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In utero and Postnatal Oxycodone Exposure: Implications for Intergenerational Effects

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> None but those who have experienced them can conceive of the enticements of science. In other studies you go as far as others have gone before you, and there is nothing more to know; but in a scientific pursuit there is continual food for discovery and wonder.

May these works provide a foundation for future studies and continue to feed scientific ideas in studies to come.

This dissertation is dedicated to Wayne and Dorothy Murdock.

Although I did not ultimately study Alzheimer’s disease, my desire to help you both led me to neuroscience, where my efforts will hopefully help in the disease of addiction.

ABSTRACT

In utero and Postnatal Oxycodone Exposure: Implications for Intergenerational Effects

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Prescription opioid abuse during and after pregnancy is a rising public health concern. Adding a layer of complexity is the role of heredity in the overall development of these exposed offspring. The present work uses a preclinical rat model mimicking oxycodone (oxy) exposure *in utero* (IUO) and postnatally (PNO) to investigate comparative and intergenerational effects in the two different exposure groups.

To understand the direct effects of IUO and PNO exposure on the F1 generation, we employed a systems biology approach encompassing proton magnetic resonance spectroscopy (^{1}H-MRS), electrophysiology RNA-sequencing, and pain assessment to elucidate molecular and behavioral changes in these offspring. ^{1}H-MRS studies revealed significant changes in brain metabolites that were corroborated with changes in synaptic currents. RNA-sequencing of the prefrontal cortex further revealed alterations in the expression of key genes associated with synaptic transmission, neurodevelopment, mood disorders, and addiction. Von Frey testing showed lower pain thresholds in both oxy-exposed groups. Further, because addictive drugs produce significant and persistent changes in the synapse, we investigated the synaptic vesicle (SV) contents of the PNO and IUO groups. To that end, we found that the expression levels of key SV proteins

associated with functional pathways and neurological disease were altered in oxy-exposed groups.

While our earlier studies characterized the effects PNO and IUO exposure have on the F1 generation, we next sought to compare the overall development between F1 offspring and their progeny, the F2 generation. We observed significant differences in phenotypic attributes of both generations in each treatment group, and RNA-sequencing of the nucleus accumbens revealed alterations in the expression of key synaptic genes in both generations. Post-validation of these genes using RT-PCR highlighted the differential expression of several neuropeptides associated with the hypocretin system, a system recently implicated in addiction. Further, behavior studies revealed anxiety-like behaviors and social deficits in both treatment groups that persisted into the F2 generation.

Collectively, our studies reveal a new line of investigation on the potential risks associated with oxy use during and after pregnancy, specifically the disruption of neurodevelopment and the intergenerational impact on behavior.

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LIST OF ABBREVIATIONS

ACSF – artificial cerebrospinal fluid

ADHD – attention deficit hyperactivity disorder

AGC – Automatic Gain Control

ALA – alanine

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

ASP – aspartate

BDNF – brain-derived neurotrophic factor

BMI – body mass index

BSA – bovine serum albumin

CaMKII – Ca^{2+}/calmodulin-dependent kinase II

cAMP – cyclic AMP

CDC – Centers for Disease Control and Prevention

CNS – central nervous system

CPP – conditioned place preference

CRE – creatine

CRH – corticotrophin-releasing hormone

CSF – cerebrospinal fluid

DEG – differentially expressed gene

DG – dentate gyrus

DOR – delta opioid receptor

Dyn – dynorphin

ECMO – extracorporeal membrane oxygenation

EPSC – excitatory post-synaptic current

F0 – parent generation

F1 – first generation

F2 – second generation

FA – formic acid

FDR – false discovery rate

FPKM – fragments per kilobase million

FSD – fat-sugar diet

GABA – gamma-aminobutyric acid

GLC – glucose

GLN – glutamine

GLU – glutamate

GLY – glycine

GO – gene ontology

GPCR – G-protein coupled receptor

HCD – Higher-energy collisional dissociation

Hcrt – hypocretin

HLSVD – Hankel Lanczos Singular Value Decomposition

HPA – hypothalamic-pituitary-adrenocortical axis

IGF – insulin-like growth factor

IPA – ingenuity pathway analysis

IUO – *in utero* oxycodone exposure

jMRUI - Java-based magnetic resonance user's interface

KOR – kappa opioid receptor

LAC – lactate

LOI – Lee's obesity index

LTD – long-term depression

LTP – long-term potentiation

mEPSC – miniature excitatory post-synaptic current

MOR – mu opioid receptor

MRI/MRS – magnetic resonance imaging/spectroscopy

MS/MS – tandem mass spectrometry

MYO – myo-inositol

NA – number of averages

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

*nN*os – nitric oxide synthase

NOWS – neonatal opioid withdrawal syndrome

NPY – neuropeptide Y

OIH – opioid-induced hyperalgesia

OPRM1 – mu opioid receptor 1

OUD – opioid use disorder

Oxy – oxycodone

PBS – phospho-buffered saline

PC – phosphorylcholine

PFC – prefrontal cortex

PKC – protein kinase C

PNO – post-natal oxycodone exposure

PSD95 – post-synaptic density protein 95

PVN – paraventricular nucleus

RARE – relaxation enhancement

RNA-seq – RNA-sequencing

SUD – substance use disorder

SV – synaptic vesicle

TAU – taurine

TBS – tris-buffered saline

TBS-T – tris-buffered saline with Tween20

tCHO – total choline

THC – Δ9-tetrahydrocannabinol

UNMC – University of Nebraska Medical Center

VAPOR – variable power RF pulses with optimized relaxation delays

VOI – volume of interest

VTA – ventral tegmental area

INTRODUCTION

Substance Use and Abuse

Substance use in the form of alcohol and plant-derived drugs has existed for thousands of years. Psychoactive drugs as well as hallucinogens were used in spiritual and religious rituals of many early cultures in order to rid the body of evil spirits.[1] Presently, many people view substance use, or drug use, as bad, and label the drug as the inherent evil of addiction. It is important to realize, however, that drugs are not inherently evil, and rather their use determines their utility and impact. The term *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," and it may lead to dependence. *Drug dependence* refers to the state in which the individual uses the drug so frequently and consistently that it would be difficult for the person to get along without the drug; *dependence* refers to the physical dependence on a substance, which can be characterized by phases of withdrawal and tolerance.[1] This dependence can ultimately lead to what we commonly term as *addiction*, which is characterized by a change in behavior due to biochemical changes that take place within the neural circuitry of the brain, leading to substance use disorder (SUD).

Substances of Abuse

Substances of abuse include legal and illegal substances that possess varying mechanisms of action and impacts in the brain. These substances are capable of inducing plastic changes in the brain that impact overall brain function and contribute to substance use disorders.[2] The three predominant classes of commonly misused or abused substances are psychostimulants, depressants, and opioids.

Psychostimulants

Psychostimulants, which include methamphetamine, cocaine, and nicotine, are the most widely abused class of drugs. Psychostimulants increase stress and hyperactivity in the central nervous system (CNS) by enhancing or mimicking catecholamine neurotransmitters such as dopamine,[3,4] contributing to the brain's susceptibility to senescence, damage, and dysregulated plasticity.[5,6] Some psychostimulants are also capable of acting on serotonin receptors to induce hallucinogenic and psychogenic effects.[7] Cocaine and methamphetamine both operate pharmacologically through presynaptic monoamine receptors and are highly addictive drugs that can cause severe withdrawal symptoms.[8,9] The mechanism of addiction of these two substances likely lies in synaptic plasticity and memory pathways.[10] Nicotine, however, acts as an agonist to the nicotinic acetylcholine receptors on dopaminergic neurons to exert its effects on the dopaminergic reward system.[11]

Depressants

Depressants like alcohol and marijuana are named for their ability to impair and depress both behavioral and mechanical functions. Alcohol, similar to nicotine in legality, is one of the most abused drugs in the world. Δ9-tetrahydrocannabinol (THC), the primary psychoactive component in marijuana, has also received significant attention recently due to its increasing social and legal acceptance worldwide. While both of these substances are canonically described as depressants, their methods of action in the brain are very different. Alcohol use affects GABA receptors, glutamate receptors, NMDA receptors, nicotinic acetylcholine receptors, and serotonin receptors,[12,13] and acute alcohol use has been shown to increase extracellular dopamine release and alter synaptic plasticity.[14] THC, on the other hand, binds to the cannabinoid 1 receptor (CB1), mediating dopamine

release in the striatum and altering neural components of salience processing and the mesolimbic pathway.[15]

Opioids

Although typically prescribed for pain management, opioids are known for their addictive potential and recent rise in abuse.[16,17] Prescription opioids include Vicodin (hydrocodone), Oxycontin and Percocet (oxycodone), Opana (oxymorphone), Kadian and Avinza (morphine), codeine, and fentanyl. Full or partial opioid agonists such as methadone and buprenorphine, respectively, are also commonly prescribed for pain or the treatment of opioid dependence.[18] In addition to prescription opioids, illegal opioids such as heroin and opium are also widely abused.[19-21] The common factor of all opioids, regardless of legality, is their action on the opioid receptors that are widely dispersed in the brain and spinal cord, allowing for complex addiction pharmacodynamics.[22]

Reward Circuitry of the Brain

Addictive drugs, such as opioids, psychostimulants, and depressants, enhance the functioning of the reward circuitry of the brain, leading to the “high” sought by drug users.[23] First discovered by Olds and Milner at McGill University in 1954,[24] the reward circuitry consists of dopaminergic neurons in the ventral tegmental area (VTA) projecting to the nucleus accumbens (NAc), prefrontal cortex (PFC), amygdala, and hippocampus (Figure 1). The NAc also receives glutamatergic innervation from the PFC, amygdala, and hippocampus.[25] Although the mechanisms of action are dependent on drug type, the initial common action of all addictive drugs is the increase in dopaminergic signaling from the VTA to the NAc, creating a strong reinforcement that may lead to maladaptive behaviors and, in some cases, compulsive consumption in the user.[26] The hypothesis of reinforcement stems from the measure of extracellular dopamine in the NAc[27] and in the observation that reinforcement in mice and rats is driven by the electrical activation of the

medial forebrain bundle, which includes axons from the VTA to the NAc,[24] or the optogenetic excitation of VTA dopamine neurons.[26,28-30] The dopamine neurons involved in this reward circuitry may be responsible for the reward prediction error that drives associative learning.[31] Within this circuitry, excessive dopamine signaling resulting from drug use may modulate gene expression and alter synaptic function circuit activity over time, resulting in the maladaptive behaviors associated with addiction.

Along with dopamine, several other neurotransmitters and neuromodulators work to regulate the specific circuitry associated with addiction. Dopamine, opioid peptides, GABA, glutamate, serotonin, acetylcholine, and endocannabinoid systems all have roles in the reward pathway at the level of either the VTA or NAc. In a healthy brain, the reward circuit is balanced with proper inhibitory control, decision making, and normal functioning of reward, motivation, stress, and memory circuits. These circuits also interact with other brain regions involved in mood regulation, such as the amygdala and hypothalamus. Drugs of abuse manipulate these circuits through the use of neurotransmitters, including dopamine, enkephalins, glutamate, GABA, norepinephrine, corticotropin-releasing factor, dynorphin, neuropeptide Y, and endocannabinoids.[32] Excess of these neurotransmitters, particularly dopamine and glutamate, can contribute to neuroinflammation by binding to the receptors expressed on microglia and astrocytes to amplify inflammatory signaling through the release of cytokines and chemokines.[33] Inflammation and gliosis, the proliferation of glial cells, are pathological features of SUDs.[34,35] SUDs also affect the release of synaptic vesicles (SVs), which are largely responsible for neurotransmitter release; cocaine[36] and alcohol[37] use disorders have highlighted alterations in the expression of proteins essential for SV release, thereby affecting synaptic transmission. These drug-induced changes to the reward pathway highlight the complexity of downstream effects of SUDs. The following sections provide more information on the roles of individual reward brain regions in SUDs.

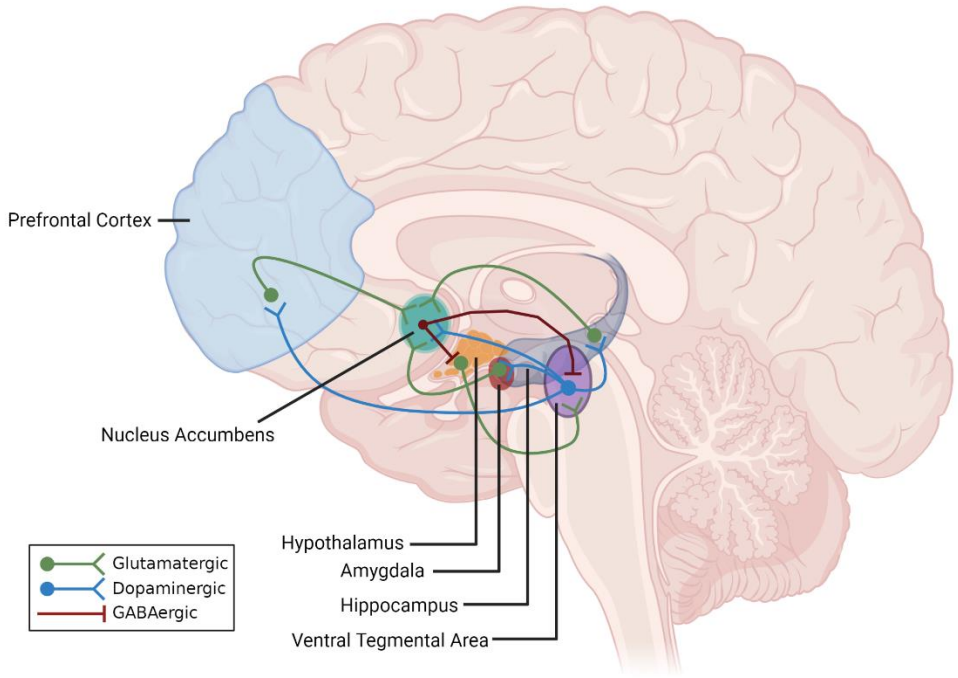


Figure 1. Simplified diagram of the major connections and areas involved in the reward circuitry of the brain. Created using Biorender.com.

Ventral Tegmental Area

The VTA is a region of the brain located near the midline of the midbrain floor that is largely composed of dopamine-producing neurons. With their diffuse connections to regions such as the NAc, amygdala, PFC, and basal ganglia, these dopamine neurons have critical roles in reward- and goal-directed behaviors.[38] Drugs of abuse activate these VTA dopamine neurons, resulting in the release of dopamine that affects many different cell populations throughout the VTA circuitry and subsequently regulates motivated behaviors.[39] The activity of these dopamine neurons is regulated by inputs from other brain regions as well as by local GABA- and glutamate-releasing neurons in the VTA. These GABA and glutamate neurons also have long-range projections to many of the brain structures that are innervated by the VTA dopamine neurons.[38] Specifically within the VTA, glutamate[40] and GABA[41] have roles in binge and intoxication, and corticotropin-releasing factor has a role in withdrawal and negative affect.[42]

Nucleus Accumbens

The NAc is located in the rostral part of the basal forebrain, just below the corpus striatum. Also called the ventral striatum, the NAc is referred to as the pleasure center of the brain, playing roles in motivation and cognitive processing of aversion.[39] The NAc receives input from VTA dopamine neurons, and the NAc has further innervations with the PFC, amygdala, hippocampus, and the basal ganglia. The NAc is composed of two regions: the core and the shell. While the NAc core is primarily responsible for the evaluation of reward and initializing reward-related motor action, the NAc shell is responsible for reward prediction and reward learning.[43] Transcerebral brain microdialysis studies have shown that dopamine transmission in the NAc was stimulated by psychostimulants, analgesics, and depressants.[44] Interestingly, NAc shell neurons make strong GABAergic connections with VTA dopamine neurons, allowing the NAc shell

neurons to inhibit VTA dopamine neuron firing.[45] Repetitive stimulation of dopamine transmission in the NAc through continued substance use causes stimuli associated with drug reward to acquire excessive incentive value and facilitate behaviors necessary for drug taking and SUDs.[44] The motivation to take more of a drug is further driven by the glutamatergic projections from the PFC, hippocampus, and amygdala to the striatal projections that increase dopamine signaling and release in the NAc.[46] Dynorphin signaling in the NAc has also been shown to have a role during withdrawal and negative affect.[47]

Prefrontal Cortex

The PFC is the section of frontal cortex at the anterior of the brain that is involved in self-awareness, complex planning, problem solving, learning and memory, executive functions, decision making, and social behavior.[48] The PFC is largely involved in controlling intense emotions and impulses and uses working memory to guide behavior and attention.[39] While it has several connections with brain regions associated with attention, action, and cognition, its reciprocal connections with the VTA and amygdala are important in the behavioral reactions to chronic drug abuse. Plastic changes within the brain associated with drug- or cue-induced reinstatement after extinction have been linked to glutamatergic pathways extending from the PFC to the NAc and GABAergic pathways from the NAc to the basal ganglia.[49,50]

Amygdala

The amygdala is a critical region of the limbic system that plays a key role in processing emotions, learning, memory, reward, attention, and arousal. The amygdala aids in the learning of the association between drug use and the subsequent “high” resulting from dopamine release. Inactivation of the amygdala prevents reinstatement cues during self-administration, and chronic exposure to drugs disrupts the projections

spanning from the amygdala to the PFC.[39] Dopamine exerts neuromodulatory control over excitatory and inhibitory transmission between the amygdala and the PFC; specifically, dopamine D1 receptor activity suppresses amygdala-evoked excitatory response in PFC neurons, and dopamine D2 attenuates amygdala-evoked inhibitory responses. Corticotropin-releasing factor,[51] neuropeptide Y,[52] and endocannabinoids[53] in the amygdala have also been implicated in withdrawal, and glutamate signaling[54] in the amygdala has been associated with drug craving.

Hippocampus

As a major structure in the limbic system, the hippocampus plays an important role in memory formation and novel object recognition. Hippocampal learning, memory, and plasticity have been shown to be heavily involved in the development and maintenance of addiction.[55] The dentate gyrus of the hippocampus contains neuronal progenitor cells that generate new neurons and contribute to neurogenesis.[39] Interestingly, most substances of abuse have been shown to suppress hippocampal neurogenesis.[56,57] Substance use has also been shown to affect hippocampal long-term potentiation (LTP)[58-61] and long-term depression (LTD).[39,62] Additionally, the hippocampus receives dopamine input from the VTA, which helps modulate hippocampal plasticity as well as learning and memory. Glutamate signaling in the hippocampus has also been linked to drug craving.[63]

Hypothalamus

The hypothalamus integrates brain function with the body's physiological needs and is thought to coordinate motivation with physiological demands.[64] Specifically, the hypothalamus's role in the hypothalamic-pituitary-adrenocortical axis (HPA) has been investigated in the context of addiction. The HPA has an essential role in normal physiological processes and in adaptation to stress, processes that are disrupted with drug abuse.[65] In response to stress and chronic substance abuse, levels of corticotropin-

releasing factor, which is released primarily by the thalamus and hypothalamus, increase, stimulating the secretion of adrenocorticotropic hormone by the anterior pituitary.[64] Of particular interest in the hypothalamus's role in the reward system are the hypocretin-containing neurons. These neurons have wide projections throughout the brain, including projections to the midbrain and the VTA. As chronic drug use and stress levels increase, more corticotropin-releasing factor is produced, activating hypocretin neurons. Sustained activation of these hypocretin neurons recruits N-methyl-D-aspartate (NMDA) receptors to VTA synapses, resulting in an increased response to glutamatergic neurons.[66] These NMDA receptors are also necessary for the induction of LTP and the promotion of burst firing in dopamine neurons, which may increase the dopamine output in regions of the VTA. Thus, the increased glutamatergic signaling by hypocretin neurons in the VTA may increase dopamine output and enhance the reinforcing properties of substances of abuse.[67]

Genetics and Epigenetics of Substance Use

As SUD is considered a chronic relapsing brain disease, it is crucial to understand the underlying causes of this disease. Mounting evidence suggests that SUDs run in families;[68,69] twin, adoption, and sibling studies implicate genetic factors are involved in the heritability of substance abuse.[70] Several human genome-wide association studies have identified loci and genes associated with substance use disorders,[69,71,72] revealing heritability estimates of substance use between 0.39 and 0.72.[71] Environmental factors, such as stress and socioeconomic status, also shape an individual's susceptibility to addiction.[73] Studies have shown that gene-environment interactions can influence behavioral phenotypes. For example, variations in the *5-HTTLPR* promoter of the serotonin transporter *SLC6A4* gene, which codes for the serotonin transporter (a target for cocaine),[74] have been shown to be moderated by parenting or mentoring relationships,

suggesting that environmental conditions can regulate genetic predispositions such as vulnerability for SUDs.[75] However, the inheritance of substance abuse cannot be explained entirely through genetic mechanisms alone; the interactions between genotype and environmental factors point toward a critical role of epigenetics in the response to substances of abuse and the development of SUDs.[76]

Epigenetics refers to the alteration of gene expression without changes to DNA sequence.[77,78] Epigenetic mechanisms provide a dynamic and heritable means of altering gene expression in response to environmental factors. Briefly, genes are composed of nucleotide base pairs in the DNA that are unique to that gene. Base pairing occurs between cytosine and guanine and between adenine and thymine. Cytosines followed by guanines in the DNA sequence are called CpGs, and they can be modified by DNA methyltransferases to add a methyl group, effectively reducing the accessibility of the gene and "silencing" its expression.[79] Methylated DNA interferes with the binding of transcriptional activators or binds to other proteins to collectively form a complex that further represses DNA accessibility.[80] Demethylation of DNA, either through DNA methyltransferase malfunction or enzymatic reaction, "reopen" accessibility to the previously suppressed gene.[79] Specific instances of DNA methylation associated with substances of abuse focus largely on epigenetic changes in the NAc. One well-established marker of repeated drug exposure is the increase in expression of the transcription factor protein ΔFosB in the NAc.[81] Acute or chronic cocaine administration has been shown to decrease methylation at the *fosB* promoter in the NAc of rodents, which co-occurs with increases in *fosB* mRNA expression.[82] Overexpression of ΔFosB has been correlated with increased sensitivity to the behavioral effects of substances of abuse and increased drug-seeking behavior, suggesting that ΔFosB may be a critical component in the long-term neural and behavioral plasticity underlying SUDs.[83]

In addition to DNA methylation, modification to histones, proteins that associate with DNA in the nucleus and condense it into chromatin, can also contribute to epigenetic changes. Histones are comprised of two copies of each of four histone proteins, H2A, H2B, H3, and H4, and modifications to the amino acid residues of these proteins can remodel the chromatin, allowing the DNA to be more tightly (heterochromatin) or loosely (euchromatin) packaged. The addition of acetyl groups to histone proteins allows the chromatin to be less condensed and more easily accessed. Acetylation of H3 and H4 subunits in the NAc following drug exposure suggests that many genes in the NAc may be primed for transcription.[84] Indeed, H4 acetylation in this region contributed to the increased expression of *fosB* in rodents.[85] In contrast, methylation of histone proteins can result in either transcriptional activation or repression, depending on which histone residue is affected.[79] For example, methylation of H3 Lysine 9 has been correlated with transcriptional repression. Interestingly, G9a, a histone methyltransferase that methylates H3 Lysine 9, is decreased in the NAc following both chronic cocaine and opiate administration.[86,87] G9a appears to oppose ΔFosB expression,[86,87] and ΔFosB represses G9a expression, creating a feedback loop that perpetuates its own expression through disinhibition. Importantly, postmortem NAc brain tissue of human cocaine users showed decreases in G9a expression[88] and increases in ΔFosB expression.[89] These modifications to the accessibility of DNA can be inherited by future generations, potentially affecting the susceptibility of these generations to SUDs.

Generational Inheritance of Substance Use

Because epigenetic inheritance can result from modifications passed down from the mother, father, or both, resulting in either maternal, paternal, or parental epigenetic inheritance, respectively, it is important to define the type of generational inheritance: multigenerational, intergenerational, or transgenerational.[90,91] Transgenerational

inheritance is the germ-line-mediated inheritance of epigenetic information between generations in the absence of direct environmental influences that lead to phenotypic variation, meaning a true transgenerational study must include at least one generation that receives no direct exposure to the stimulus.[92] While the definition of transgenerational inheritance is fairly straightforward, defining inter- and multi-generational inheritance is slightly more difficult.[90] If the F0 drug use occurs in males or in females prior to pregnancy, the germ cells, which will become the F1 generation, are exposed to the drug. Because both the F0 and the F1 generations are directly exposed to the drug, their inheritance pattern is considered intergenerational. In this scenario, the F2 generation would be the first generation not exposed to the drug, resulting in transgenerational inheritance. Alternatively, if the F0 female is exposed to the drug during pregnancy or postpartum, the somatic and germ cells of the F1 offspring receive direct exposure to the drug *in utero* or postpartum via the breastmilk.[93] Since the F1 germ cells are exposed to the drug, and the F2 offspring originate from those germ cells, the first generation without direct exposure to the drug is F3. In these cases, a study spanning from F0 to F3 would be considered transgenerational, as F3 is the first generation without direct exposure; a study spanning from F0 to F2 would be considered multigenerational.

Multigenerational inheritance is the coincident direct exposure of multiple generations to an environmental factor promoting alterations in the multiple generations exposed.[94] Therefore, in the scenario of drug exposure during F0 pregnancy, the relationship among F0, F1, and F2 would be multigenerational, as the effects of direct exposure to the drug span more than two generations. The paired relationships between F0-F1 and F1-F2, however, would be intergenerational, as these pairs span only two generations post-direct drug exposure. A depiction of multi-, inter-, and trans-generational types of inheritance is shown in Figure 2.

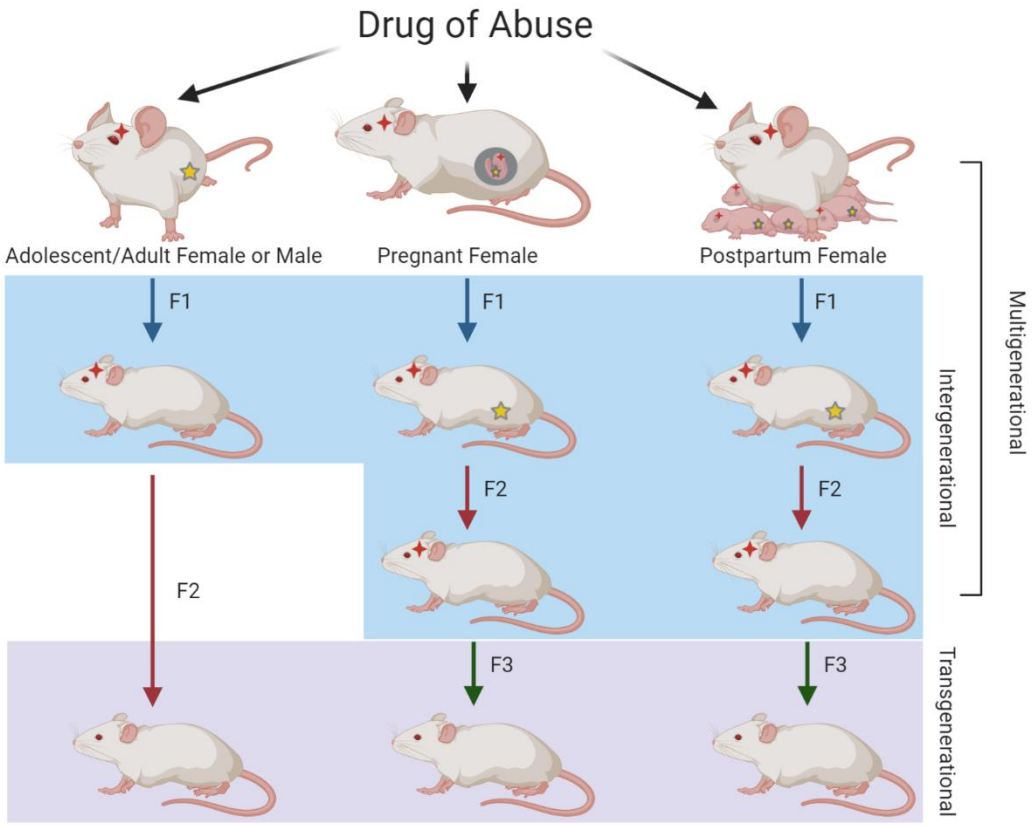


Figure 2. Illustration of multi-, inter-, and trans-generational relationships resulting from parental drug exposure. When F0 males or females are exposed to drugs of abuse in adolescence or adulthood, the germ cells that give rise to the F1 generation are directly exposed, resulting in intergenerational transmission. Because the germ cells of the F1 generation were not directly exposed, the F2 generation represents transgenerational transmission. When F0 pregnant or postpartum breastfeeding females are exposed, the F1 generation and their germ cells that will give rise to the F2 generation are also directly exposed, resulting in intergenerational transmission. For both of these scenarios, the F3 generation is the first without direct drug exposure and therefore represents transgenerational transmission. As F0-F2 generations have direct drug exposure in the case of pregnant or postpartum females, the relationship among the three generations represents multigenerational transmission. Red stars represent direct exposure to that particular generation while yellow stars represent direct exposure to the germ cells. Figure reproduced from Odegaard, Pendyala, and Yelamanchili, 2021.[91]

Opioid Use

Opiate analgesics have a long history of clinical use in the treatment of chronic pain. First derived from opium in 1803, morphine became widely used with the technological innovation of the perfected hypodermic syringe in 1853, which allowed for faster delivery of morphine into the blood or tissue. The American Civil War (1861-1865), the Prussian-Austrian War (1866), and the Franco-Prussian War (1870) also rapidly increased the use of morphine for the reduction of pain and relief from dysentery, often leading to a dependence later deemed “soldier’s disease” or the “army disease.”[1] In England, morphine was first recommended for treatment of cancer pain in the 1950s.[95] Taken orally or by injection, morphine is still widely used today for acute and chronic pain management. Unfortunately, the pain-relieving nature of opioids contribute to their illicit and excessive use, affecting the behavior of the user.[96] Recently, the increased abuse of prescription and non-prescription opioids has resulted in a severe public health crisis throughout large swaths of America.[97-99] In 2017, over two-thirds of drug-overdose deaths resulted from opioid abuse,[100] and opioid overdose-attributed deaths have tripled since the turn of the new millennium.[101] Of the opioids used today, perhaps the most notorious are morphine, heroin, and oxycodone. Chemical structures for morphine (CID 5288826), heroin (CID 5462328), and oxycodone (CID 5284603) as published on PubChem[102-104] are shown in Figure 3.

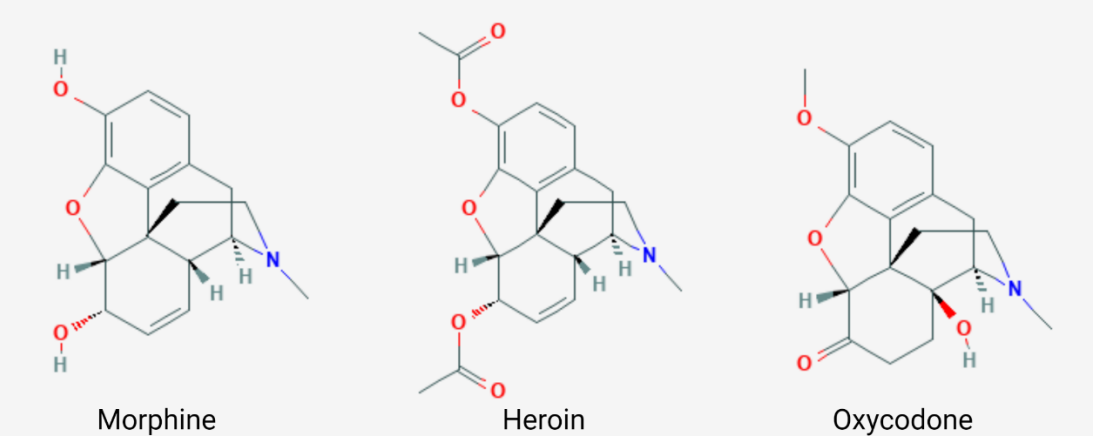


Figure 3. Chemical structures of morphine, heroin, and oxycodone, as published by PubChem.

Routes of Administration

The route of administration of opioids has important implications on an individual's health, including risk of dependence, susceptibility to infection, and experience of any route-specific health complications.[105] The most common routes of administration for opioids are oral, intravenous, intramuscular, and subcutaneous.[106] Varying routes of administration are chosen based on the ease and tolerability of administration as well as the ability to target specific sites of opioid receptors for localized or generalized effects.[107] Although it is the simplest and well-tolerated route, oral administration of opioids may not always be an option, particularly when oral access is limited. Oral administration and the subsequent first pass metabolism may also affect the function of the drug. For example, oral administration of fentanyl results in extensive hepatic metabolism, affecting the utility of the drug.[107] To remedy this, opioids have been prepared in immediate- or slow-release forms. Immediate-release preparations have an onset of action of 20-30 minutes, with analgesia obtained in 45-60 minutes and a duration of action of 3-4 hours. Slow-release preparations allow for a higher initial dose to be administered and a sustained plasma level with a duration of action of 12-24 hours. The onset of action, however, takes 3-4 hours to peak, which is much slower than that of the immediate-release option.[107] Intravenous administration allows for a faster onset of pain relief that is effective and suited to perioperative use. Disadvantages of this route include the need for cannula insertion and its maintenance. Intramuscular and subcutaneous administrations allow an injected opioid to be absorbed into the systemic circulation of the body. Opioid absorption of intramuscular injections may vary, however, depending on tissue perfusion; low perfusion rates may result in poor uptake into the systemic circulation and lead to inadequate analgesia. Subcutaneous injections are preferable to intramuscular injections as they can be administered via an indwelling cannula and do not risk nerve injury that may accompany intramuscular injection.[107] Pharmacological advancements continue to

address the safety and efficacy of opioids to allow for alternative routes of administration that are best suited to individual patients and their conditions.

Mechanism of Action

Whether used licitly or illicitly, opioids activate three types of receptors (mu, MOR; delta, DOR; kappa, KOR). These receptors are dispersed throughout the CNS and peripheral tissues. MOR are generally found in the brainstem and medial thalamus. They are required for supraspinal analgesia, respiratory depression, euphoria, sedation, and physical dependence. DOR are located in the brain and may be responsible for psychomimetic and dysphoric effects. KOR are located in the limbic and diencephalic areas, the brain stem, and the spinal cord, and they are responsible for spinal analgesia, sedation, dependence, and dysphoria.[108]

As G-protein coupled receptors (GPCRs), opioid receptors are activated when opioid agonists bind to the receptor and activate the G-protein (Figure 4). The activated G-protein then moves to its target, like an enzyme or an ion channel, where these targets can alter phosphorylation via the inhibition of cyclic AMP (cAMP). cAMP, as a second messenger, contributes to the activation of protein kinases that can impact gene transcription proteins as well as gene transcription.[108] Opioid receptors on the presynaptic terminals of nociceptive fibers can indirectly inhibit voltage-dependent calcium channels, decreasing cAMP levels and blocking the release of pain neurotransmitters, which results in analgesia. Opioids can antagonize NMDA receptors and activate descending serotonin and norepinephrine pain pathways in the brainstem. The stimulation of the NMDA receptors may also result in neuropathic pain and the development of tolerance.[108] Opioids can also activate the receptors on GABA neurons, thereby inhibiting the release of GABA in the VTA and allowing dopaminergic neurons to increase firing, resulting in extra dopamine in the NAc and creating a pleasurable sensation.[108] The NAc is largely affected

by the binding of opioids to their receptors in the dopaminergic reward system and undergoes changes in density of the dendritic spines,[96] effectively altering the plasticity of the dendritic spines during nervous system development. This neural plasticity is critical in the development of SUDs and other ingrained behaviors.

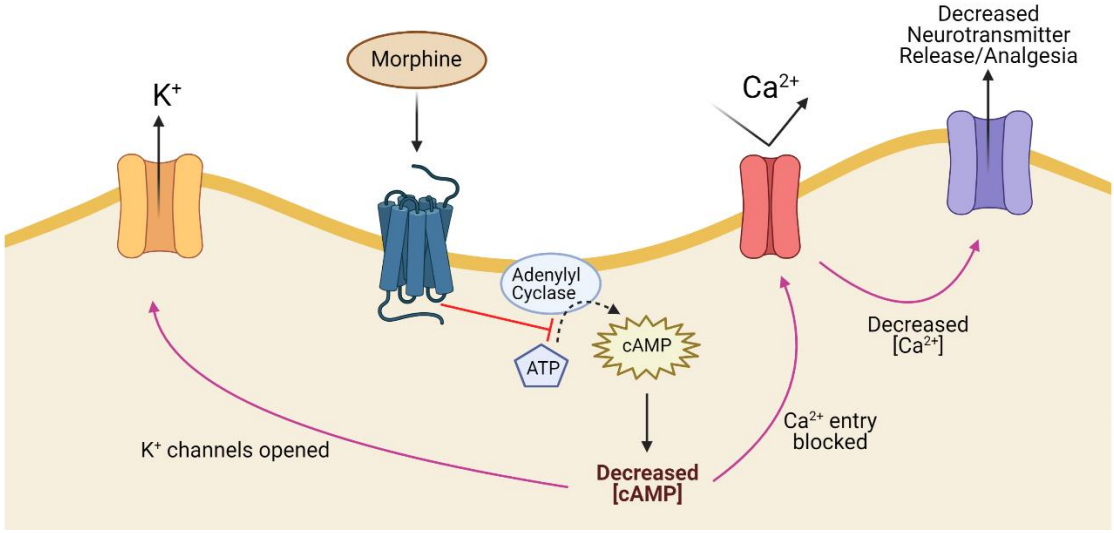


Figure 4. Simplified mechanism of action of opioids using morphine as an example of an opioid receptor agonist. Created using Biorender.com.

Morphine

Morphine ($C_{17}H_{19}NO_3$) is a potent analgesic primarily used in hospitals to combat severe pain. Structurally, morphine is a benzylisoquinoline alkaloid with two ring closures that is considered part of the phenanthrene group of opioids.[108] Approximately 40-50% of an orally administered dose reaches the CNS; this occurs within 30 minutes for an immediate release form of morphine and within 90 minutes for any extended release form.[108] The half-life of morphine is approximately 2-3 hours.[109] While the initial stage of morphine metabolism is carried out by CYP450, morphine is predominantly metabolized by glucuronidation by UGT2B7 to produce morphine-6-glucuronide and morphine-3-glucuronide in a ratio of 6:1.[110] Glucuronidation occurs almost immediately after morphine enters the serum in both hepatic and extra hepatic sites.[111] Morphine-3-glucuronide in high enough concentrations is believed to potentially lead to hyperalgesia,[112] and morphine-6-glucuronide is believed to be responsible for the majority of morphine's analgesic effects.[113] Demethylation of morphine via CYP3A4 and CYP2C8 produces normorphine.[114]

While morphine may be effective for pain relief, there are potential adverse side effects such as tolerance and addiction as well as molecular alterations.[115] Morphine's powerful analgesic effects stem from its role as an opioid receptor agonist. By binding to MOR and KOR, morphine blocks the transmission of nociceptive signals, signals pain-modulating neurons in the spinal cord, and inhibits primary afferent nociceptors to the dorsal horn sensory projection cells.[116] Morphine also activates the reward pathway by binding to receptors in the VTA and NAc, leading to an influx of dopamine in the synapse.[117]

Heroin

The result of a chemical modification to morphine, heroin ($C_{21}H_{23}NO_5$) is about three times as potent as its parent drug.[1] Unlike morphine, which can be prescribed, heroin is an illicit substance. Like other opioids, heroin acts agonistically to the three opioid receptors.[118] Heroin activation of MOR contributes to analgesic effects, respiratory depression, and euphoria;[119] KOR activation contributes to a degree of analgesia as well,[119] and DOR activation is more involved in spinal analgesia. When injected, heroin is metabolized by sequential deacetylation of two separate ester bonds to form 6-monoacetylmorphine, a more potent MOR agonist than morphine,[119] which can further be metabolized to morphine and morphine metabolites.[118] When taken orally, heroin undergoes first-pass metabolism to morphine via deacetylation. Therefore, unlike intravenous administration, oral ingestion does not cause a rapid onset of effects conferred by 6-monoacetylmorphine.[120] The preferred method of injecting heroin, coupled with impaired judgment can lead to needle sharing, which can increase the risk of contracting infectious diseases such as HIV and hepatitis.[121]

Oxycodone

Structurally similar to morphine, oxycodone (oxy; $C_{18}H_{21}NO_4$) has been established as a potent and widely abused prescription opioid. Designed for pain management, oxy operates primarily as a full agonist to MOR.[122] The binding of oxy to MOR subsequently inhibits neurotransmitter release by decreasing cAMP production.[122] Importantly, oral doses of extended release oxy are thought to be twice as potent as a similar dose of morphine,[123] contributing to its popularity and abuse. The bioavailability of oxy is high in oral dosage, with a half-life of 2.5-3 hours. Like morphine, oxy is metabolized by glucuronidation by CYP3A4 and CYP3A5 to noroxycodone, and by CYP2D6 to oxymorphone.[124] Both oxymorphone and noroxycodone can be further metabolized to

noroxymorphone by CYP3A4 and CYP2D6. While oxy itself contributes the majority of the analgesic effect, its metabolites also act as opioid agonists. Oxy and its metabolites can diffuse across the blood brain barrier or may be actively transported by an unknown mechanism.[125] With oxy's utility as a treatment for moderate to severe pain in pregnant women, it is important to note than oxy and its metabolites can also pass through the breastmilk as well as through the placenta to be distributed to the offspring.[126]

Generational Inheritance and Opioid Abuse

The ongoing opioid epidemic in the United States and worldwide is characterized by an increase in use of licit and illicit opioid substances, such as prescription opioids, illicit heroin, and illicitly-produced fentanyl. While steps have been taken by the FDA to limit opioid abuse and misuse, the potential for addiction remains an issue, especially when individuals turn to cheaper, illicit opiates as an alternative to prescription opioids.[127] Further, opioids are commonly prescribed to postpartum women, often in abundance,[128,129] making pregnant women a particularly vulnerable group in the opioid epidemic. With regard to generational studies and opioid exposure, studies involving *in utero* exposure to opioids are particularly critical, as these may result in neonatal opioid withdrawal syndrome (NOWS) or neonatal abstinence syndrome (NAS). Neonates with NOWS/NAS exhibit high-pitched cries, tremors, difficulty feeding, hypertonia, and breathing issues.[130] In the context of *in utero* opioid exposure and NOWS/NAS, the fetal origins of adult disease hypothesis, first posited by Dr. David Barker in 1995,[131] is particularly interesting. This hypothesis states that risk factors from intrauterine environmental exposures affect fetal development during sensitive periods, and increase the risk of specific diseases in adult life.[132] Indeed, prenatal exposure to opioids has been associated with smaller head size, lighter birthweights, and shorter body lengths in neonates.[133-138] Moreover, not only do young adults exposed to heroin prenatally exhibit

cognitive and motor function deficits,[139,140] but they may also have an 8-fold increased risk of depression, a 3-fold increased risk of attention disorders, and a 16-fold increased risk of substance use disorders.[141] As substance use disorder is considered a disease, it is critical to understand how the generational effects of opioid exposure manifest in not only newborns, but adults as well.

Animal Studies

Several animal studies using rats and mice have been conducted to determine multi-, inter-, and transgenerational effects of opioid exposure, with most studies being conducted using morphine, heroin, and oxycodone. The studies discussed in this section involve maternal, paternal, parental, and perinatal exposure paradigms. Maternal and paternal studies were conducted in adolescent and adult models. Maternal models are also part of the perinatal paradigms as the opioids were administered to the mother and passed to the offspring through the placenta (*in utero* exposure) or via the breastmilk (postnatal exposure). In parental paradigms, both parents were exposed to opioids.

Morphine

A number of studies using rats have been conducted to determine the generational effects of parental morphine use on offspring. Chronic morphine exposure during adolescence has been shown to significantly impact male reproductive parameters, as evidenced by reduced seminal vesicle and testes weights,[142] and delay sexual maturation.[143] Additionally, offspring born to males with adolescent morphine exposure had several endocrine deficits in adulthood. Specifically, adult male offspring born to morphine-treated males had lower serum testosterone and luteinizing hormone levels and higher adrenal weights.[142] In female offspring, serum corticosterone and β-endorphin levels were significantly higher, but no differences in reproductive endocrine status were observed. Interestingly, F1 and F2 males descended from adolescent morphine-exposed

dams had blunted corticosterone secretion, an effect that was specific to offspring from females exposed to morphine during adolescence as opposed to those exposed during adulthood.[144] These males also had significantly lower levels of paraventricular nucleus (PVN) corticotropin releasing hormone (CRH). F2 males also exhibited dysregulated MOR expression in the PVN.[144] Interestingly, on a fat-sugar diet (FSD), F1 males born to adolescent morphine-exposed females had increased weight gain, fasting glucose, insulin, and corticosterone levels, as well as increased neuropeptide Y (NPY), a potent orexigen.[143] Maternal opioid exposure in adolescence appears to interact with F1 offspring diet to further disrupt hypothalamic and neuroendocrine systems that influence sexual maturation, energy metabolism, synaptic plasticity, and possibly immune function.[143]

Interestingly, dams with adolescent morphine exposure were shown to have reduced levels of prolactin secretion postpartum, suggesting that morphine use during the pubertal period may affect prolactin secretion into adulthood, potentially affecting the maternal behavior and subsequent behaviors of the offspring.[145] Indeed, Johnson *et al.* found that adolescent exposure to morphine did affect maternal behavior, with exposed mothers spending less time nursing and grooming pups and more time away from the nest and engaging in self-directed behavior.[146] Perhaps as a result of maternal effects, female offspring of exposed dams exhibited increased anxiety-like behavior.[147,148] Further, male and female offspring appeared to develop a tolerance for the sedative effects of morphine more quickly than offspring born to untreated dams.[147,148] Male offspring also demonstrated decreased rough and tumble play behaviors, while females exhibited a tendency toward increased rough and tumble play, demonstrating sex-specific effects of maternal adolescent morphine exposure on the F1 generation.[146] To determine changes in the reward system, Vassoler *et al.* used conditioned place preference (CPP) and locomotor sensitization. F1 animals showed increased sensitivity to morphine, and females showed increased locomotor activity when compared to males.[149] Additionally, all

F1 animals born to morphine-exposed dams had decreased MOR protein expression in the VTA, while only females had an increased expression of MOR in the NAc. This differential expression of MOR in the NAc and VTA may contribute to the increased sensitivity to morphine seen in these offspring born to morphine-exposed mothers.[149]

Transgenerational effects of maternal adolescent morphine exposure have also been reported. Male offspring of morphine-exposed dams had previously shown enhanced locomotor sensitization in response to repeated morphine exposure.[147] As dopamine agonists have been shown to affect locomotor sensitization, Byrnes *et al*. sought to investigate the effect of quinpirole, a dopamine D2/D3 receptor agonist, on locomotor sensitization in F1 and F2 male progeny of morphine-exposed F0 dams.[150] Quinpirole treatment resulted in an increased locomotor response and also increased plasma corticosterone levels in both generations. Repeated administration of quinpirole resulted in increased expression of KOR and dopamine D2 receptor in the NAc of the males in both generations. Together, these data may suggest the presence of functional and transgenerational neuroadaptations within the NAc that are transmitted across two generations following maternal adolescent exposure to opiates.[150]

The effects of adolescent morphine exposure are not limited to females, however. Paternal, maternal, and parental adolescent morphine exposure have been shown to increase anxiety-like behavior in male offspring, depressive-like behavior in female offspring, and enhanced morphine consumption in both sexes during adolescence.[151] Interestingly, parental exposure to an enriched environment reduced anxiety- and depressive-like behaviors and voluntary morphine consumption in the offspring, offering a potential preventative measure in the development of these traits in future offspring.[151] Further, paternal adolescent morphine exposure also altered pain perception[152] and several electrophysiological properties in the locus coeruleus of male progeny.[153] Additionally, F0 male and female parents chronically exposed to morphine in adolescence

had increased levels of hippocampal TNF-α, an inflammatory cytokine; F1 male and female offspring of these parents showed the same increase in hippocampal TNF-α.[154] Fathers, but not mothers, also had lower levels of S100B, another key protein in neuroinflammation. This decrease in S100B was also reported in the male and female offspring, suggesting that adult exposure to morphine can induce hippocampal modifications intergenerationally.[154] Additionally, males born to morphine-exposed fathers exhibited morphine tolerance and decreased firing of VTA dopamine neurons.[155]

Adolescent morphine exposure in males may also impact future substance abuse of the F1 generation. Male and female F1 progeny have delayed acquisition and decreased intake of cocaine. In addition, they have blunted self-administration levels compared to control rats. Female offspring also had increased levels of morphine intake during acquisition and increased self-administration for oxycodone.[156] Surprisingly, even following acquisition of morphine self-administration, males and females still demonstrated a blunted effort for cocaine. Brain-derived neurotrophic factor (BDNF), an important factor in hippocampal synaptic plasticity, neurogenesis, and modulation of learning and memory, was increased in the prefrontal cortex (PFC) in F1 male and female offspring. Together, these data identify systems that are vulnerable to the adolescent parental exposure and how they may impact drug use of future generations.

Prenatal morphine exposure studies in animals have offered insight into the developmental effects on learning and memory. Prenatal morphine exposure has been shown to affect hippocampal neuron viability and expression of proteins crucial for synaptic plasticity. Increased hippocampal neuron apoptosis, decreased CaMKII (Ca^{2+}/calmodulin-dependent kinase II; essential for the induction of synaptic potentiation and memory formation) activity, and decreased BDNF expression have been reported in offspring that were prenatally exposed to morphine.[61,157] Prenatal morphine exposure has also been shown to decrease post-synaptic density protein 95 (PSD-95; implicated in the

regulation of synaptogenesis, synaptic plasticity, learning and memory) expression in the hippocampus.[158,159] Prenatal exposure also reduced the magnitude of the expression of nitric oxide synthase (*n*NOS), LTD (a mechanism of learning and memory), and phosphorylated $CREB^{Serine\text{-}133}$ (an important transcription factor for mammalian learning and memory) in the hippocampal CA1 region of post-natal day 14 (P14) pups. Interestingly, prenatal coadministration of dextromethorphan, an antitussive drug that has been shown to mitigate morphine tolerance and withdrawal,[160,161] during pregnancy and throughout lactation significantly attenuated these changes in the hippocampus, presenting a potential therapeutic strategy for combatting these effects of prenatal morphine exposure.[158]

Intriguingly, as another cellular process underlying learning and memory, LTP has also been reported to be reduced in offspring prenatally exposed to morphine.[58-61] Niu *et al.* suggested that depressed LTP in the morphine group may denote a change in GABAergic inhibition; indeed, a loss of GABA-containing neurons in the dentate gyrus (DG) area of the morphine group was observed.[59] Subsequent behavioral tests to elucidate the impact of these molecular changes have shown altered long- and short-term learning and memory tasks,[157,158] impaired working and cued reference memory,[159] impaired spatial learning and memory,[59,61,162] and enhanced maintenance but impaired acquisition of contextual fear memory[60] in rats prenatally exposed to morphine. Further, prenatally exposed offspring exhibited reduced anxiety-like behavior and no differences in locomotor activity,[60] contrary to what was reported in the adolescent morphine studies mentioned previously. Intriguingly, postnatal exercise and enriched environment was shown to improve deficits in spatial learning, hippocampal LTP, and BDNF levels caused by prenatal morphine exposure.[61]

Adult parental exposure prior to pregnancy and gestation has also been shown to affect behavior and hippocampal signaling. F0 males and females exposed to morphine

as adults yielded F1 offspring that exhibited anxiety-like behavior[163,164] and dendritic retraction in the DG of the hippocampus in adulthood.[164] Further, CRH levels in the plasma and CSF (cerebrospinal fluid), along with CRH receptor 1 mRNA levels, were also increased in F1 progeny while protein kinase C (PKC) levels were decreased.[163] Additionally, insulin-like growth factor (IGF)-2 signaling in the granular zone of the DG was downregulated in these F1 animals. Interestingly, overexpression of IGF-2 prevented anxiety-like behaviors in these offspring. Further, exposure to an enriched environment during adolescence corrected the reduction of hippocampal IGF-2 expression, normalized anxiety-like behavior, and reversed dendritic retraction in the adult offspring.[164] This study suggests that IGF-2 and an enriched environment may be potential forms of intervention to prevent anxiety and brain atrophy in the offspring of parental opioid exposure.

Further, males born to parents with adult morphine exposure exhibited significant memory impairments and tolerance to morphine that were more prominent with maternal morphine exposure than either paternal or parental exposure.[165] Additionally, maternal adult morphine exposure resulted in diminished spatial memory in males in the F1 and F2 generations.[166] Hippocampal *Mecp2*, a transcriptional repressor, and *Hdac2*, a histone deacetylase important in cognitive function, were also significantly upregulated in the males of both generations.[166] These changes were not seen in the female F1 and F2 progeny, suggesting sex-specific alterations resulting from maternal adult morphine exposure. Ellis *et al.* have also shown that paternal morphine exposure produces sex-specific impairments in object recognition memory in female offspring.[167] Additionally, offspring born to parents with adult morphine exposure had decreased object recognition, possibly due to decreased histone H3 acetylation and ΔFosB levels in the PFC and hippocampus.[168] Interestingly, nociception was reduced in offspring born to adult morphine-exposed parents, and these offspring also had a low neuronal firing rate and enhanced opioid receptor expression in the NAc.[169]

Furthermore, F1 male progeny of morphine-exposed parents had a greater preference for morphine and more anxiety-like behavior; these effects were not seen in the F2 generation.[170] F1 male progeny also exhibited increased expression of D1 and D5 dopamine receptors in the PFC and NAc; D5 and D2 receptors were decreased in the hippocampus. The D4 dopamine receptor was augmented in striatum and hippocampus and decreased in the PFC.[170] Alterations in dopamine receptor expression within the reward system may be one mechanism responsible for these behavioral changes in F1 offspring. Interestingly, injection of SCH23390, a dopamine D1 receptor antagonist, into the PFC and the hippocampus improved spatial memory that was previously reported to be impaired in males born to parents with adult morphine exposure.[171]

Heroin

Rat models of parental heroin exposure have provided insight into how heroin exposure in the F0 generation can affect subsequent generations. In one transgenerational study of adolescent paternal exposure, F0 male exposure resulted in smaller F1 litter sizes as well as heightened anxiety and increased aggression in both F1 and F2 offspring; these effects appeared to subside in the F3 generation.[172] While paternal heroin exposure has only recently been explored, several studies of prenatal heroin exposure have been done in mice models. For example, Zhu *et al*. found that heroin-exposed pups had a marked reduction in birth weight, and the postnatal weight gain in these pups was significantly lower than that of controls, particularly in female pups.[173] These female pups also showed a significant increase in ambulation and rearing, suggesting that prenatal heroin exposure could result in a sex-specific delay of postnatal development and learning.[173]

With regard to learning, prenatal heroin exposure has been shown to affect the hippocampus and memory-associated behaviors. Prenatal exposure to heroin in mice

resulted in increased expression of hippocampal *caspase-3* and *Bax* and decreased expression of *Bcl-2*, which together may enhance neuronal apoptosis and impair hippocampus-dependent learning and memory.[174] Interestingly, prenatal heroin exposure was also shown to decrease dendrite length and branch number in the somatosensory cortex.[175] Further, mice prenatally exposed to heroin displayed behavioral deficits in eight-arm and Morris mazes[174,176] and exhibited impaired short-term spatial memory as adults.[175] These mice also had increased synaptic activity in cholinergic hippocampal cells.[176] Additional research found that these hippocampus-related behavioral deficits may be linked to the cholinergic receptor-mediated translocation of PKC isozyme PKCγ, which has a role in learning and memory.[177-179] Interestingly, the alterations in cell signaling and subsequent behavior deficits found in prenatal mice models were also reported with pre-hatch heroin exposure in chickens, where heroin exposure affected imprinting behavior and PKCγ.[176]

Oxycodone

Several studies of perinatal oxycodone have been conducted in rats to deduce the negative effects of oxycodone exposure *in utero* or postnatally. One study of prenatal oxycodone exposure found that rat pups exposed *in utero* had congenital malformations of the face, mouth and vertebrae as well as intrauterine growth retardation.[180] In adolescence, these animals had lower body and kidney weights. Additionally, endothelin receptor A expression was higher at P1 but appeared to return to baseline by P7, P14, and P28. Conversely, endothelin receptor B expression was lower in the exposed offspring at P1 and P7, returned to baseline at P14, and was increased by P28. As the endothelin receptors are important in normal development of the CNS, the alterations in their expression levels in these offspring may suggest a delay in CNS development.[180]

Additionally, prenatal oxycodone exposure rat models have shown alterations in cardiovascular responses as well as behavior. Rats exposed to oxycodone *in utero* showed subtle alterations in stress cardiovascular response that subsided with age. In adulthood, these rats exhibited impaired memory of stress conditioning learned with adolescent tail shock testing, suggesting the presence of a memory deficit associated with prenatal oxycodone exposure.[181] Indeed, prenatal oxycodone exposure has been shown to impair spatial learning and memory.[182] Prenatal oxycodone exposure rats made a greater number of reference memory errors in the beginning of radial arm maze testing. They also had a deficit in memory retention when assessed by T-maze five days post-acquisition training. In Morris water maze, prenatally exposed rats exhibited poor acquisition during long inter-trial intervals only, with no deficit reported during short inter-trial intervals. Exposed rats also had an increased latency to find and greater distance traveled to the platform in the Morris water maze.[182] Using several spatial memory tasks, this study by Davis *et al.* indicated that prenatal oxycodone exposure consistently impairs learning and memory.[182] Interestingly, other behavioral paradigms have linked prenatal oxycodone exposure and hyperactivity in adult rats, which is consistent with hyperactivity problems identified in children exposed to opiates *in utero.*[183]

In an oxycodone self-administration study, offspring exposed *in utero* demonstrated region-specific effects of oxycodone exposure on MOR-1 at P1; specifically, MOR-1 expression was significantly decreased in the midbrain and forebrain of exposed females.[184] Further, the number of ultrasonic vocalizations by pups exposed to oxycodone *in utero* varied over time depending on oxycodone intake and pup development. For example, on P3, higher oxycodone intake was associated with fewer vocalizations, while by P9 higher oxycodone intake was associated with a greater number of vocalizations. Maternal oxycodone self-administration and dose-dependent alteration of the maternal-

offspring dyad may have resulted in sex- and region-specific effects on early measures of neurodevelopment.[184]

Our own laboratory studies have shown that prenatal oxycodone exposure has recently been shown to alter the cargoes of extracellular vesicles (EVs), key players in cell-cell communication.[185] Through RNA-sequencing, distinct miRNA signatures were identified in EVs isolated from P14 rat brains. The gene targets of the dysregulated miRNAs identified in offspring exposed to oxycodone *in utero* (IUO) or postnatally (PNO) were largely related to the regulation of key functional pathways associated with brain development, with the more impacted group being the IUO. Additionally, treatment of primary neurons with these EVs isolated from IUO brains caused significant reductions in dendritic spine density compared with treatment of PNO or control brain-derived EVs. Dopamine D1 expression was also increased in synaptosomes isolated from P14 IUO brains, potentially a result of the upregulated miR-504 cargo of IUO EVs, which may also play a role in the loss of dendritic spines.[185]

Human Studies

Most human studies regarding generational effects of opioid exposure combine data from different opioid exposure cases together and consider them as one opioid-exposure group. Additionally, most of these studies discuss prenatal opioid exposure and its consequences on the children as they age. Such prenatal exposure studies focus on the immediate outcomes in the F1 children, making a majority of these studies intergenerational. Indeed, short-term consequences of opioid exposure are well-characterized,[186] but the long-term, intergenerational consequences require further study. This section will discuss combined opioid exposure studies, where different exposures to morphine, heroin, oxycodone, etc. have been grouped into one opioid exposure group, as

well as opioid-specific studies, which consist mainly of heroin with or without polysubstance abuse.

Recently, *in utero* opioid exposure has been significantly associated with higher risks of fetal growth restriction, preterm birth, lack of normal development, childhood conduct disorder or emotional disturbance in preschool children, and attention-deficit/hyperactivity disorder (ADHD) in older children.[187] A longitudinal study of 8,509 mother-child pairs included 454 mother-child pairs exposed to opioids and 8,055 control mother-child pairs not exposed to opioids. As most of the sample included urban, low-income, and minority ethnic groups, the higher incidence rates of NAS serve to highlight the plight of prenatal opioid exposure in urban, low-income populations in the inner cities of the United States.[187] Infants born to these mothers had lower gestational age, lower birthweight, and smaller size for gestational age. Within the first six years of age, exposed children had a higher risk of diagnosis with conduct disorders, emotional disturbance, and deficits in physiological development; after six years of age, the likelihood of being diagnosed with ADHD increased in these children. This study adeptly describes the opioid epidemic as an intergenerational problem.[187]

A recent meta-analysis of publications spanning from 1993 to 2018 showed the extent of neurodevelopmental outcomes reported in children born to opioid-dependent mothers.[188] Exposed children had lower infant cognitive and psychomotor scores, lower general cognition/IQ and language scores, and higher parent-rated internalizing, externalizing, and attention problems. Overall, this analysis showed that prenatal opioid exposure increases the risk of adverse neurodevelopment in children at least through middle childhood. Important to note, however, is that this meta-analysis only covered three school-aged reported outcomes, limiting the ability to assess longer-term impacts of prenatal opioid exposure.[188] More importantly, this limitation highlights an overall limitation

in the field; more longitudinal human studies of prenatal opioid abuse are critical in understanding long-term intergenerational effects of opioid exposure.

In an fMRI study of prenatal drug exposure, which included prenatal exposure to heroin, Geng *et al.* found that prenatal drug exposure was related to decreased memory performance and altered brain activation during memory encoding.[189] Interestingly, the deficits in memory in adolescents with prenatal drug exposure may stem from variations in encoding rather than in retrieval processes, meaning that potential interventions should focus on encoding rather than retrieval to improve memory performance in these children. This study conducted in 5- to 14-year-old children showed that prenatal exposure to drugs, heroin among them, has long-term effects on memory.[189] Interestingly, a 2010 neuroimaging study found that prenatally-exposed children had smaller intracranial and brain volumes as well as thinner cortex of the right anterior cingulate and lateral orbitofrontal cortex. The volumes of the anterior cingulate, orbitofrontal cortex, and accumbens area were associated with cognitive ability and behavior problems.[190]

Providing a paternal/maternal consideration of intergenerational effects beyond prenatal exposure, a recent study by Griesler *et al.* investigated the intergenerational patterns of nonmedical prescription opioid (NMPO) use in adolescents (12- to 17-year-olds) and their parents, providing a paternal/maternal consideration of intergenerational effects beyond prenatal exposure. Maternal use of NMPO had a stronger association with adolescent use than paternal use.[191] Use of other drugs also affected both parental and offspring NMPO use. In addition to parental drug use, the quality of the parent-child relationship, adolescent drug use of non-NMPO substances, risk of drug use, drug use by peers, depression, and delinquency were each associated with adolescent NMPO use. These authors therefore suggested that parent-based interventions targeted at NMPO use among youth should not only address parental NMPO use but should also promote positive parenting practices, such as monitoring and reduced conflict.[191] In a similar study

considering prescription opioids, Kerr *et al.* found that paternal opioid misuse and maternal opioid use were associated with the child's substance use; further, parental prescription opioid misuse or use also predicted child use of alcohol, marijuana, or tobacco by adolescence.[192]

In an older study that assessed attentional performance and cardiac reactivity to attentional demand, boys (aged 7 to 12) who were exposed to opioids *in utero* were compared to boys whose mothers began using illicit substances after the child's birth (environmental controls), and boys whose mothers were non-drug users.[193] Vagal tone, a measure of heart-rate variability, was measured during an attention task. When distractors were added to the task, opioid-exposed boys failed to suppress vagal tone. Interestingly, both groups with parental opioid exposure made fewer correct responses on this task, indicating that environmental influences, in addition to prenatal opioid exposure, impact attentional performance. The authors concluded that normal physiological responses to increased attentional demand may be impaired in opioid-exposed male children.[193]

Of the opioids discussed in this review, heroin appears to be the most studied in human subjects. Illicit heroin use during pregnancy has been associated with several adverse effects in mothers and infants.[188] Prenatal exposure to heroin resulted in reduced head circumference, birth length, and birth weight,[194] but it has not been associated with congenital malformations.[195] Additionally, one small retrospective study showed that 93.9% of heroin-exposed infants developed drug withdrawal symptoms within 24 h of birth.[196] This withdrawal, or NAS, may impact language and cognition development.[197]

In another interesting study, children (5-6 years of age) born to heroin-dependent mothers were compared to those born to heroin-dependent fathers and three control groups. Out of 83 children born to heroin-dependent mothers, five had significant neurological damage. Of the 76 children born to heroin-dependent fathers, six had significant neurological damage.[198] Further, the children with maternal heroin exposure

had lower birthweight and lower head circumference than controls. Parental heroin dependence also resulted in higher hyperactivity, inattention, and behavioral problems in the children. Interestingly, children of heroin-dependent mothers that had been adopted at an earlier age functioned similarly to controls while those raised by their birth mothers functioned significantly lower. This study implicates the role of home environment in the developmental outcomes of children born to heroin-dependent parents.[198]

Indeed, prenatal drug abuse, unstable parental care, and low birthweight may contribute to the vulnerability of exposed offspring.[140] A study of 17- to 21-year-old individuals prenatally exposed to heroin and poly-substance abuse revealed lower cognitive functions that appeared to be partially mediated by lower birthweights.[140] Further, the individuals exposed to the least amount of drugs and who had more stable parental care had the best cognitive scores compared to those with unstable homes or mothers who used more drugs during pregnancy.[140] Interestingly, parental factors have previously been shown to play a role in the increased risk of substance use disorders reported in children of heroin-dependent parents.[141] Nygaard *et al.* continued their investigation of prenatal heroin and polysubstance abuse by studying the mental health of these individuals.[199] Similar to the findings by Vidal *et al.*,[141] a higher proportion of these individuals reported lifetime experiences with major depressive disorder, alcohol abuse, and ADHD.[199] These individuals also scored higher on the aggressive behavior scale and had more sexual partners and were younger at their sexual debut.[199]

Summary and Conclusions

Overall, SUD resulting from biochemical alterations that occur within the reward circuitry of the brain is often characterized by maladaptive and compulsive behaviors of the user. In recent years, opioid use disorder (OUD), specifically, has been deemed an epidemic in the United States. As pregnant women continue to be a vulnerable population

within this epidemic, potentially impacting future generations, understanding multi-, inter-, and trans-generational inheritance is critical. As current human studies of prenatal opioid exposure have yet to extend to long-term inter- or trans-generational studies, preclinical animal models are working to fill in the knowledge gaps. While most animal studies have considered the generational impacts of morphine exposure, the need to investigate other opioid exposures is paramount. As an opioid commonly prescribed to women for pregnancy pain, oxycodone is an important opioid to investigate in the context of generational effects. The present work, presented as three chapters, investigates and characterizes the effects of pre- and post-natal oxycodone exposure on the F1 and F2 generations to fill the knowledge gaps surrounding this important opioid and its generational impact. Chapter One describes the effects of oxy exposure on the F1 generation, specifically with regard to biochemical, electrophysiological, and behavioral changes; this chapter includes data first published in *Translational Psychiatry*.[138] Chapter Two further explores the impact of oxy exposure on the F1 generation through the characterization of synaptic vesicle proteomes. Lastly, Chapter Three characterizes the intergenerational effects of oxy exposure in both F1 and F2 generations with regard to physical development, gene expression, and behavior; this chapter contains data first published in *Frontiers in Cell and Developmental Biology*.[200] These chapters are followed by an overall discussion to encompass all findings and future avenues of this research.

Materials

Antibodies

Primary Antibodies

Name	Company
GAPDH *Monoclonal, mouse, 1:2000*	Invitrogen (Carlsbad, CA, USA)
LAMTOR4 *Monoclonal, rabbit, 1:750*	Cell Signaling (Danvers, MA, USA)
MEGF8 *Polyclonal, rabbit, 1:1000*	Bioworld Technology (St. Louis Park, MN, USA)
PSD95 *Monoclonal, mouse, 1:2000*	Invitrogen (Carlsbad, CA, USA)
PSMC6 *Monoclonal, mouse, 1:1000*	Abcam (Cambridge, UK)
SNAP25 *Polyclonal, rabbit, 1:2000*	Sigma Aldrich (St. Louis, MO, USA)
SYP *Polyclonal, rabbit, 1:5000*	Invitrogen (Carlsbad, CA, USA)

Secondary Antibodies

Name	Company
anti-mouse IgG *peroxidase-labelled, 1:5000*	Thermo Fisher Scientific (Waltham, MA, USA)
anti-rabbit IgG *peroxidase-labelled, 1:2000; 1:10000*	Thermo Fisher Scientific (Waltham, MA, USA)

Chemicals and Reagents

Name	Company
Acetonitrile	Thermo Fisher Scientific (Waltham, MA, USA)
ATP-Mg	Thermo Fisher Scientific (Waltham, MA, USA)
Bovine Serum Albumin	Thermo Fisher Scientific (Waltham, MA, USA)
$CaCl_2$	Thermo Fisher Scientific (Waltham, MA, USA)
Cs-methanesolfonate	Thermo Fisher Scientific (Waltham, MA, USA)
EGTA	Thermo Fisher Scientific (Waltham, MA, USA)
Formic Acid	Thermo Fisher Scientific (Waltham, MA, USA)
Glucose	Thermo Fisher Scientific (Waltham, MA, USA)
GTP-Na_2	Thermo Fisher Scientific (Waltham, MA, USA)
HEPES	Thermo Fisher Scientific (Waltham, MA, USA)
KCl	Thermo Fisher Scientific (Waltham, MA, USA)
L-ascorbic acid	Thermo Fisher Scientific (Waltham, MA, USA)
Mass Spectrometry-grade Trypsin	Thermo Fisher Scientific (Waltham, MA, USA)
$MgSO_4$	Thermo Fisher Scientific (Waltham, MA, USA)
NaCl	Thermo Fisher Scientific (Waltham, MA, USA)
NaH_2PO_4	Thermo Fisher Scientific (Waltham, MA, USA)
$NaHCO_3$	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)
Oxycodone hydrochloride	Sigma Aldrich (St. Louis, MO, USA)
Phosphocreatine	Thermo Fisher Scientific (Waltham, MA, USA)
Picrotoxin	Thermo Fisher Scientific (Waltham, MA, USA)
Protease Inhibitor	MilliporeSigma (Burlington, MA, USA)

Sucrose	Thermo Fisher Scientific (Waltham, MA, USA)
SuperSignal West Pico Chemiluminescent Substrate	Thermo Fisher Scientific (Waltham, MA, USA)
Tetraethylammonium chloride (TEA-Cl)	Thermo Fisher Scientific (Waltham, MA, USA)
Thiourea	Thermo Fisher Scientific (Waltham, MA, USA)
Tris-buffered Saline (TBS)	Thermo Fisher Scientific (Waltham, MA, USA)
Tween20	Thermo Fisher Scientific (Waltham, MA, USA)

Buffers and Solutions

Name	Composition
Antibody Solution (Western and Dot blots)	*0.75 g BSA* *50 mL TBS-T*
Artificial Cerebrospinal Fluid (ACSF)	*124 mM NaCl* *2.5 mM KCl* *1.25 mM NaH_2PO_4* *24 mM $NaHCO_2$* *12.5 mM Glucose* *2 mM $CaCl_2$* *2 mM $MgSO_4$*
Blocking Solution (Western and Dot blots)	*5 g Nonfat Powdered Milk* *100 mL TBS-T*
Mass Spectrometry Solvent B	*0.1% Formic Acid in 80% Acetonitrile*
NMDG-based ACSF	*92 mM NMDG* *2.5 mM KCl* *1.25 mM NaH_2PO_4* *30 mM $NaHCO_2$* *20 mM HEPES* *0.5 mM $CaCl_2$* *10 mM $MgSO_4$*

	2 mM Thiourea *5 mM L-ascorbic acid* *3 mM Na-pyruvate*
Patch Pipette Solution *(pH = 7.35; osmolality = 282 mOsm)*	*120 mM Cs-methanesolfonate* *10 mM HEPES* *8 mM* TEA-Cl 5 mM ATP-Mg *0.5 mM GTP-Na* *5 mM Phosphocreatine* *0.5 mM EGTA*
SV Homogenization Buffer	*320 mM Sucrose* *4 mM HEPES* *Protease Inhibitor Tablet*
TBS-T	*900 mL deionized water* *100 mL 10X TBS* *1 mL Tween20*

Methods

Animals

Male and female Sprague Dawley rats were obtained from Charles River Laboratories Inc. (Wilmington, MA, USA) and group housed in a 12-h light–dark cycle and fed *ad libitum*. 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.

Oxycodone Treatment

The development of the IUO treatment paradigm was adapted from a previously published study,[182] and the overall treatment paradigm previously established in our lab was followed.[185] A schematic of the treatment regimen can be found in Figure 5. Briefly, nulliparous female (64–70 days of age) Sprague Dawley rats were treated with oxycodone HCl dissolved in saline or saline vehicle via oral gavage. An ascending dosing procedure was used wherein doses of 10 mg/kg/day of oxy were orally-gavaged for 5 days followed by a 0.5-mg/kg/day escalation for 10 days until reaching a final dose of 15 mg/kg/day, after which females were mated with proven male breeders. The treatment regimen continued throughout mating, gestation, and parturition until weaning (P21). 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. For the PNO paradigm, dams were orally-gavaged with 15 mg/kg/day of oxy only after parturition until weaning. Upon weaning of the pups, dams were euthanized by isoflurane overdose followed by decapitation using a guillotine. To elucidate intergenerational effects in the IUO and PNO groups, untreated F1 females (P70) from each condition were mated with male breeders naïve to the experiment. F2

pups were housed with their mothers until weaning (P21). All data presented are from complete litters per condition and include both male and female offspring.

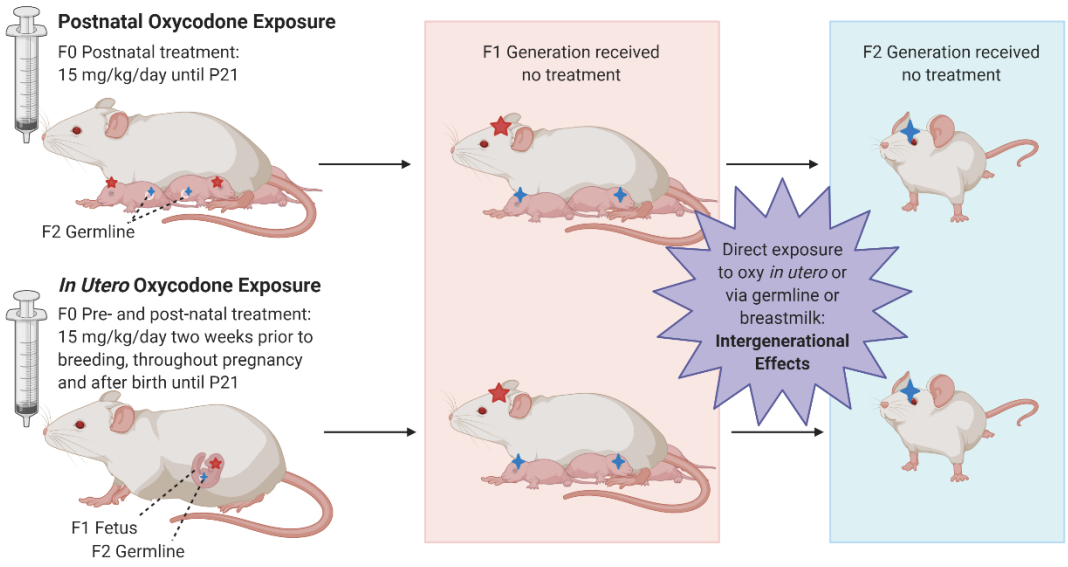


Figure 5. Schematic of dosing procedure for IUO and PNO dams and oxy exposure of F1 (red star) and F2 (blue star) generations. The intergenerational effects of oxy exposure resulting from *in utero*, germline, or breastmilk exposure are studied in the F1 and F2 generations.

MRI/MRS Acquisitions (Chapter One)

Basis Set

For these experiments, P17 and P30 F1 pups were used for *in vivo* localized ^{1}H-MRS. Animals were set in a plastic holder and anesthetized by inhalation of 1-1.5% isoflurane in 100% oxygen and maintained 40-80 breaths/minute. The duration of a study for a single animal was about 1 h. MRI and 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, if necessary, performed manually to achieve a water line width of 10–15 Hz. The water signal was suppressed by variable power RF pulses with optimized relaxation delays (VAPOR).[201] MR images were acquired for anatomical reference using a multi-slice rapid acquisition with relaxation enhancement (RARE) sequence (Effective TE = 36 ms, Rare Factor = 8, TR = 4200 ms, Number of Averages = 2, Scan Time = 3m21s; FOV = 20x20 mm^2, Matrix Size = 256x256, Spatial Resolution = 0.078125 x 0.078125 mm^2, Number of Slices = 29, Slice Thickness = 0.5 mm). ^{1}H-MRS data sets were obtained using semiLASER localization with timing parameters (TE/TR = 40/4000 ms, 576 averages, 2048 points) from a 2x5.187x1.557 mm3 (16.15 µl) VOI located in the hippocampus. Pulse types and specifications: Excitation: hermite 90˚, duration = 0.7 ms, bandwidth = 5400 Hz; 1st & 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 and NAA. For the water suppression module, the spoiler strength matrix was calculated automatically. Spoiler strength was 35% and 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 for the quantitation process.

Unsuppressed water spectra were obtained with identical metabolite spectra parameters except for the following: TR = 1s, number of averages (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 variable power RF pulses with optimized relaxation delays (VAPOR)[201] 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), Creatine (CRE), Gamma-Aminobutyric acid (GABA), Glucose (GLC), Glutamine (GLN), Glutamate (GLU), Glycine (GLY), Lactate (LAC), Myo-inositol (MYO), N-Acetyl Aspartate (NAA), Phosphorylcholine (PC), Taurine (TAU), and total choline (tCHO). 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[202] sequences at 40 ms TE, maintaining the phantom at 38 °C with a circulating water jacket during spectral acquisition. The set of metabolites spectra form what is called a *metabolite basis set,* which was used as *prior-knowledge* in the quantification process. In all groups, n = 6 for all metabolites except LAC (IUO n = 4).

All MRS data were processed and quantified using LCModel (LCMODEL Inc., CA).[203] All pre-processing was done automatically using a home-built software programmed in Matlab (MathWorks, Natick, MA). *fid(s)* signals were first transformed to the frequency domain using Fourier Transformation and spectra of the signals were obtained. Then, a *truncation* operation was used to remove the first 68 points of the *fid* that correspond to zero data introduced by the digital electronics of the system before the meaningful data stream. This was followed by a *zero filling* operation to add back 68 zero-value points at the end of the *fid* in order to maintain the signal with the 2,048 original number of points. Then, a 5-Hz *apodization* operation was applied in order to reduce noise

and truncation artifacts. All spectra were then zero-order phased and first-order phased using a time of -0.226 ms. Finally, the *fid(s)* from the water-unsuppressed acquisitions were corrected for frequency shifts using the NAA peak from the basis sets as reference and then summed for the metabolite concentration measurement. Metabolite spectra from phantoms were amplitude-normalized and corrected for frequency shifts using the reference peaks *3-(trimethylsilyl)-1-propane-sulfonic acid* and *sodium formate*. Residual water was then removed using the HLSVD filter in the Java-based magnetic resonance user's interface (jMRUI) package.[204] Finally, the resulting metabolites spectra were made into a metabolite basis set file and used as prior knowledge for the quantitation algorithms in LCModel. The water-unsuppressed "water" spectra and the water-suppressed "metabolite" spectra were then submitted to LCModel for quantitation. Concentration values of individual metabolites were output in spreadsheet for statistical analysis.

Electrophysiology (Chapter One)

Coronal hippocampal brain slices were prepared from F1 P17 pups (n = 6) using the "protected recovery" method.[205] Briefly, rats were euthanized by CO_2 asphyxiation and decapitation, and brains were rapidly dissected into a slush of artificial cerebrospinal fluid (ACSF) and continuously bubbled with a mixture of 5% CO_2 and 95% O_2. The cerebellum was removed with a razor blade, and the brain was affixed to the cutting chamber using cyanoacrylate glue. 250-micron-thick coronal brain sections through the hippocampus were cut using a vibrating microtome (Leica VT1000S) and hemisected into right and left halves through the midline before being transferred to a net submerged in an N-methyl-D-glucamine (NMDG)-based ACSF, warmed to approximately 30 °C and bubbled with 5% CO_2 and 95% O_2. After a 10- to 15-minute incubation in the NMDG ACSF, slices were transferred to a chamber containing room temperature ACSF and allowed to recover for one hour before beginning patch clamp experiments.

For whole-cell recording, slices were positioned in a recording chamber on an upright fixed-stage microscope (Olympus BX51WI) and superfused by a gravity-fed system with ACSF warmed to 29-31 °C using an in-line solution heater at approximately 4 mL/min. The ACSF was supplemented with 60 µM picrotoxin. A concentric bipolar stimulating electrode was positioned in the *stratum radiatum* to stimulate Schaffer collateral axons using a 0.1 ms current delivered at 0.1 Hz from an isolated pulse stimulator (A-M Systems Model 2100). CA1 pyramidal neurons were targeted for whole-cell recording with patch pipettes pulled from thin-walled borosolicate glass on a Sutter P-1000 micropipette puller. The patch pipettes had a resistance of 5-8 MΩ when filled with solution (materials table). Reported voltages were corrected for a 10 mV liquid junction potential. The intensity-response profile of the evoked excitatory post-synaptic currents (EPSCs) for each cell was determined by the average of 3-10 responses obtained at each stimulus strength (50-225 µA). The AMPA/NMDA ratio was measured as the ratio of the peak of the inward EPSC recorded at -70 mV to the outward EPSC amplitude at 50 ms post-stimulus at a holding potential of +40 mV. Miniature EPSCs (mEPSCs) were recorded in the absence of a stimulus and were detected and analyzed using MiniAnalysis (Synaptosoft). mEPSC frequency for each recorded cell was determined as the median of the instantaneous frequencies of all detected events for that cell. Due to variability in the number of cells patched and recorded, respective samples sizes are provided in Figures 8 and 9.

Total RNA extraction, Quality control, library preparation, small RNA-Seq

F1 P14 PFC (Chapter One)

Total RNA from prefrontal cortex (PFC) tissue was isolated from the randomly selected pups (n = 6) from each treatment group at P14 using the Direct-Zol RNA kit (Zymo Research, CA, USA) based on the manufacturer's protocol. Samples were sent to

UNMC's Next Generation Sequencing (NGS) core. RNA-seq libraries were generated beginning with 1 µg of total RNA from each sample using the TruSeq V2 RNA sequencing library kit from Illumina following recommended procedures (Illumina Inc., San Diego, CA). Resultant libraries were assessed for size of insert by analysis of an aliquot of each library on a BioAnalyzer instrument (Agilent Technologies, Santa Clara, CA). Each library contained a unique indexing identifier barcode allowing the individual libraries to be multiplexed together for efficient sequencing. Multiplexed libraries (18 samples per pool) were sequenced on a single flow cell of the NextSeq550 DNA Analyzer (Illumina) to generate a total of approximately 28 million 75-bp single reads for each sample.

F1 and F2 P14 NAc (Chapter Three)

Total RNA from nucleus accumbens (NAc) tissue was isolated from the randomly selected male or female pups of each treatment group at P14 using the Direct-Zol RNA kit (Zymo Research, CA, USA). RNA samples were sent on dry ice to LC Sciences (Houston, TX, USA) for sequencing. Transcriptomes from all samples were merged to reconstruct a comprehensive transcriptome using a proprietary Perl script of LC Sciences (Houston, Texas, U.S.A.). Following transcriptome reconstruction, FPKM (Fragments Per Kilobase Million) reads were evaluated by StringTie, and differentially expressed genes (DEGs) were evaluated by edgeR. Potential hits having ±1.5 fold expression and $p < 0.05$ were validated using relevant TaqMan probes by real-time PCR (RT-PCR). Further analysis into the Hcrt system was similarly done using RT-PCR TaqMan probes. Delta-delta Ct method[206] was used to calculate fold change and statistical significance.

Bioinformatic Analyses (Chapters One, Two, and Three)

In Chapter One, differentially expressed genes (up- and down-regulated) between SAL and PNO, SAL and IUO, and PNO and IUO were chosen for further functional characterization using ClueGO plug-in module[207] in Cytoscape software.[208] The 'biological

process' option in Clue-Go analysis was used to visualize the categories of DEG functions in each comparison. In Chapter Two, proteins were identified as differentially expressed if the FDR-corrected P-value was ≤ 0.05 and log2 fold change was ≥ 2. Heatmaps of the top 25 proteins in each comparison were plotted using the function heatmap.2 in the R (version 3.6.0) package *gplots*. Gene Ontology (GO) analysis of differentially expressed proteins were performed using the Cytoscape plugin ClueGO.[207] Biological process, molecular function, and KEGG pathways were included for GO enrichment analysis. Enriched disease-associated pathways were identified using the Ingenuity Pathway Analysis (IPA) software (Ingenuity® Systems, CA, USA, www.ingenuity.com). Canonical pathway analysis in IPA was performed by comparing the differentially expressed proteins against known canonical pathways (signaling and metabolic) within the IPA database. In Chapter Three, functional relevance of DEGs (up- and down-regulated) was evaluated using the Cytoscape plug-in ClueGO.[207] A kappa score level threshold of 0.4 was used to restrict the GO network connectivity with three as minimum and eight as maximum level of the genes in each GO term.

Synaptic Vesicle Isolation (Chapter Two)

To investigate the effects of *in utero* and post-natal oxy exposure on synaptic transmission, we isolated synaptic vesicles (SVs) following the protocol designed by Ahmed *et al.,*[209] with minor modifications. Briefly, one hemisphere of P14 rat brains (~500 mg) from each group (n = 6) was homogenized in 9 ml of ice-cold homogenization buffer using a tight-fitting glass-Teflon homogenizer with ten strokes at 2k – 3k RPM. 100 µl of homogenate was saved for use in western blot. The homogenized mixture was centrifuged at 2,700 RPM (1,000 *g*) for 11 min at 4 °C, and the pellet (P1; cell fragments and nuclei) was discarded. The supernatant was centrifuged at 11,000 RPM (15,000 *g*) for 16 min at 4 °C. The resulting supernatant (S2) was collected in a 15-ml tube. The pellet (P2;

synaptosomes) was washed carefully in 1 ml of homogenization buffer, leaving the brown center of the pellet (mitochondria) remaining on the tube. 100 µl of P2 was saved for use in western blot. The resuspended P2 was transferred to a tight-fitting glass-Teflon homogenizer, and 9 ml of ice-cold double distilled water was added to perform osmotic lysis. Osmotic lysis was accomplished by performing three strokes at 2,500 RPM. 50 µl of 1 M HEPES and protease inhibitors were added immediately after the last stroke. The osmotic lysis product was centrifuged at 12,000 RPM (17,000 *g*) for 19 min at 4 °C in a swinging Ti41 ultracentrifuge rotor. The resulting supernatant (LS1) was combined with S2. The LS1/S2 mixture was centrifuged at 20,000 RPM (48,000 *g*) for 29 min at 4 °C in a swinging Ti41 ultracentrifuge rotor. The resulting supernatant (CS1) was transferred to a tight-fitting glass-Teflon homogenizer and homogenized using 3 – 5 strokes at 2k – 3k RPM. To ensure disruption of SV clusters, the homogenized CS1 was drawn through a 20-gauge hypodermic needle attached to a 10-ml syringe and expelled through a 27-gauge needle. 5 ml of CS1 was carefully layered onto a 5.5-ml 0.7 M sucrose cushion and centrifuged at 38,000 RPM (133,000 *g*) for 1 hour at 4 °C in a swinging Ti41 ultracentrifuge rotor.

After centrifugation, 350-µl fractions were collected, starting from the top of the gradient, resulting in 30 350-µl fractions. The white pellet at the bottom of the gradient was resuspended in 200 µl of homogenization buffer. 50 µl of each fraction and the resuspended pellet were collected for use in dot blot. The remaining resuspended pellet was homogenized using a handheld homogenizer. Fractions 16 – 30 and the pellet were combined and centrifuged at 245,000 *g* for 2 h and 35 min at 4 °C in a swinging Ti41 ultracentrifuge rotor. These fractions were used based on purity verification using dot blotting. The supernatant was discarded, and the pellet was resuspended in 200 µl of 1x PBS. The ultracentrifugation tube was washed again with 100 µl of 1x PBS to ensure complete reconstitution of the pellet. The resuspended SVs were drawn through a 27-

gauge hypodermic needle attached to a 1-ml syringe and expelled. 100 µl of the SV was saved for western blotting. SVs were stored at -80 °C. Protein quantification of the isolated SVs was carried out using Pierce BCA protein assay (Thermo Scientific, IL, USA). A schematic of the SV isolation process can be found in Figure 6.

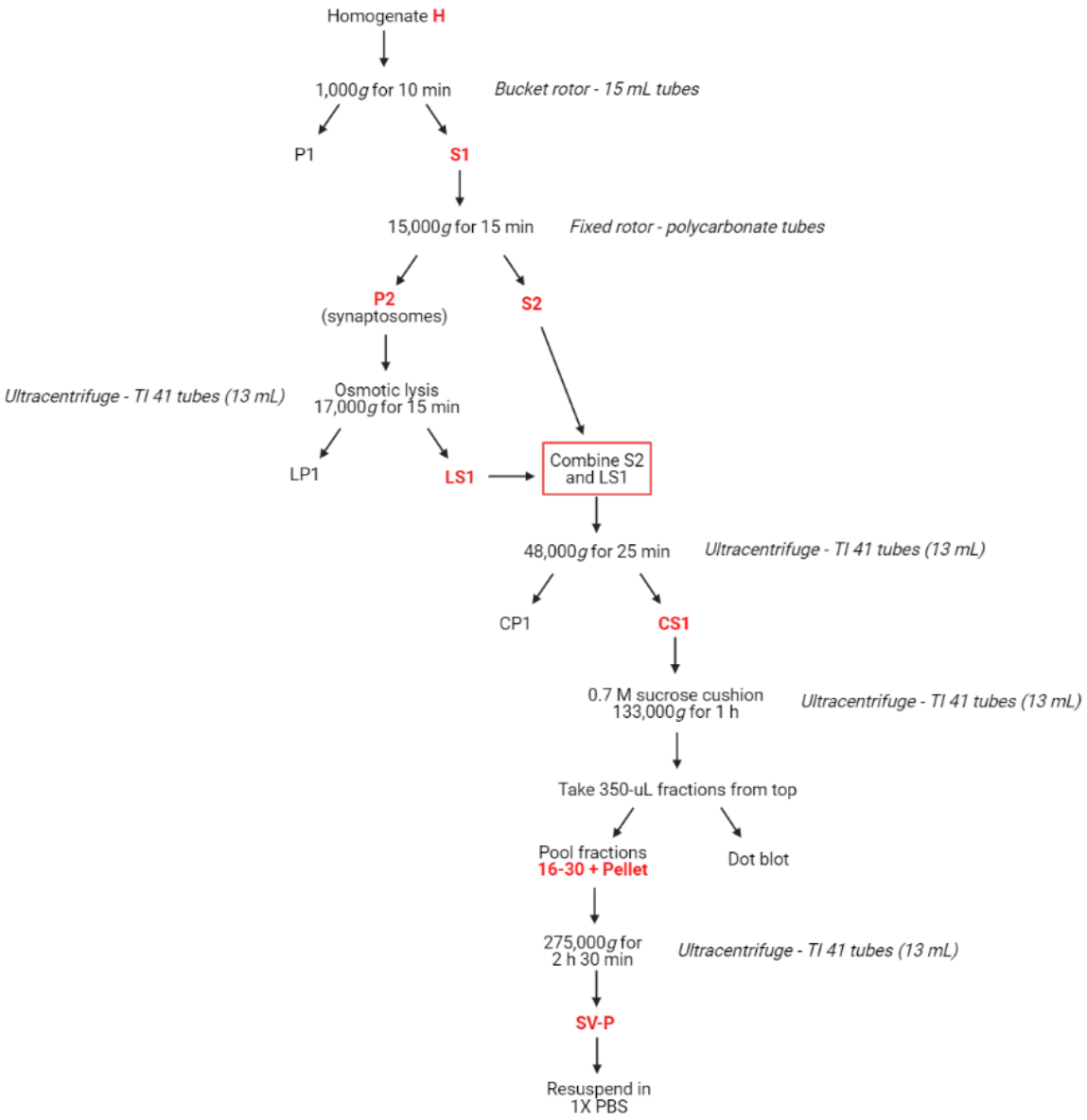


Figure 6. Schematic of synaptic vesicle isolation adapted from Ahmed *et al.*[209]

Dot Blot (Chapter Two)

To ensure the purity of the isolated SVs, 2 µl of each of the 30 separated fractions and the pellet from the SV isolation were blotted onto a nitrocellulose membrane separated into grids. Samples were added slowly so the area of the absorbed drop of sample was 2 – 4 mm in diameter. The membrane was allowed to dry before blocking non-specific sites using 5% BSA in TBS-T for 30 minutes to 1 hour. The membrane was incubated with primary antibody diluted in 0.1% BSA in TBS-T for 30 min at room temperature. Anti-PSMC6, a proteasome marker, was used to determine fraction purity and ensure no fractions with proteasome contaminants continued into the sucrose cushion step of the isolation protocol. After incubation, the membranes were washed three times with TBS-T for 5 min each. The secondary antibody was diluted according to the manufacturer's protocol in 0.1% BSA in TBS-T and added to the membrane for 30 min at room temperature. Secondary antibodies were HRP-conjugated anti-rabbit IgG. The membrane was washed three times with TBS-T for 5 min each. Membranes were allowed to sit in SuperSignal West Pico Chemiluminescent Substrate for 1 min before imaging with the Azure cSeries Imager (Azure Biosystems, CA, USA).

Western Blot (Chapter Two)

SVs (10 – 20 µg) from each animal were loaded into 4–12% Bis-Tris wells (Invitrogen, MD, USA) under reducing conditions, followed by transfer to a nitrocellulose membrane using iBlot2 (Invitrogen) and immunodetection. Nonfat milk (5%) was used to block nonspecific antibody binding. After blocking, membranes were incubated overnight at 4 °C with primary antibody. Primary antibodies included GAPDH, PSD95, SYP, VGLUT1, and SNAP25. MEGF8 and LAMTOR4 were additionally selected from the proteomic analysis for post-validation. Secondary antibodies were HRP-conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG. Blots were developed using Azure

cSeries Imager (Azure Biosystems, CA, USA) with SuperSignal West Pico Chemiluminescent Substrate.

Mass Spectrometry (Chapter Two)

50 µg of SV protein per sample (n = 6) per group was taken and detergent was removed by chloroform/methanol extraction. The protein pellet was re-suspended in 100 mM ammonium bicarbonate and digested with MS-grade trypsin overnight at 37 °C. Peptides cleaned with PepClean C18 spin columns (Thermo Scientific) 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 PepMap 100 75 µm x 2 cm C18 LC Columns (Thermo Scientific) at a flow rate of 4 µl/min, then separated with a Thermo RSLC Ultimate 3000 (Thermo Scientific) on a Thermo Easy-Spray PepMap RSLC C18 75 µm x 50cm C-18 2 µm column (Thermo Scientific) with a step gradient of 4–25% solvent B 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. Eluted peptides were analyzed by a Thermo Orbitrap Fusion Lumos Tribrid (Thermo Scientific) mass spectrometer in a data-dependent acquisition mode. A survey full scan MS (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. The most intense ions with charge state 2 – 6 were isolated in 3-sec cycle and fragmented 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. Protein identification was performed by searching MS/MS data against the Swiss-Prot *Rattus norvegicus* protein database downloaded in May, 2019, using the in-house mascot 2.6.2 (Matrix Science, MA, USA) search engine. The search was set up for full tryptic peptides with a maximum of two missed cleavage sites. Acetylation of protein N-terminus

and oxidized methionine were included as variable modifications and carbamidomethylation of cysteine was set as fixed modification. The precursor mass tolerance threshold was set at 10 ppm and maximum fragment mass error was 0.02 Da. The significance threshold of the ion score was calculated based on a false discovery rate (FDR) of ≤ 1%. Qualitative analysis was performed using progenesis QI proteomics 4.1 (Nonlinear Dynamics, MA, USA).

Phenotypic Measurements (Chapter Three)

Body measurements included weight, body length, and head size circumference and were obtained from the complete litters at P1, P7, P14, and P30. Body mass index (BMI) and Lee's Obesity Index (LOI) were calculated as described by Novelli *et al*.[210]

Behavioral Studies

Von Frey (Chapter One)

Von Frey experiments were conducted (n = 4) from each group at P17, and the same animals were tested at P75. The test was commenced as the rats placed four paws comfortably on the mesh floor and the plantar were clearly visible. The examiner randomly picked the left or right hind paw as the first evaluated paw during each assessment. A monofilament Von Frey hair was applied exactly vertical on the plantar surface until the hair buckled and the shape of the hair was held for 5 s. The specific value of the forces chosen were: 0.6, 1.0, 1.4, 2.0, 4.0, 6.0, 8.0, 10.0 and 15.0 g. The cut-off force was set as 15.0 g because the paw would be lifted if the next force (26.0 g) was applied. During the measuring process, each force was applied 10 times with an interval of at least 5 s to allow the animal to recover from the previous stimuli. Noxious responses were determined if any of the following robust reflex responses occurred: paw retracting, paw withdrawal, or paw licking. Once there were 4 positive responses in 10 applications, the force was determined

as the mechanical withdrawal threshold. The mechanical withdrawal threshold for both of two paws was recorded.

Social Testing (Chapter Three)

Social testing consisting of social novelty and social preference were carried out in P60-65 F1 and F2 male and female rats from the different treatment groups using an in-house built chamber. Briefly, a 90x40x40-cm acrylic chamber was divided into three 30x40x40-cm compartments. Left and right compartments contained 15x15x30-cm isolation cubes with evenly-drilled holes spaced 1 cm apart along the entirety of the cube.

To evaluate social novelty, a naïve 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 naïve 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.

Marble Burying (Chapter Three)

Marble burying was tested on all three groups on male and female pups between ages P65-P70. A rat cage (929 cm^2, 43.18x21.59x20.32 cm) contained a leveled 5-cm layer of ¼" corncob bedding (Envigo #7097), and 20 standard glass marbles (15 mm diameter, 5.2 g) were lightly placed in a 5x4 arrangement along 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 2/3 of a marble was under the bedding.

Data and Statistical Analyses

All data represented in this thesis are reported as mean ± SEM. Data in each analysis were normally distributed. Significant differences were computed using Welch's t-test (Electrophysiology and Western blot) and two-way ANOVA (Von Frey, MRI/MRS data, social testing) followed by Tukey's test with a significance criterion of $p \leq 0.05$. Significant intergenerational differences were computed using two-way ANOVA followed by Tukey's test with a significance criterion of $p \leq 0.05$. Main interactions in intergenerational studies were further analyzed independently within generations using one-way ANOVA followed by Tukey's test with a significance criterion of $p \leq 0.05$. Data were analyzed using the Graph Pad Prism software (La Jolla, CA, USA).

CHAPTER ONE

A Holistic Systems Approach to Characterize the Impact of Pre- and Post-natal Oxycodone Exposure on Neurodevelopment and Behavior

Abstract

Increased risk of oxycodone (oxy) dependency during pregnancy has been associated with altered behaviors and cognitive deficits in exposed offspring. However, a significant knowledge gap remains regarding the effect of *in utero* and postnatal exposure on neurodevelopment and subsequent behavioral outcomes. Using a preclinical rodent model that mimics oxy exposure *in utero* (IUO) and postnatally (PNO), we employed an integrative holistic systems biology approach encompassing proton magnetic resonance spectroscopy (^{1}H-MRS), electrophysiology, RNA-sequencing, and Von Frey pain testing to elucidate molecular and behavioral changes in the exposed offspring during early neurodevelopment as well as adulthood. ^{1}H-MRS studies revealed significant changes in key brain metabolites in the exposed offspring that were corroborated with changes in synaptic currents. Transcriptomic analysis employing RNA-sequencing identified alterations in the expression of key genes associated with synaptic transmission, neurodevelopment, mood disorders, and addiction in the treatment groups. Furthermore, Von Frey analysis revealed lower pain thresholds in both exposed groups. Given the increased use of opiates, understanding the persistent developmental effects of these drugs on children will delineate potential risks associated with opiate use beyond the direct effects in pregnant women.

Introduction

Over the last few years, the increasing trend in opioid abuse has become a major public health crisis across the globe. As a result, this steep increase in abuse of prescription opioids, which include both licit and illicit opioids, has resulted in the opioid epidemic.[211] Whilst this epidemic has traversed different groups in the society, pregnant women are a particularly vulnerable group since they are prescribed opioids such as morphine, buprenorphine, and methadone, all of which have been shown to cross the placenta,[212-214] potentially impacting the developing fetus. Limited data exist regarding the effects of *in utero* (IUO) or postnatal (PNO) exposure to oxycodone (oxy), however. Oxy is prescribed for multiple types of pain and can bind to mu- and kappa-opioid receptors.[215] Oxy easily passes through the blood-brain barrier, thus allowing higher concentrations to accumulate in the brain,[216-218] subsequently contributing to its analgesic properties and risk for dependency and addiction.

Several studies[219] have been conducted with rodent models to investigate the detrimental effects of gestational opioid use on neurodevelopment of the offspring, but a gap in knowledge exists regarding the effects of IUO or PNO oxy exposure on synaptogenesis. We have previously identified novel miRNA signatures related to neurodevelopment contained within brain-derived extracellular vesicles of PNO and IUO offspring,[185] and our current study aims to investigate metabolic, synaptic, molecular, and behavioral alterations in these exposed offspring. Using a Sprague Dawley rat model previously established by our labs,[138,185] we employed proton magnetic resonance spectroscopy (^{1}H-MRS) to measure biochemical changes of main brain metabolites in the hippocampus. Additionally, we identified synaptic alterations in the hippocampus through the use of electrophysiology experiments. Further, RNA-sequencing (RNA-seq) was conducted on tissue RNA isolated from the prefrontal cortex (PFC) to determine changes in gene expression, particularly in genes related to neurodevelopment, disease states,

and mood disorders. The hippocampus and the PFC are key regions involved in substance abuse disorders and the negative emotional state associated with withdrawal;[220] indeed, systemic opioid exposure has been shown to attenuate hippocampal afferent-driven activity in the PFC.[221] For these reasons, we have investigated both regions in this study to identify alterations in either area of the brain during the early developmental period spanning from post-natal day 14 (P14) to P17, which corresponds with peak synaptogenesis.[222] In our final experiments, we employed Von Frey tests to elucidate any lasting impacts of early-life oxy exposure on pain thresholds. The comprehensive and systematic approach used in this study allows for thorough research into pre- and post-natal oxy abuse, a critical step in closing the knowledge gap surrounding this commonly used opioid analgesic.

Results

Quantitation of metabolites in oxy-exposed pups

Brain metabolites are spatiotemporally regulated during development.[223] However, it is unknown whether IUO or PNO exposure influences the expression levels of these metabolites in the offspring. Accordingly, we conducted ^{1}H-MRS scans on the brain hippocampus of P17 and P30 saline, PNO, and IUO groups (Figure 7). We found that IUO or PNO treatment did affect metabolite concentrations in these animals (Metabolite: $F_{(13, 208)} = 96.75$, $P < 0.0001$; Treatment: $F_{(2, 208)} = 5.520$, $P = 0.0046$; Interaction: $F_{(26, 208)} = 2.093$, $P = 0.0023$). Specifically, we identified higher levels of neurotransmitter aspartate (ASP) and glutamate (GLU) in both PNO and IUO groups. However, N-acetyl aspartate (NAA), the second most abundant metabolite in the brain, was significantly elevated in the IUO group. Additionally, taurine (TAU) concentration was elevated in the PNO group but was significantly lower in the IUO offspring compared to controls. At P30, ^{1}H-MRS scans revealed that both PNO and IUO groups had lower levels of NAA than controls, and IUO

levels were lower than those of PNO, similar to when they were measured at P17 (Metabolite: $F_{(13,\ 194)}$ = 101.5, P < 0.0001; Treatment: $F_{(2,\ 194)}$ = 3.746, P = 0.0253; Interaction: $F_{(26,\ 194)}$ = 2.710, P < 0.0001). Intriguingly, TAU levels were higher in the IUO group compared to both PNO and control groups (Fig 2B). Together, these data point to alterations in key metabolite levels in both the PNO and IUO groups that are more pronounced in the latter.

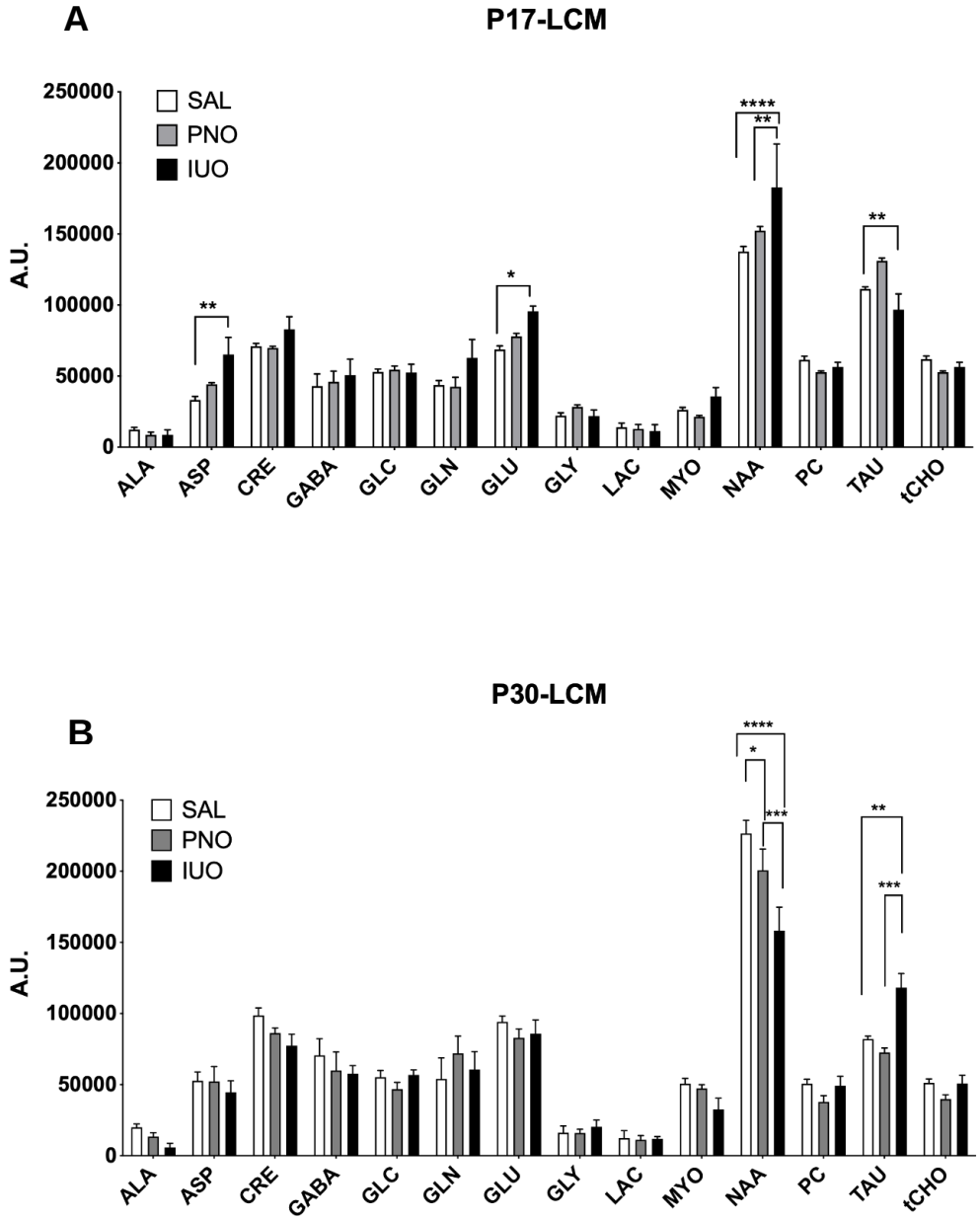


Figure 7. Metabolite concentrations in the brain measured by ^{1}H-MRS at P17 and P30 in PNO and IUO animals. Measured metabolites included Alanine (ALA), Aspartate (ASP), Creatine (CRE), Gamma-Aminobutyric acid (GABA), Glucose (GLC), Glutamine (GLN), Glutamate (GLU), Glycine (GLY), Lactate (LAC), Myo-inositol (MYO), N-Acetyl Aspartate (NAA), Phosphorylcholine (PC), Taurine (TAU), and total choline (tCHO). A) In all groups at P17, n = 6 for all metabolites except Lac (n = 4 in the IUO group). IUO animals showed higher concentrations of Asp, Glu, and NAA compared to controls. IUO had a lower concentration of Tau than controls. B) For all groups at P30, n = 6 except GABA (IUO n = 5), GLU (SAL n = 5), and GLY (SAL n = 5). For P30 ALA, n = 3 for each group except IUO (n = 5). For LAC, n = 4 for each group. IUO and PNO had lower concentrations of NAA, and IUO offspring had a higher concentration of Tau compared to control and PNO animals. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Synaptic alterations in IUO and PNO offspring

CA1 synapses were monitored in hippocampal slices of control, PNO, and IUO rats (Figures 8 and 9). Input-response curves showed that the post-synaptic currents in the PNO group cells were smaller than control (p = 0.02, p = 0.017; Figure 8). Although the AMPA/NMDA ratio in PNO rats appeared reduced compared to controls, the difference was not significant (p = 0.07; Figure 8). The paired pulse ratio (PPR) of post-synaptic currents did not significantly differ among the three groups, suggesting no change in presynaptic vesicle release probability (Figure 8). When measuring miniature excitatory post-synaptic currents (mEPSCs), we found that, although the frequency and amplitude of the currents did not differ between groups, the PNO mEPSCs had slightly faster decay kinetics (p = 0.0048; Figure 9), which is consistent with altered AMPA receptor subunit composition. Together, these data point to altered synaptic maturation in the PNO offspring.

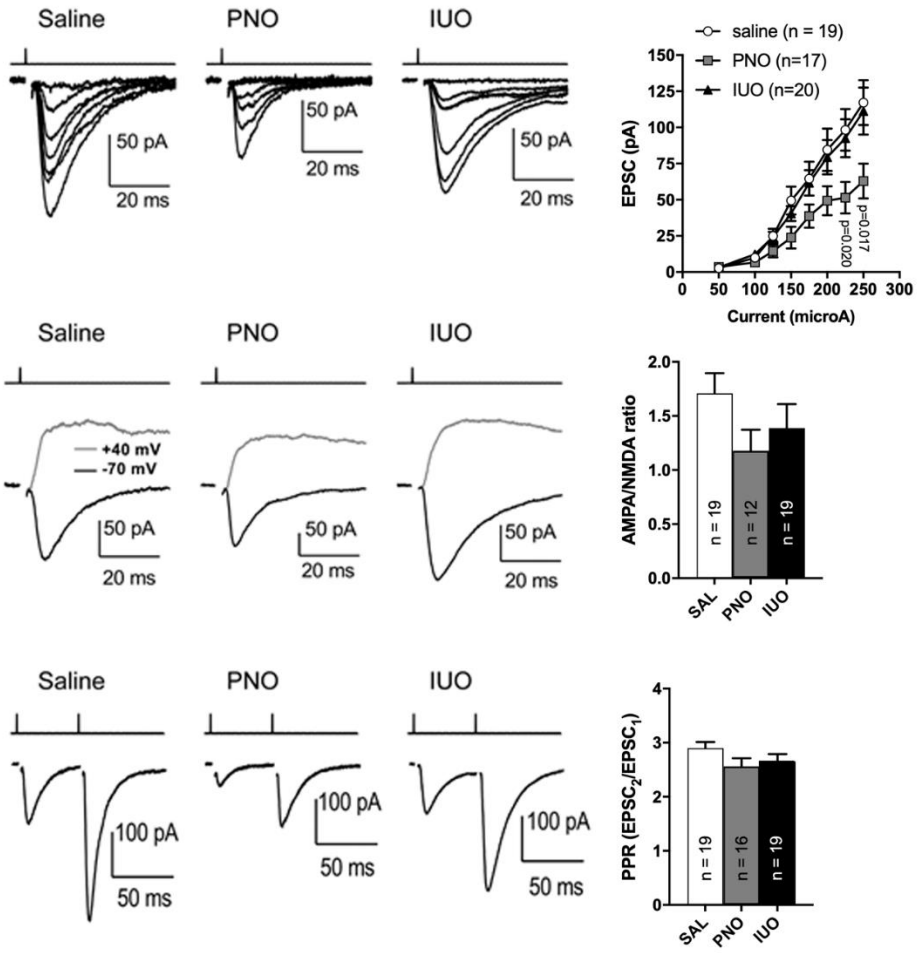


Figure 8. Evoked EPSC monitored in hippocampal slices of control, PNO, and IUO rats. Evoked EPSC data; Top: A series of EPSCs recorded in each of the three treatment conditions in response to a series of stimulus strengths (50-225 μA). Stimulus timing is marked above the traces. Group data show the intensity-response profiles for cells recorded from Saline, PNO, and IUO rats. Middle: AMPA receptor and NMDA receptor-mediated EPSCs recorded by voltage-clamping CA1 pyramidal cells at -70 and +40 mV, respectively. The AMPA receptor component was measured at the peak of the inward EPSC while the NMDA receptor component was measured 50 ms post-stimulus. Group data of AMPA/NMDA ratios show there was no significant difference between the groups. Bottom: Paired pulse traces showing the characteristic synaptic facilitation in response to a pair of pulses separated by 50 ms. Group data show the paired pulse ratio was not significantly different between treatment conditions. Sample sizes of recorded cells are shown in the bars of each graph; all data represented as mean ± SEM.

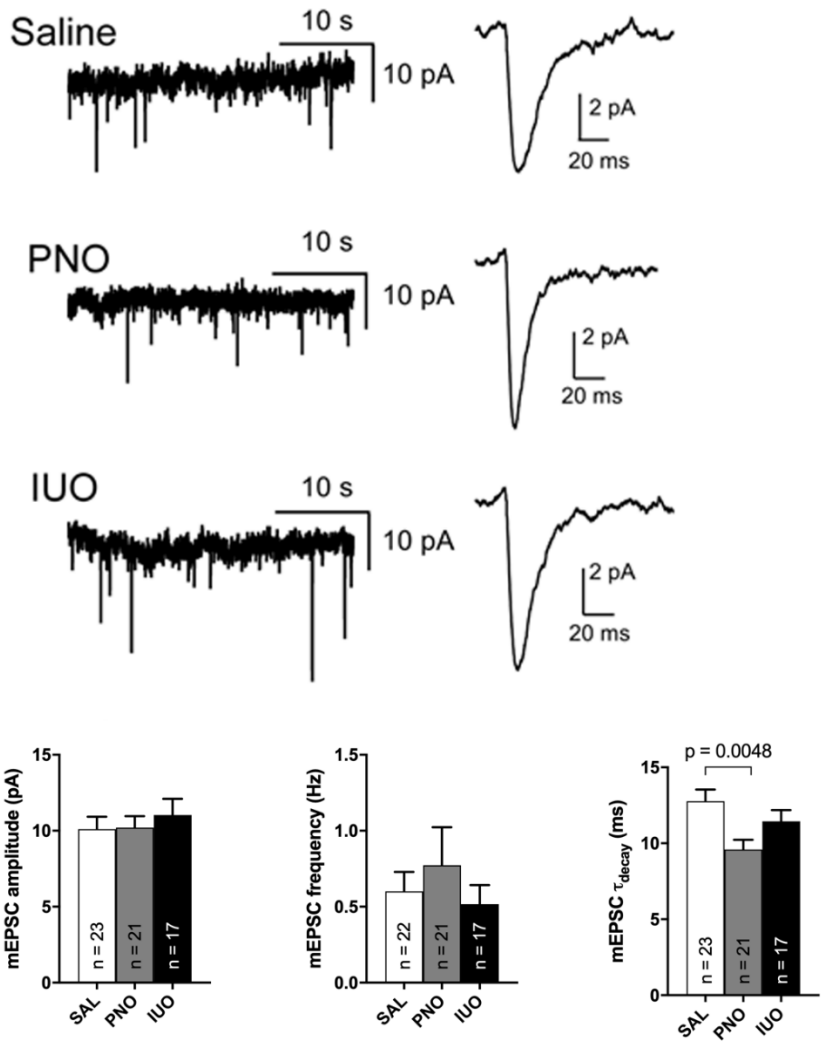


Figure 9. mEPSC monitored in hippocampal slices of control, PNO, and IUO rats. Traces of mEPSCs recorded in the absence of stimulation followed by mEPSCs waveforms from individual cells. These were obtained by averaging all detected events in individual cells. Group data show that mEPSC amplitudes and frequencies were not significantly different between treatment groups. Group data of mEPSC decay time constants (τ_{decay}), show that mEPSCs in the PNO group decayed more quickly than in control. Sample sizes of recorded cells are shown in the bars of each graph; all data represented as mean ± SEM.

RNA-seq highlights gene expression changes in oxy-exposed pups

To further understand the molecular causes associated with changes in synaptic currents, we performed RNA-seq analysis on the PFC (Figure 10A). Employing a criteria of 1.5 fold change and $p < 0.05$, we found 62 genes (20 up and 42 down) between saline and PNO and 161 genes (78 up and 83 down) between saline and IUO. When comparing the PNO and IUO groups, we found 1,465 genes (1,199 up and 266 down). We found three genes (*Sytl2* – Synaptotagmin-like 2, *Vwa5b1* – Von Willebrand Factor A Domain-Containing Protein 5B1, and a predicted gene AABR07042623.1) were differentially regulated among all three groups.

Next, the enriched biological pathways associated with these differentially expressed proteins were determined using Clue-GO analysis (Figure 10B). Notably, pathways involved in synaptic transmission and morphine, nicotine, and alcohol addictions were significantly enriched in the two treatment groups. Furthermore, the opioid signaling pathway was affected in the PNO and IUO offspring. A number of the genes affected in the IUO and PNO pups were associated with diseases and psychotic disorders (Table 1). To summarize, oxy exposure can significantly affect neurodevelopment in exposed offspring by inducing changes in key genes and pathways during synaptogenesis that could persist during late adolescence and adulthood.

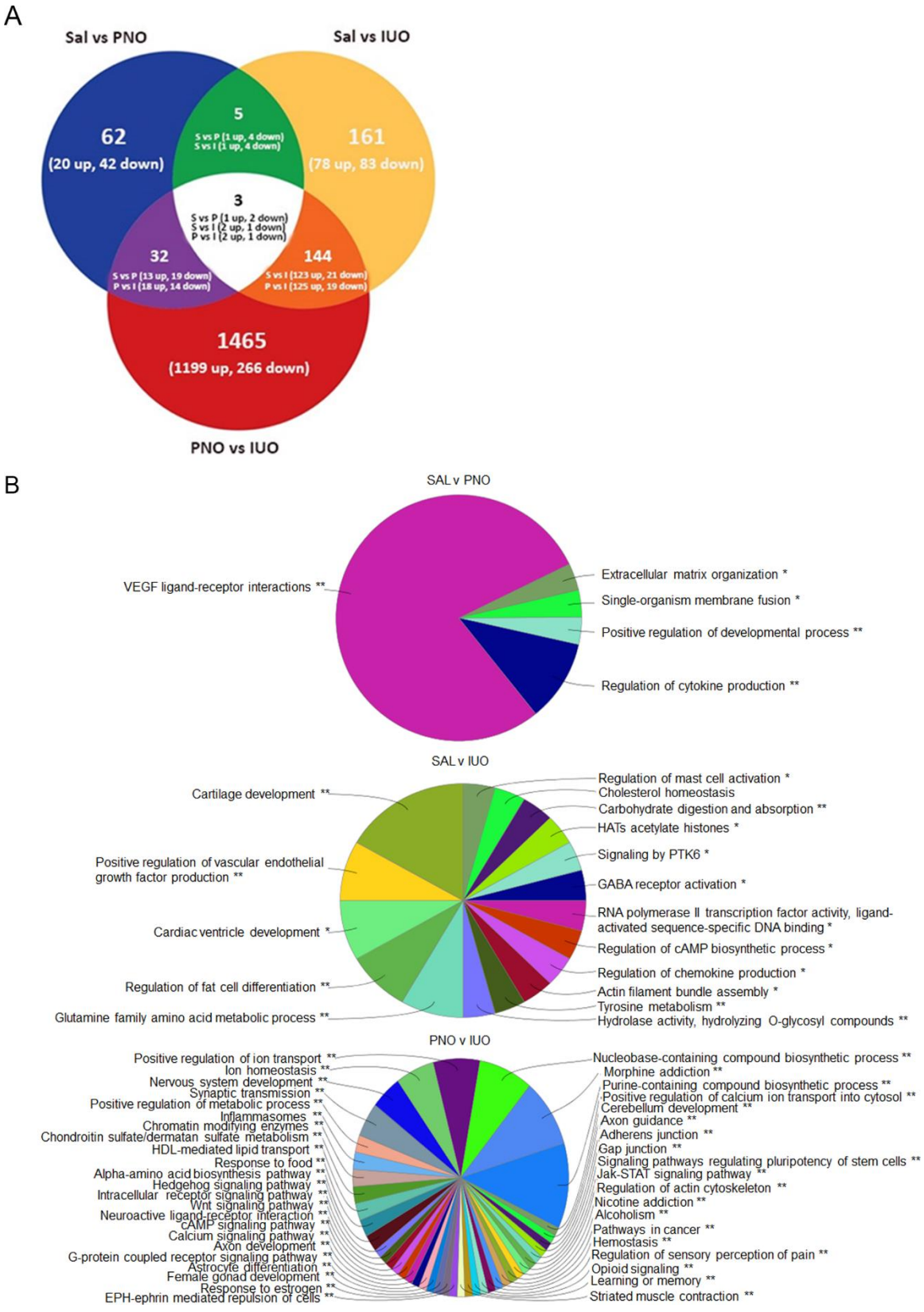


Figure 10. Differentially expressed genes at P14 identified using RNA-Seq. RNA was

isolated from the PFC regions of P14 brains (n = 6) for each group. Mapping of biological processes in the PFC. Clue-Go pie diagrams resulting from RNA-seq data show enriched biological processes involved in developmental, neurological, and psychological disorders, which are more impacted in the IUO offspring. The asterisks represent the group term p-value representing each category. * $p < 0.05$, ** $p < 0.01$.

Table 1. Differentially expressed genes identified in PFC from both IUO and PNO groups that are related to diseases or psychotic disorders. A criterion of ±1.5 fold change, $p < 0.05$, and FDR < 5% was used.

Disease or Psychotic Disorder	Genes Associated with the Disorder
Alcohol-related birth defect	NTRK2
Alcohol withdrawal syndrome	GAD2
Anhedonia	OPRK1, PDYN
Cannabis dependence	GABRA2
Chronic schizophrenia	CYP3A4
Clinical depression	ITGAL, NPY
Cocaine dependence	OPRK1, PDYN, DRD3, GABRA2, OPRM1, NPY
Delirium	DRD3
Depression, postpartum	OPRM1
Dysphoric mood	PDYN, OPRM1
Dysthymic disorder	NTRK2
Fetal alcohol spectrum disorders	BCL2
Susceptibility to schizophrenia	MYH9, PLP1
Manic symptom	DNAH8, NR3C2, CSRP1
Non-organic psychosis	OPRM1, TBX1, CDH17, PDE10A, CSNK1E, DLX1, SP8, SP3
Paranoid schizophrenia	NTRK2
Psychoses, drug	DPYSL2, DRD1
Recurrent depression	DRD1, DRD3
Schizoaffective disorder	GRIA1, NPY, PCDH11Y, GABRB1
Schizophrenia, catatonic	CELSR1
Seasonal affective disorder	HTR2C, PER3, NPY, RORA
Severe depression	ESR2
Wernicke-Korsakoff syndrome	ALDH2

Pain sensitivity in oxy-exposed pups

One key pathway we identified from our RNA-Seq analysis was regulation of sensory perception of pain. Because oxy is prescribed for pain management, we investigated whether PNO or IUO exposure has lasting effects on pain sensitivity. Von Frey testing was conducted at P17 (pups exposed to oxy via the breastmilk) and on the same animals at P75 (adulthood) after a sustained absence of oxy exposure (Figure 11). While no significant differences in the pain threshold were observed in the PNO or IUO at P17, both groups displayed a significantly lower pain threshold than controls at P75 (Age: $F_{(3, 242)} = 455.3$, $P < 0.0001$; Treatment: $F_{(2, 242)} = 88.13$, $P < 0.0001$; Interaction: $F_{(6, 242)} = 31.18$, $P < 0.0001$). These data suggest a lasting impact of early life oxy exposure on pain sensitivity during adulthood.

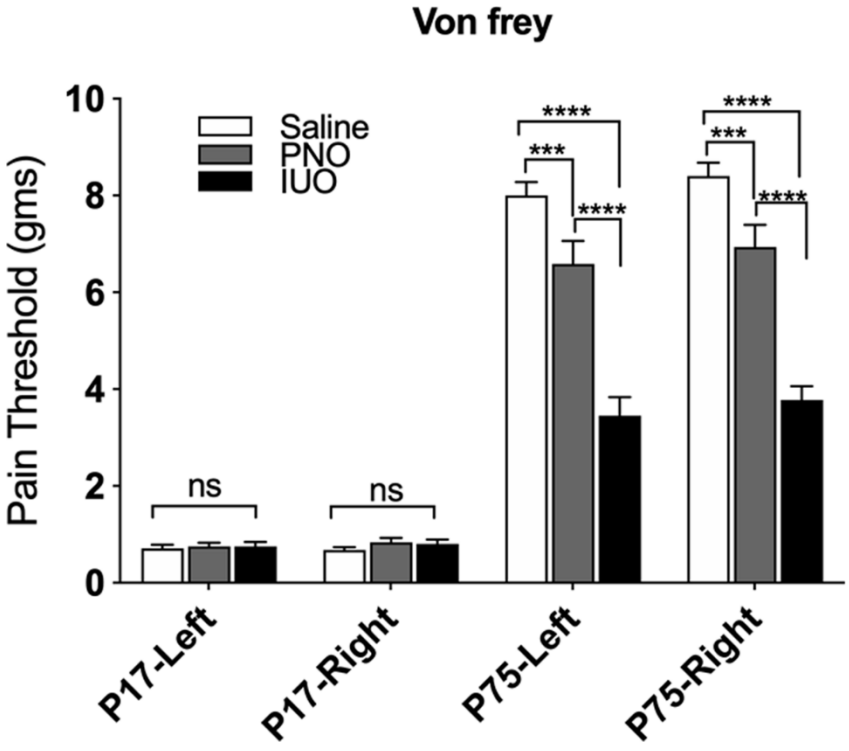


Figure 11. Measurement of pain thresholds using the Von Frey test. Animals from each group (n = 4) were tested at P17 and again at P75 to determine changes in pain thresholds. At P75, the oxy-exposed groups have lower pain thresholds than controls. Additionally, IUO pups had lower pain thresholds than PNO pups. All data represented as mean ± SEM. *** $p < 0.001$, **** $p < 0.0001$.

Discussion

While previous studies have reported poor neurodevelopmental outcomes in offspring exposed to opioids,[180,182,183,224] a comprehensive analysis comparing changes in neurodevelopment with pre- and post-natal exposure to drugs has not been evaluated. In the present study we show for the first time a comparative analysis on alterations in metabolic, synaptic, molecular, and behavioral changes in offspring exposed to the oxy pre- and post-natally. Additionally, the PNO and IUO groups are clinically relevant.[138,185] The dose of oxy (15 mg/kg) used in our study has been shown to be well-tolerated in animals and mimics the development of chronic analgesia in humans.[225,226] Importantly, the PNO group established by our studies represents neonates in the neonatal intensive care unit (NICU) that may be exposed to high-dose opiates through infusions. Infants supported by extracorporeal membrane oxygenation (ECMO) and mechanical ventilation are exposed to opiates for sedation and analgesia,[227] and in some cases these infants may require support for three weeks or longer, exposing the newborns to potent opiates for an extended period of time and contributing to the increase in neonatal abstinence syndrome diagnoses.[228,229] Our PNO group provides a high-dose opiate exposure comparable to the high-dose opiate exposure neonates in the NICU experience, thus allowing us to fill a gap in knowledge regarding the implications of these opiate drips on the neurodevelopment of newborns.

The use of both PNO and IUO groups in this study was critical to elucidate the extent of oxy exposure effects on neonates. While the PNO group was exposed to oxy only via the breastmilk, the IUO group was exposed via placental concentrations of oxycodone throughout gestation as well as via the breastmilk. Opiates have been shown to pass into the placenta and act on fetal opioid receptors.[212-214] Oxy as a postoperative analgesic has been reported in the literature for postpartum pain or caesarian sections in lieu of morphine drips.[230,231] Because both PNO and IUO groups experienced postnatal

exposure via the breastmilk, it is important to note that the degree of exposure of an infant to a drug passed through the breastmilk depends on the concentration of the drug in the milk, the amount of milk ingested, and the rate of elimination from the infant.[232] A human study by Seaton *et al*. has shown that oxycodone is concentrated in the breastmilk, and offspring exposed via the breastmilk may receive less than 10% of a typical oral therapeutic infant dose (0.1–0.2 mg/kg).[233] Despite this low dose, infant exposure to oxy via the breastmilk has been associated with sedation and central nervous system depression,[234] and a number of animal studies have also revealed deficits in behavior and development associated with perinatal opioid exposure.[180,182,183,224]

The effects of pre- and post-natal oxy use on brain chemistry have not been clearly elucidated in the literature. Therefore, we used ^{1}H-MRS to investigate the biochemical changes present in the brains of P17 and P30 rats in the PNO and IUO groups. At P17, the IUO group had higher signals for ASP and GLU compared to controls and higher signals of NAA compared to controls and PNO. The IUO group also had lower levels of TAU than controls. GLU and ASP regulate a majority of the excitatory synaptic neurotransmission in the brain,[235] and their enhanced expression may point to excitotoxicity and possibly enhanced excitatory signaling in the brain. Additionally, higher levels of ASP may suggest more ASP is available to react with acetyl-CoA to make NAA,[236] which was also elevated in the IUO group at P17. By P30, both PNO and IUO groups had lower levels of NAA, and IUO had higher levels of TAU than both PNO and saline controls. In a human study of opiate-dependent individuals, NAA and GLU were present in lower concentrations in the opioid-using cohort than the controls.[237] In our study, we see that the level of NAA for both PNO and IUO groups is lower at P30 than at P17, and the GLU levels are not significantly different from the control group. Interestingly, NAA is believed to be present in neurons and has been used as a measure of neuronal loss or dysfunction.[238] Lower levels of NAA have been reported in studies of brain ischaemia,[238,239] multiple

sclerosis,[240] and several more neurodegenerative diseases.[241] The lower levels of NAA in both the PNO and IUO at P30 may indicate neuronal damage or loss in synaptic abilities in these oxy-exposed groups as they age, even without further exposure to oxy. Intriguingly, a recent study by Ward *et al*. suggests that NAA may also be a marker of CNS injury after opioid dependence and withdrawal,[242] so the lower concentration of NAA in PNO and IUO offspring at P30 may also indicate withdrawal. By P30, TAU is present in higher concentrations in the IUO compared to both PNO and control groups. TAU has a role in brain development, and TAU deficiency can lead to a delay in cell differentiation and migration in the cerebellum, pyramidal cells, and visual cortex.[243] Hernandez-Benitez *et al.* have shown that TAU promotes neural development in the embryonic brain as well as in adult brain regions,[244] so the lower levels of TAU may point to neurodevelopmental deficits.

Based on the observation of increased GLU levels in our MRS study, we investigated the extent of synaptic changes in IUO and PNO animals. Glutamate receptors play a role in mediating the reward pathway involved in drug addiction,[245] and they are also involved in opiate-induced neural and behavioral plasticity.[246-248] AMPA receptors, one type of ionotropic glutamate transporter, are crucial for opioid withdrawal during development.[249-254] While we saw a reduction in the AMPA/NMDA ratio in the PNO rats, the difference was not significant. Additionally, there were no differences in the paired-pulse ratio of post-synaptic currents, suggesting no changes in vesicle release. Interestingly, PNO mEPSCs had slightly faster decay kinetics, which is consistent with altered AMPA receptor subunit composition. Intriguingly, no significant effects were seen in the IUO group, which is interesting because the IUO pups were exposed to oxy for a much longer period than PNO pups. Possible reasons include the potential loss of neurons given the longer exposure to oxy[255,256] and the higher glutamate levels in the IUO pups compared to the PNO group, as evidenced by ^{1}H-MRS. Thus, our study for the first time

lends insight into the synaptic changes associated with PNO exposure and its effects on altered glutamatergic signaling.

Recent studies employing high-throughput technologies have further provided new insights into the molecular underpinnings associated with chronic oxy dependency. These include alterations in key genes associated with integrated stress response in the brain,[257] induction of apoptotic signaling in neurons by promoting demyelination,[224] alterations in reward related genes,[258] axon guidance molecules,[259] inflammation/immune related genes,[226] neurotransmitter receptor genes[260] as well as expression of synaptic plasticity genes,[261] including key sex-specific neuroplasticity-related genes.[262] Similarly, our RNA-seq data showed alterations in pathways associated with synaptic transmission, axon guidance, inflammasomes, and genes associated with the reward system. Among others, genes affecting synaptic transmission and axon development included *Egfr*, *Adrb2*, and *Ntrk*. Interestingly, Fan *et al.* found that chronic oxy exposure leads to axonal degeneration in rat brains.[224] Chronic oxy exposure altered the white matter of the rats through deformation of axonal tracks, reduction of the size of axonal fascicles, loss of myelin basic protein, and accumulation of amyloid precursor protein.[224] Importantly, human studies of infants prenatally-exposed to opioids have shown alterations in the white matter, such as punctate white matter lesions or white matter signal abnormalities on structural MR imaging,[263,264] so the results of our RNA-seq align with what is known in the field for both animal models and human studies. Interestingly, our RNA-seq data also showed differences in glutamatergic synapse genes, with seven genes being differentially expressed in the IUO and PNO groups: *Adrb2, Egfr, Grik2, Npy2r, Ntrk1, Ntrk2,* and *Oxtr*. Combined with our electrophysiology results, our RNA-seq results further suggest alterations in glutamatergic signaling within the reward pathway of these exposed offspring. In addition, our studies suggest PNO and IUO exposure not only alter gene expression but also may increase the risk of developing other diseases, particularly renal

disease. Genes associated with renal adysplasia, renal cancers, renal failure, and several other renal diseases were differentially regulated in the PNO and IUO groups. Interestingly, depletion of TAU has been shown to play a role in renal dysfunction.[243] In human studies, opioid use has been associated with acute kidney injury, particularly in the case of opioid overdose.[265] The altered gene expression and lower levels of TAU in the reward system of the IUO offspring during early development may warrant further exploration into the potential of these offspring to develop renal diseases as adults, particularly because P30 IUO animals had significantly higher levels of TAU than PNO or controls. Further, several genes in our analysis are also involved in other substance use-related disorders, such as nicotine, cocaine, and cannabis dependence, morphine addiction, and fetal alcohol spectrum disorders. Opioid use has also been associated with mental health disorders, with a higher proportion of adolescents exposed prenatally to opioids having experiences with major depressive episodes, alcohol abuse, and attention deficit hyperactivity disorder.[199] Genes associated with depression, anxiety disorders, schizophrenia, and obsessive compulsive disorder were all enriched in both PNO and IUO offspring, suggesting a higher risk for such disorders in these offspring.

Oxy is generally prescribed for pain management, therefore we sought to determine how pre-/post-natal oxy exposure may affect the pain thresholds in PNO and IUO animals. When assessed by the Von Frey filament test at P17, the PNO and IUO pups did not exhibit a difference in pain threshold compared to saline controls. However, during adulthood (P75), we saw a significantly lower pain threshold in the IUO when compared to both PNO and saline offspring. The PNO group also had a lower pain threshold when compared to saline controls. In a study of neonatal morphine exposure, P40 rats exhibited a lower pain threshold than the controls, but the pain threshold approached control levels by P50 when tested using Von Frey.[266] Because we see similar results continuing up to P75, pre- and post-natal exposure to oxy may alter normal

synaptic development involved in nociception. Indeed, a number of genes shown to be differentially regulated in both PNO and IUO in our RNA-seq data are associated with pain, such as *Adrb2*, *Cck*, *Htr2c*, *Npy2r*, *Oprk1*, *Oprm1*, and *P2rx3*. Further analyses of these genes could possibly lend more mechanistic insights into the pain etiology in these offspring.

In summary, our study using a holistic systems approach shows a comparative analysis on alterations in metabolic, synaptic, molecular, and behavioral changes in offspring exposed to the prescription opioid oxy pre- and post-natally. Importantly, these changes not only impact the overall development during early stages but also persist into adulthood.

CHAPTER TWO

Distinct synaptic vesicle signatures associated with pre- and post-natal oxycodone exposure

Abstract

The current opioid crisis ravaging all segments of the society continues to pose a rising public health concern. Importantly, dependency on prescription opioids such as oxycodone (oxy) during and after pregnancy can have a significant impact on the overall brain development of the exposed offspring, particularly at the synapse. A significant knowledge gap that still remains is the identification of distinct synaptic signatures associated with these exposed offspring. Accordingly, the overall goal in the current study was to identify distinct synaptic vesicle (SV) signatures. Using Sprague Dawley rats to develop an animal model that mimics oxycodone exposure *in utero* (IUO) and postnatally (PNO), we employed a quantitative mass spectrometry-based proteomics platform to examine changes in the synaptic vesicle proteome and associated functional pathways in postnatal day 14 (P14) offspring. MEGF8, associated with Carpenter syndrome, was downregulated in the IUO offspring while LAMTOR4, associated with the Ragulator complex involved in lysosomal signaling and trafficking, was found to be upregulated in the PNO groups. The respective differential expression of these proteins was further validated by western blot. Our current study shows that exposure to oxy *in utero* and postnatally impacts the SV proteome in the exposed offspring. Moreover, the identification and validation of key treatment-specific SV markers could further help elucidate their role in inducing synaptic alterations during brain development in the exposed offspring.

Introduction

In recent years, widespread abuse of prescription opioids and dramatic increase in the availability of illicit opioids have created what is known as the opioid epidemic.[211] Pregnant women are a particularly vulnerable group since they are prescribed opioids such as morphine, buprenorphine, and methadone, all of which have been shown to cross the placenta,[212-214] potentially impacting the developing fetus. Limited data exist regarding the effects of *in utero* (IUO) or postnatal (PNO) exposure to oxycodone (oxy), however. Oxy is prescribed for multiple types of pain and can bind to mu- and kappa-opioid receptors.[215] Oxy also passes through the blood-brain barrier, allowing higher concentrations of oxy to accumulate in the brain,[216-218] which can contribute to the analgesic properties as well as the risk for dependency and addiction.

Several studies[219] have been conducted with rodent models to investigate the detrimental effects of gestational opioid use on neurodevelopment of the offspring, but a gap in knowledge exists regarding the effects of IUO or PNO oxy exposure on synaptogenesis. Addictive drugs, such as opioids, produce significant and persistent changes in the synapse, which may explain their long-term effects.[267] One way in which to study changes at the synapse is through the use of synaptic vesicles (SVs). SVs, small vesicles 40-50 nm in diameter, are found in the presynaptic terminal and act as a store for neurotransmitters.[209] Upon arrival of an action potential, SVs docked and primed at the active zone of the presynaptic plasma membrane fuse with the membrane and release neurotransmitters into the synaptic cleft, after which the vesicles are retrieved via endocytosis, thus restoring the presynaptic vesicle pool.[268] With around 5% of the total protein concentration of the mammalian central nervous system being contributed by SVs, understanding the protein composition of these vesicles and its influence on the trafficking of SVs is essential in understanding neurotransmitter release.[209,268] Mass spectrometry and proteomic strategies have been used to compare protein expression profiles of parts

of the synapse, including SVs, to create a coherent map of the synapse proteome.[269] Such analyses of the synaptic proteome are critical in understanding the role of synaptic proteins in disease states such as drug addiction, as proteomic analysis allows for the global view of drug-induced changes within a specific proteome. While we have previously identified novel miRNA signatures related to neurodevelopment contained within brain-derived extracellular vesicles of PNO and IUO offspring,[185] the present study aims to further investigate the effect of oxy exposure on the induction of alterations in the synaptic vesicle (SV) proteome.

Using a pre- and postnatal oxy exposure Sprague Dawley rat model previously established in our lab,[185] we employed a mass spectrometry-based proteomic approach to examine changes in the SV proteome resulting from *in utero* and postnatal oxy exposure. Subsequent pathway analysis of SV signatures highlighted a number of functional pathways affected by the differential expression of these SV signatures. The proteomics-based approach used in this study allows for in-depth research into the changes of SV signatures associated with perinatal oxy exposure. Further, the identification of associated functional pathways and disease states affected by the protein expression changes in these vesicles elucidates potential downstream effects that may continue to affect the development of IUO and PNO offspring.

Results

Purity and validation of synaptic vesicle samples

To ensure sufficient protein content, SVs were isolated from one complete hemisphere of the brain (~500 mg) removed from IUO, PNO, and control P14 animals. To verify our product contained only purified SVs, we performed dot blot and western blot analyses on isolated fractions collected during different stages of our protocol. To ensure no proteasome contaminants remained in our sample, we conducted a dot blot analysis

using the proteasome marker anti-PSMC. Of the 30 fractions and the pellet collected from our protocol, we found the distribution of proteasome contaminants was higher in fractions 1 – 15 compared to fractions 16 – 30 and the pellet (Figure 12A). Thus, only fractions 16 – 30 and the pellet were used for our SV isolation protocol.

Further, we validated the purity of our SVs through western blot analysis. We compared the expression levels of known synaptic proteins present in the homogenate, synaptosome (P2), and SV fractions (Figure 12B). Known integral membrane proteins present on SVs were enriched in the final SV fractions. Proteins not specific to the SV were enriched in the homogenate and P2 fractions. After validating the purity of our SV product, samples from each group were for mass spectrometry.

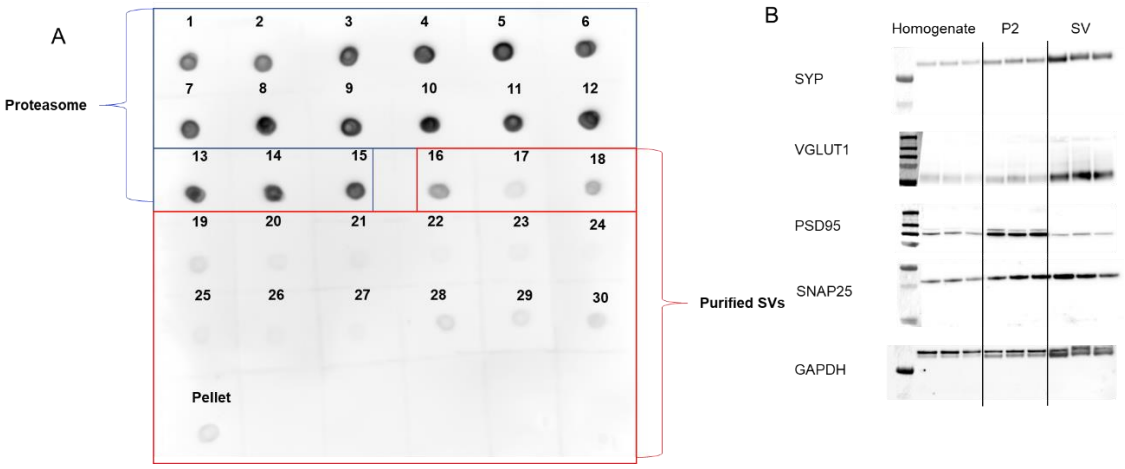
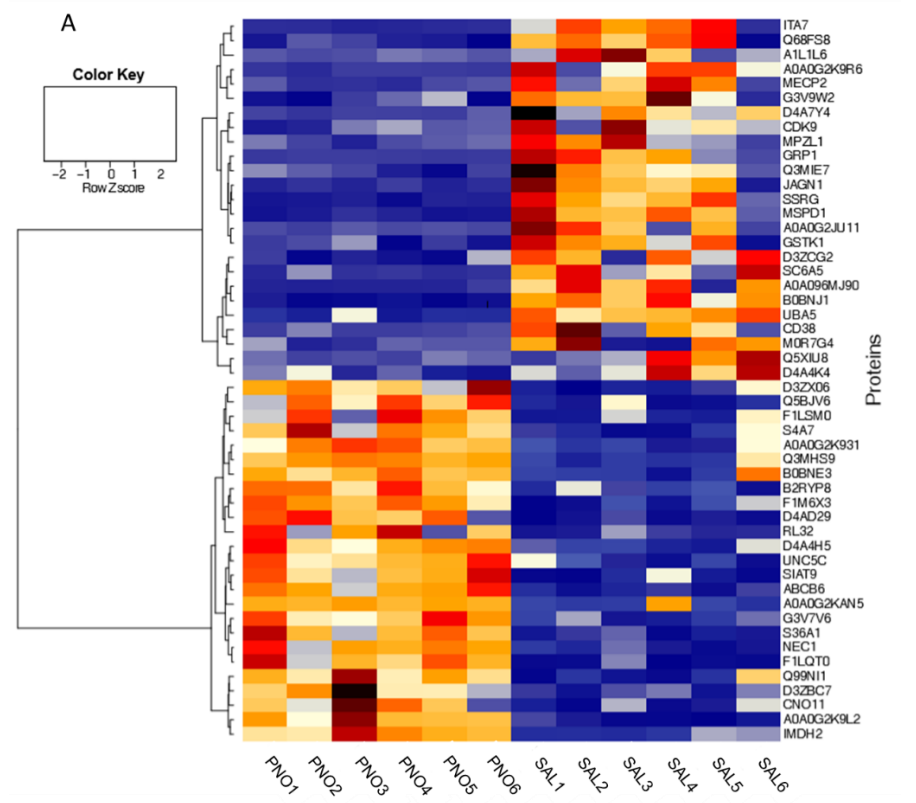


Figure 12. SV isolation purity and validation of SV proteins. A) Dot blot depicting SV purity using the proteasome marker anti-PSMC. B) Western blots further demonstrating the concentration of synaptic proteins in the purified SVs compared to other collected fractions (homogenate and P2).

Differential expression of synaptic vesicle proteins

To examine changes in the SV proteome resulting from *in utero* and postnatal oxy exposure, purified SVs were sent for proteomic analysis using quantitative mass spectrometry. A number of proteins were found to be differentially expressed in both the IUO and PNO groups when compared to saline controls (Figure 13). A Clue-GO analysis of the differentially expressed proteins highlighted several pathways that were affected in the PNO and IUO groups. In the PNO group, cell-cell adhesion, SNARE binding, axon guidance, vesicle docking, synaptic membrane adhesion, and regulation of axon guidance were among the biological processes affected by differential expression of SV proteins (Figure 14A). In the IUO group, glutamate receptor signaling, endosome transport, regulation of synapse assembly, and vesicular transport were affected (Figure 14B).

Additionally, a number of the differentially expressed proteins were uniquely up- or down-regulated in the IUO and PNO groups (Figure 15). Of the differentially expressed proteins in the PNO group compared to controls, 288 were upregulated and 62 were downregulated. In the IUO group, 205 proteins were upregulated while 25 proteins were downregulated. Further, of the up-regulated proteins in both PNO and IUO comparisons with controls, 48 proteins were common. Of the downregulated proteins, three proteins were common. Furthermore, when comparing the upregulated proteins of the IUO with the downregulated proteins of the PNO, five proteins were common. When comparing the upregulated proteins of the PNO with the downregulated proteins of the IUO, only one protein was common.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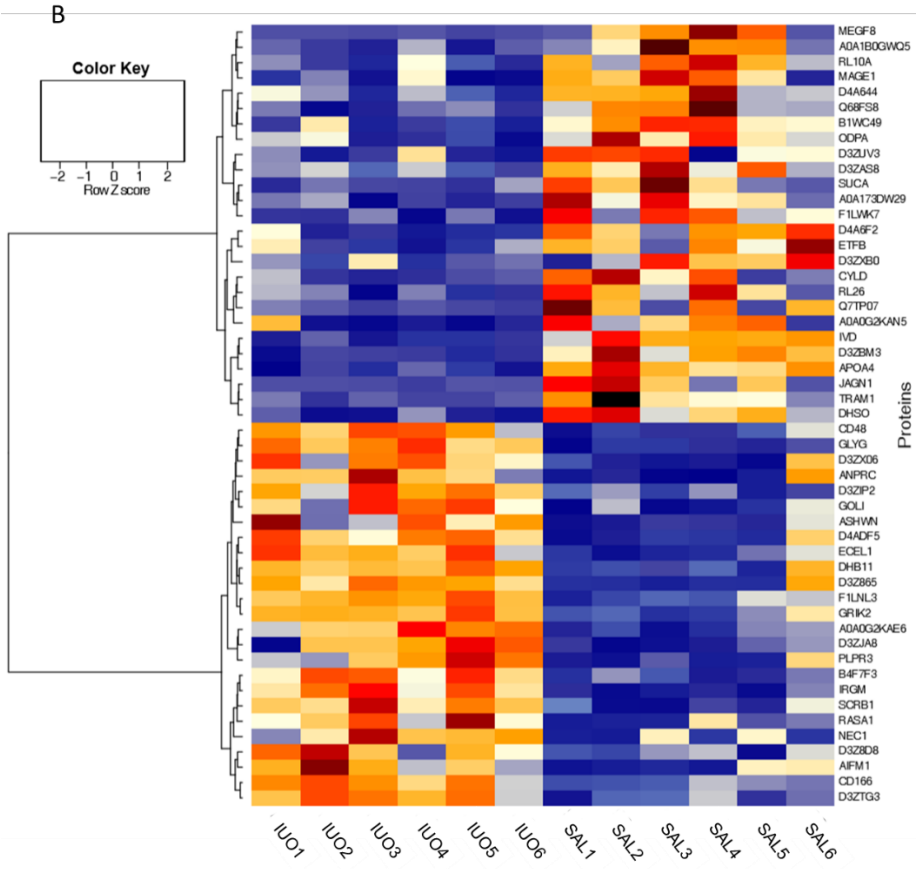
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Proteins
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IUO2
IUO3
IUO4
IUO5
IUO6
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Figure 13. Differential expression of SV proteins isolated from one complete brain hemisphere of P14 IUO, PNO, and control rats. A) Quantitative mass spectrometry revealed a number of proteins differentially expressed in PNO groups compared to saline controls. B) Quantitative mass spectrometry revealed a number of proteins differentially expressed in IUO groups compared to saline controls.

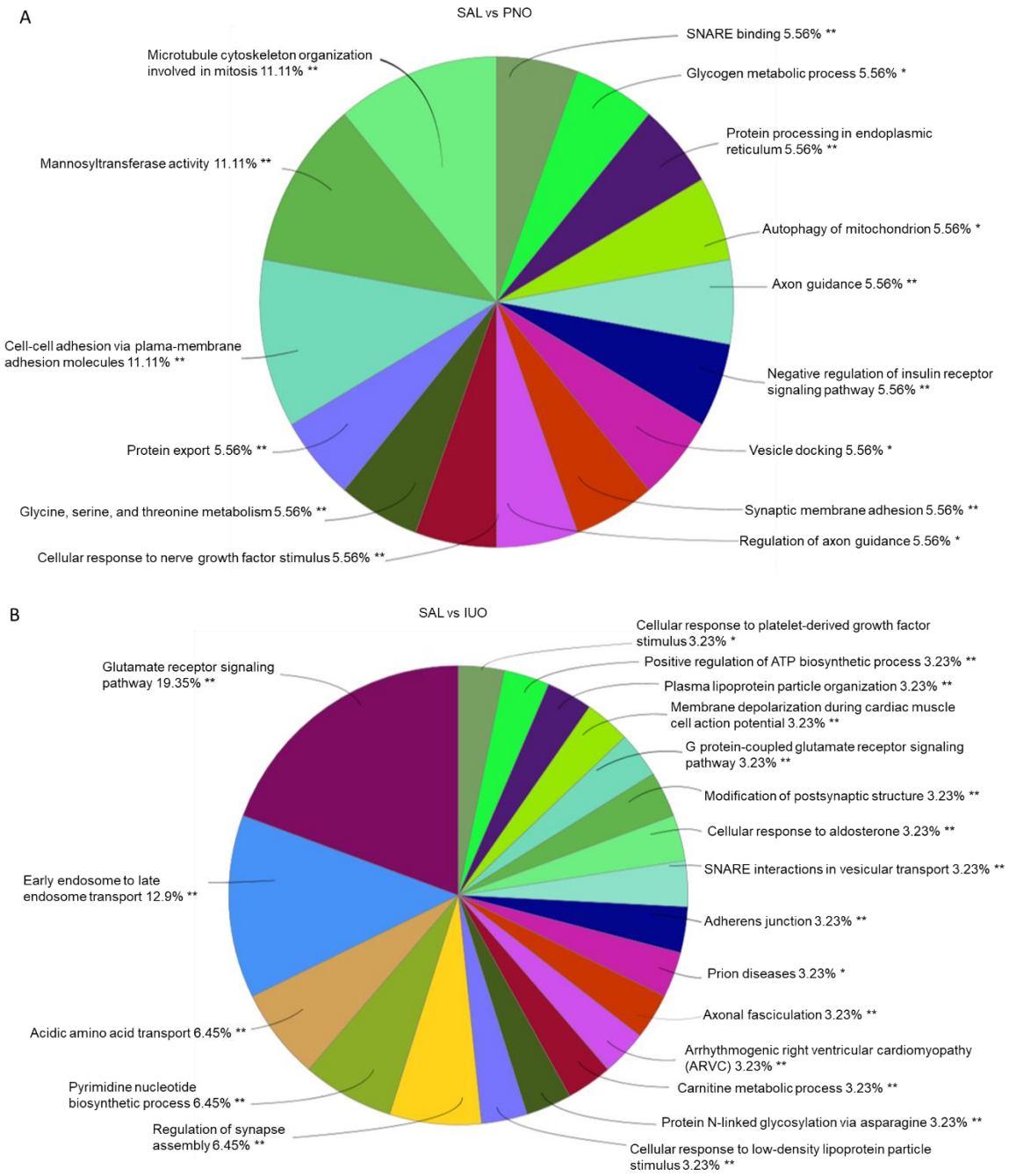


Figure 14. Differential expression of SV proteins isolated from one complete brain hemisphere of P14 IUO, PNO, and control rats. A) ClueGo representations of differentially expressed proteins in the PNO group compared to control. B) ClueGo representations of differentially expressed proteins in the IUO group compared to control. Asterisks represent the group term p-value of each category. *p < 0.01, **p < 0.001.

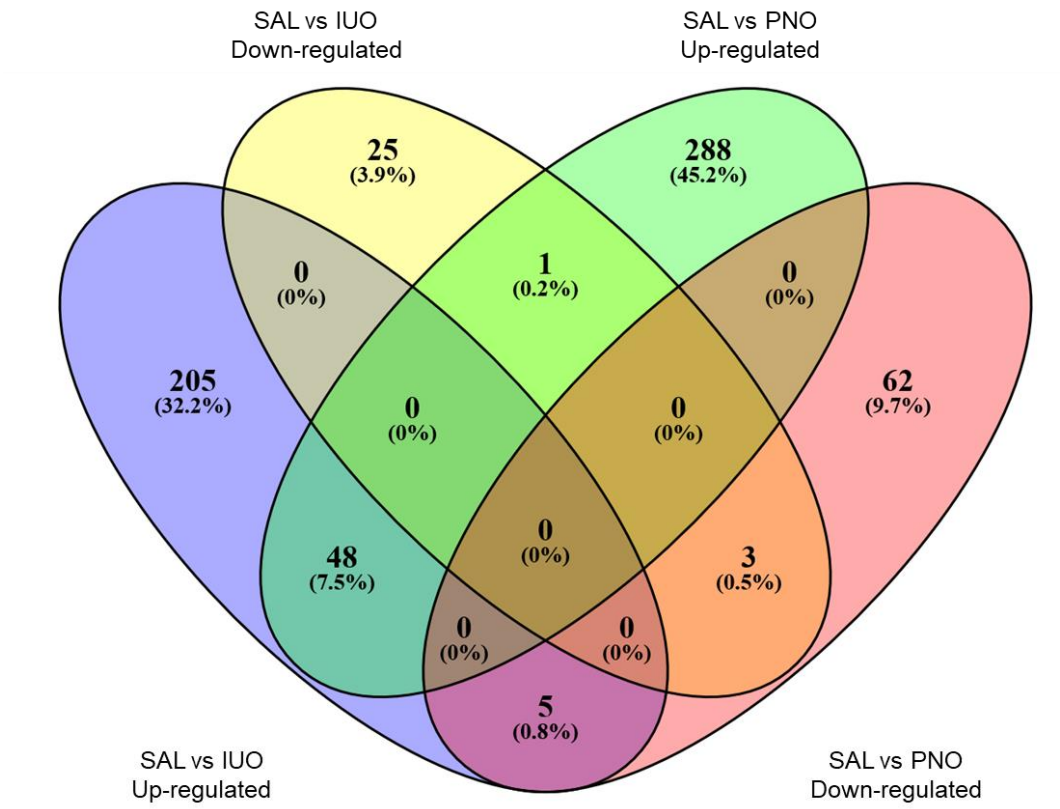


Figure 15. Uniquely up- or down-regulated proteins found in each comparison conducted among the groups.

Further, enriched disease-associated pathways were identified using the Ingenuity Pathway Analysis (IPA) software. Top canonical pathways affected in the PNO group compared to saline control included the synaptogenesis pathway, clathrin-mediated endocytosis signaling, Huntington's Disease signaling, insulin receptor signaling, and axonal guidance signaling. Additionally, pathways associated with neurological disease, hereditary disorders, psychological disorders, and skeletal and muscular disorders were also affected in the PNO group (Figure 16A). Physiological and molecular pathways, such as those involved in nervous system development and function, behavior, cellular development and cellular growth and proliferation, were also affected in these animals. A number of pathways associated with heart failure, liver dysfunction, and renal issues were also impacted by PNO treatment. In the IUO group, the top canonical pathways that were affected by oxy exposure included the synaptogenesis pathway, clathrin-mediated endocytosis signaling, caveolar-mediated endocytosis signaling, and glutathione redox reactions (Figure 16B). Pathways associated with neurological disease, inflammatory response, and respiratory disease were among the disease-associated pathways affected by IUO treatment. Physiological and molecular functions, such as nervous system development, behavior, and cell-cell signaling, were also found to be affected in the IUO group. Further, pathways associated with cardiac, liver, and renal abilities were affected with IUO treatment. Overall, both IUO and PNO animal SV proteomes denoted several differentially regulated proteins that impact biological pathways, particularly those associated with several disease states.

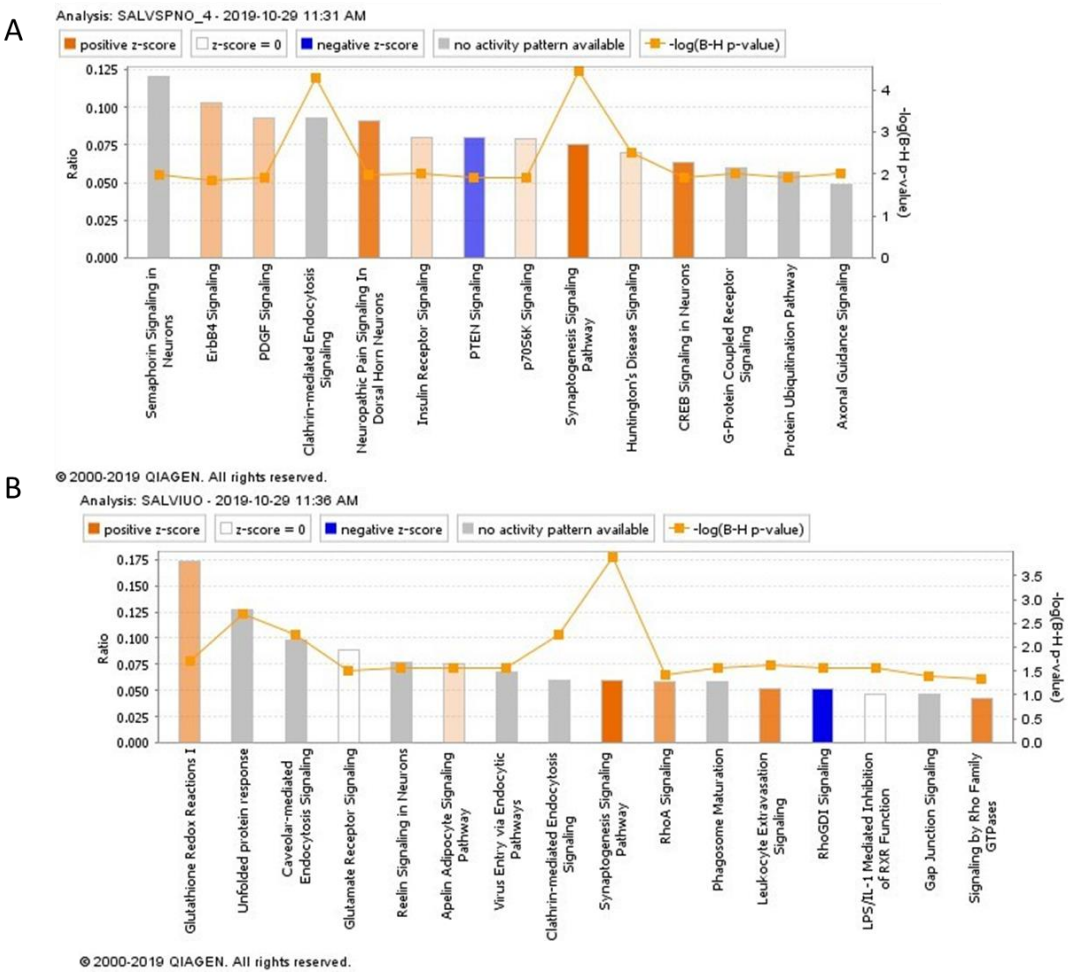


Figure 16. Enriched disease-associated pathways identified using Ingenuity Pathway Analysis (IPA) software. A) Pathways enriched in the PNO group compared to saline controls. B) Pathways enriched in the IUO group compared to saline controls.

Further, the IPA revealed analysis-ready hits that were significantly up- or down-regulated in the groups. Of these, MEGF8, which is associated with Carpenter's syndrome,[270,271] was downregulated in the IUO SVs, and LAMTOR4, a regulator of microglial lysosomes,[272] was upregulated in the PNO SVs. Our western blots validated these results, showing a lower expression of MEGF8 in IUO SVs and a higher expression of LAMTOR4 in PNO SVs compared to controls (Figure 17).

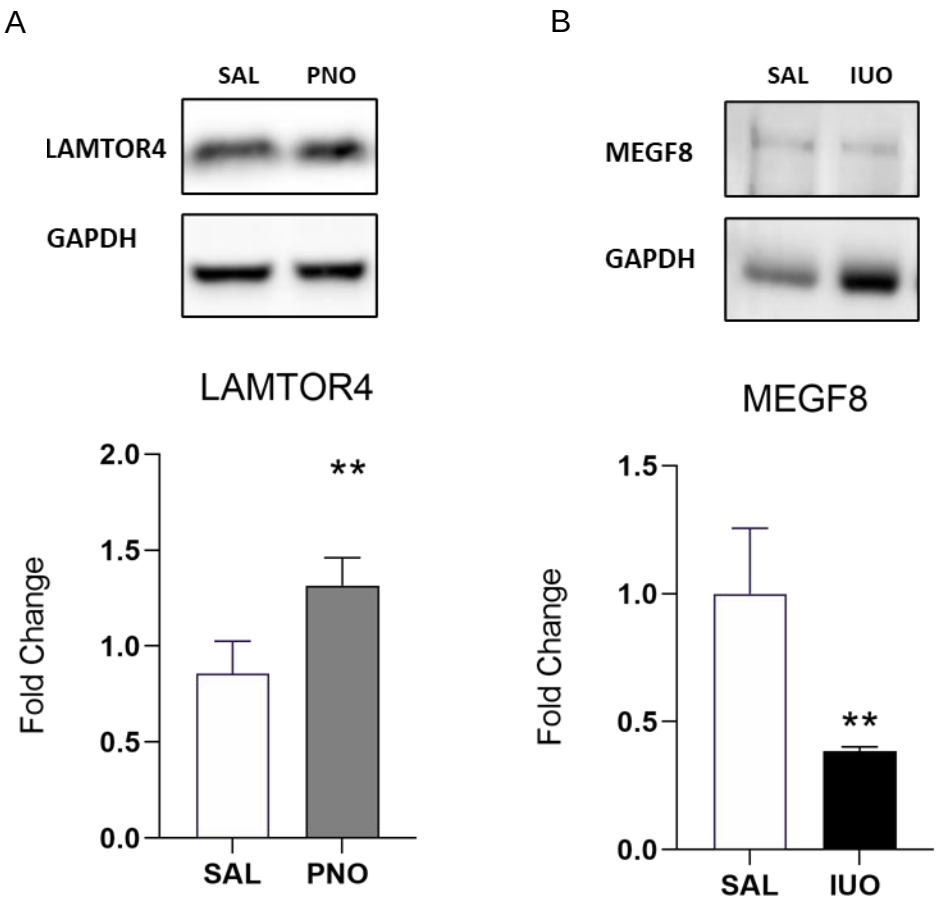


Figure 17. Post-validation of LAMTOR4 and MEGF8 expression in SVs isolated from one complete brain hemisphere of P14 IUO, PNO, and control rats. A) Validation of LAMTOR4 upregulation in PNO SVs. B) Validation of MEGF8 downregulation in IUO SVs.

Discussion

Drugs of abuse, such as amphetamine, cocaine, nicotine, and morphine, have been shown to alter the structure of dendrites and dendritic spines on cells in regions of the brain associated with reward, judgment, and inhibitory control.[267] Robinson *et al.* suggested that this structural plasticity associated with exposure to drugs of abuse reflects a reorganization of patterns of synaptic connectivity that alters their operation, thus contributing to the detrimental effects drug use, namely addiction.[267] Alterations in the synaptic abilities in one area of the brain may result in strengthening or weakening connections with other brain regions, possibly driving distinct aspects of addictive behavior.[273] Chronic morphine studies have shown alterations in gene expression, synapse morphology, synaptic transmission, and synaptic protein profiles,[274] but studies regarding oxy are still lacking. With regard to synaptic plasticity, our lab has previously shown that the number of dendritic spines decreased with PNO and IUO exposure.[185] In the same study, we identified novel extracellular vesicle signatures associated with PNO or IUO treatment that may impact synaptogenesis in these offspring. The present study sought to further this investigation of the impact of PNO and IUO exposure on synaptic development through the use of synaptic vesicles, identifying novel SV signatures associated with PNO and IUO exposure. We also found that the differentially expressed proteins associated with the SVs impact functional pathways as well as disease states. As mentioned in our previous work, the PNO and IUO groups are clinically relevant,[185] thus lending translational significance to our study.

From the purified SVs isolated from one intact brain hemisphere of P14 PNO and IUO rat brains, a number of functional pathways and disease states were suggested to be affected by the differentially regulated protein contents of the SVs. Of the several pathways impacted by PNO and IUO exposure, axon guidance and axon fasciculation were particularly interesting. Human studies have shown that prenatal opioid exposure

can result in abnormal tract development in newborns that persists at 12 to 15 years of age.[264,275] Additionally, infants born with prenatal opioid exposure had alterations in the white matter, such as punctate white matter lesions or white matter signal abnormalities on structural MR imaging.[263] In preclinical studies, chronic oxy exposure altered the white matter of rats through deformation of axonal tracks, reduction of the size of axonal fascicles, loss of myelin basic protein, and accumulation of amyloid precursor protein.[224] Proteomic investigations of morphine-regulated changes in the synapse have shown signaling, vesicle trafficking, cytoskeletal proteins, energy metabolism, signal transduction, synaptic transmission, and cell adhesion all to be affected.[158,274,276-279] Similar pathways were shown to be affected in both PNO and IUO groups of our study, specifically pathways associated with vesicle docking, membrane adhesion, signaling, synapse assembly, and vesicular transport.

Increasing evidence suggests the opioid system controls a number of aspects related to the pathophysiology of neurological disorders.[280] Opioid use has also been associated with mental health disorders, with a higher proportion of adolescents exposed prenatally to opioids having experiences with major depressive episodes, alcohol abuse, and attention deficit hyperactivity disorder.[199] In our PNO group, Huntington's disease signaling was affected by oxy exposure. Interestingly, in patients with Huntington's disease, individuals who abused substances had a significantly earlier age of motor onset.[281] Additionally, both PNO and IUO exposures were associated with a number of renal complications. In human studies, opioid use has been associated with acute kidney injury, particularly in the case of opioid overdose.[265] With regard to cardiac diseases, which were associated with both PNO and IUO groups, a recent study found that coronary artery disease was significantly higher in patients with opioid use than controls, suggesting opioid use may be an important risk factory in this disease.[282] Further, opiate consumption has been linked to hypertension and diabetes mellitus, and evidence suggests that chronic

opioid use may increase the risk of cardiovascular diseases.[283] Further, chronic morphine use was associated with increased risk of both renal and hepatic damage.[284] Liver disease associated with PNO and IUO groups may stem from the metabolism of oxy in the liver. Verna *et al.* suggest that the metabolic pathways involved in opioid metabolism may contribute to or worsen liver injury.[285] δ-opioid receptors, which contribute significantly to cellular development and are abundant in liver tissue, have been shown to affect the initiation and progression of liver disease.[286] Additionally, histopathologic examination of hepatocytes from rat models of chronic opioid use exhibited sinusoidal dilatation, perivenular ballooning degeneration extending to the midzonal region, perivenular necrosis, hemorrhage, and focal microvesicular steatosis.[284] Taken together, these results highlight the importance of understanding the long-term effects of perinatal exposure to opioids, as a number of complications and disease states have been associated with the toxic effects of opioids.

The downregulation of MEGF8 and upregulation of LAMTOR4 revealed in our IPA in the IUO and PNO SVs, respectively, were particularly interesting. MEGF8 is a single-pass type I membrane protein that contains several EGF-like domains.[271] Defects in the gene that encodes MEGF8 result in Carpenter syndrome, which is an autosomal recessive multiple congenital malformation disorder characterized by multisuture craniosynostosis and polysyndactyly, umbilical hernia, cryptorchidism, and congenital heart disease.[270] Interestingly, loss of MEGF8 disrupts axon guidance in the peripheral nervous system, leading to limb, heart, and left-right patterning defects.[287] As mentioned before, human newborns with prenatal opioid exposure had alterations in the white matter,[263] and preclinical models of chronic oxy administration have revealed deformation of axonal tracks, reduction of the size of axonal fascicles, loss of myelin basic protein, and accumulation of amyloid precursor protein.[224] Perhaps the downregulation of MEGF8 may contribute to these deficits in axonal development reported with prenatal opioid exposure.

LAMTOR4 is part of the Rag complex, a scaffold protein complex comprised of LAMTOR subunits 1 – 5 that senses amino acids and lipids and integrates growth factor signaling.[288] This “Ragulator” complex also controls the activity of the mTOR complex 1 (mTORC1) on the lysosome.[289] Interestingly, mTORC1 is required for the initiation and maintenance of chronic pain and opioid-induced tolerance/hyperalgesia.[290] Additionally, it has been suggested that prenatal opioid exposure is the next neonatal inflammatory disease.[291] Intriguingly, LAMTOR4 is essential for microglial development; zebrafish lacking LAMTOR4 were shown to have a reduction in microglia.[272] Further investigation in to LAMTOR4 and the Ragulator complex in the context of PNO exposure is needed to understand its role in opioid exposure.

Overall, our study found that IUO and PNO oxy exposure alter the proteome of SVs, impacting the synaptic abilities of these offspring. Taken together, our results suggest that *in utero* and postnatal exposure to oxy can alter the proteome of SVs, thus influencing signaling pathways and potentially increasing the vulnerability of these offspring to disease in the future.

CHAPTER THREE

Characterization of the intergenerational impact of *in utero* and postnatal oxycodone exposure

Abstract

Prescription opioid abuse during and after pregnancy is a rising public health concern. While earlier studies have documented that offspring exposed to opioids *in utero* have impaired neurodevelopment, a significant knowledge gap remains in comparing the overall development between offspring exposed *in utero* and postnatally. Adding a layer of complexity is the role of heredity in the overall development of these exposed offspring. To fill in these important knowledge gaps, the current study uses a preclinical rat model mimicking oxycodone (oxy) exposure *in utero* (IUO) and postnatally (PNO) to investigate comparative and intergenerational effects in the two different treatment groups. While significant phenotypic attributes were observed with the two treatments and across the two generations, RNA sequencing revealed alterations in the expression of key synaptic genes in the two exposed groups in both generations. RNA sequencing and post-validation of genes using RT-PCR highlighted the differential expression of several neuropeptides associated with the hypocretin system, a system recently implicated in addiction. Further, behavior studies revealed anxiety-like behaviors and social deficits that persisted even in the subsequent generations of the two treatment groups. To summarize, our study for the first time reveals a new line of investigation on the potential risks associated with oxy use during and after pregnancy, specifically the disruption of neurodevelopment and intergenerational impact on behavior.

Introduction

The widespread abuse of prescription opioids and a dramatic increase in the availability of illicit opioids have created what is commonly referred to as the opioid epidemic.[211] As a particularly vulnerable group, pregnant women are prescribed opioids such as morphine, buprenorphine, and methadone to alleviate pregnancy and postpartum pain, all of which have been shown to cross the placenta.[212-214] Data collected by the Centers for Disease Control and Prevention (CDC) from a 2008-2012 study found that more than a third of reproductive-aged women enrolled in Medicaid (39%) and more than a quarter of those with private insurance (28%) filled a prescription for an opioid pain medication each year.[292] Additionally, postpartum women, regardless of delivery method or pain measurement, commonly receive similar amounts of opioids upon discharge from the hospital.[128,293] The lack of standardization in prescribing patterns of these opioids contributes to a sizable amount of leftover medication, which can lead to nonmedical use of the prescriptions.[129,293] Among the prescription opioids, oxycodone (oxy) has recently emerged as a serious contender for widespread abuse. A postoperative analgesic, oxy has been reported in the literature for postpartum pain or caesarian sections in lieu of morphine drips,[230,231] potentially exposing neonates to this powerful opioid.

Several studies[219] have been conducted with rodent models to investigate the detrimental effects of gestational opioid use on neurodevelopment of the offspring, but few of these studies consider oxy. Additionally, these prior studies relied on self-administration or continuous release pumps for drug administration, and very few have used oral delivery to mimic the usual route of administering pain medication. Orally-administered analgesics are the most common form of pain relief prescribed after caesarian sections,[294,295] and oral oxy administration has been shown to be safer than and as effective as intravenous administration of other opioids.[230,296,297] Previous oxy studies have shown deficits such as behavioral impairments and disruption in both *OPRM1* and endothelin receptor expression

during development in offspring exposed to oxy *in utero.*[180,182-184] However, these studies focused primarily on prenatal oxy exposure, leaving a large gap in the knowledge regarding postnatal oxy exposure.

Opiates have been shown to pass into the placenta and act on fetal opioid receptors.[212-214] Opioids also accumulate in the breastmilk.[298] The degree of exposure of an infant to a drug passed through the breastmilk depends on the concentration of the drug in the milk, the amount of milk ingested, and the rate of elimination from the infant.[232] A human study by Seaton *et al*. showed that oxy is concentrated in the breastmilk, and offspring exposed via the breastmilk may receive less than 10% of a typical oral therapeutic infant dose (0.1–0.2 mg/kg).[233] Despite this low dose, infant exposure to oxy via the breastmilk has been associated with sedation, central nervous system depression, and neonatal toxicity,[234,299-301] and a number of animal studies have also revealed deficits in behavior and development associated with perinatal opioid exposure.[180,182,183,224] The full extent of postnatal oxy exposure effects is not yet known particularly in regard to central neural synaptic function and gene expression. Further, pre- and postnatal opioid abuse can result in several phenotypic consequences across multiple generations of offspring, despite no previous exposure to drugs.[302] Currently, a gap in knowledge exists regarding the long-term, intergenerational effects of oxy exposure during the perinatal period on future generations.

The present study was performed to determine the intergenerational effects of *in utero* and postnatal oxy exposure on development in both the F1 and F2 generations. This study was conducted using a Sprague Dawley rat model our labs have previously established.[185] This model consists of two groups of pregnant F0 dams that had been orally administered oxy. The first group was treated daily with an oral administration of oxy before, during, and after pregnancy, thereby exposing the F1 pups to oxy *in utero* (IUO) throughout fetal and postnatal development. The second group was treated daily with an

oral administration of oxy only after giving birth, exposing the F1 pups to oxy postnatally (PNO). The F2 generation was descended from the germlines of F1 dams exposed to oxy via the breastmilk or *in utero*, allowing us to elucidate intergenerational effects. Importantly, both the PNO and IUO groups are clinically relevant.[185] The IUO dams represent women who exhibit chronic opioid use before, during, and after pregnancy, and the PNO group represents children exposed to opioids after their mothers are prescribed medication post-caesarian section. Additionally, the PNO group represents neonates in the neonatal intensive care unit (NICU) that may be exposed to high-dose opiates through infusions. Infants born with heart defects and persistent pulmonary hypertension are exposed to opiates for sedation and analgesia while being supported by extracorporeal membrane oxygenation (ECMO) and mechanical ventilation.[227] Infants may be supported by ECMO for three weeks or longer, exposing the newborns to potent opiates for an extended period of time and contributing to the increase in diagnoses of neonatal abstinence syndrome.[228,229] Little is known regarding the implications of these opiate drips and how they may impact neurodevelopment in newborns. Our PNO group provides a high-dose opiate exposure to the pups that is comparable to the high-dose opiate exposure neonates in the NICU experience, establishing clinical relevance for the inclusion of this group in future studies.

The present study uses a holistic integrated systems biology approach to determine the intergenerational effects of pre- and postnatal oxy exposure on development in both the F1 and F2 generations descended from oxy-exposed mothers. Our overall hypothesis was that F0 maternal oxy use and F1 *in utero* and postnatal oxy exposure result in developmental impairments (physical, molecular, behavioral) in the F1 offspring that persist in the F2 generation. The comprehensive and systematic approach used in this study allows for thorough research into intergenerational effects of pre- and

postnatal oxy abuse, a critical step in closing the knowledge gap surrounding this commonly used opioid analgesic.

Results

Oxy induces intergenerational developmental differences in exposed pups

To determine whether oxy exposure affects physical development in the PNO and IUO pups, we measured head size, body weight, and body length on postnatal days (P) 1, 7, 14, and 30 in both generations (Figure 18). Additionally, we calculated the BMI and LOI to estimate obesity and body fat, respectively (Figure 19). By measuring at these time points, we can assess perinatal development through periadolescence.[303] Additionally, these time points correspond with human aging from infant, young child, childhood, and preadolescence.[304]

At P1 in the F1 animals, PNO pups weighed less than controls. While there were no differences in F1 weights at P7, a dramatic decline was observed in both PNO and IUO groups at P14 that persisted through P30. Interestingly, F2 PNO and IUO animals showed a significant increase in body weights compared to the saline controls at each time point (Figure 18A). While no significant changes in body length were seen in the F1 pups at P1, P7, and P14, there was a marked reduction in the body length at P30. In the F2 pups, the PNO offspring had longer body lengths than both the saline and IUO groups at P1 and longer body lengths than controls at P14 (Figure 18B). Head size circumference was markedly affected in the F1 and F2 IUO offspring at birth while F2 PNO pups had larger head size circumference than both IUO and controls at P1 (Figure 18C).

BMI and LOI measurements were significantly different in the oxy-exposed groups of both generations. The F1 PNO group had both lower BMI (Figure 19A) and a lower score on the LOI (Figure 19B) than the saline and IUO groups at P1, suggesting this group may be underweight. Intriguingly, in the F2 animals, both the BMI (Figure 19A) and LOI

(Figure 19B) indicated obesity and greater body fat in the IUO at P1. Additionally, while there were no differences in BMI in the F1 P7 animals, the F2 PNO and IUO groups had larger BMI than the controls at P7, indicating obesity (Figure 19A). At P14, F1 PNO had a lower BMI than the saline controls while F2 IUO had a larger BMI than PNO (Figure 19A). We did not observe any differences in BMI (Figure 19A) in either generation at P30.

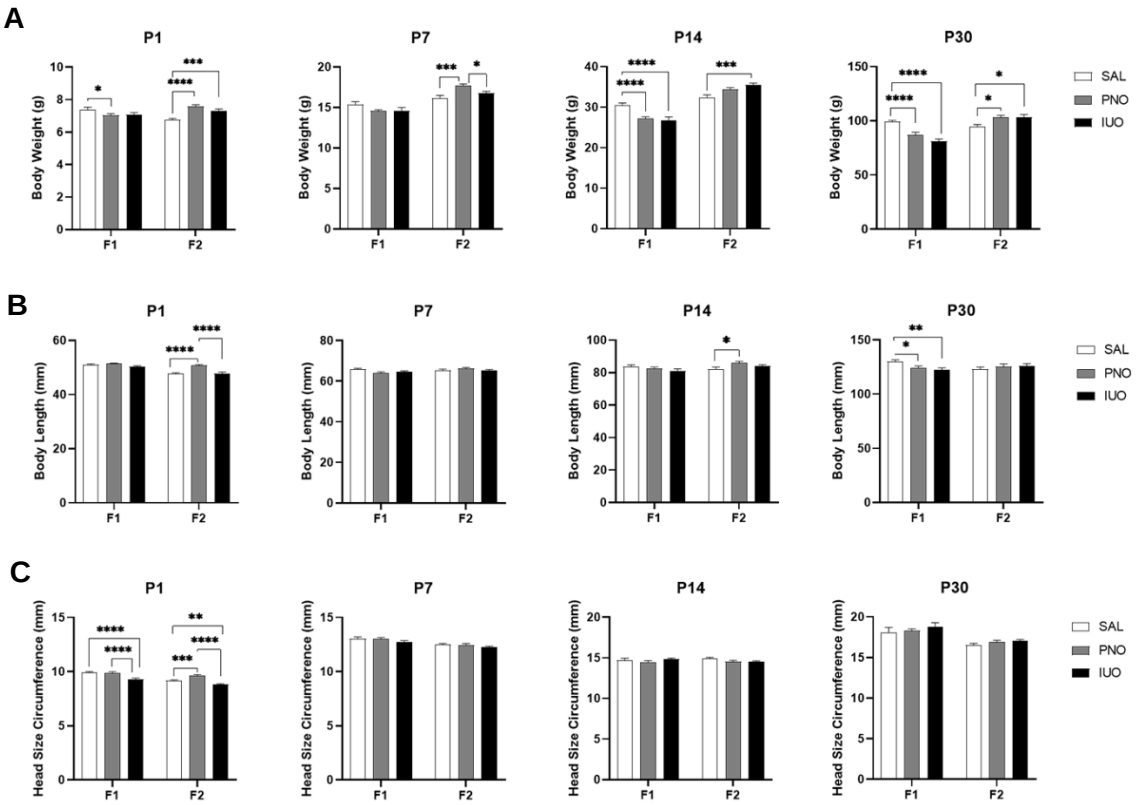


Figure 18. Phenotypic measurements. A-C) Alterations in physical development patterns in both the IUO and PNO offspring as observed through body weight, body length, and head size circumference. *p < 0.05; ***p < 0.001; ****p < 0.0001 as determined by Two-Way ANOVA followed by a post-hoc Tukey's test.

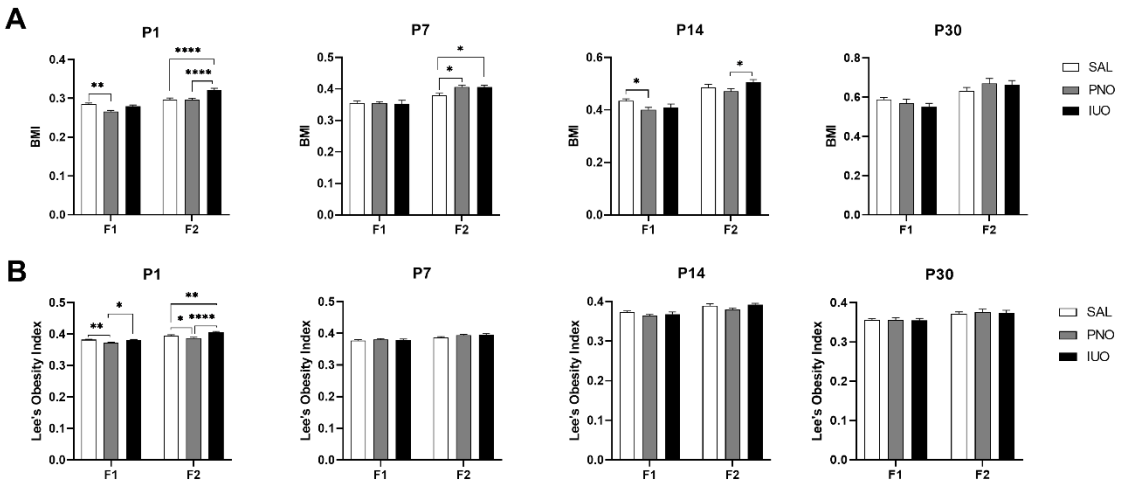


Figure 19. Phenotypic measurements. Alterations in physical development patterns in both the IUO and PNO offspring as observed through body mass index (BMI; A) and Lee's Obesity Index (LOI; B). *p < 0.05; **p < 0.01; ****p < 0.0001 as determined by Two-Way ANOVA followed by a post-hoc Tukey's test.

RNA-seq identified distinct gene signatures and molecular pathways in the two generations

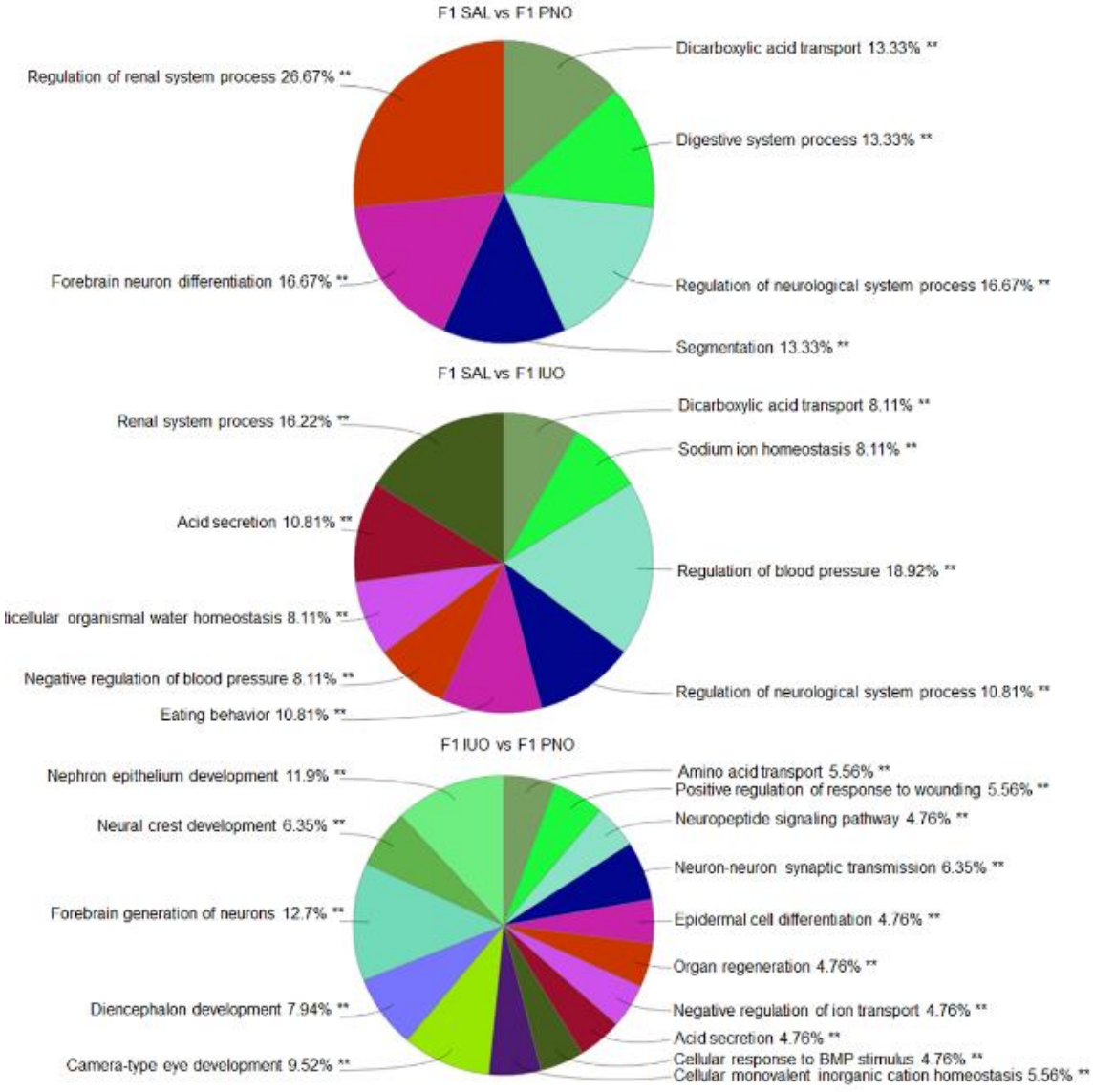
Based on the significant physical attributes observed at P14 in the F1 and F2 generations, we performed RNA-Seq analysis on the nucleus accumbens (NAc). The NAc was chosen given its association with the reward pathway. RNA-Seq analysis showed several up- and down-regulated genes among the IUO, PNO, and saline groups in both the F1 and F2 generations. Using Clue-GO analysis (Figure 20), we found that pathways involved in the regulation of neurological system process, forebrain and neural crest development, and synaptic transmission were affected by the differentially expressed genes in the F1 animals. In the F2 animals, we identified pathways involved in fear response, neurodevelopment, cell signaling, digestive processes, and heart rate regulation that were affected by the differentially expressed genes. Further, when comparing the same F1 group comparisons with the F2 comparisons, we found that a number of genes were consistently differentially regulated in both generations (Figure 21A). Of the differentially regulated genes in both the F1 and F2 generations of saline compared to IUO, 23 genes were common. For saline compared to PNO, 15 genes were commonly differentially regulated between the generations. Lastly, when comparing PNO and IUO, 10 genes were consistently differentially regulated in the F1 and F2 generations.

Potential hits based on a fold change value of ±1.5 that were involved in behavior and development were selected for post-validation using RT-PCR; we found six genes successfully post-validated in the F1 and F2 generation (Figure 21B). Interestingly, of the genes that were post-validated in both F1 and F2, *Pmch, Hcrt*, and *Avp* were significant in both generations. *Hcrt* and *Avp* also had similar expression trends among the experimental groups in both F1 and F2. Intriguingly, the F1 IUO had significantly higher expression of *Pmch* compared to both groups, while the PNO had significantly higher expression in the same F2 comparison. These three genes are important in behavior and

development and, based on their differential expression, may contribute to the differences observed between the F1 and F2 offspring.

Because the hypocretin (*Hcrt*) expression trends were the same in both generations, we sought to further investigate this system (Figure 21C). We found that *Hcrtr1* expression was significantly higher in the IUO group in both generations. *Nptx2*, a protein expressed in orexin neurons, was also highly expressed in IUO compared to PNO and controls in both generations. In the F1 generation, the PNO also high a higher expression of *Nptx2* compared to controls. Further, *Pdyn*, the precursor for dynorphin (an inhibitory neuropeptide co-released with hypocretin), showed higher expression levels in IUO than both PNO and controls in the F1 generation; both IUO and PNO offspring had higher levels of *Pdyn* than controls in the F2 generation.

A


F1 SAL vs F1 PNO
Dicarboxylic acid transport 13.33% **
Digestive system process 13.33% **
Regulation of neurological system process 16.67% **
Segmentation 13.33% **
Forebrain neuron differentiation 16.67% **
Regulation of renal system process 26.67% **
F1 SAL vs F1 IUO
Dicarboxylic acid transport 8.11% **
Sodium ion homeostasis 8.11% **
Regulation of blood pressure 18.92% **
Regulation of neurological system process 10.81% **
Eating behavior 10.81% **
Negative regulation of blood pressure 8.11% **
licellular organismal water homeostasis 8.11% **
Acid secretion 10.81% **
Renal system process 16.22% **
F1 IUO vs F1 PNO
Amino acid transport 5.56% **
Positive regulation of response to wounding 5.56% **
Neuropeptide signaling pathway 4.76% **
Neuron-neuron synaptic transmission 6.35% **
Epidermal cell differentiation 4.76% **
Organ regeneration 4.76% **
Negative regulation of ion transport 4.76% **
Acid secretion 4.76% **
Cellular response to BMP stimulus 4.76% **
Cellular monovalent inorganic cation homeostasis 5.56% **
Nephron epithelium development 11.9% **
Neural crest development 6.35% **
Forebrain generation of neurons 12.7% **
Diencephalon development 7.94% **
Camera-type eye development 9.52% **

B

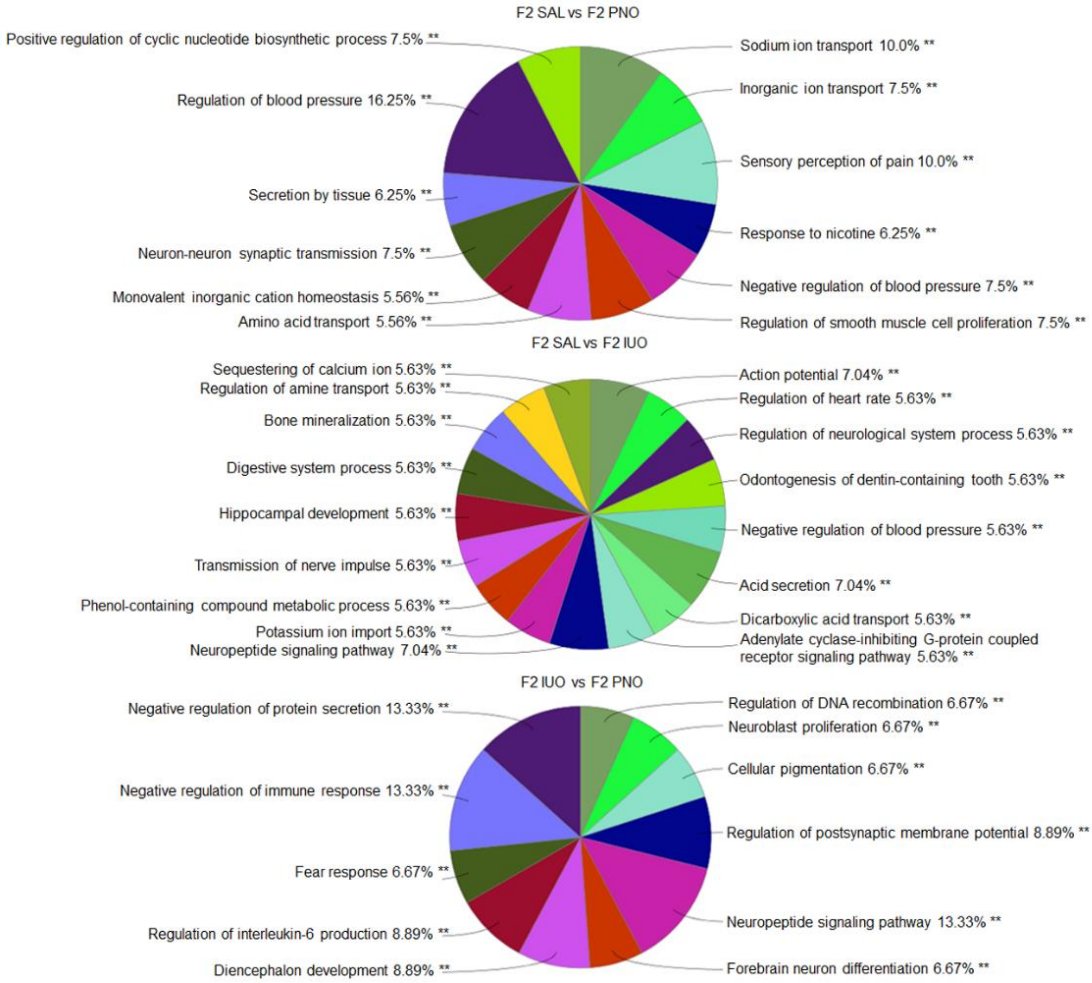


Figure 20. RNA-seq analysis on P14 Nucleus Accumbens (NAc) of F1 and F2 animals. A) Clue-Go pie charts depicting affected biological processes in F1 generations resulting from differential gene expression between groups. B) Clue-Go pie charts depicting affected biological processes in F2 generations resulting from differential gene expression between groups. Results are shown as the percent of genes per group. Asterisks represent the group term p-value of each category. **p < 0.001.

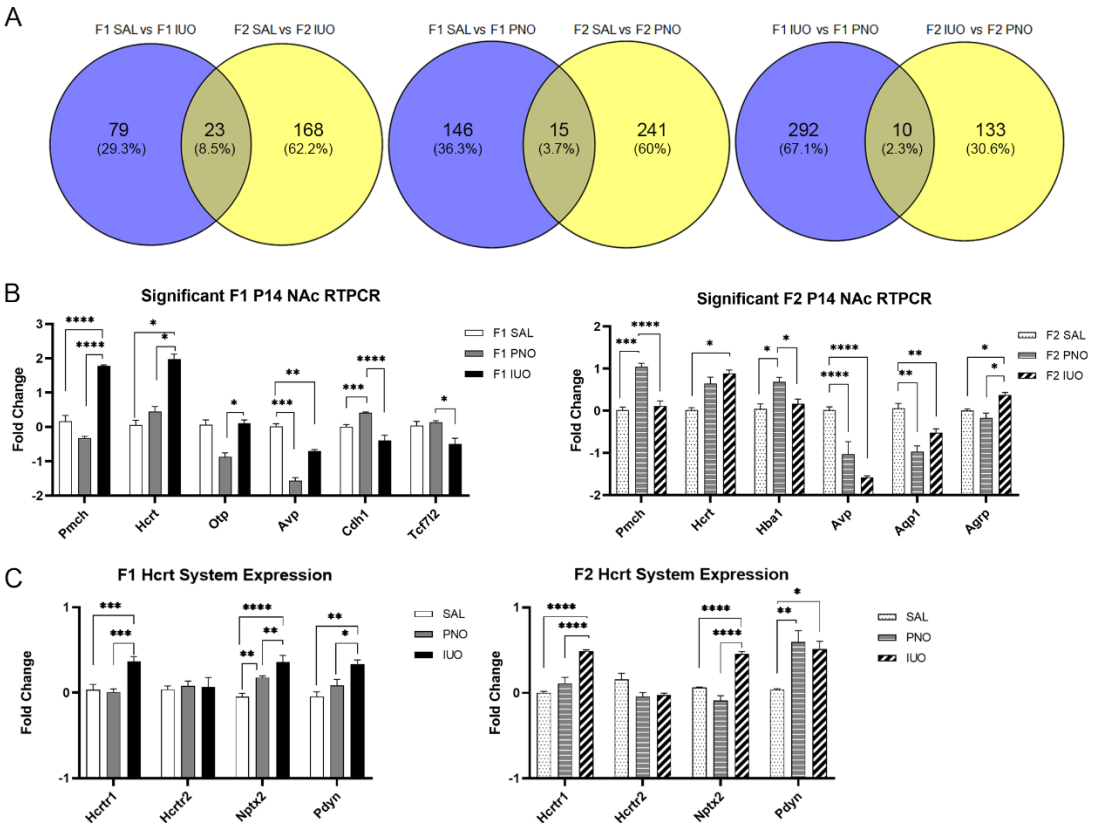


Figure 21. RNA-seq analysis on P14 Nucleus Accumbens (NAc) of F1 and F2 animals. A) Venn diagram depicting the total number of genes affected in the comparison of treatment groups of each generation. B) Of the genes post-validated in the F1 NAc samples, *Pmch, Hcrt, Otp, Avp, Cdh1, Oxt*, and *Tcf7l2* were significant. Of the genes post-validated in the F2 NAc samples, *Pmch, Hcrt, Hba1, Avp, Aqp1*, and *Agrp* were significant. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 as determined by One-Way ANOVA followed by a post-hoc Tukey's test. C) Further investigation of the hypocretin system neuropeptide expression levels found *Hcrtr1*, *Nptx2*, and *Pdyn* were upregulated in the IUO group of both generations. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 as determined by Two-Way ANOVA followed by a post-hoc Tukey's test.

Behavioral deficits continue in the next generation of oxy-exposed pups

Early life insults can significantly impact developmental and behavioral outcomes exhibited during adulthood. Based on our RNA-seq data that highlighted several genes associated with behavioral responses, we examined if oxy exposure induces intergenerational behavioral deficits during adulthood in both IUO and PNO pups. Accordingly, we performed social interaction and marble burying tests in adult animals (P60-70).

In the F1 generation, social novelty tests revealed no significant differences in social interactions of the PNO and IUO groups (Figure 22). However, in the F2 generation, IUO offspring spent more time with the cagemate than the PNO group and less time with the naïve animal than both the control and PNO groups (Figure 23A). Although not significant, the IUO demonstrated a higher tendency to enter the cagemate chamber than the other two groups (Figure 23A). Additionally, when considering the number of contacts with the naïve animal, the IUO displayed fewer contacts with the naïve animal (Figure 23A), suggesting a social deficit and hesitancy to spend more time with or interact with the unknown animal. Similar to the F1 social novelty test results, F1 social preference tests revealed no significant differences in social interactions exhibited by the PNO and IUO groups (Figure 22). In the F2 social preference task, IUO animals spent less time with the naïve animal than did either of the other groups (Figure 23B), and IUO had more contacts with the toy than had either the PNO or control animals, further suggesting social deficits in the F2 generation.

Marble burying tests, which measure repetitive stereotypy, compulsive behaviors, and anxiety-like behavior,[305] showed that both the PNO and IUO groups in both generations buried more marbles than the controls, suggesting heightened anxiety and compulsivity in these animals (Figure 23C). Interestingly, IUO animals in the F2 group buried more marbles than the PNO, suggesting that the F1 IUO exposure may result in

more pronounced intergenerational anxiety and compulsive behaviors than F1 PNO exposure. In summary, *in utero* and postnatal oxy exposure can significantly alter behavioral outcomes that persist into the next generation.

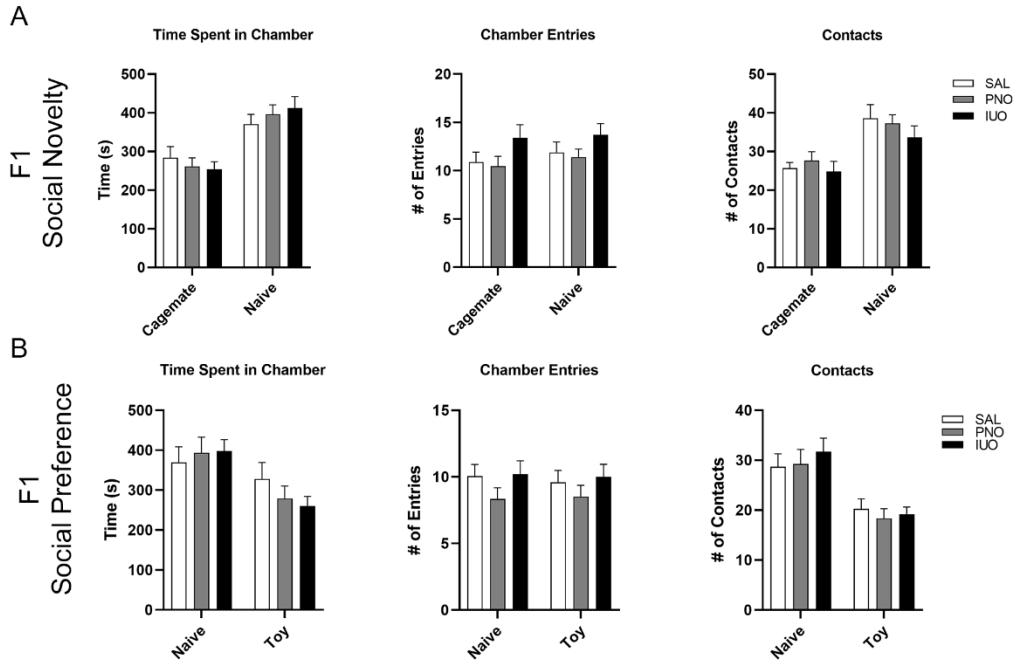


Figure 22. Social novelty and preference results for F1 animals. A) During the social novelty test, F1 animals in each group had no significant differences in time spent in each chamber, number of chamber entries, or number of contacts with the naïve or cagemate animals. B) During the social preference test, F1 animals in each group had no significant differences in time spent in each chamber, number of chamber entries, or number of contacts with the naïve animal or toy.

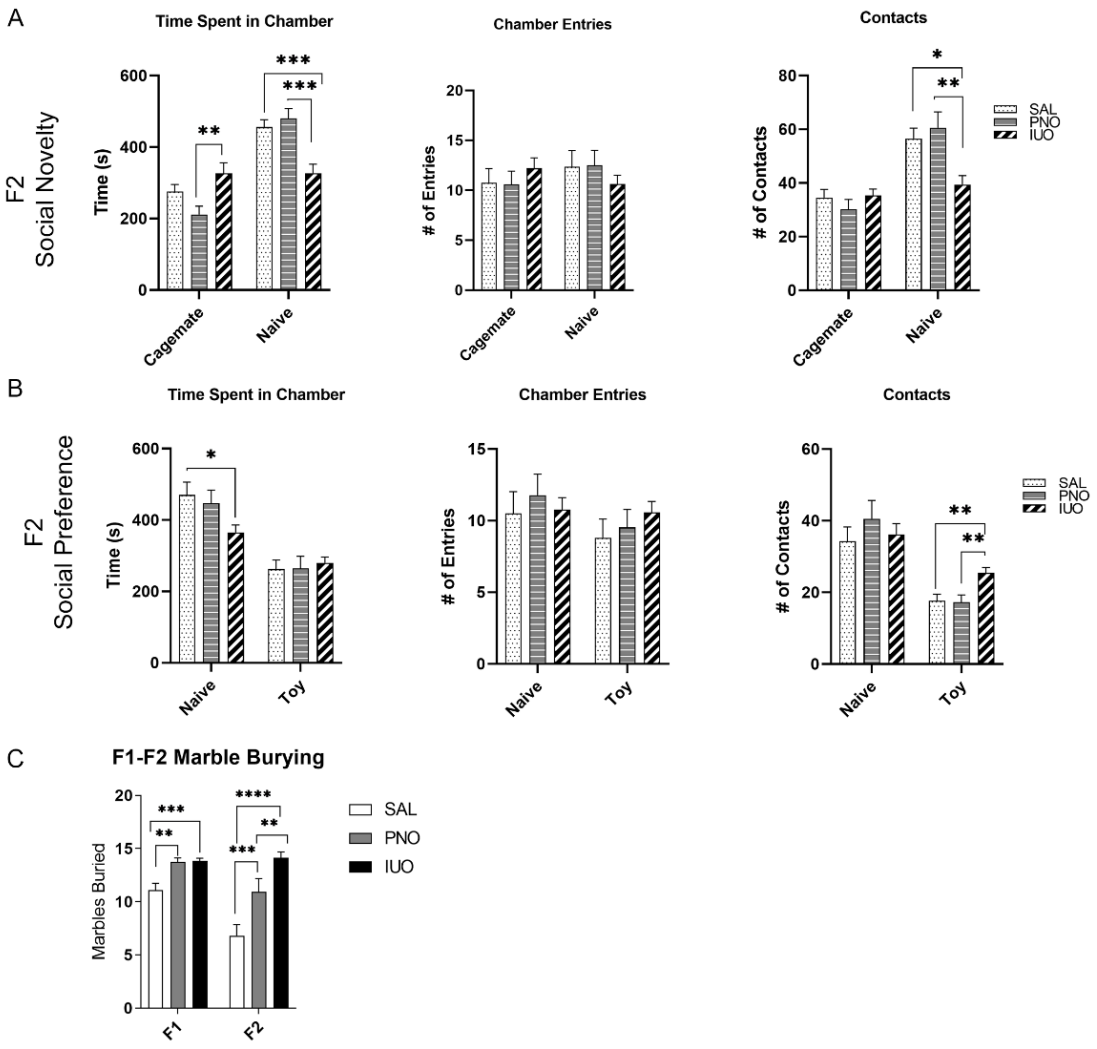


Figure 23. F2 Social behavior tests and F1-F2 marble burying. A) F2 social novelty testing revealed that IUO offspring spent less time with naïve animals and more time with the cagemate than the other groups. IUO also had fewer contacts with the naïve animal. B) F2 social preference testing showed IUO spent less time with the naïve animal than the other groups, and IUO had more contacts with the toy than did the other groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ as determined by Two-Way ANOVA followed by a post-hoc Tukey's test. C) Marble burying tests in both generations showed increased burying activity in the PNO and IUO groups. **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ as determined by Two-Way ANOVA followed by a post-hoc Tukey's test.

Discussion

The model system and dose of oxy (15 mg/kg) used in our study mimicked a chronic prescription opiate-dependent woman during gestation and parturition. This dose has been shown to be well-tolerated in animals and mimic development of chronic analgesia in human subjects experiencing breakthrough pain.[225,226] A gap in knowledge exists regarding the implications of how perinatal oxy exposure may impact neurodevelopment in newborns and affect long-term adult behaviors in an intergenerational manner. Intergenerational transmission of traits and phenotypes results from direct exposure of the F0 parent and F1 offspring to a stressor,[306] such as oxy. When the oxy exposure occurs *in utero*, as in the IUO group, the F1 fetus and its germlines are directly exposed. Additionally, when exposed via the breastmilk, which occurs in both the PNO and IUO groups, the germlines are also exposed. The exposure of the germline within the F1 offspring results in an intergenerational transmission to the F2 generation. Studying the intergenerational effects of oxy exposure *in utero* and postnatally can highlight the long-term adverse consequences that extend beyond the mother into future generations.

Opioid use during pregnancy has been associated with smaller head sizes, lighter birthweights, and shorter body lengths in newborns.[133-137] Our data dovetail with this trend, showing that *in utero* oxy exposure affects birthweight, body length, and head development. A rat study using prenatal exposure to morphine yielded similar results, with offspring during the preweaning period having a lower body weight as well as a reduction in brain and cerebellar weights and widths.[307] Similarly, a study using buprenorphine showed delayed offspring development, decreased body weight, decreased body length, and lower pain sensitivity.[308] In our F1 generation, body length appeared to be delayed at P30 when compared to controls. Interestingly, the F2 PNO pups, specifically, had longer bodies at P1 and P14, but all F2 body lengths were comparable at P30. As for head size,

decreased head size and body weight have been reported in a study of NICU neonates.[309] In our study, IUO head size circumference in both generations was smaller compared to controls at P1; head size circumference was comparable to controls at the remaining time points, however.

With regard to weight, *in utero* morphine exposure has also been shown to produce weight deficits that persisted in rats through adulthood,[310] much like what we observed in the F1 PNO and IUO groups. Eriksson *et al.* showed that rats exposed to prenatal morphine treatment not only had lower birthweights than controls but also gained less weight than controls until P19.[311] In our study, F1 PNO and IUO pups appeared to maintain the weight deficit through P30 and did not approach weight levels comparable to saline controls. Our study also identified differences in BMI and LOI during early development. Intriguingly, IUO and PNO in the F2 generation had heavier body weights at all time points compared to controls. Additionally, while the F1 generation trended toward lower BMIs early in development, the F2 generation had higher BMI scores. In other studies considering underweight mothers and the weight gain of their offspring, maternal undernutrition improves the metabolic health of the next generation[312] and can lead to weight gain and obesity in the next generation,[313] much like what we observed in our overweight F2 generation compared to their underweight F1 mothers.

After post-validation of our RNA-seq results, the upregulation of *Hcrt* in the IUO groups of both generations led us to investigate the expression of key genes in the hypocretin system. Hypocretins are involved in arousal, but they are also involved in drug addiction and reward-related behaviors.[314] Hypocretin activation of Hcrtr1 is critical for morphine withdrawal, and NAc activation during withdrawal is dependent on Hcrtr1 function.[315] Not only do our data show an increase in *Hcrt* expression in IUO pups of both generations, which is associated with acute opiate withdrawal,[316,317] but the expression of *Hcrtr1* in the NAc is also increased significantly in the IUO, further suggesting these IUO

pups may be experiencing acute withdrawal. Additionally, Nptx2, which regulates the clustering of AMPA receptors at the synapse, has been shown to increase following opiate withdrawal.[318] The IUO animals in both generations exhibited an increase in *Nptx2* expression, again suggesting withdrawal in these animals. Lastly, our IUO animals had higher levels of *Pdyn* expression in both generations. Chronic exposure to drugs of abuse results in the upregulation of the dynorphin (*Dyn*)/KOR system, and this system has been shown to contribute to psychiatric disorders such as anxiety, depression, and addiction.[319,320]

In addition to highlighting the hypocretin system, our RNA-seq data also highlighted several genes associated with behavioral responses; therefore we sought to investigate the impact of IUO and PNO exposure on social behaviors. In human studies, prenatal opioid exposure has been associated with increased social problems and difficulty with sociability.[309,321] In our social novelty and preference tasks, we did not observe any significant differences in social behaviors among the F1 groups; however, the F2 IUO group appeared to exhibit deficits in social interaction behaviors. In the literature, conflicting results exist regarding the impact of *in utero* and postnatal opioid exposure on social behaviors in animal models. In one study of prenatal exposure to buprenorphine, methadone, and morphine, social interactions in exposed rats were impaired, similar to what has been reported in human studies.[322] In contrast, other studies have shown that prenatal morphine exposure increased social behaviors and resulted in less social avoidance.[323,324] Differences in results may be due to different experimental designs, such as behavior chambers and scoring or the dosing schedule of the animals. For example, many reported behavioral tests for prenatal opioid exposure studies have tested social interaction and play behavior in open chambers where the animals can interact freely.[322-324] Our social preference and novelty tests used a three-chambered apparatus that did not allow for free interaction or play between the test rat and the

cagemate or naïve animal. As for treatment differences, both Hol *et al*. and Niesink *et al*. treated rats with morphine during the last week of gestation and showed increased play behavior in the offspring.[323,324] Najam and Panksepp, however, showed that early postnatal treatment causes a delay in achieving control levels of play behavior in rats.[325] Our treatment paradigms started prior to mating and continued until weaning (IUO) or started only after giving birth and continued until weaning (PNO). Alterations in rodent behavior post-treatment may depend on the stage of development at which the offspring are exposed to treatment, and social problems resulting from intrauterine[309] or adolescent[303] exposure may resolve with age. Our social behavioral tests occurred during young adulthood (P60-65), so perhaps any existing social deficits returned to baseline by the time of testing. The antisocial behavior exhibited by the F2 IUO animals may be attributed to the downregulation of *Avp* highlighted in our NAc RNA-seq and post-validation data. AVP systems have been shown to modulate social behaviors in rats,[326] and blocking AVP receptors resulted in significantly decreased investigation of novel objects.[327] Therefore, the regulation of *Avp* and its effects on social behavior in the context of perinatal opioid exposure may be an important avenue of study for future work.

In addition to social behaviors, we investigated compulsive and anxiety-like behaviors using marble burying tests in both generations. In our study, both the PNO and IUO groups in F1 buried more marbles than the control group, suggesting the presence of anxiety-like and obsessive-compulsive phenotypes. This pattern persisted into the F2 generation, demonstrating intergenerational effects of maternal opioid exposure on subsequent generations. Importantly, the hypocretin system has been implicated in anxiety disorders.[328] Hcrtr1 antagonists have been shown to attenuate anxiety-like behaviors.[329] Further, Hcrtr1 activity is anxiogenic, while Hcrtr2 activity is anxiolytic.[330] The increased expression of *Hcrt* and *Hcrtr1* may contribute to the anxiety-like behaviors exhibited by both generations. Further, prenatal exposure to morphine and methadone

has been shown to increase anxiety-like behaviors in light-dark transition and elevated plus-maze tests in both male and female rats.[322] Additionally, Rohbani *et al*. found that parental morphine exposure affected compulsive grooming and anxiety-like behaviors through marble burying in the offspring.[331] In the case of the F2 IUO, the F1 germ line that would produce F2 was directly exposed, contributing to the intergenerational effect we saw on anxiety-like behavior in this group. In a study using marble burying to assess autism-like and anxiety-like behaviors, increased marble burying occurred in F1, F2, and F3, suggesting that these behaviors may exist through transgenerational epigenetic inheritance.[332] Our F2 animals exhibited the same anxiety-like behaviors as the F1 animals; thus, extension of this study to F3 may elucidate whether transgenerational inheritance is involved in this behavioral phenotype. It is important to note that, while the number of marbles buried in the F1 oxy-exposed groups was roughly similar, the F2 IUO buried significantly more marbles than the F2 PNO, suggesting the IUO may be the more severely impacted of the two groups.

Overall, our study is the first to identify intergenerational effects of pre- and postnatal oxy exposure on development and behavior, thus prompting caution, as both routes of exposure can lead to developmental deficiencies that may persist into the next generation.

DISCUSSION AND FUTURE DIRECTIONS

Discussion

The findings reported in this thesis are novel and contribute greatly to the field of perinatal opioid exposure. By using the Sprague Dawley rat model to mimic oxy exposure *in utero* and postnatally, we have elucidated lasting developmental, biochemical, molecular, and behavioral alterations in exposed progeny. The findings reported for the F1 generation of oxy-exposed offspring as well as the intergenerational impacts of oxy exposure described in these results provide a sufficient baseline upon which future transgenerational studies may build to further investigate physical development, synaptic alterations, behavior, neurological disease, neuroinflammation, and more.

Within the F1 generation itself, oxy exposure had several developmental impacts. Much like what has been observed in other studies of prenatal opioid exposure,[310,311] both the PNO and IUO groups had lower body weights and shorter body lengths than controls. The IUO were also born with smaller head size circumferences than either PNO or control pups. A possible factor impacting head size circumference may be the metabolite TAU; IUO pups had lower TAU levels than either PNO or control animals. Interestingly, Hernandez-Benitez *et al.* have shown that TAU promotes neural development in the embryonic brain as well as in adult brain regions,[244] so it may be that this deficit in TAU impacted the development of the brain and contributed to the smaller head size circumference of P1 IUO pups. Further, as the IUO pups aged, we found that TAU levels increased, which coincided with the return of head size circumference measurements to a comparable size with control and PNO offspring at P30. To further explore this possibility, future studies should aim to investigate TAU levels of P1 pups to discover whether the TAU deficiency is present from birth to P14, or whether it develops during the first two weeks of postnatal development.

Through electrophysiology, RNA-seq, and SVs, our studies of the F1 generation of oxy-exposed animals have demonstrated alterations in synaptic abilities of these offspring. Glutamate receptors have a role in mediating the reward pathway, particularly AMPA receptors, which are crucial for opioid withdrawal during development.[249-254] Our studies suggest altered AMPA subunit composition in the P14 PNO pups but no adverse effects in the IUO group, possibly due to possible neuronal loss due to longer oxy exposure and increased GLU levels in these pups.[255,256] Although no receptor signaling alterations were identified in the IUO group through electrophysiology, we found that *Nptx2* expression in the NAc was increased in both the IUO and PNO groups at P14. Intriguingly, Nptx2 regulates the clustering of AMPA receptors at the synapse and has been implicated in withdrawal.[318] Nptx2 has been consistently associated with AMPA-mediated excitatory synaptogenesis;[333] therefore, as P14 is the peak of synaptogenesis in rats, it may be interesting to further evaluate synaptogenesis and the role of AMPA receptors and Nptx2 in the PNO and IUO offspring during early development, particularly in the PNO offspring that may have altered AMPA subunit composition according to our electrophysiology results. Further synaptic alterations were revealed through our RNA-seq studies of both the PFC and the NAc of IUO and PNO animals. These studies showed alterations in genes associated with pathways involved in synaptic transmission, axon guidance, inflammasomes, and the reward system. Chronic oxy exposure has previously been shown to contribute to axonal degeneration as well as alter white matter through deformation of axonal tracks, reduced axonal fascicle size, loss of myelin basic protein, and the accumulation of amyloid precursor protein.[224] Intriguingly, loss of MEGF8, which was downregulated in the P14 IUO SVs, disrupts axon guidance in the peripheral nervous system.[287] As human studies of infants exposed to opioids prenatally have shown punctate white matter lesions and white matter signal abnormalities in structural MR imaging,[263,264] further investigation of MEGF8 loss in the IUO group would be interesting to elucidate any

lasting effects of IUO exposure into adulthood. Additionally, investigating the axonal structure of P14 IUO brains would also be an intriguing follow-up study to relate our SV data showing MEGF8 downregulation and our RNA-seq data suggesting axon guidance pathway alterations.

An interesting aspect of our F1 studies revealed an enrichment of the sensory pain pathway as well as a lower pain threshold in IUO and PNO animals in adulthood (P75). This lowered pain threshold may be the result of opioid-induced hyperalgesia (OIH), a state of nociceptive sensitization caused by exposure to opioids.[334] Increased hyperalgesia has been reported previously in prenatal opioid exposure studies and, as reported from studies using cross-fostering at birth, may be due to effects incurred during gestational exposure to opioids.[219] The molecular mechanisms of OIH are not well understood,[334] but the involvement of the central glutaminergic system and dynorphins are particularly interesting in the context of our study. NMDA receptors have been shown to play a large role in OIH; inhibiting NMDA receptors with antagonist MK-801 blocked morphine tolerance and thermal hyperalgesia.[335] Further, hyperalgesia has been associated with alterations in subunit composition of AMPA receptors.[336] Fittingly, our P17 electrophysiological studies revealed alterations in AMPA subunit composition in PNO rats. As the P17 rats did not demonstrate any differences in pain thresholds, it may be interesting to extend our electrophysiological studies to P75 to further investigate AMPA and NMDA receptor function in the context of OIH in the IUO and PNO animals. As for dynorphins and OIH, spinal dynorphins have been shown to increase with continuous infusions of μ-receptor agonists.[337] Interestingly, prodynorphin, the precursor to dynorphin, was increased in the F1 IUO animals at P14. Notably, LAMTOR4, which was upregulated in the SVs of our P14 PNO animals, may also influence OIH through its role in the Ragulator complex, which controls the activity of mTORC1. mTORC1 is required for the initiation and maintenance of chronic pain and OIH.[290] As LAMTOR4 was only upregulated

in the PNO group at P14, it would be advantageous to further investigate mTORC1 and LAMTOR4 in both PNO and IUO groups at P75 to not only establish whether the differential expression of LAMTOR4 in PNO still exists, and if so, whether it exists outside of SVs, but also to discern whether the hyperalgesia found in our Von Frey study is dependent on mTORC1.

When considered together, the ^{1}H-MRS and RNA-seq studies used in our research provided insight into the susceptibility of the F1 generation to future diseases and mental disorders. In the PFC RNA-seq data from the F1 generation, we found that genes associated with depression, anxiety, schizophrenia, and obsessive compulsive disorder were enriched in both PNO and IUO offspring. Interestingly, in our marble burying study, F1 IUO and PNO buried more marbles than controls, indicating heightened anxiety and compulsive behavior. A large number of genes associated with renal diseases were also highlighted. Further, the depletion of TAU in our IUO group may further impact possible renal dysfunction.[243] It is also worth noting that, although not significant, the trend of higher CRE and MYO levels in the F1 P17 IUO offspring may indicate gliosis in these animals.[338] Reactive gliosis, or neuroinflammation, involves the accumulation of microglia and astrocytes immediately following the occurrence of a CNS injury.[339] Interestingly, elevated levels of CRE and increased gliosis have been identified in studies of multiple sclerosis.[340,341] Also within the realm of gliosis, LAMTOR4, which was upregulated in the SVs of our P14 PNO animals, is essential for microglia development; improper LAMTOR4 and Ragulator function lead to abnormal lysosomal accumulation within the microglia, rendering the microglia incapable of digesting debris that may contribute to neurological diseases such as Alzheimer's disease.[272] Interestingly, studies have reported higher MYO levels in patients with Alzheimer's disease and dementia.[342-344] Further, several genes associated with Alzheimer's disease (*Psen1, Chrnb2, Dpysl2, Amfr, Pcdh1x, Npy, Bcl2*) were also highlighted in the IUO and PNO groups in the bioinformatic analysis of our PFC

RNA-seq data. Furthermore, the IPA of our SV contents suggested an increase in CRE in the IUO pups as well as highlighted neurological disease as a top hit in both IUO and PNO groups; however, in our ^{1}H-MRS study of P30 IUO, CRE and MYO levels were lower than controls. More investigation is needed to determine whether gliosis during P14 is increased, how an upregulation in LAMTOR4 affects gliosis in the PNO offspring, and whether an increase in gliosis during early development has any lasting effects into adulthood in the IUO offspring, including any impact on the expression of genes related to Alzheimer's disease.

Although not investigated as thoroughly as the F1 generation, the F2 generation demonstrated the impact of oxy exposure on development, gene expression, and behavior in an intergenerational manner. Like the F1 generation, F2 IUO P1 pups had smaller head size circumferences than either PNO or saline pups. In the F1 generation, this smaller head size circumference may be due in part to the lower levels of TAU, we are unsure whether this is the case for the F2 generation. Extending the ^{1}H-MRS studies into the F2 generation may offer more insight into the smaller head size circumference of P1 IUO pups in the F2 generation. In contrast to the F1 generation, the F2 pups in the IUO and PNO groups weighed significantly more than saline counterparts at P1, P7, P14, and P30. Further, the P1 F2 IUO offspring had higher BMI and LOI scores than the saline and PNO offspring, and both oxy-exposed groups had higher BMI scores than saline pups at P7. Because the F1 mothers were underweight as they approached adulthood, it may be possible the F2 generation was affected in a compensatory way. Maternal undernutrition has been shown to improve the metabolic health of the next generation,[312] often leading to weight gain and obesity in these offspring.[313] Future studies may aim to further investigate this effect by cross-fostering the pups to dams that did not have IUO or PNO exposure themselves.

While our intergenerational study did not include studies on synaptic signaling, it did provide insight into the molecular effects of oxy exposure in the NAc. The most influential finding from this intergenerational study was the upregulation of *Hcrt* in the IUO of both F1 and F2 generations. Hypocretins are involved in drug addiction and reward-related behaviors,[314] and activation of Hcrtr1 is critical for morphine withdrawal and NAc activation during withdrawal.[315] The increased expression of *Hcrt* and *Hcrtr1*, along with the increased expression of *Nptx2*, in the IUO pups is associated with acute opiate withdrawal.[316-318] Investigations into withdrawal in the IUO offspring, specifically those in the F1 generation that are actively exposed to oxy during development, using an opioid antagonist such as naloxone may allow us to verify addiction and withdrawal through a behavioral test. Additionally, *Pdyn* was highly expressed in F1 and F2 IUO animals. As dynorphins have been associated with OIH,[334] and as the F1 oxy-exposed groups appear to exhibit OIH, extending the Von Frey testing to the F2 generation may yield interesting insight into the possibility of lasting hyperalgesia effects in the F2 generation. Further investigation of the hypocretin system and related genes in terms of epigenic modification would be the next step for this study to determine the extent of these effects and whether they are inherited from the mother.

The social behavior studies in the F1 and F2 generations provided us with interesting findings regarding the F2 generation. In human studies, prenatal opioid exposure has been associated with increased social problems and difficulty with sociability.[309,321] While our F1 animals exhibited no differences in social behaviors, the F2 IUO group demonstrated deficits in social interaction behaviors. Because alterations in rodent behavior post-treatment may depend on the developmental stage at which the rats are exposed to treatment, social problems resulting from intrauterine[309] or adolescent[303] exposure may resolve with age. Our social behavioral tests occurred during young adulthood (P60-65), so perhaps any existing social deficits in the F1 generation returned

to baseline by the time of testing. Perhaps implementing different social testing paradigms or testing at an earlier age may shed more light on any social deficits in the F1 animals. As for the antisocial behavior exhibited by the F2 IUO animals, the downregulation of *Avp* highlighted in our NAc RNA-seq and post-validation data may play a role. AVP systems have been shown to modulate social behaviors in rats,[326] and blocking AVP receptors resulted in significantly decreased investigation of novel objects.[327] As *Avp* was also downregulated in the F1 IUO animals, yet these animals showed no social deficits, studying the AVP system in both generations while also employing different social behavior paradigms may yield more information on whether this system truly affects social behavior in these oxy-exposed animals.

As a number of genes associated with anxiety and obsessive-compulsive disorder were highlighted in the F1 P14 RNA-seq as well as in the F1and F2 P14 NAc RNA-seq, we employed marble burying tests in both generations to study these behaviors. In our study, both the PNO and IUO groups in both F1 and F2 generations buried more marbles than the control group, suggesting the presence of anxiety-like and obsessive-compulsive phenotypes that may be inherited intergenerationally. Importantly, the hypocretin system has been implicated in anxiety disorders.[328] Hcrtr1 antagonists have been shown to attenuate anxiety-like behaviors.[329] Further, Hcrtr1 activity is anxiogenic, while Hcrtr2 activity is anxiolytic.[330] The increased expression of *Hcrt* and *Hcrtr1* may contribute to the anxiety-like behaviors exhibited by both generations. In addition to the hypocretin system, Abraham *et al.* suggest the involvement of KOR; activation of KOR with agonist U50488 significantly increased the number of marbles buried, suggesting KOR-mediated increases in anxiety-like or stress-induced compulsive behaviors.[345] Additionally, chronic exposure to drugs of abuse results in the upregulation of the *Dyn*/KOR system, and this system has been shown to contribute to psychiatric disorders such as anxiety, depression, and addiction.[319,320] Importantly, from our NAc RNA-seq data of F1 and F2 generations,

we do see an upregulation of *Pdyn* expression in the oxy-exposed groups. Further, prenatal exposure to morphine and methadone has been shown to increase anxiety-like behaviors.[322] Parental morphine exposure has also been shown to affect compulsive grooming and anxiety-like behaviors in the offspring.[331] In a study using marble burying to assess autism-like and anxiety-like behaviors, increased marble burying occurred in F1, F2, and F3, suggesting that these behaviors may exist through transgenerational epigenetic inheritance.[332] Our F2 animals exhibited the same anxiety-like behaviors as the F1 animals; thus, extension of this study to F3 may elucidate whether transgenerational inheritance is involved in this behavioral phenotype.

Based on the findings of our intergenerational study of IUO and PNO exposure groups in which we identified the differential expression of key genes involved in the hypocretin system as well as physical and behavioral effects of oxy exposure, the extension of this study to a transgenerational model is critical. Moreover, performing electrophysiology, ^{1}H-MRS, SV isolation and characterization, and pain testing would be advantageous in further defining intergenerational effects of oxy exposure in the F2 IUO and PNO groups. As the findings in our intergenerational study regarding the F1 generation may provide more insight into those studies performed solely in the F1 generation, so too may we extend the F1 studies into the F2 generation to further explore these changes. The study of the generational impact of oxy exposure may be strengthened by the investigation of transgenerational effects and any possible epigenetic alterations that contribute to the generational inheritance of phenotypic, molecular, or behavioral alterations exhibited by the IUO and PNO offspring.

Future Directions

Although we employed a comprehensive approach in investigating the effects of pre- and post-natal oxy exposure, there are several ways in which our studies can improve

in the future. Our approach focused on the maternal effects of oxy use. However, a growing body of research considers the paternal effects of opioid use on the offspring. Paternal use of opioids such as morphine and heroin have been shown to have sex-specific effects on object recognition memory,[167] increased anxiety and aggression,[172] and altered pain perception and signaling.[152,153] As our study has shown that pre- and post-natal exposure to oxy via the mother have led to developmental, behavioral, and molecular alterations, extending our study to include a parental exposure group may also lend critical insight into intergenerational inheritance of these alterations. Additionally, our studies may also benefit from monitoring maternal behavior and nutritional intake to determine the type of environment and nutrition the pups had in the three different conditions. The weight deficits reported in the F1 IUO and PNO groups may be the result of altered maternal care or nutritional intake. Morphine exposure has been shown to disrupt maternal behavior,[346] and proper nutritional status of the mother may be impacted by substance use, thus impacting the breastfeeding offspring.[347,348] By investigating maternal effects as well as nutrition, we can better understand the consistent weight deficits exhibited by the F1 oxy-exposed animals. Further, because maternal undernutrition may improve the metabolic health of the next generation[312] and lead to weight gain and obesity in the next generation,[313] understanding the persistently low weights of the F1 generation may provide more information relevant to the overweight F2 generation.

Another important aspect to include in future studies regarding the mother would be the establishment of addiction. Without testing withdrawal, our groups cannot be defined as “addicted” but instead are referred to as “exposed.” Although our studies were conducted in the context of oxy exposure, it may be beneficial to extend our study to cover addiction as well. Using a naloxone treatment on the dams and monitoring behavior would allow us to discern whether our dosing regimen is sufficient to establish addiction. In the case of exposed offspring, determining the concentration of oxy in the breastmilk would

be important in understanding exposure versus addiction in the pups. Once we determine the amount of oxy pups receive, withdrawal in the pups can be measured at weaning with a naloxone treatment and subsequent monitoring of withdrawal behaviors, allowing us to consider our findings in the context of addiction rather than exposure.

Perhaps the most prominent finding from our study that warrants further investigation is the role of the hypocretin pathway in the intergenerational effects of perinatal oxy exposure. Because of its role in the regulation of motivation, arousal, and stress, the hypocretin system has become a promising target for the treatment of substance use disorder. Hypocretins, also known as orexins, are neuropeptides derived primarily from hypocretin-containing neurons in the lateral hypothalamus. Hypocretin-1 (orexin-A) and hypocretin-2 (orexin-B) act through hypocretin receptor types 1 (Hcrtr-1, or OX1R) and 2 (Hcrtr-2, or OX2R), respectively, and have a number of physiological roles, such as the regulation of the sleep-wake cycle, feeding behavior, energy homeostasis, and reward-seeking.[349] Hypocretin signaling dysfunction has been associated with narcolepsy, insomnia, depression, stroke, and, importantly, addiction.[314,349,350] Studies of alcohol,[351] opioid,[352] and cocaine[353] use have shown Hcrtr-1 involvement in addictive behaviors. Because nearly half of all hypocretin neurons express mu-opioid receptors, the main target of opioids,[354,355] targeting the hypocretin system may reduce opioid intake, craving, and relapse.[352] Indeed, recent studies have shown that Hcrtr-1 blockade attenuates drug-seeking behavior in rat self-administration studies.[351,353] Further, activation of Hcrtr-1 is critical for morphine withdrawal, and the activation of the NAc during withdrawal is dependent on Hcrtr-1 function.[315] Hcrtr-1 antagonism has effectively reduced motivation for oxy, fentanyl, and remifentanil.[352] Recently, suvorexant, an FDA-approved treatment for insomnia that blocks the activity of both Hcrtr-1 and Hcrtr-2, has been applied in clinical trials[356,357] for the treatment of sleep, craving, and stress states in patients with substance use disorders, including opioid use disorder; however, no data are published

yet. Because we have identified an upregulation of the hypocretin system in both the F1 and F2 generations of oxy-exposed pups, the addition of a treatment with an Hcrtr-1 antagonist, such as suvorexant, may mitigate this upregulation. This would be particularly interesting if our studies also extended into the susceptibility of the IUO and PNO offspring to future drug use and addictive behaviors. To best explore the impact of hypocretin system upregulation with PNO and IUO exposure may require the use of a self-administration study. Extending our intergenerational study to include a self-administration model of oxy addiction for the adult F1 and F2 pups would allow us to study any predispositions to oxy addiction, withdrawal, and, by using an Hcrtr-1 antagonist, further investigate how the upregulation of the hypocretin system and the blocking of Hcrtr-1 impact addiction and withdrawal in these oxy-exposed animals.

As Hcrtr-1 antagonists like suvorexant are only recently being investigated as potential therapeutics for opioid use disorder, it may be interesting to consider the effects of other therapeutic interventions for maternal opioid use on our IUO and PNO groups. Methadone, a synthetically derived agonist that binds to mu-, kappa-, and delta-opioid receptors, exerts morphine-like effects and, therefore, is the most commonly used pharmacotherapy for opioid dependence, including pregnant women.[322] Buprenorphine, a semisynthetic thebaine derivative that selectively binds to the mu-opioid receptor as a partial agonist, the nociception receptor as a full or partial agonist, and the kappa-opioid receptor as an antagonist, has also been used to treat opioid dependence during pregnancy.[322] Although used for treatment of opioid use in pregnant women as well as for infants with NOWS, methadone and buprenorphine are not without their own faults. Animal studies have shown that buprenorphine and methadone maintenance therapy produce detrimental side effects on cognitive function and social behaviors[322] as well as influence the severity anxiety-like behaviors.[358] Human studies have shown that newborns exposed to buprenorphine prenatally exhibited mild symptoms of NOWS[359] that were not as severe

as those identified in newborns exposed to high-dose methadone.[360] Prenatal methadone exposure was also associated with weakness in language and cognition.[197] Contrarily, other human studies have revealed no significant differences in neurobehavioral outcomes between newborns exposed to buprenorphine versus those exposed to methadone.[361] It may therefore be interesting to include a buprenorphine or methadone treatment group in our IUO and PNO studies to determine how treatment with these drugs may impact the development of the oxy-exposed pups.

Further, Jantzie *et al.* have recently shown that perinatal methadone has a distinct systemic and neuroinflammatory signature.[291] Based on these findings, the authors posit that prenatal opioid exposure may be the next neuroinflammatory disease. Further investigation into the possible increase in gliosis in the F1 IUO suggested by our ^{1}H-MRS and RNA-seq studies may allow us to identify whether prenatal oxy exposure can also be associated with neuroinflammation. By isolating plasma from our IUO groups during the perinatal period and adulthood and by measuring the concentration of inflammatory cytokines and chemokines, we could investigate sustained neuroinflammation in these animals. If prenatal oxy exposure also has distinct neuroinflammatory signatures, perhaps the use of anti-inflammatory drugs may mitigate the changes our F1 studies have found. Further, implementing this investigation in the context of inter- or trans-generational inheritance may also yield more information about the extent of prenatal opioid exposure as a neonatal inflammatory disease.

Overall, our studies provide a solid groundwork on which future studies can build to further investigate the generational effects of perinatal oxy exposure. Since we have characterized maternal oxy use and the effects on F1 and F2 offspring, the addition of paternal exposure groups would add to our knowledge of the generational effects of oxy exposure by considered exposure from either parent. The hypocretin pathway is becoming increasingly relevant as a possible treatment for opioid use, as evidences by ongoing

clinical trials. The investigation of therapeutics such as buprenorphine, methadone, and the more recent suvorexant may aid in the identification of the most effective treatment for opioid-using mothers or newborns diagnosed with NOWs. Investigating prenatal oxy exposure in the context of a neonatal inflammatory disease would also be a novel and intriguing possibility for future work. Taken together, the further investigation of each of these avenues of research would provide us a more complete understanding of perinatal oxy exposure and the severity of any subsequent generational consequences.

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