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# Prenatal buprenorphine exposure reduces expression of myelin proteins in neonatal longs-Evans rat

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# PRENATAL BUPRENORPHINE EXPOSURE REDUCES EXPRESSION OF MYELIN PROTEINS IN NEONATAL LONGS-EVANS RATS

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## Approval of Thesis

We, the faculty supervising the work of Christopher James Grahe, affirm that the thesis, *Prenatal Buprenorphine Exposure Reduces Expression of Myelin Proteins in Neonatal Longs-Evans Rats*, meets the high academic standards for original scholarship and creative work established by the Biomedical Sciences department and the Joan C. Edwards School of Medicine. This work also conforms to the requirements and formatting guidelines of Marshall University. With our signatures, we approve the manuscript for publication.


Dr Larry Grover, Department of Biomedical Sciences, Committee Chairperson Date


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# Table of Contents

# List of Tables

# List of Figures

## List of Abbreviations

ADHD - Attention-deficit hyperactivity disorder

ALS – Amyotrophic lateral sclerosis

AMP – Adenosine monophosphate

BSID-II - Bayley Scales of Infant Development, Second Edition

BSI-III - Bayley Scale of Infant Toddler Development III

CNPase - 2',3'-cyclic nucleotide 3'-phosphodiesterase

CNS - central nervous system

CPP - Conditioned place preference

DA – Dopamine

DOP - Delta Opioid receptor

ECL – Enhanced chemiluminescence

fMRI – Functional magnetic resonance imaging

GABA – Gamma amino butyric acid

GD – Gestational Day

GOF – Gain of function

GPCRs - G-protein coupled receptors

HDAC - Histone de-acetylation

HIV - Human Immunodeficiency Virus

IBQ-R - Infant Behavior Questionnaire Revised

ICV – Intracranioventricular

IV – Intravenous

ITSP - Infant Toddler Sensory Profile

kD – Kilodalton

KOP – Kappa Opioid receptor

KIF5A - Kinesin family member 5A

LOF - Loss of function

MAP – Mitogen-activated protein

MBP - Myelin basic protein

MOP – Mu Opioid receptor

MOUD - Medications for OUD

MS – Multiple sclerosis

n – sample number

NAcc - Nucleus accumbens

NAPLAN - National Assessment Program: Literacy and Numeracy

NAS - Neonatal abstinence syndrome

NG2 - Neuron/glial antigen 2

NICU - Neonatal intensive care unit

NMS - National Minimum Standard

NOP – Nociceptin Opioid receptor

Olig2 – Oligodendrocyte transcription factor 2

OPC - Oligodendrocyte Precursor Cells

OTM - Oral transmucosal

OUD - Opioid use disorder

PBN - Parabrachial nucleus

PD – Post natal day

REEL-3 - Receptive-Expressive Emergent Language 3

SAMHSA – Substance Abuse and Mental Health Services Administration

SOX10 - SRY-related HMG-box 10

TCA – Trichostatin A

VEP - Visual evoked potentials

Vglut2 - Vesicular glutamate transporter 2

VTA - Ventral tegmental area

# Abstract

In the USA and especially in WV, there has been a surge in the rise of opioid use disorder (OUD), and with it a rise in neonatal abstinence syndrome (NAS). What makes this rise in NAS so unfortunate is that the Medications for OUD (MOUD) do not prevent the development of NAS. Although the relationship between MOUD and NAS is still unclear, it is thought that buprenorphine, one of the main substances used to treat OUD in pregnant women, may feed into the development of NAS, which may affect mental and physiological development and cause other health problems. Via understanding the developmental impact of NAS, we hope to pave the way for future NAS treatments and a brighter future for the children of the USA.

The central hypothesis of this proposal is that developmental issues caused by prenatal buprenorphine exposure are due to reduced expression of white matter development proteins, such as NG2 and MBP, and changes in neuronal proteins such as Kif5A, and that these changes cause deficits in the white matter parts of the brain. To study this hypothesis, we used a rat in utero buprenorphine exposure model to investigate the impact of this opioid on the post-natal time course of protein expression in two white matter tracts, the optic nerve and cerebellum.

Preliminary Western blot analysis indicates that there is a significant interaction between post-natal age and treatment for multiple proteins involved in myelination. Both white matter tracks also showed significant increases of KiF5A late in post-natal development. This data indicates that in utero buprenorphine impacts white matter development promoting a change in the protein expression timelines. If these developmental changes are maintained throughout the life span, they could explain some of the issues associated with the long-term impact of NAS.

# Chapter 1

In the United States and especially in West Virginia, an epidemic of opioid use disorder (OUD) has hit a fever pitch, resulting in many varied and serious problems. There are approximately 2.1 million US citizens aged 12 years and older with opioid use disorder, and 47,600 fatal opioid overdoses as of 2017 (Florence et al., 2021). To make matters worse, the OUD epidemic has claimed many lives, a topic that Gomes et al. (2018) cross-examined in a previous study from 2001 to 2016. According to Gomes, "Over the 15-year study period, [335,123] opioid-related deaths in the United States met our inclusion criteria, with an increase of 345% from 9,489 in 2001 (33.3 deaths per million population) to [42,245] in 2016 (130.7 deaths per million population)." Most of those deaths were observed in young adults within the range of 25-34 years old. Altogether, the deaths due to opioid use disorder accounted for a staggering 1,681,359 years of life lost.

It's not just the deaths that are concerning from a medical and quality-of-life standpoint, either. From 1992 to 2012, reports of prescription opioid use among pregnant women have escalated from 2% to 28%--a 14-fold increase (Goldfarb et al., 2020). This is alarming because of a single well-documented observation: substances consumed by a pregnant mother can exert unwanted influences on their developing fetus. It is the reason we have begun discouraging pregnant women from smoking or consuming alcohol, and it is also why medicine commercials advise against taking their medications if one is pregnant or planning to become pregnant, but OUD is such a problem that it continues to escalate despite this knowledge. The incidence of maternal opioid-related diagnoses throughout the US has escalated 131%, with West Virginia taking the brunt of the increases (Hirai et al., 2021). However, while the specific numbers per 1000 births of both opioid-related conditions was not as high in most states as it was in West

Virginia in 2017, the percent change in both conditions from 2010 and 2017 was remarkably high. Even though the only other state to have a percent increase in NAS incidence within that time frame was Oklahoma, many states had such a high percent increase that the mean percent change for the USA as a whole was 82%. Meanwhile, not only did the whole USA have an average increase of 131% in maternal OUD from 2010 to 2017, but West Virginia in particular had one of the higher individual percent increases. This paper's goal is to dissect these conditions and the proteins that may be involved in their development, starting with explaining what an opioid is so that we may establish a baseline to discuss our experimental results.

In basic terms, an opioid is any substance, endogenous or exogenous, that binds to and activates opioid receptor proteins in the brain and body. These opioid receptors are 7-transmembrane G-protein coupled receptors (GPCRs) whose active states bind agonists with high affinity and activate heterotrimeric G proteins, triggering downstream intracellular signaling pathways. The conformational state of a GPCR, including the opioid receptors, is controlled not only by its agonist occupying the orthosteric site but also by endogenous substances acting at other, allosteric sites on the receptor (Livingston & Traynor, 2018).

Aside from being commonly found in different parts of the body, including the brain, spinal cord, and even the digestive tract, opioid receptors are widely studied due to their crucial role in pain management, drug abuse/addiction, and mood disorders (Shang & Filizola, 2015). Three major subtypes of opioid receptors have been found so far: Delta (DOP), Mu (MOP), and Kappa (KOP), each activated by endogenous peptides such as endomorphins, enkephalins, and dynorphins. However, these are not strictly the only possible ligands that can trigger opioid receptors, as they are also activated by naturally occurring alkaloids and other synthetic and semi-synthetic small-molecule ligands. Although there is a fourth subtype, the nociceptin opioid

receptor (NOP), which is phylogenetically related to the other three, it does not bind the same ligands, and thus it will not be discussed as thoroughly in this paper. However, even despite all three opioid receptor proteins sharing the same core 7-transmembrane domain, each is coded by a distinct gene and has distinct terminal domains (Wei & Loh, 2011). As such, each opioid receptor protein has different ligand binding affinities, signal transduction pathways, and pharmacological effects. MOPs are found in the cerebral cortex, thalamus, periaqueductal gray matter, and rostral ventromedial cortex, and they are responsible for the hedonic, rewarding effects of opioids, although they also cause constipation and respiratory depression (Wang, 2019). KOPs are found in the hypothalamus and periaqueductal gray matter and are responsible for the anti-reward properties of opioids, such as diuresis, nausea, and dysphoria. DOPs are found in the basal ganglia, pontine nucleus, and amygdala and are responsible for anxiolytic effects of opioids. All three subtypes appear to have analgesic effects when stimulated, making the appeal of opioid drugs obvious. Even though this is a report on the expression of non-opioid-receptor proteins in varying areas of the brain, and thus we will not be investigating the activity of these receptors in our procedures, it is still important to understand the mechanisms of action of opioids, so that we may see how this research is relevant to current knowledge.

Even despite the extensive research into OUD and its influence on the brain uncovering these novel receptors, the precise circuitry of opioid pathways in the brain is still unclear (Galaj & Xi, 2021). It was initially thought that abuse liability of opioids was derived from drug reward effects, mediated primarily by dopaminergic (DA) neurons in the ventral tegmental area (VTA). Early electrophysiological and microdialysis studies indicated that MOP stimulation directly inhibits GABAergic neurons of the VTA, leading to rapid disinhibition of neighboring DA neurons and release of dopamine into the nucleus accumbens (NAcc). This hypothesis was

supported by a series of behavioral studies, including self-administration of MOP agonists directly into the VTA and conditioned place preference (CPP) for environments associated with intra-VTA infusions of MOP agonists. However, there is also evidence that refutes this DA disinhibition hypothesis, such as DA-deficient mice (i.e. mice who are unable to synthesize dopamine) developing robust CPP for morphine when given morphine in conjunction with either caffeine or the dopamine precursor 1-dihydroxyphenylalanine (Hnasko et al, 2005). Another example refuting the DA disinhibition hypothesis is that lesioning of DA terminals in the NAcc with 6-OHDA failed to alter heroin self-administration (Gerrits et al, 1984).

Further complicating matters for the DA disinhibition hypothesis, a recent study by Corre et al (2018), using c-Fos immunohistochemistry and fiber photometry, showed that a single injection of heroin can activate DA neurons in the medial part of the VTA and increase dopamine release in the medial shell of the NAcc. This suggests that not all neurons in the VTA are activated by opioids, and it implies that other unidentified neural substrates may also be involved in opioid action. However, the striking caveat here is that chemogenetic inhibition of the DA neurons in the VTA inhibits heroin self-administration, providing supporting evidence for the role of VTA DA neurons in opioid reward. Meanwhile, taking an optogenetic approach, it was found that dopamine transporter (DAT)-cre mice transfected with an inhibitory halorhodopsin in VTA DA neurons readily learned to lever-press for IV heroin self-administration (Galaj & Xi, 2021). However, this self-administration was significantly reduced with optogenetic inhibition of the transfected neurons. Thus, the study provided evidence both for and against the DA disinhibition hypothesis; however, since there was striking evidence that inhibition of the DA neurons impaired self-administration, the possibility that dopamine is involved in the euphoric effects of opioid substances only appears all the more probable. Either

way, this study providing both evidence for and against the DA disinhibition hypothesis indicates that opioid addiction is a far more complex neurological disorder than simply the brain needing a foreign substance to function normally, a theme that seeps into all of the following points of information about OUD.

Regardless of how the underlying neuronal mechanisms work, however, OUD always involves the same core set of factors (Kosten & Baxter, 2019): Opioids are consumed in larger doses or over a longer period of time than intended, and a lot of time is spent obtaining, using, or recovering from opioids. The patient has a strong desire or "craving" to use opioids, and any patients who wish to quit using opioids routinely find their attempts to discontinue opioid use to be unsuccessful. Their continued opioid use causes them copious social, financial, and even physical problems, and while they are aware of them, the patient still continues to use opioids. Lastly, physiological signs of OUD include tolerance to opioids, where continuous use of the drugs diminishes the effect from the same dose; and opioid withdrawal symptoms, unpleasant side-effects that appear after ceasing drug use. In the modern era, we have treatments to combat the withdrawal symptoms, but it is still very much an uphill battle against OUD as a whole.

According to the SAMSHA (2021), opioid receptor ligands come in three distinct classes: full agonists (those that activate the opioid receptor fully in a dose-dependent manner, such as methadone), partial agonists (those that plateau at a lower threshold of intrinsic activity than full agonists, such as buprenorphine), and antagonists (those that bind to the receptor without activating it at all, such as naltrexone; rather than binding to the receptor as if to activate it, opioid receptor antagonists block agonists from binding to the receptor). It is the antagonists and partial agonists that interest pharmacists and medical researchers the most, for they are presently in use as medications to help treat and prevent OUD. When opioid drugs are being discussed, it

is typically the full agonists that are being mentioned, as those are the ones with the strongest effects; in contrast, a partial agonist will only be able to activate the receptor to a certain degree. Partial agonists are thus considered for treatment of OUD because the lower degree of receptor activation equates to a lowered risk of overdose; antagonists, meanwhile, are considered for OUD treatment and prevention because of their ability to completely block opioid agonists from affecting the patient. However, OUD is still a rampant disease since opioid misuse and abuse continues to be a prevalent problem in America.

The mechanisms underlying neurological disorder caused by opioid addiction are not yet clear (Feng et al., 2012). However, what is known about the neurophysiology of OUD runs as follows: The neuronal basis of positive reinforcement relies on the activation of dopaminergic neurons, resulting in an increase in dopamine release in the mesolimbic brain structures. Meanwhile, certain aspects of opioid dependence and withdrawal are also related to noradrenergic and serotonergic systems, as well as both excitatory and inhibitory amino acid and peptidergic systems. In addition, an important role in neurochemical mechanisms of opioid reward, dependence, and vulnerability to addiction has been ascribed to the activation of endogenous opioid peptides, particularly those activating via the Mu and Kappa opioid receptors.

At the onset of OUD, most people use opioids to experience euphoric feelings or to control pain; for these purposes, the use of opioids to control pain for longer than prescribed by the doctor is referred to as “misuse,” and the intentional seeking of euphoric feelings is referred to as “abuse” (Hagemeier, 2018). However, regardless of the reason why the user continues to use opioids, tolerance develops quickly, and patients rapidly lose control of their opioid intake; combined with a craving to minimize withdrawal symptoms, opioid dependence develops as a direct consequence (Wang, 2019). Euphoria fades easily as tolerance develops, but the

withdrawal symptoms persist, including muscle aches, bone pains, runny nose, excessive yawning, diarrhea, abdominal cramps, agitation, anxiety, and sweating. While the withdrawal symptoms will eventually go away as the body readjusts to no longer consuming opioids, they still serve as a potent form of negative reinforcement to incentivize OUD sufferers to continue using opioids.

Receptor internalization when β-arrestin binds leads to desensitization to opioids. The desensitized receptors recover over time, taking anywhere from minutes to hours, depending on the agonist, after the stimulus has been withdrawn, and the endocytosed receptors are recycled to the plasma membrane in a resensitized state (Martyn et al., 2019). If this desensitization is not recovered from, however, the desensitized state may become permanent, and tolerance follows from this. While this is a considerable factor in opioid tolerance, according to Feng et al. (2012), "Several important processes have been identified including upregulation of cAMP/PKA and cAMP response element-binding signaling and perhaps MAPK cascades in opioid sensitive neurons, which might not only influence tolerance and withdrawal, but also synaptic plasticity during the cycles of intoxication and withdrawal." Intracellular molecules of signal transmission that are also involved in opioid tolerance and dependence include G proteins, cyclic AMP, MAP kinases, and some transcription factors. The latter link in this chain of reactions modifies the expression of target genes, which may make them responsible for long-lasting neuroplasticity induced by opioids. Regardless of how tolerance develops, however, it always leads to the same end result: OUD sufferers find that they receive less and less of an effect from the same dose of the drugs that they are taking, so they attempt to compensate for this by increasing the dose. When this occurs, the flood of drugs entering the user's system activates the desensitized

neurons more strongly, which can prove deadly when considering that opioids have respiratory-depressive effects.

However, as mentioned briefly above, tolerance is not the only motivator for an OUD sufferer to continue taking more and more opioids. The big negative reinforcer is withdrawal symptoms, which contribute to the underlying basis of opioid dependence by forcing the user to consume more opioids in order to avoid them. Symptoms include piloerection, myalgia (pain), diarrhea, nausea/vomiting, pupillary dilation, photophobia (light sensitivity), insomnia, tachypnea (faster breathing), tachycardia, sweating, hypertension, and hyperthermia (Shah & Huecker, 2022).

Despite the research into OUD formation, the mechanisms of the withdrawal symptoms are much better understood than the formation of OUD. For example, Zhang et al. (2020) found that MOP receptor-containing neurons in the thalamus, medial habenula, and parabrachial nucleus (PBN) are mostly glutamatergic, and that the neurons that express MOP also express Vglut2, which allows the MOP receptors to be selectively deleted on glutamatergic neurons. When the MOP receptors on glutamatergic neurons were deleted, Zhang et al. (2020) then induced opioid withdrawal by injections of naloxone. While the unmodified opioid-treated group showed the expected deviations from the unmodified control group (higher teeth chattering, headshakes, tremors, and anxiety in the open-field test), the modified opioid-treated groups showed withdrawal scores closer to the unmodified control group. This implies that the glutamatergic neurons of the PBN, thalamus, and medial habenula are vital to the onset of withdrawal symptoms. Likewise, Wu et al. (2020) found that Wnt5b is produced and accumulated in dorsal root ganglia following repeated opioid exposure, and this Wnt5b is released into the dorsal hippocampus following withdrawal. Inhibiting the synthesis or

downstream signaling of Wnt5b largely suppressed withdrawal behaviors in the study by Wu et al. However, these studies are still highly theoretical, and it may be a long time before the rationale behind these experiments paves the way for new medications for opioid use disorder (MOUD).

Aside from the reinforcing effects caused by opioids and opioid withdrawal, there exist several barriers that limit the access of OUD patients to treatment (Farnsworth et al., 2021). Chief among these is the myth that individual willpower should be sufficient to discontinue the addiction, which continues to persist despite the vast breadth of evidence to refute this point. This misconception creates a stigma against OUD sufferers that may encourage them to hide their drug misuse or abuse and make them reluctant to seek medical care. In addition, an estimated 25% of OUD sufferers are living below the poverty line and 20% lack insurance, compared to the general population with 12.5% living below the poverty line and 8% uninsured. Even among patients who do receive treatment for OUD, there still exist barriers to effective recovery. Firstly, while Medicaid expansion under the Affordable Care Act of 2010 provides insurance benefits to people suffering from OUD, some states exclude methadone treatment, a common form of pharmacological intervention, from the list of benefits that Medicaid can provide. Secondly, OUD patients who happen to be African-American or Hispanic are less likely to be granted access to buprenorphine therapy than Caucasian patients, as well as being less likely to have insurance and more likely to experience delays in treatment than Caucasian patients. When combined with the possibility that some of these minority patients may also live below the poverty line, it does not bode well for their possible outcomes in the OUD epidemic in the USA.

It is not only the individuals suffering from OUD that are suffering financially, however. The US economy itself also suffers greatly from the OUD epidemic. The overall economic burden totaled approximately $1.021 trillion dollars in 2017 (Florence et al., 2021). $470.975 billion, slightly less than half of these costs, were attributed to OUD itself. Of that $470.975 billion, $31.308 billion were from health care to assist people suffering from OUD, mostly from private insurance and Medicaid. $3.534 billion were spent on treatment to help patients overcome OUD, with most of that being paid for by the state and local governments. $14.819 billion were criminal justice spending, with $6.209 billion spent on police protection, $2.819 billion in legal fees for the courts, $5.445 billion to fund correction facilities, and the remaining $378 million to recover or replace property lost due to the crimes. $31.311 billion of the costs attributed to OUD itself were from lost productivity between increased disability, reduction of productive time, and individuals incarcerated for drug-related crimes. The last of these non-fatal costs of OUD were $390.003 billion, the value of the quality of life lost to OUD sufferers. The other half of the OUD-associated costs, $549.691 billion, were attributed to deaths from opioid use disorder. The lives lost had a statistical value of $480.737 billion, with an additional $68.694 billion lost in productivity, and $260 million spent on health care for the dying.

As one can imagine, these staggering financial costs stretch thin the already limited resources of the United States. A study by Loudin et al. (2017) found that overall incidence of neonatal abstinence syndrome (NAS, which we will discuss later in this paper) has quadrupled in recent years, from 1.5 per 1000 births in 1999 to 6 per 1000 births in 2013. On its own, this would already be a depressing figure of our agonizing battle against drug use and its consequences, but when you consider that West Virginia has the highest rate of NAS at 33.4 per 1000 births, plus the fact that about a quarter of all OUD sufferers are poor, compared to 12.5%

of all Americans (Farnsworth et al., 2021), it becomes a grim omen for the current trajectory of the US economy. Although the neonatal intensive care unit (NICU) is a slightly cheaper option than medically treating NAS patients, a dedicated area for the treatment of NAS was significantly cheaper, clocking in at a median of $17,688 per patient in hospital charges for a dedicated unit, compared to $90,601 per patient for medical treatment and $68,750 per patient for the NICU (Loudin et al., 2017).

There are a multitude of other risks involved in OUD besides the withdrawal symptoms, which may contribute to the staggeringly high body count. Morphine abuse inhibits T-helper 17 cells and, at the same time, enhances the activity of T-regulatory cells, which could be linked to immune suppression. (Malafoglia et al., 2021). Mu receptor activation has also been shown to regulate macrophage function, including nitric oxide production and phagocytosis. In addition, morphine can completely attenuate neutrophil migration to the site of inflammation, and opioid consumption can block these cells from destroying bacteria. These factors combine to yield a disconcertingly high risk of pathogenic infection, as well as increasing the severity of many infections, which compounds on the next point in a devastating way. People who inject opioids intravenously may suffer from infectious diseases as well as OUD (Farnsworth et al., 2021). Infections may occur as a result of inoculation with resident flora, such as by licking needles clean or neglecting to clean the skin prior to injection; sharing needles between users, risky sexual practices, or as a result of contamination during the drug manufacturing processes. Common pathogens that infect OUD sufferers include Hepatitis B Virus, Hepatitis C Virus, Human Immunodeficiency Virus (HIV), *Streptococcus* and *Clostridium* bacteria, *Mycobacterium tuberculosis*, and *Candida* fungi. These infections can cause seriously debilitating damage to the patient's systems and potentially kill them, especially when taken together with the

aforementioned immune system interference by MOP receptor agonists such as morphine. Thus, it is little wonder that many people die from OUD each year. However, it gets worse. According to Wu et al., "tissue inflammation and nerve injury induce [MOP] constitutive activity, which causes latent sensitization in the dorsal horn (DH) of the spinal cord that can be unmasked by [MOP] antagonism" (2020). This increased sensitization of the DH of the spinal cord leads to increased sensitivity to pain (Uta et al., 2021), which can impact overall quality of life. This pain is also compounded by the withdrawal symptoms, which may lead OUD sufferers to continue misusing opioids to keep their pain under control.

In order to combat the rising prevalence of OUD, scientists all around the world have been researching potential cures and treatments for the condition. Medications for opioid use disorder can be classified into three groups: full agonists, partial agonists, and antagonists (Wang, 2019). Methadone is a full MOP agonist with a longer half-life than heroin in an opioid-tolerant patient. Patients who use methadone for opioid replacement therapy do not experience withdrawal symptoms and cravings, which reduces the likelihood of relapse. Buprenorphine, meanwhile, is a partial MOP agonist. It does not stimulate the MOPs to the same degree as methadone, thus reducing the likelihood of respiratory depression, thus serving as a safer alternative. Buprenorphine also does not provide the same amount of euphoria, making it less addictive to patients. Naloxone and naltrexone, on the other hand, are universal opioid receptor antagonists, competing with agonists for multiple opioid receptor subtypes without activating the receptors. Antagonists do not have any of the pharmacological side effects associated with agonists, such as sedation, analgesia, and euphoria, making them preferable for use as medications to treat OUD. In particular, Naloxone's high affinity for MOP is useful to prevent respiratory and mental depression in opioid overdose patients, and long-term injectable

naltrexone is used to decrease heroin use by blocking both MOPs and KOPs. However, there is a chance that overuse of opioid receptor antagonists can cause some of the very same withdrawal symptoms that OUD sufferers experience when not taking opioids.

Although MOUD sounds like the miraculous solution to all of the USA's problems with OUD, there are still several problems with the regimen. According to Wakeman et al. (2020), "Medication for opioid use disorder (MOUD) is effective and improves mortality, treatment retention, and remission, but most people with OUD remain untreated. Many parts of the United States lack access to buprenorphine prescribers, and only a few addiction treatment programs offer all forms of MOUD." Thus, although MOUD is an effective treatment regimen, a good portion of it goes unused and untapped, leading to the escalation in OUD that we see today. Thus, various alternative approaches to OUD treatment have been put into practice in the meantime. According to Wakeman et al. (2020), the most common treatment pathway was non-intensive behavioral health, followed by inpatient detoxification or residential services and buprenorphine or methadone. A lack of treatment altogether was more common than naltrexone or intensive behavioral therapy. However, MOUD treatment with buprenorphine or methadone reduced serious opioid-related acute care use by 26% at 12 months, and patients treated with methadone or buprenorphine for longer than 6 months overdosed less often than those who received shorter durations of treatment or no treatment. In essence, MOUD may not be a "magic bullet" that can instantly solve the problem of OUD in America, especially considering that many patients require treatment for an additional condition that may promote OUD such as chronic pain. However, when MOUD is added to an existing treatment, it can certainly make a major contribution to the probability of a more positive treatment outcome.

Even despite the efficacy of MOUD, one factor continues to threaten the overall health and well-being of Americans whose family struggled with OUD. According to Vasan et al. (2021), "maternal opioid use rates during pregnancy have more than quadrupled in the last decade. According to the Centers for Disease Control, from 2008–2012, approximately 1 in every 3 pregnant women filled an opioid prescription." As was already mentioned, these statistics are shocking because of the observation that the developing fetus is affected by substances that the mother consumes. However, far more disconcerting is that MOUD is the recommended means for treating maternal OUD, and the most common treatment selected for this condition is buprenorphine. What makes this observation so worrying is that opioid replacement therapy with buprenorphine does nothing to prevent the development of what has been called neonatal abstinence syndrome, or NAS (Jones et al., 2010). In fact, the instance of NAS has only escalated in recent years. According to Hirai et al. (2021), "Between 2010 and 2017, the estimated rate of NAS significantly increased from 4.0 … to 7.3 … per 1000 birth hospitalizations, representing… a relative increase of 82%."

In fact, some studies may implicate MOUD in the development of NAS in the first place. One study by McGlone et al. (2013) examined infants who were born to drug-misusing mothers who had been prescribed methadone as a MOUD treatment. The infants' flash visual evoked potentials (VEPs) were measured with electrodes and recorded, from within the first 72 hours of life, and with flashes delivered with a hand-held light-emitting diode stimulator. The procedure was repeated a minimum of 30 times per average to check reproducibility, and each recording session took about 30 minutes to complete. After the data was analyzed, it was found that the methadone-exposed infants were less likely to show VEP signals with healthy development markers than control infants, and overall, the methadone-exposed group had more immature and

atypical VEPs and fewer mature responses. Even after correcting for head circumference, maternal cigarette smoking, and excess alcohol exposure in utero, these differences continued to persist within the data. Thus, it is reasonable to assume that methadone treatment leads to abnormal flash VEPs, and thus problems in processing visual stimuli, in infants exposed to methadone in utero. Although this study may have been confounded by mothers who consumed drugs other than methadone, mainly other opiates but including amphetamines, cocaine, and even cannabis, it is still a noticeable mark against the overall efficacy of MOUD.

As for what NAS is, it is characterized by disturbances in the central nervous system (CNS). Clinical manifestations vary, from mild tremors, frequent yawning, and irritability to weight loss, fever, and seizures (McQueen & Murphy-Oikonen, 2016). Their timing of onset can vary but is usually within a few days of birth. The reasons for this variation are poorly understood and believed to involve many factors, including the pharmacokinetic half-life of the opioid used, exposure to other substances, maternal stress and diet, genetics, and even the extent of care that the neonates receive. The effects of opioid exposure in utero are unclear, but it is widely agreed that there are subtle and long-lasting, even permanent, consequences. For example, a study by Oei et al. (2017) sampled 605,094 children born in New South Wales, Australia, between July 1, 2000 and December 31, 2006. This cohort was divided into three groups, one with NAS, one control group, and one big group for the rest of the population, which was effectively a second control group. Each group started school in the calendar year that they turned 6 years of age, and each child was tested by a National Assessment Program: Literacy and Numeracy (NAPLAN), which scored students' performance at grades 3, 5, and 7 in each of the following five areas: Reading, Numeracy, Writing, Grammar, and Spelling. Each grade level had a predetermined National Minimum Standard (NMS), and students who fell short of the NMS were considered to

be behind on developing the necessary skills to proceed to the next level of education. Oei et al found that children with NAS fell below the NMS on the NAPLAN over twice as often as those in both the Control group and the general population, across all three grades and across all five areas. Thus, it is perfectly reasonable to assume that NAS leads to delays in the development of the human child's brain, at least with regards to a student's capacity for reading, writing, and arithmetic.

However, that is not the only area in which NAS puts children at risk. Altered development of the immune system leads to primed immune cells and sustained hyper-reactivity of the immune system (Vasan et al., 2021). Impaired nervous system development leads to fragmenting of neural networks and disruptions of anatomical connectivity. Both sets of factors lead to neurocognitive impairment of persons who were exposed to opioids in utero, lasting all the way into adulthood. Children who suffered in-utero opioid exposure are in general at a higher risk of adverse neurodevelopment at least into middle childhood, including an increased rate of ADHD diagnoses from ages 10-14. In addition, Mental Development Index scores on the Bayley Scales of Infant Development, Second Edition (BSID-II) was significantly lower in children with NAS than in healthy children, at both 18 months and 36 months (Hunt et al, 2008). Furthermore, these neurophysiological defects are associated with increased prefrontal cortex activation in fMRI scans with increasing working memory demands (Vasan et al., 2021). This is significant because the effects of in utero opioid exposure might not manifest until the children begin attending school, when working memory demands meet their highest points in a child's life. Combined with the lowered Mental Development Index (Hunt et al, 2008), this can cause serious problems in school, including stress over poor performance and strained relationships with teachers and peers.

All of this sounds bad already, but when adding this to what we know about neuroplasticity, it can be outright catastrophic. According to N.V. Gulyaeva (2017),

> Neuroplasticity (brain plasticity or neural plasticity), a remarkable capacity of the brain to change and adapt, implies physiological changes in the brain resulting from interactions of the organism with the environment. This dynamic process allowing to adapt to different experiences and to learn is also a factor in recovery from brain injury, since rehabilitation is aimed at rebuilding connections between neurons, "rewiring" of the brain. The specificity of brain organization… and the key role of brain (*sic*) in animal survival explain the necessity of plasticity providing for adaptive changes in brain structure and functions… Neuroplasticity can be observed on multiple scales, with adaptive behavior, learning, and memory being at the top of neuroplasticity hierarchy. The basis of this pyramid is shaped of molecules and their interactions, which underlie subcellular/synaptic, cellular, and neuronal circuit and network levels. (237/365)

Thus, if the molecular interactions at the very bottom of the neuroplastic hierarchy are disrupted, such as by introduction of extra, exogenous molecules that mimic the activity of endogenous molecules, neuroplasticity will go haywire, causing detrimental changes in the way the brain works.

However, it is not only that the opioids themselves are interfering with the usual functions of neuroplasticity. A study by Hamilton et al. (2010) found that dopamine increased the excitability of the dendrites of dentate granule cells, both in human and rat brains. Dentate granule cells, while lacking in excitability and synaptic plasticity under normal circumstances, are nonetheless important mediators of hippocampal function (Lopez-Rojaz & Kreutz, 2016). Thus, increasing the excitability of dentate granule cell dendrites may have unexpected effects on

hippocampus signaling, which could potentially disrupt learning and memory. On the other hand, if making dentate granule cell dendrites more excitable led to beneficial effects on learning and memory, the possibility of thinking more clearly might attract more users to the drugs, as is observed with Ritalin addiction. In addition, even though this study did not involve opioids, it still serves to establish the base principle that dopamine is involved in certain forms of neuroplasticity. From this knowledge and the observation that many drugs of abuse, potentially including many opioids, work through altering levels of dopamine in the brain's synapses, we can deduce that drug abuse has the potential to cause serious disturbances in neuroplasticity, which may lead to some of the deleterious effects observed in conditions such as NAS.

In order to discern how NAS affects the brain, we must first consider how the brain develops in a healthy neonate. Cellular precursors of the brain and spinal cord develop early into embryogenesis, through a process called neurulation. (Rice & Barone Jr, 2000) The notochord, a cellular rod that defines the primitive axis of the embryo, is integrated into the vertebral system and induces the overlying ectodermal tissue to form the neural plate, the precursor to the central nervous system, at approximately 2 weeks gestation in humans. At GD 18 in humans, the neural plate invaginates along the central axis, forming the neural groove with neural folds on each side. By the end of the third week of gestation, these folds have begun to fuse together, forming the neural tube near the anterior end of the notochord; fusion progresses both cranially and caudally, in a zipperlike manner.

After complete fusion, the neural tube separates from the overlying ectoderm, which becomes a contiguous surface over the back of the embryo and differentiates into the epidermis. A population of cells separates from the ectoderm, forming the precursors to the sensory ganglia of the spinal and cranial nerves, Schwann cells (cells that cover nerves of the peripheral nervous

system), the meninges covering the brain and spinal cord, and some skeletal and muscular components of the head, among other structures. The neural tube begins to close in the area of the hindbrain above the origin of the notochord and proceeds both anteriorly and posteriorly. This creates a caudal-to-rostral gradient in the development of the brain. Neural tube formation is complete at approximately GD 10.5-11 in rats and from GD 26 to 28 in humans. The anterior neuropore closes first, then the posterior neuropore.

Beginning early in the second week of gestation in rodents (GD 7 in mice, GD 9.5 in rats) and the first month of gestation in humans, specific areas of the CNS begin to form, through neurogenesis (the creation of new neurons) and the migration of cells in the forebrain, midbrain, and hindbrain (Rice & Barone Jr, 2000). There follows a sequence of developmental processes including proliferation, migration, differentiation, synaptogenesis, apoptosis, and myelination; most of these processes continue into postnatal days, but of these listed, only differentiation and synaptogenesis continue into adolescence. It is the prenatal and early postnatal days of development that matter the most for the proper development of the brain and nervous system, as those are the days where the brain's developmental processes are either already happening or yet to happen; thus, the alteration of these delicate processes by exogenous chemicals can cause noticeable aberrations in the development of the brain, which can persist well into adulthood.

These developmental missteps are not simply superficial quirks, either. Preclinical studies such as Vasan et al. (2021) have shown significant deficits of cognitive control and executive function in juvenile rats following perinatal opioid exposure, which is directly translatable to humans. These deficits, in humans, can include impaired motor control, impaired cognition, inattention, hyperactivity, and increased risk of developing attention-deficit hyperactivity disorder (ADHD) (Goldfarb et al., 2020). Thus, in utero opioid exposure causes considerably

more problems than NAS, as children who were born after prenatal opioid exposure tend to have trouble with paying attention and thinking properly. These impairments, which may not even manifest until children begin attending school (Vasan et al., 2021), can affect an afflicted person's performance in just about every aspect of their lives, leading to heightened stress levels that only exacerbate their hyperactivity and cognitive impairments.

In order to prevent such drawbacks from causing serious reductions in the quality of life of NAS patients, we must consider ways to treat or prevent such impairments. One way to advance upon this knowledge is to consider the possible proteins involved in the development of the brain, which is what the study present in this paper is investigating. The proteins of interest in this present study include kinesin family member 5A (KIF5A), myelin basic protein (MBP), neuron/glial antigen 2 (NG2), and 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase). KIF5A is an axon motor protein that was identified during an unpublished RNAseq study in Summer 2021, while the other three were chosen for their relevance to the development of the white matter of the brain, which was heavy in the areas we were examining in this study.

Changes in the expression of these genes may manifest through many pathways, but the most common one is by epigenetic modulation of the genes, rather than direct alteration of the genetic code by the opioids. Epigenetic modulation is basically the means by which the body's cells control gene expression without altering the code of the genes themselves, by changing the structure of associated histone proteins to allow or restrict access to the genes in a context-dependent manner (Browne et al., 2020). The most common of these modulations is histone de-acetylation (HDAC); using various drugs such as NaBut and TSA to inhibit the HDAC process either systemically, through intracranioventricular (ICV) administration, or in the NAcc specifically, Browne et al was able to increase the strength of morphine-induced CPP and

locomotor sensitization, as well as reducing the rate of CPP extinction. While Brown et al's study did not examine the results of altered epigenetic modulation of cerebellar tissue, it is worth keeping the results in mind, as they also argued that the earliest, most transient changes in gene transcription induced by drugs and drug-related stimuli set the stage for more persistent transcriptional alterations later in life.

KIF5A is essential for GABA(A) receptor transport (Nakajima et al., 2012). Deletion of the KIF5A gene is embryonic-lethal in test rodents. In humans, loss of function (LOF) mutations in KIF5A may cause ALS (Nicolas et al., 2018), and missense mutations have been linked to hereditary spastic paraplegia and Charcot-Marie-Tooth disease (Hares et al., 2021). However, certain ALS-related mutations may also lead to a reduction in autoinhibition, leading to a gain of function (GOF) mutation that leads to anomalous cellular transport, reducing the survival probability of the afflicted neurons (Baron et al., 2022). These LOF and GOF mutations are concerning because, since epigenetic regulation affects the amount of a protein expressed, it is theoretically possible that toxic LOF or GOF effects could occur by altering the epigenetic regulators ensuring that KIF5A is expressed at just the right level. KIF5A is also present in high levels in cerebrospinal fluid in patients with multiple sclerosis (MS).

According to Widder et al. (2019), "Myelin basic protein (MBP) is a structural protein that associates with opposing leaflets of the cytoplasmic side of the oligodendrocyte membrane and ensures a high compaction of the myelin sheath." In simpler terms, MBP keeps the myelin sheath from floating off of the nerve axons, allowing signals to be conducted easily without misfire, in a manner similar to the rubber coating around electrical cables. Altering the structure of MBP is also correlated with incidence of MS (Widder et al., 2019), which makes sense considering that MS is a disease where the body attacks its own myelin sheaths. The odd thing

about MBP is that, although epigenetic modulation, such as increased availability of oligodendrocyte transcription factors and histone protein demethylation and deacetylation, is sufficient to increase promoter activity, there is not a single modulation that can influence gene expression on its own (Liu et al., 2010). However, combining multiple epigenetic treatments was sufficient to increase the activity of MBP even in non-myelinating cells. While it is not entirely out of the question for opioids or OUD to induce multiple epigenetic changes at once in affected cells, the requirement of multiple epigenetic changes in order to increase MBP activity does offer some degree of protection against potential toxicity.

NG2 glia are also called Oligodendrocyte Precursor Cells (OPCs), are highly abundant at birth, and are ubiquitous in the adult brain (Zhang et al., 2021). NG2 glia are also the only glial cells that exhibit neuron-like properties, such as long-term potentiation at synapses and receiving input from glutamatergic and GABAergic neurons. Activation of NG2 glia induces GABA release and, thus, anxiety-like effects. In addition, NG2 overexpression is demonstrated in a wide variety of cancers, including melanoma, glioblastoma, lymphoid leukemia, and diffuse intrinsic pontine glioma (Yadavilli et al., 2015). As epigenetics govern the amount of a protein expressed in a cell, it is entirely likely that altering these control mechanisms could increase the user's risk for these various cancers, lowering their average lifespan and overall quality of life. Speaking of epigenetics, another study by Gotoh et al. (2018) found that transcription factors Sox10 and Olig2 bind to and activate the enhancer region of the NG2 gene, increasing expression and allowing the gene product to be produced at sufficient levels. Thus, it is not entirely unreasonable to assume that opioid-related dysregulation of these transcription factors could lower the amount of NG2 in the patient's cells to unhealthy levels, leading to other deleterious effects.

Alternative splicing of the CNPase gene can yield two different isoforms of CNPase, both of which are abundantly expressed in the cytoplasmic compartment of non-compacted myelin; the second isoform is also expressed in low levels outside the nervous system (Raasakka et al., 2015). CNPase has the ability to convert 2′,3′-cAMP to adenosine, thus protecting the neurons from the toxic effects of 2',3'-cAMP and its metabolites (Verrier et al., 2013). Oligodendrocytes, which are rich in CNPase, are far more efficient at this process than neurons. In addition, CNPase also has functions in cellular signaling, as CNPase levels were markedly elevated in activated microglia, and these increased levels of CNPase can inhibit the production of proinflammatory mediators (Yang et al., 2014).

However, according to Raasakka et al. (2015):

> CNPase is a potential autoantigen in multiple sclerosis, and variability in CNPase expression levels has been linked to neurological and psychiatric disorders, including Alzheimer's disease, Down syndrome, schizophrenia, and schizophrenia-related catatonic depression. CNPase-deficient mice develop axonal swelling and degeneration, which further leads to progressive motor deficiencies and premature death. In these mice, the inner tongue of myelin is most notably deformed, although myelin appears morphologically normal. (1/15)

In other words, while CNPase is a helpful, even necessary protein, its levels in the brain and body must be tightly regulated in order to prevent horrible diseases from manifesting.

Several prior studies have also examined the relationship between prenatal opioid exposure and brain development. For example, Jones et al. (2010) conducted a study where they examined the infants of pregnant women who underwent opioid replacement therapy for OUD, with one group being administered methadone and another being administered buprenorphine. What they

found was that, while buprenorphine administration did significantly lower the doses of morphine and shorten the hospital stays of the buprenorphine group compared to the methadone group, there was no significant difference in the number of NAS cases between the buprenorphine and methadone groups. Thus, while buprenorphine may lower the severity of NAS in the instances it does occur, it does nothing to lower the incidence of NAS or prevent it from developing. Even then, the study did not examine the underlying mechanisms of NAS development. Later, Kaltenbach et al. (2018) would conduct a study on 175 opioid-dependent pregnant women with a singleton fetus. Tests utilized in this study were growth measurements, the Bayley Scale of Infant Toddler Development III (BSI-III, a thorough assessment of cognitive development), Receptive-Expressive Emergent Language 3 (REEL-3, used for gauging the child's linguistic development), Infant Toddler Sensory Profile (ITSP, a measure of sensory processing ability and its function in daily life), and Infant Behavior Questionnaire Revised (IBQ-R, which measures an infant's behavior across 14 different areas on a scale of 1/never to 7/always). Infants treated with in utero buprenorphine scored a considerably lower mean on the REEL-3 and IBQ-R tests than infants treated with in utero methadone, but infants treated for NAS scored a higher mean than infants who were not treated for NAS. This implies that while NAS does impact a child's linguistic and behavioral performance, treatments can rescue their performance to some extent. However, the study did not delve into the underlying mechanisms behind the pathophysiology of NAS. Gibson et al. (2022) also conducted a study into the consequences of perinatal methadone exposure in the neonatal rat brain. Said study found that, in the corpus callosum and the cerebellum, less oligodendrocyte differentiation and more apoptosis was observed in the methadone-treated rats than the control rats. However, this study did not examine the proteomic angle underlying the changes observed in the study. In addition, the study

by Gibson did not examine the influence of perinatal buprenorphine exposure on differentiation and apoptosis in any other brain area. Across all of these studies, there remains a considerable gap in the knowledge in which the underlying protein expressions would be considered, and that gap is the area in which the present study will be contributing.

One study, however, has also offered contributions to the area we are presently investigating. Oberoi et al. (2019) performed a study of their own in this subject, and their findings were that rats treated with methadone from gestational day 7 had reduced expression in both CNPase and MBP in the hippocampus, cerebral cortex, and cerebellum; while MBP expression was still lowered in the brainstem, CNPase expression was elevated. According to Oberoi et al. (2019), "an increasing trend of [CNPase] expression may reflect an increase in immature oligodendrocytes undergoing differentiation. … In the cortex and hippocampus, expression levels of [CNPase]… and MBP were all significantly lower in [methadone]-exposed groups, indicating impairments of both immature and mature oligodendrocytes." However, while that study investigated the effects of prenatal methadone exposure, we are investigating the effects of prenatal buprenorphine exposure; this is relevant to our rationale because methadone is a full MOP agonist while buprenorphine is only a partial agonist (SAMHSA, 2021). In addition, Oberoi et al did not investigate the effects of prenatal methadone treatment on GAPDH, KIF5A, or NG2, only CNPase and MBP.

In our efforts to analyze the effects of prenatal buprenorphine exposure on white matter development, we considered the differences between injection and oral administration of buprenorphine. In a study by Houston et al. (2021), subcutaneous injection of buprenorphine in male and female Sprague-Dawley rats yielded a blood buprenorphine level that peaked after 8 hours, then tapered off within 48 hours, after dosing. Meanwhile, in an experiment with *Cavia*

*porcellus* guinea pigs, Sadar et al. (2018) found that oral transmucosal (OTM) administration yielded a lower maximum plasma concentration of buprenorphine than intravenous administration. The OTM-treated guinea pigs also had less drug exposure from the same dose compared to intravenously treated guinea pigs. In effect, this meant that oral treatment led to considerably weaker effects of the drug than intravenous injection, and despite the possibility that guinea pig biology is substantially different than rat biology, this observation still stands across species. This is the primary reason why many recreational opioids such as heroin and morphine are injected intravenously rather than taken as pills or other oral means. However, we chose to administer buprenorphine to our lab animals orally because that was the most common method of administration for patients undergoing MOUD treatment in a clinical setting.

As for the rationale behind this study, it is a most pressing matter to the health and safety of US citizens. As was mentioned earlier, the USA is enduring an epidemic of opioid use disorder, which severely weighs on the economy (Florence et al., 2021, & Hagemeier et al., 2018) and poses a threat to the unborn children of OUD patients (Hirai et al., 2021). Children who are exposed to opioids in utero are likely to contract NAS, and that condition will have serious, long-reaching repercussions for the child's physical and mental development (Vasan et al., 2021). Our goal for this study is to examine the proteins of white matter so that we may better understand the underlying mechanisms behind NAS, and through that we may find better treatment options for NAS sufferers.

# Chapter 2

## Hypothesis

### Specific Aims Overview

The purpose of this study is to examine how exposure to buprenorphine in utero affects the development of white matter of the cerebellum and optic nerve in neonatal rats. The cerebellum is rich in white matter, and it is involved in motor coordination and balance; therefore, altering the development of this brain region should result in impaired capacity to move in a coordinated manner. The central hypothesis of this proposal is that prenatal buprenorphine exposure leads to reduced expression of white matter development proteins, such as NG2 and MBP, as well as neuronal proteins such as Kif5A, and that these reductions cause deficits in the white matter parts of the brain, such as the optic nerve and cerebellum.

#### *Aim 1: To Investigate the Effects of Prenatal Buprenorphine Exposure on White Matter Protein Expression*

An unpublished summer 2021 RNAseq study on the optic nerve from our lab identified several genes, of which expression was altered after in utero exposure to buprenorphine. However, an RNAseq study on the cerebellum has yet to be performed. This proposal aims to determine whether similar decreases and increases in white matter development proteins in the optic nerve occur in the cerebellum of rats exposed to buprenorphine in utero.

# Chapter 3

# Materials and Methods

## Animal Preparation

We used timed-gestation adult female Long-Evans rats because they were not albino. Albino rats develop abnormalities in visual processing areas early in life (Welniak-Kaminska et al., 2019); since we were investigating the optic nerve, a component of the visual system, we selected a non-albino strain of rats to avoid visual system dysfunction. Only female rats were purchased because of the intent to examine the effects of in utero opioid exposure, which would require pregnant rats; pups were examined regardless of sex, although the numbers of each sex of pups were recorded.

Buprenorphine-treated rats were administered 2 mg/kg/day of buprenorphine solution, delivered orally in sweetened condensed milk solution. Control rats were administered the vehicle milk solution only. Rats were initially allowed 5 mL of milk solution, to condition them to drink the solution; once the rats had consumed nearly the entire volume of solution, the solution was increased to 10 mL. From Gestation Day 7 until euthanasia, animals were allowed access to the milk solution for one hour every day, and after the hour was up, the milk solution remaining was measured and recorded. Animal body weights are measured weekly, and volume of buprenorphine stock is adjusted accordingly.

Cages were kept in a 12-hour light-dark cycle, with bedding and ad libitum access to food and water. Bedding was changed every time rat pups were collected, and the uncollected rats were transferred to clean cages. All treatments and conditions were in accordance with Marshall University IACUC protocols.

**Sample Collection and Preparation**

Pups of age groups 3, 7, and 10 days were sacrificed by decapitation; pups of age groups 14, 17, 22, and 25 days were sedated by CO2 inhalation and then decapitated. The younger three groups were decapitated without sedation because rats at 10 days of age and younger do not have sufficient nervous system development to feel pain, which combined with a great resistance to CO2 would have made sedation by CO2 inhalation pointless. After euthanasia, brains were extracted and dissected to isolate the cerebellum and optic nerve. The cerebellum samples were rapidly frozen in liquid nitrogen and homogenized in NP40 lysis buffer, using an ultrasonic cell disruptor. The samples were then centrifuged at 14,000 x g, and the pellet debris was discarded.

**Western Blot**

A Bradford assay was used to determine the protein concentration of the supernatant, and proteins were separated by polyacrylamide gel electrophoresis, with equal amounts of protein loaded per lane. These amounts maximized at 40 µg, but younger animals had too dilute of protein concentrations to make this possible, resulting in amounts as low as 5 µg. After electrophoresis, the proteins were transferred to nitrocellulose membranes, which were then stained with Ponceau S to verify equal loading and allow normalization to total protein. After Ponceau S Staining, the blots were probed with antibodies against KIF5A, CNPase, NG2, MBP, and GAPDH. The blots were then imaged using Enhanced Chemiluminescence (ECL) on a BioRad ChemiDoc and analyzed in Biorad ImageLabs software.

**Table 1**

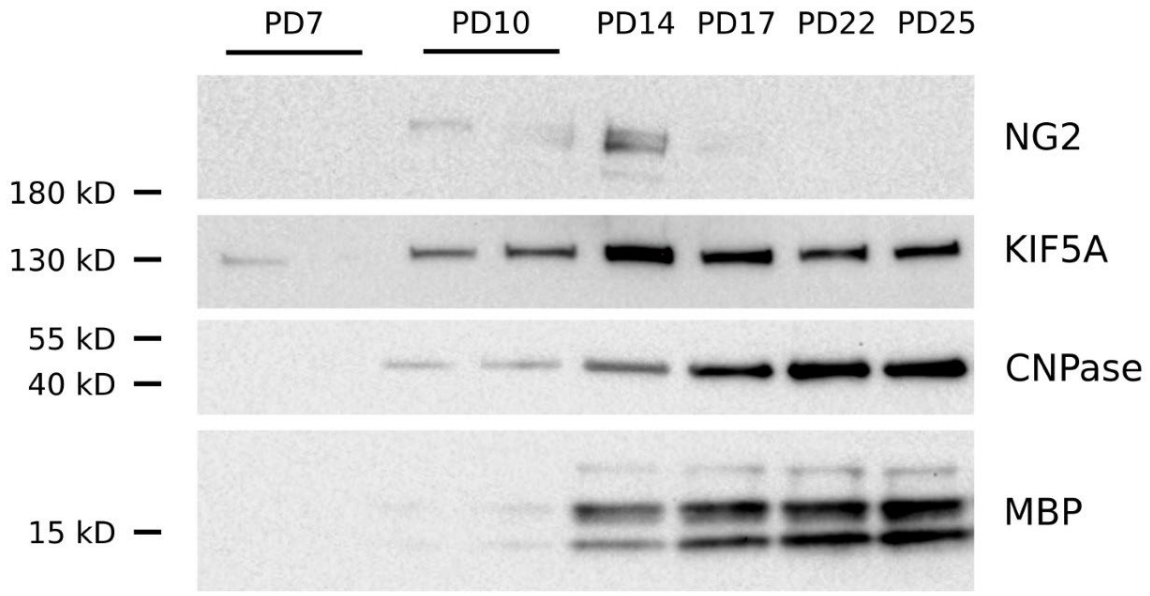
*Antibodies Used*

| **Antibody** | **Dilution** | **Source** | **Vendor** | **Catalog No.** |
|---|---|---|---|---|
| Anti-Kif5A | 1:1000 | Rabbit | Bethyl | A304-343A-M |
| Anti-CNPAse | 1:1000 | Rabbit | Cell Signaling Technologies | 5664 |
| Anti-MBP | 1:500 | Rabbit | Cell Signaling Technologies | 78896 |
| Anti-NG2 | 1:500 | Rabbit | Cell Signaling Technologies | 52635 |
| Anti-GAPDH | 1:500 | Mouse | ThermoFisher | MA5-15738 |
| Anti-Rabbit Peroxidase-Conjugated | 1:10,000 | Goat | Sigma-Aldritch | A0545 |
| Anti-Mouse Peroxidase-Conjugated | 1:10,000 | Goat | Sigma-Aldritch | A9917 |

*Note.* This table details all of the antibodies used during the Western blotting process. Included is the name of the antibody, the dilution, the source of the antibody, and the company that made the antibody.

**Figure 1**

*Sample Western Blot*




*Note.* An example of a Western Blot prepared for analysis, with visible Kif5A, CNPase, and slightly visible MBP. Image was taken from a BioRad ChemiDoc.

**Image Analysis and Statistics**

Western Blots were analyzed in BioRad ImageLabs software, with the data from the analysis ported into Microsoft Excel. Band density was normalized to total protein and expressed as a percentage of the control, and line graphs were then made with the data using 2-Way ANOVA in GraphPad PRISM 9 software.

# Chapter 4

# Results

**Table 2**

*Dam Results*

| **Dam** | **Male Pups** | **Female Pups** | **Total Pups** | **Avg. Bup Use (mg/kg/day)** |
|---|---|---|---|---|
| Dam 8 (Control) | 1 | 5 | 6 | 0 |
| Dam 9 (Control) | 5 | 9 | 14 | 0 |
| Dam 11 (Control) | 3 | 5 | 8 | 0 |
| Dam 18 (Control) | 7 | 1 | 8 | 0 |
| Dam 24 (Control) | 8 | 6 | 14 | 0 |
| Dam 26 (Control) | 3 | 5 | 8 | 0 |
| Dam 27 (Control) | 10 | 4 | 14 | 0 |
| Dam 31 (Control) | 5 | 7 | 12 | 0 |

| | | | | |
|---|---|---|---|---|
| Dam 32 (Control) | 4 | 6 | 10 | 0 |
| Dam 35 (Control) | 2 | 9 | 11 | 0 |
| Dam 40 (Control) | 8 | 6 | 14 | 0 |
| Dam 43 (Control) | 9 | 5 | 14 | 0 |
| Dam 52 (Control) | 7 | 6 | 13 | 0 |
| Dam 55 (Control) | 2 | 4 | 6 | 0 |
| Dam 10 | 5 | 3 | 8 | 1.8 |
| Dam 12 | 1 | 6 | 7 | 2.0 |
| Dam 17 | 7 | 5 | 12 | 1.9 |
| Dam 33 | 6 | 2 | 8 | 1.8 |
| Dam 34 | 0 | 0 | 0 | 1.6 |
| Dam 38 | 1 | 5 | 6 | 1.8 |
| Dam 39 | 7 | 7 | 14 | 1.8 |
| Dam 41 | 6 | 5 | 11 | 1.9 |
| Dam 50 | 7 | 4 | 11 | 1.48 |
| Dam 51 | 0 | 0 | 0 | 1.91 |

| | | | | |
|---|---|---|---|---|
| Dam 53 | 6 | 3 | 9 | 1.89 |
| Dam 54 | 4 | 6 | 10 | 1.28 |

*Note.* This table details the results regarding the Dams examined in this experiment, including average buprenorphine consumption and number of pups. Dams 52 and 55 were control dams, and so they did not receive any buprenorphine; all other dams consumed anywhere from 1.28 to 1.91 mg/kg of buprenorphine per day on average. Dam 51 had no offspring; outside of that, the lowest total number of children was from Dam 55 at 6 pups, 4 females and 2 males. Other dams had 9-13 pups total.

**Table 3**

*Average Weight by Age and Treatment Group*

| **Age (Postnatal Day)** | **Control Group Average Weight** | **Treated Group Average Weight** | **Male Pups** | **Female Pups** | **Male to Female Ratio** |
|---|---|---|---|---|---|
| 3 | 9.5 g (n=10) | 7.73 g (n=11) | 12 | 9 | 4:3 |
| 7 | 14.93 g (n=15) | 13.46 g (n=13) | 12 | 16 | 3:4 |
| 10 | 20.33 g (n=12)* | 20.14 g (n=7) | 11 | 8 | 11:8 |
| 14 | 29.375 g (n=8) | 32.00 g (n=7) | 9 | 6 | 3:2 |
| 17 | 37.43 g (n=14) | 33.21 g (n=14) | 14 | 14 | 1:1 |
| 22 | 55.45 g (n=11) | 46.50 g (n=10)* | 8 | 14 | 4:7 |
| 25 | 61.375 g (n=8) | 56.33 g (n=6) | 9 | 5 | 9:5 |

*Note.* This table details the average weight in grams of the rat pups from each age group, with control and treated groups weighed separately. Only the rats euthanized at that age group were counted. For both control and treated groups, the average weight increased with age;

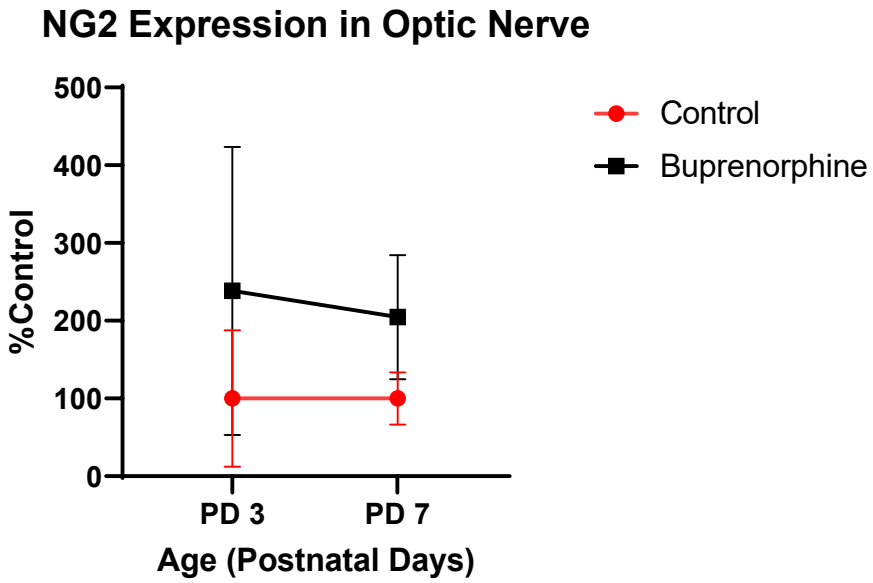
however, the treated group was several grams lighter than the control group in most measurements.

*One sample was missing from the lab notes, and thus weight data was omitted.

The following figures show the results of analysis of our blots, by graphing the mean expression levels as a percentage of the mean control expression levels (%Control), normalized to total protein, and performing two-way ANOVA in GraphPad PRISM 9. For NG2 (Figure 3), no significant effects of age, treatment, or the interaction between age and treatment could be observed at any postnatal age. For CNPase (Figure 4), expression in the buprenorphine-treated group started at about 750% on PD10 before dropping to about 200% of control expression on PD 14, then falling further to levels close to control expression on PD17 and staying within that range for the rest of the study. For the 18-kD strain of MBP (Figure 5), expression in the buprenorphine-treated group started at about 10% on PD 14, then rose near control levels on PD 17 before skyrocketing to about 400% of control expression levels on PD 22. A similar trend was observed in the 22-kD strain of MBP (Figure 6), although it was determined that none of these results were statistically significant. Kif5A provided the most interesting results (Figure 7), starting at about 210% of control expression on PD3, then lowering to slightly above control expression on PD 7, staying slightly above control expression up through PD 17, and finally hitting record highs of nearly 415% of control expression on PD 22.

**Figure 2**

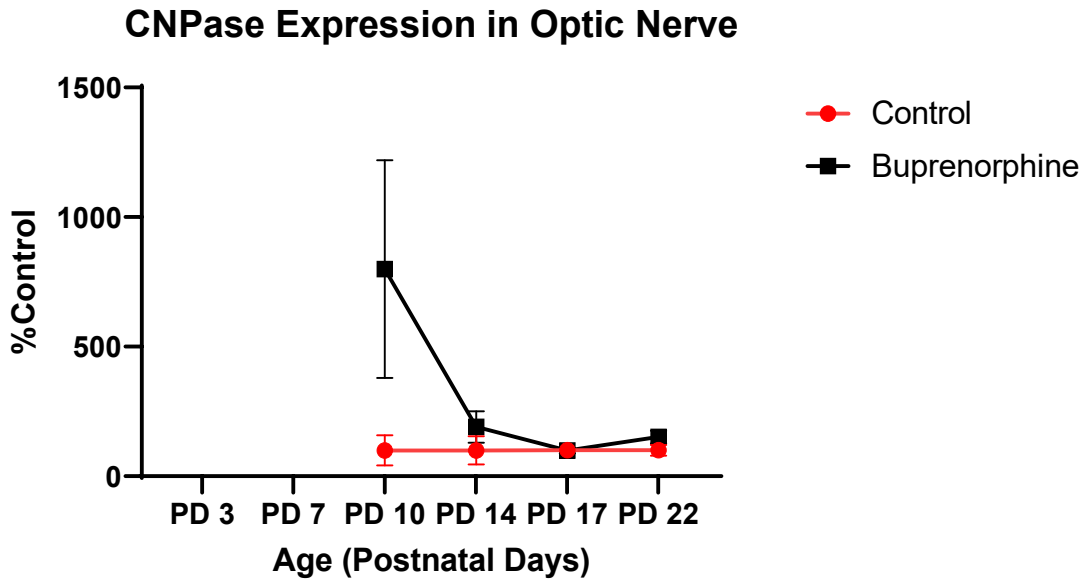
*NG2 Expression in the Developing Optic Nerve*




*Note.* No significant effects could be discerned for age, treatment, or the interaction of age and treatment on the expression of NG2 in the developing Optic Nerve. However, it is very likely that this is due to our limited sample size and range of time courses in this study, as the future results will show. For Buprenorphine samples, all n = 5; for Control, all n=4. Error bars represent standard error of the mean.

**Figure 3**

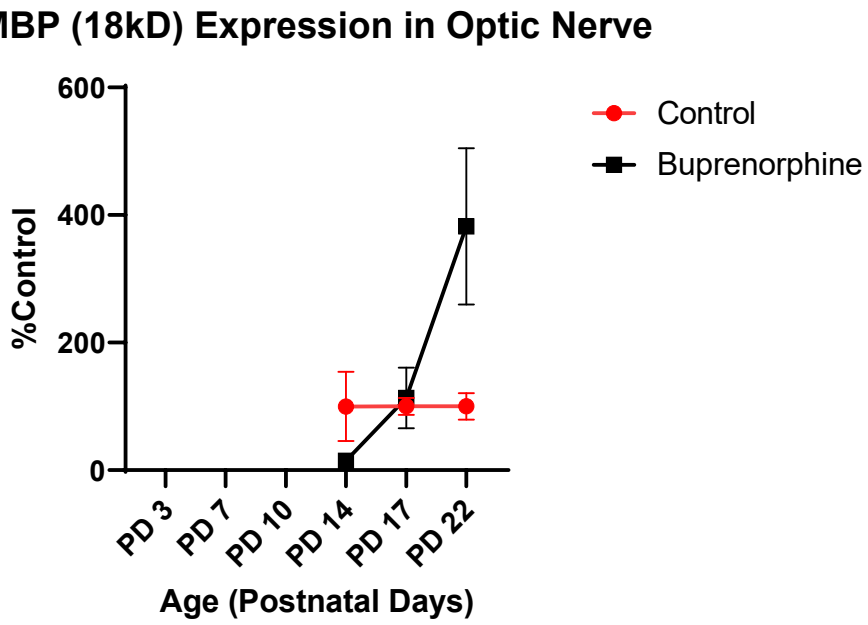
*CNPase Expression in the Developing Optic Nerve*




*Note.* Although no significant time points could be identified, the analysis results show a significant influence of the interaction of age and treatment on CNPase expression (p=0.0472). For Control samples PD 10, 14, 17, and 22, n = 5, 4, 4, and 5, respectively. For Buprenorphine samples PD 10, 14, 17, and 22, n = 4, 5, 5, and 4, respectively. Error bars represent standard error of the mean.

**Figure 4**

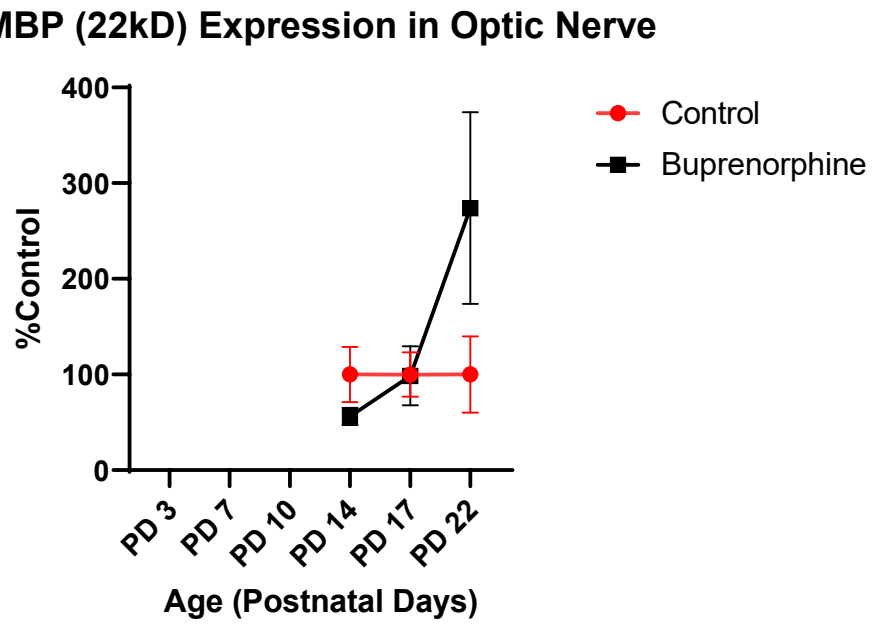
*MBP (18 kD) Expression in the Developing Optic Nerve*




*Note.* Again, no significant time points could be identified in this part of the study. However, not only did the interaction between age and treatment have a very significant effect on 18-kD MBP expression in the optic nerve ($p=0.0076$), but age alone also had a significant effect on the expression of 18-kD MBP in the optic nerve ($p=0.0190$). For Control samples, PD 14, 17, and 22 had n = 4, 4, and 5, respectively. For Buprenorphine samples, PD 14, 17, and 22 had n = 5, 5, and 4, respectively. Error bars represent standard error of the mean.

**Figure 5**

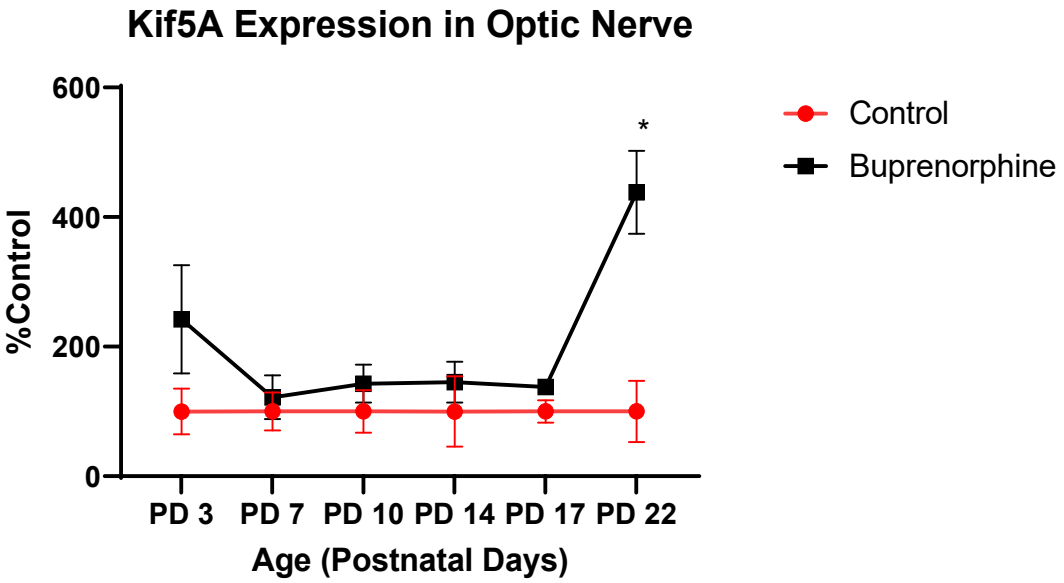
*MBP (22 kD) Expression in the Developing Optic Nerve*




*Note.* Again, no significant time points could be identified in this study. However, no significant effect of age, treatment, or the interaction of age and treatment could be identified. For Control samples, PD 14, 17, and 22 had n = 4, 4, and 5, respectively. For Buprenorphine samples, PD 14, 17, and 22 had n = 5, 5, and 4, respectively. Error bars represent standard error of the mean.

**Figure 6**

*Kif5A Expression in the Developing Optic Nerve.*

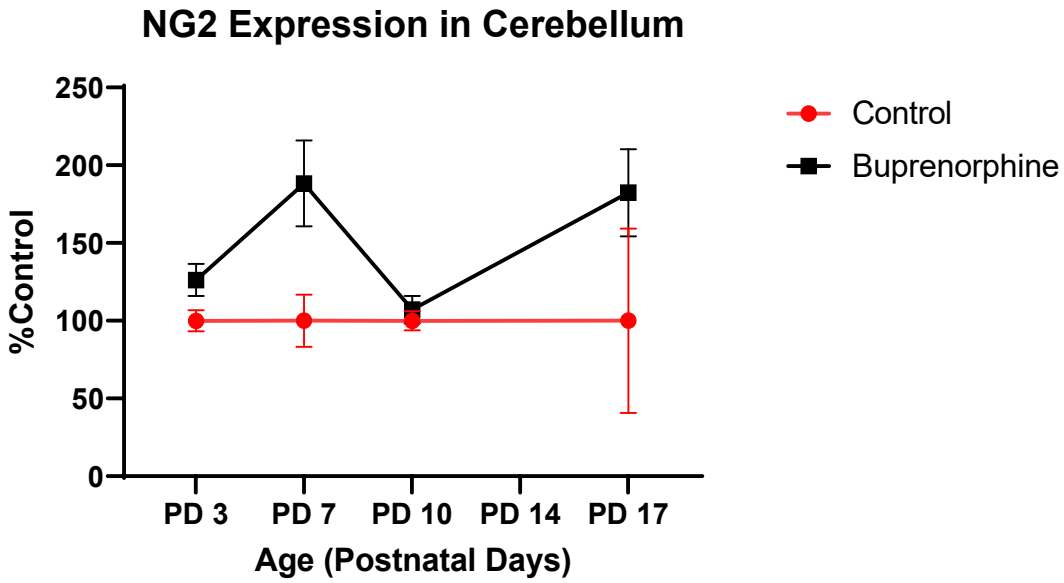



*Note.* The only significant difference in this case was at the PD 22 time point (p=0.0336). However, despite there being only one significant time point, there exists a significant effect of age (p=0.0311), treatment (p=0.0045), and the interaction between age and treatment (p=0.0074) on the expression of Kif5A in the developing optic nerve. For Control samples, PD 3, 7, 10, 14, 17, and 22 had n = 4, 4, 5, 4, 4, and 5, respectively. For Buprenorphine samples, PD 3, 7, 10, 14, 17, and 22 had n = 5, 5, 4, 5, 5, and 4, respectively. Error bars represent standard error of the mean.

The next group of figures details the results of our analysis of blots from the developing cerebellum. NG2 (Figure 8) started at about 125% of control expression on PD 3, rose to nearly 200% of control expression on PD 7, declined to control levels on PD 10, then returned to nearly 175% of control levels on PD 17. PD 14 data was omitted because of a failure of blot transfer to nitrocellulose membrane. Meanwhile, no significant effect of age, treatment, or the interaction between age and treatment could be observed for CNPase (Figure 9), since the Standard Error of the Mean was so massive. 18-kD MBP (Figure 10) expression levels were at about 30% of the controls on PD 10, rose to 150% of control levels on PD 14, and fell to nearly 75% of control levels on PD 17. Then, on PD 22, 18-kD MPB expression levels rose to nearly 150% of control expression, returning to control expression levels on PD 25. Again, a similar trend was observed in 22-kD MBP (Figure 11), with buprenorphine-treated expression starting at nearly 10% of control expression on PD 10, spiking to nearly 200% on PD 14, falling to about 80% of control expression levels on PD 17, rising to about 250% of expression levels on PD 22, and returning to near control levels on PD 25. Kif5A (Figure 12) expression levels in buprenorphine-treated rats started at nearly 125% of controls on PD 3, normalized to near control levels on PD 7, and remained near control expression levels through PD 14. On PD 17, Kif5A expression levels then escalated to nearly 160% of control expression, which continued into PD 22 before returning to control levels for PD 25.

**Figure 7**

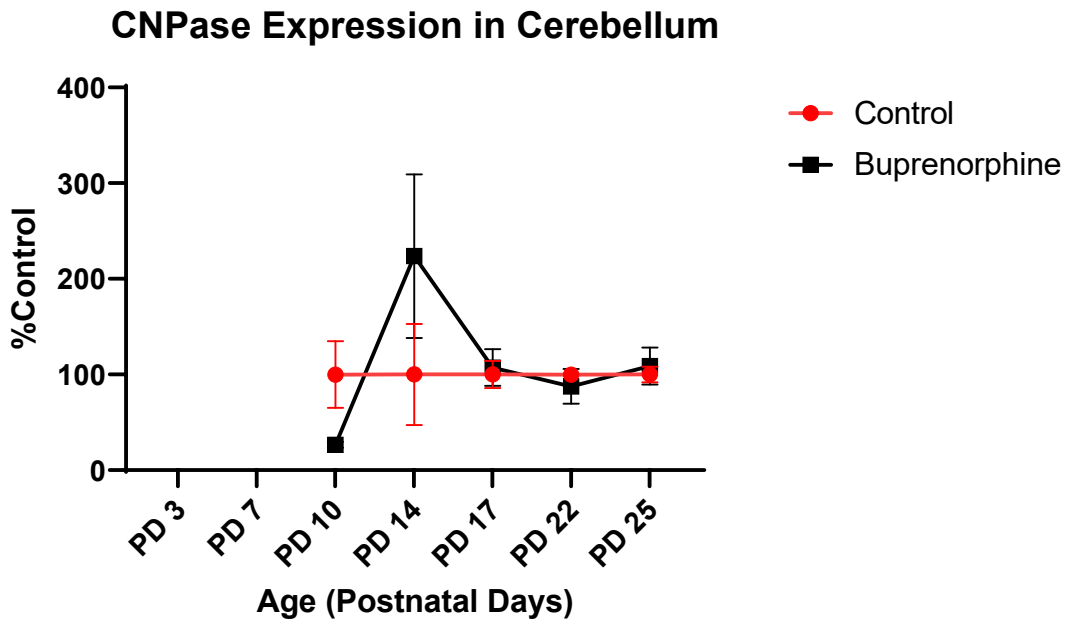
*NG2 Expression in the Developing Cerebellum*




*Note.* Again, no significant time points could be determined. However, despite the lack of significant time points, there was a significant effect of treatment (p=0.0466) on NG2 expression in the cerebellum. No significant effect of the interaction between age and treatment could be determined. For Control samples, PD 3, 7, 10, and 17 had n = 4, 4, 6, and 5, respectively. For Buprenorphine samples, PD 3, 7, 10, and 17 had n = 5, 4, 3, and 4, respectively. For both samples, PD 14 was omitted because of a failure of the transfer of the polyacrylamide gel electrophoresis results to nitrocellulose membrane. Error bars represent standard error of the mean.

**Figure 8**

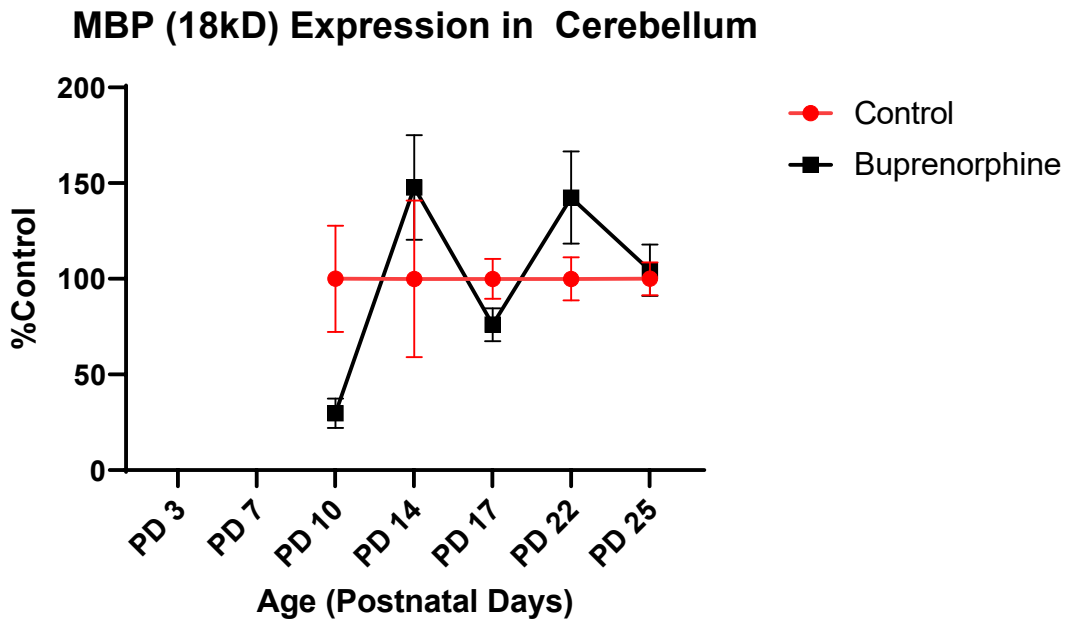
*CNPase Expression in the Developing Cerebellum*




*Note.* Again, no significant time points could be specifically identified. There was also no significant effect of age, treatment, or the interaction between age and treatment on cerebellar CNPase expression, that could be identified in this study. For Control samples, PD 10, 14, 17, 22, and 25 had n = 5, 6, 11, 7, and 8, respectively. For Buprenorphine samples, PD 10, 14, 17, 22, and 25 had n = 4, 7, 11, 10, and 6, respectively. Error bars represent standard error of the mean.

**Figure 9**

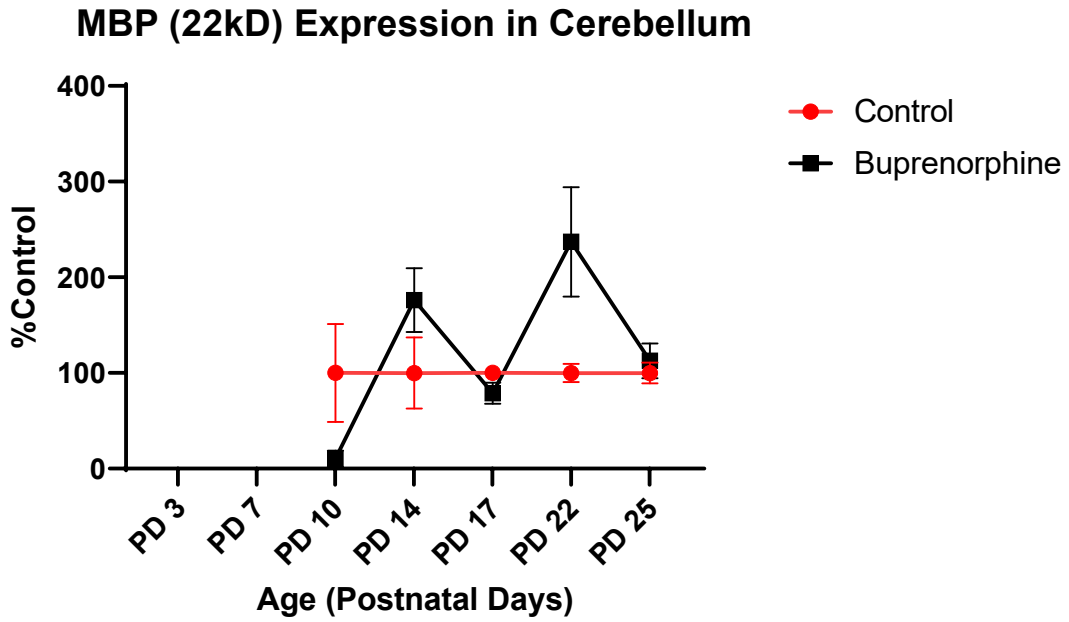
*MBP (18 kD) Expression in the Developing Cerebellum*




*Note.* Although no significant time points could be identified, this study did identify a significant effect of the interaction between treatment and age (p=0.0485). For Control samples, PD 10, 14, 17, 22, and 25 had n = 5, 6, 11, 7, and 8, respectively. For Buprenorphine samples, PD 10, 14, 17, 22, and 25 had n = 4, 7, 11, 10, and 6, respectively. Error bars represent standard error of the mean.

**Figure 10**

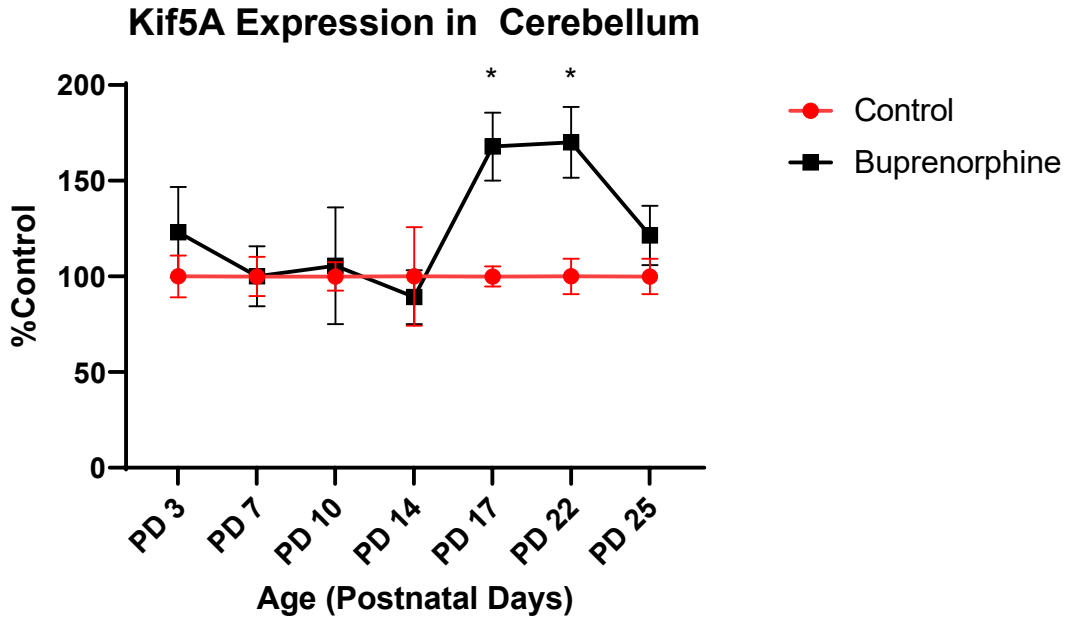
*MBP (22 kD) Expression in the Developing Cerebellum*




*Note.* In this analysis, a significant effect of both age (p=0.0382) and the interaction of age and treatment (p=0.0125) was observed. No significant time points could be identified, however. For Control samples, PD 10, 14, 17, 22, and 25 had n = 5, 6, 11, 7, and 8, respectively. For Buprenorphine samples, PD 10, 14, 17, 22, and 25 had n = 4, 7, 11, 10, and 6, respectively. Error bars represent standard error of the mean.

**Figure 11**

*Kif5A Expression in the Developing Cerebellum*




*Note.* The PD 17 (p=0.0231) and PD 22 (p=0.0336) timepoints were identified as statistically significant. In addition, a strongly significant effect of treatment (p=0.0050) was observed on Kif5A expression in the cerebellum. For Control samples, PD 3, 7, 10, 14, 17, 22, and 25 had n = 8, 14, 11, 6, 11, 7, and 8, respectively. For Buprenorphine samples, PD 3, 7, 10, 14, 17, 22, and 25 had n = 10, 12, 7, 7, 11, 10, and 6, respectively. Error bars represent standard error of the mean.

# Chapter 5

## Discussion

Strangely, no overall trend could be observed within our NG2 data (Figure 3). However, our sample size for the NG2 optic nerve samples was rather small, even compared to the other areas of the study (only 5 Buprenorphine-treated samples and 4 Control samples were available for both PD 3 and 7 of NG2). Thanks to the small sample size, it is likely that there is a significant effect of treatment on the expression of NG2. In fact, it is still significantly possible that further testing of more samples across more postnatal ages would yield similar results to those found in other proteins.

In the cerebellum, the trend for NG2 was quite different from its trend in the optic nerve. In treated rats, NG2 started close to control expression levels at PD 3, spiked to nearly 200% of control at PD 7, reduced to near control levels at PD 10, and then ascended back to almost 175% of control at PD17. As NG2-rich glia are considered oligodendrocyte precursor cells (Zhang et al., 2021), this increase may be explained as the rat still undergoing copious myelination, which may be aberrant in nature and lead to disruptions in intercellular signaling.

In the developing optic nerve, CNPase yielded no significant time points but had a significant effect of the interaction of age and treatment. Under this significant effect, CNPase expression in buprenorphine-treated optic nerve tissue started at ludicrously high levels (nearly 800% of control expression) at PD10, then declined to more reasonable levels (closer to 250%) by PD14. However, CNPase expression levels in the cerebellum yielded a different trend. In buprenorphine-treated cerebellum, CNPase expression started at about 25% of control expression on PD 10, peaked at over 200% of control expression on PD 14, then declined to levels close to control expression on PD 17 and stayed close to control expression on PD 22 and 25. Although

no significant effect of age, treatment, or the interaction between age and treatment could be observed on cerebellar CNPase expression, it is likely that the large standard error of the mean caused enough overlap between buprenorphine and control samples to discount a significant effect.

These increases in CNPase activity may imply any of several negative effects. According to Raasakka et al. (2015), ”CNPase is a potential autoantigen in multiple sclerosis, and variability in CNPase expression levels has been linked to neurological and psychiatric disorders, including Alzheimer’s disease, Down syndrome, schizophrenia, and schizophrenia-related catatonic depression.” While we did not examine the behavioral effects of these increases in this present study, there is one other possibility that we may be able to measure in a future study. Verrier et al. (2013) found that increased CNPase expression in activated microglia inhibited production of proinflammatory mediators. Thus, it is likely that aberrant increases in CNPase may inhibit the inflammatory response in afflicted rats and impair the ability of the brain to fight off infections and other intrusions. Meanwhile, Oberoi et al. (2019) observed that “an increasing trend of [CNPase] expression may reflect an increase in immature oligodendrocytes undergoing differentiation.” This increase in immature oligodendrocyte differentiation may explain some of the vision problems that NAS patients have been observed to experience (Jones et al., 2010), as it implies that oligodendrocytes are differentiating prematurely and thus creating defective myelination cells, leading to areas of the optic nerve with too much or too little myelination. It is possible that this last point may be a possible reason for the association between altered CNPase expression levels and psychiatric disorders: the defective myelination is causing damage to the nerve connections, such as killing large numbers of neurons in Alzheimer’s disease or interfering with brain signaling in schizophrenia.

MBP comes in both 18 kD and 22 kD strains, with two contradictory trends observed across both tissue types. In the developing optic nerve, while no significant effects of age, treatment, or the interaction between the two were observed on 22-kD MBP, a significant effect of the interaction between age and treatment was observed on 18-kD MBP. 18-kD MBP in treated rats started at about 0% of control expression on PD 14. On PD 17, MBP's expression level elevated to near control expression, and on PD 22, 18 kD MBP expression rose to about 400%. Meanwhile, in the developing cerebellum of buprenorphine-treated rats, MBP starts at below control expression levels on PD 10, with 18 kD at 30% of control expression and 22 kD at 10%. On PD 14, the expression levels of both proteins spike to all-time highs, with 18 kD MBP at nearly 150% of control expression and 22 kD MBP at nearly 200%. Then, on PD 17, 18 kD MBP falls down to about 75% of control expression, and 22 kD MBP to about 80%. Both proteins demonstrated significant effects of the interaction between treatment and age, foretelling serious health problems in these cases. As MBP is the protein that keeps the myelin sheathes of neurons attached to their axons (Widder et al., 2019), reduced expression levels may foretell poor myelination and, thus, deficiencies in axon conduction velocity. However, it has also been observed that aberrant MBP expression is connected to MS incidence (Widder et al., 2019), so the increased expression of MBP in the later days is not necessarily a good sign either.

Finally, Kif5A in the developing optic nerve and in the developing cerebellum displays unique trends. In the optic nerve of buprenorphine-treated rats, Kif5A starts at nearly 200% of control expression on PD3, before declining to about 125% of control expression around PD 7. From PD 10 to PD 17, Kif5A expression remains close to 150% of control expression, before peaking at over 400% of control expression on PD 22. Meanwhile, in the cerebellum of buprenorphine-treated rats, Kif5A starts at nearly 125% of control expression on PD 3, stays

close to control expression levels until PD 14, and peaks at over 150% of control expression on PD 17 and 22, returning to control levels on PD 25. Both tissue types showed significant effects of treatment, which has bad implications for the patients. Excessive Kif5A activity can reduce autoinhibition and lead to reduced survival probability of neurons (Baron et al., 2022), whereas loss of Kif5A activity can lead to ALS (Nicolas et al., 2018), hereditary spastic paraplegia, and Charcot-Marie-Tooth Disease (Hares et al., 2021), with outright deletion of the Kif5A gene leading to epilepsy and outright fatal effects (Nakajima et al., 2012).

# Chapter 6

## Conclusions

The purpose of this research paper was to investigate the effects of prenatal buprenorphine exposure on the development and protein expression of white matter-rich parts of the brain, such as the optic nerve and cerebellum. In doing so, we examined the changes in expression of NG2, CNPase, MBP (both 22 kD and 18 kD strains), and Kif5A in both areas between buprenorphine-treated and control rats. What we found was that CNPase started out with elevated expression levels in the optic nerve before returning to near control expression levels and staying close to control levels for the rest of the study. Meanwhile, MBP starts out low in all buprenorphine-treated samples, then began rising; in the optic nerve, both strains of MBP continued rising until they were far above control levels, whereas in the cerebellum, both strains of MBP rose to a peak above control levels before falling below control levels, then peaking again at a later day and returning to control expression levels. Kif5A provided the most interesting results, always being above control expression levels in the optic nerve and staying close to control expression until a massive peak in the cerebellum. Regardless, these proteins have yielded many interesting results, results which may be useful in the future to determine specific protein, cellular, or even genetic targets to assist in the treatment of NAS and conditions branching off from it. For example, MBP could serve as a target for treatments that reduce aberrant myelination or alleviate the symptoms of MS.

## Future Directions

Obviously, knowing the changes in protein expression levels is not sufficient to win the battle against NAS and its destructive effects. We need to conduct behavioral testing to determine if these defects in protein expression can lead to defects in motor skills, memory, and cognitive development, such as those seen in incidences of NAS. It would also help to address whether these deficits could be observed in other parts of the brain, including less white matter-rich areas such as the orbitofrontal cortex. Another possible future direction that might be suggested would be to assess the overall protein activity, to confirm that it is the altered protein expression that may be causing problems in rats suffering from NAS. We should also consider more closely examining the levels of NG2 expression in the optic nerve after prenatal buprenorphine exposure, as that part of our present study was hampered by lack of data. Lastly, while we have plenty of behavioral data on incidences of humans with NAS suffering from cognitive, sensory, and motor deficits, we have woefully little data on how their brain function is being altered to cause these deficits. Thus, a final step would be to conduct electroencephalography studies on live NAS patients, or perhaps with informed consent, take samples of the brain tissue of expired infants for future testing.

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# Appendix: IRB Approval Letter

MARSHALL UNIVERSITY
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Office of Research Integrity

March 30, 2023

Christopher Grahe
Biomedical Sciences
Marshall University

Dear Christopher:

This letter is in response to the submitted thesis abstract entitled *"Prenatal Buprenorphine Exposure Reduces Expression of Myelin Proteins in Neonatal Longs-Evans Rats."* After assessing the abstract, it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Institutional Animal Care and Use Committee (IACUC) has reviewed and approved the study under protocol #764. The applicable human and animal federal regulations have set forth the criteria utilized in making this determination. If there are any changes to the abstract, you provided then you would need to resubmit that information to the Office of Research Integrity for review and a determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director