280. Zhang, Z., et al., *iTRAQ-based proteomic profiling reveals protein alterations after traumatic brain injury and supports thyroxine as a potential treatment.* Mol Brain, 2021. **14**(1): p. 25.

281. Vercellino, I. and L.A. Sazanov, *The assembly, regulation and function of the mitochondrial respiratory chain.* Nature Reviews Molecular Cell Biology, 2022. **23**(2): p. 141-161.

282. Murphy, M.P., *How mitochondria produce reactive oxygen species.* Biochem J, 2009. **417**(1): p. 1-13.

283. Daniels, T.E., E.M. Olsen, and A.R. Tyrka, *Stress and psychiatric disorders: The role of mitochondria.* Annual review of clinical psychology, 2020. **16**: p. 165-186.

284. Morella, I.M., R. Brambilla, and L. Morè, *Emerging roles of brain metabolism in cognitive impairment and neuropsychiatric disorders.* Neuroscience & Biobehavioral Reviews, 2022: p. 104892.

285. Dillin, A., et al., *Rates of behavior and aging specified by mitochondrial function during development.* Science, 2002. **298**(5602): p. 2398-2401.

286. Nikolaou, P., et al., *Monitoring methadone milk concentrations of methadone-maintained women in postpartum period for breastfeeding safety.* Toxicology Letters, 2009(189): p. S264.

287. Bogen, D.L., et al., *Estimated infant exposure to enantiomer-specific methadone levels in breastmilk.* Breastfeeding Medicine, 2011. **6**(6): p. 377-384.

288. Jansson, L.M., et al., *Methadone maintenance and breastfeeding in the neonatal period.* Pediatrics, 2008. **121**(1): p. 106-114.

289. Baykara, S. and K. Alban, *The effects of buprenorphine/naloxone maintenance treatment on sexual dysfunction, sleep and weight in opioid use disorder patients.* Psychiatry Research, 2019. **272**: p. 450-453.

290. Parvaresh, N., et al., *A study of the sexual function, sleep, and weight status of patients after 6 months of methadone maintenance treatment.* Addiction & health, 2015. **7**(1-2): p. 24.

291. Shah, K., et al., *Association of opioid use with cardiometabolic disease risk factors: evidence from the 2009-2018 National Health And Nutrition Examination Survey.* Cureus, 2020. **12**(6).

292. Jennings, A., et al., *Elucidating the Relationship Between Maternal Diabetes and Neonatal Abstinence Syndrome: A 2017-2018 Project WATCH Study in Rural Appalachia.* Adv Neonatal Care, 2022. **22**(3): p. 239-245.

293. Menni, F., et al., *Neurologic outcomes of 90 neonates and infants with persistent hyperinsulinemic hypoglycemia.* Pediatrics, 2001. **107**(3): p. 476-479.

294. Disher, T., et al., *Pharmacological Treatments for Neonatal Abstinence Syndrome: A Systematic Review and Network Meta-analysis.* JAMA Pediatrics, 2019. **173**(3): p. 234-243.

295. Young, L.W., et al., *Eat, Sleep, Console Approach or Usual Care for Neonatal Opioid Withdrawal.* N Engl J Med, 2023. **388**(25): p. 2326-2337.

296. Brenna, J.T., *Long-chain polyunsaturated fatty acids and the preterm infant: a case study in developmentally sensitive nutrient needs in the United States.* Am J Clin Nutr, 2016. **103**(2): p. 606s-15s.

297. Fetterman Jr, J.W. and M.M. Zdanowicz, *Therapeutic potential of n-3 polyunsaturated fatty acids in disease.* American journal of health-system pharmacy, 2009. **66**(13): p. 1169-1179.

298. Heaton, M.B., J.J. Mitchell, and M. Paiva, *Amelioration of Ethanol-Induced Neurotoxicity in the Neonatal Rat Central Nervous System by Antioxidant Therapy.* Alcohol: Clinical and Experimental Research, 2000. **24**(4): p. 512-518.

299. Illenberger, J.M., et al., *Suvorexant, an FDA-approved dual orexin receptor antagonist, reduces oxycodone self-administration and conditioned reinstatement in male and female rats.* Frontiers in Pharmacology, 2023. **14**: p. 1127735.

300. Eisenstein, T.K., *The role of opioid receptors in immune system function.* Frontiers in immunology, 2019. **10**: p. 2904.

301. Oliveros, G., et al., *Repurposing ibudilast to mitigate Alzheimer's disease by targeting inflammation.* Brain, 2022. **146**(3): p. 898-911.

302. Jacobsen, J.H., L.R. Watkins, and M.R. Hutchinson, *Discovery of a novel site of opioid action at the innate immune pattern-recognition receptor TLR4 and its role in addiction.* Int Rev Neurobiol, 2014. **118**: p. 129-63.

303. Wu, N.C. and J.J. Wang, *Ibudilast, a Phosphodiesterase Inhibitor and Toll-Like Receptor-4 Antagonist, Improves Hemorrhagic Shock and Reperfusion-Induced Left Ventricular Dysfunction by Reducing Myocardial Tumor Necrosis Factor α.* Transplant Proc, 2020. **52**(6): p. 1869-1874.

304. Cooper, Z.D., et al., *The effects of ibudilast, a glial activation inhibitor, on opioid withdrawal symptoms in opioid-dependent volunteers.* Addict Biol, 2016. **21**(4): p. 895-903.

305. Li, X., et al., *Ibudilast Attenuates Folic Acid-Induced Acute Kidney Injury by Blocking Pyroptosis Through TLR4-Mediated NF-κB and MAPK Signaling Pathways.* Front Pharmacol, 2021. **12**: p. 650283.

306. Chand, S., et al., *A comprehensive study to delineate the role of an extracellular vesicle-associated microRNA-29a in chronic methamphetamine use disorder.* Journal of Extracellular Vesicles, 2021. **10**(14): p. e12177.

307. Charntikov, S., et al., *Ibudilast reverses the decrease in the synaptic signaling protein phosphatidylethanolamine-binding protein 1 (PEBP1) produced by chronic methamphetamine intake in rats.* Drug and alcohol dependence, 2015. **152**: p. 15-23.

308. Moore, D., et al., *Extracellular vesicle associated microRNA-29a elicits microglial inflammation and synaptodendritic injury during chronic methamphetamine abuse.* Journal of Extracellular Vesicles, 2018. **7**: p. 108-108.

309. Oki, K., et al., *Trends of Antiplatelet Therapy for the Management of Moyamoya Disease in Japan: Results of a Nationwide Survey.* Journal of Stroke and Cerebrovascular Diseases, 2018. **27**(12): p. 3605-3612.

310. Wilbur, C. and E.A. Yeh, *Improving Outcomes in Pediatric Multiple Sclerosis: Current and Emerging Treatments.* Pediatric Drugs, 2019. **21**(3): p. 137-152.

311. Coiro, P., et al., *Impaired synaptic development in a maternal immune activation mouse model of neurodevelopmental disorders.* Brain, behavior, and immunity, 2015. **50**: p. 249-258.

312. Almond, D. and J. Currie, *Killing Me Softly: The Fetal Origins Hypothesis.* J Econ Perspect, 2011. **25**(3): p. 153-172.