EFFECTS OF PRENATAL OPIATE EXPOSURE

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EFFECTS OF PRENATAL OPIATE EXPOSURE

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Editorial: Effects of Prenatal Opiate Exposure

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Editorial on the Research Topic

Effects of Prenatal Opiate Exposure

Non-prescription opioid abuse continues to to be a growing problem in the United States. According to the National Survey on Drug Use and Health (NSDUH)—2014: 4.3 million Americans engaged in non-medical use of prescription painkillers in the last month; ∼1.9 million Americans met criteria for prescription painkiller use disorder based on their use in the past year; and, 1.4 million people used non-medical prescription painkillers for the first time in the past year (1). Opioid overdoses increased 30 percent from July 2016 through September 2017 in 52 areas in 45 states (2). It is estimated that everyday, more than 115 people in the United States die after overdosing on opioids (3). The smallest subjects afffected by this crisis are newborn infants suffering from Neonatal Abstinence Syndrome (NAS). It is estimated that between 50 and 94% of infants with *in utero* exposure to opiates will have postnatal manifestations of withdrawal (4, 5). The national incidence of NAS has increased from 3.4 to 5.8 per 1000 live births between 2009 and 2012 (6). Neonatal ICU admissions for NAS increased from 7/1000 live births in 2004 to 27/1000 in 2013 (7). However, these statistics obscure the regional variation in the distribution of NAS, with some units seeing very few patients per year while others are experiencing epidemic numbers of affected infants.

In this Research Topic issue of *Frontiers*, we bring together a diverse panel of scientific researchers to give historical perspectives, basic science work, clinical investigation, and a look at the future of NAS research. We would like to offer our thanks to *Frontiers* for the opportunity to bring you this topic and to the readers, for your interest in this topic, and the work of all contributors of the included authors.

HISTORICAL PERSPECTIVES

Gomez-Pomar and Finnegan give a comprehensive history of the exploding opioid epidemic. They describe over 4000 years of drug history from opium use by ancient Sumerians, to the first pharmacologic marketing of morphine in 1827, to the use of synthetic opioids and addiction we see today. Parallel to the increase in opioid addiction has been the burgeoning number of NAS cases. They go on to describe the current state of diagnosis, assessment and management of NAS.

BASIC SCIENCE RESEARCH

The article by Hauser and Knapp provides a basic science perspective on how opioid drugs disrupt neuronal and glial maturation in the central nervous system. The authors describe the tendency for opioids to inhibit and delay growth of neurons and associated CNS cells in specific brain regions, providing a plausible explanation for the clinical presentation of infants with NAS and adults

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with addiction. Sithisarn et al. give an accounting of their experience with an animal model of *in utero* oxycodone exposure. They found rats with *in utero* oxycodone exposure exhibited excessive hyperactivity as adults. They did not find the same cognitive impairment we see in human studies of *in utero* opioid exposure, but this may be due to methodological issues (dose, timing of exposure). Thus, further work in this area is warranted.

CLINICAL INVESTIGATIONS

Pandey et al. describes a case series of twins with prenatal substance exposure and their subsequent discordance or concordance of clinical presentation. Abu Jawdeh et al., describe their finding of preterm infants and respiratory control. Interestingly, although clinicians usually consider preterm infants to be not likely to display symptoms of NAS, the authors found substance exposed infants spent more time with lower SpO_2 than unexposed infants, suggesting that these infants may be displaying symptoms not previously suspected to be NAS related. Similarly, the work by Reynolds et al., explores another previously undescribed area of physiology, looking at non-nutritive suck in NAS. The fact that swallow-breath coordination in infants affected by NAS is similar to preterm infants and not a separate pathologic entity, seems to be in line with the disruption of neuronal and glial maturation described by Hauser and Knapp. How or if these infants catch up to unaffected infants remains to be determined. The work by Subedi et al. may also be related to Hauser and Knapp's findings. They found that infants affected by NAS have elevated plasma brain-derived neurotrophic factor (BDNF). Given that prenatal opioid exposure disrupts neuronal and glial growth and maturation, it is not out of the question to hypothesize that BDNF levels may be upregulated in an effort to stimulate new growth and development in the area. Further investigation is needed. Palla et al. describe their experience in the assessment of infants with seizure-like activity as one presenting clinical manifestation. Video Electroencephalography (vEEG) or standard EEG recordings were reviewed in detail. Epileptic seizures were rare, noted in 7.5% of their series. VEEG demonstrated disturbed non-rapid eye movement (REM) sleep with frequent arousal, jittery movements, and sleep myoclonus. VEEG would be useful prior to initiation of anti-convulsant medication in infants presenting clinically with seizure like activity. The final clinical science paper is offered by Devlin et al., who describe the successful work by her group to decrease pharmacologic treatment and length of stay for infants with NAS. They compared a new treatment algorithm to historic controls and found decreased need for treatment and length of hospital stay for infants with their new protocol, demonstrating significant financial savings.

FUTURE OF NAS RESEARCH

Cole et al., gives us a review of the pharmacogenomics of NAS and how a better understanding of how genetics intersects with substance exposure can lead to novel methods of identifying at-risk patients and potentially better treatment practices. Finally, as we look to the future of NAS research, Westgate and Gomez-Pomar give us a framework to assess the value of the current and newly developing tools to describe the severity of NAS symptoms and suggest a formative modeling framework to judge these tools.

Unfortunately, in spite of many studies related to NAS, there is still a lot to learn so the knowledge can be applied to the prevention and management of infants with NAS. The problem of NAS requires a multifaceted approach that must be taken into consideration by those involved in its research and in the care of these children and their families and those involved in policy in shaping the landscape of opioid addiction.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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The Epidemic of Neonatal Abstinence Syndrome, Historical References of Its' Origins, Assessment, and Management

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Neonatal abstinence syndrome (NAS) refers to a constellation of signs that are present in some newborn infants resulting from the abrupt cessation of passive transfer of maternal opioids used during pregnancy. The classic NAS refers to infants born to mothers who used opioids during pregnancy, but the term has broadened to include infants whose mothers have used or abused other psychoactive substances during pregnancy that contribute to the expression of the syndrome. Pregnant women who use opioids do so illicitly, and/or as medically prescribed for pain relief, and/or as medication assisted treatment for opioid dependence. The first case of NAS in infants and the subsequent treatment (or lack thereof) was reported in 1875 and was called Congenital Morphinism. By 2012, the incidence of NAS increased to more than 30 per 1,000 hospital live births, along with an increase in the number of infants being treated pharmacologically for NAS, resulting in an increase in the length of stay and healthcare expenses. We present historical references on NAS, the various factors and events that led to its increasing prevalence and today's current epidemic. We also review the current tools to assess infants with NAS and treatment options in its management.

Keywords: drug withdrawal, neonatal abstinence syndrome, neonate, opioid, methadone, buprenorphine

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> "The one who does not remember history is bound to live through it again." [Georges Santayana, Spain (1863–1952)]

THE EARLY DAYS OF *IN UTERO* EXPOSURE TO OPIOIDS

The first description of opium use goes back 6,000 years; then known as "the plant of joy" (1) being first isolated by Sumerians around 2,000 years BC (2). Initially used to cause euphoria (probably as part of religious rituals), it was found that it could also be used to help people die painlessly and to calm crying children (1, 2). Medical use of opium included treatment of a variety of illnesses such as: pain (headaches, menstrual cramps, etc.), respiratory (bronchitis, asthma, cough), infections (fever, leprosy), and mental problems (melancholy) (1). As opium use spread throughout Europe, China and India, its abuse and addiction appeared and advanced but it wasn't until 1803, after the isolation of its main component, morphine, and the expansion of its medical use, that addiction became a public health problem (1, 2).

Morphine was marketed by Merck & Co., Inc. in 1827 to be used for pain relief and alcoholism treatment (3). In 1853, morphine became the first drug to be used intravenously for pain control

during minor surgeries (1, 2). However, morphine was found to be as addictive as opium, and efforts were made to synthetize an opium derivative that did not cause addiction. The assumption was that if potency was high, less quantity should be needed resulting in less addiction (2, 3). This effort resulted in the development of heroin by Heinrich Dreser in 1898, a drug five times more potent than morphine (1, 2). Heroin was distributed by Bayer (2) AG (1898–1910) as an over-the-counter medication to treat colds, sore throats, pneumonia, and tuberculosis (1). Secondary to the belief that heroin was not addictive, it was also proposed as treatment for morphine-addicted patients.

The introduction of opium in the US started between 1850 and 1870 with the arrival of Chinese citizens during the California Gold Rush (1–3). Its use rapidly extended to all American major cities (1). From 1900 to 1924, heroin was distributed in the US as an over-the-counter medication for the treatment of colds and the flu and was marketed as safe to be given to pregnant women and infants (1). Opium was initially consumed using smoking devices, but because of the poor reputation of smokers (most likely Chinese laborers and white criminals), consumption spread to the middle and upper classes mostly after the availability of oral and intravenous forms (4). There were about 300,000 opioid addicts at the beginning of the twentieth century, and two thirds of those consumers were women who started using prescription opioids for a variety of illnesses and continued using them afterward (3, 4). Opiate abuse was also exacerbated by poor governmental control regarding the sale and distribution of narcotics (4).

At first, public perception of opium addicts was much positive than that of alcoholics, influencing the widespread use of morphine and heroin (4). However, these attitudes started to change at the beginning of the twentieth century, as negative perceptions of who addicts were, and how they became addicted, appeared (4). Concomitantly, physicians became more aware of the addictive effects of morphine and heroin and its deleterious effects on their patients, which resulted in fewer new cases of medical addiction (4–7).

The first attempt to regulate opiates in the US came in 1875, by the city of San Francisco, who enacted the Opium Den Ordinance, which declared public smoking (opium dens) illegal (1). Around that time, alarmed physicians raised their voices to remind their colleges about the dangers of opiate addiction and to challenge the proposed standards of care that used heroin for many afflictions including the treatment of addicts (8). This prompted the establishment of more controls across the states (7). By 1914, the federal government passed the Harrison Narcotic Act (4, 7) which required anyone who imported, produced, sold or dispensed "narcotics" to register and keep records of their operations. The Act also allowed authorities to prosecute unregistered distributors. The Harrison Act was later replaced by the 1970 Controlled Substances Act (7) which has been enforced by the Drug Enforcement Administration since its creation in 1973.

The term opioid was introduced in the late 1950s to refer to the synthetic narcotics (1). Oxycodone is a semisynthetic opioid synthesized from thebaine, an opioid alkaloid found in the Persian poppy, and one of the many alkaloids found in the opium poppy (9). Oxycodone was initially synthetized in 1916 in Germany and used for pain control, especially during World War II. In the post war time, and after the negative image of heroin, oxycodone was introduced in the US. It has since become one of the best selling prescription drugs in the country (1). By 1939, meperidine, the first synthetic opioid, was created; this was followed by the synthesis of methadone in 1946 (2, 10). Due to a nationwide effort to create treatment programs for opiate addiction, methadone was first used as a maintenance medication in the 1950s (10). Buprenorphine was discovered in 1966 and found to be beneficial for the treatment of opioid dependence as well as methadone (10, 11). Buprenorphine use was approved by the Food and Drug Administration in 1985. However, physicians were not permitted to prescribe buprenorphine in treatment settings other than Opioid Treatment Programs until the Drug Addiction Treatment Act of 2000 (part of the Children's Health Act of 2000) (10–12).

The use of opioids for pain control in terminally ill patients started in 1948 after Cicely Saunders founded the Hospice Care movement in London (13). Ms. Saunders introduced the concept of a pain-free dignified death using opioids to prevent pain instead of using them as treatment. This movement was adopted by the US and, in 1984, the Compassionate Pain Relief Act was passed allowing physicians to legally treat terminally ill patients with heroin (1). However, in 1986 a new call was made to use chronic opioid treatment for non-malignant pain, based on the incorrect assumption that opioids can be used chronically without causing serious addiction (14, 15).

INADEQUATE PRESCRIBING PRACTICES LEADING TO A NATIONAL EPIDEMIC

Opioid prescriptions shifted to the treatment of chronic pain resulting in a steep increase in the abuse of prescribed opioids with a reported 259 million prescriptions for opioid medications in 2012 (16, 17). Approximately half of all emergency room visits related to drug misuse in 2011 are related to nonmedical use of prescription drugs, 40% of these visits related to opioid pain relievers (18). Therefore, treatment admissions have more than quadrupled between 2002 and 2012, along with deaths related to overdose (11). In 2014, there were almost 19,000 deaths related to the overdose of prescription opioids (19). As of 2015, there are an estimated two million people living in the US with a substance abuse disorder related to prescribed opioids (11).

However, due to stricter controls on prescribed opioids (18), there has been a shift to heroin and other options easier to find (11, 20). Evidence also shows that individuals who abuse opioids orally, will eventually switch to injection routes thus resulting in additional health risks related to the injections (20–23). Admissions for prescribed opioid abuse decreased from 2004 to 2013, meanwhile, there was an increase in the number of individuals using injection and smoking/inhalation methods (21). In 2015, there were an estimated 591,000 people with heroin use disorder and approximately 13,000 deaths related to overdose (11); more than five times the number in 2002 (22).

Children and adolescents have not escaped this epidemic; from 1997 to 2012 more than 22,000 children were treated for opioid poisoning (16). Those among the ages 0–5 years experienced the largest number of exposures to opioids, mostly from

unintentional sources (accidental ingestion or therapeutic error) (16). However, among teenagers the majority of opioid exposure was intentional, being more significant among boys (16). A survey from 2015 found that 1 in 23 high school seniors reported misusing opiates (18) and research has also shown that teens and adolescents who abuse prescription medications are also more likely to use other drugs (18).

Sex differences related to substance abuse, like cause of consumption and drug metabolism, make women a unique group (24). As described earlier, opioid consumption was already common among women during the nineteenth century (4), possibly relating to women being more likely to suffer from chronic pain and being more susceptible to craving and relapse (24). Drug abuse and deaths due to overdose are more common in men; however, the rate of overdose is increasing more sharply in women compared to men (18). Misuse of prescription drugs is also more common among men, except in adolescents; adolescent girls, age 12–17, surpass boys in the use of nonmedical prescription drugs (18). Deaths related to overdose of prescription pain relievers are also increased more rapidly in women compared to men; reaching an overwhelming 18 women dying per day due to overdose in 2010 (24).

Opioids use during pregnancy results in complications to the newborn known as neonatal abstinence syndrome (NAS) (25–29). From 1995 to 2009, opioid prescriptions for pain management in pregnant women doubled (11). In a cohort of more than 100,000 pregnant women exposed to opioids, nearly 96% were non-maintenance prescriptions (30). Women who are pregnant or have young children may not look for addiction treatments or drop off from treatment due to the fear of legal repercussions or that they will be seen as unable to take care of their children (12, 24, 31). Stopping opioids during pregnancy is not an option as it is associated with poor neonatal outcomes and may result in fetal demise (11, 31).

Currently, there is no federally approved medication treatment for pregnant women with an opioid disorder; however, methadone and buprenorphine (category C drugs as per the Food and Drug Administration) appear to be effective and safe options during pregnancy (24, 31). Still, knowledge gaps persist concerning methadone pharmacodynamics, pharmacokinetics and the lack of guidelines for adequate dosing during pregnancy (12).

NEONATAL ABSTINENCE SYNDROME

Prior to 1875, infants born to women who were opioid dependent were thought to be unaffected because morphine use among women was associated with sterility and loss of sexual desire (32). However, in 1875 several cases of deceased infants born from mothers dependent on morphine were reported (3, 33, 34). This condition was called Congenital Morphinism and it was described in full term infants who appeared normal at birth then began crying inconsolably on the third day of life; some of these infants were reported to develop generalized seizures (32, 33). Due to a lack of knowledge of the cause of these signs, NAS was frequently fatal to newborns (3, 33, 34). As early as 1901, it was recognized that this was the result of the infant withdrawing from the cessation of the passive transfer of maternal morphine and that providing the infant with medication would ease his/her signs (33, 34). Later on, infants were given opium in small quantities to treat their symptoms with reports of success (34).

Due to the low-molecular-weight and lipid solubility of opioids, they pass freely through the placenta and to the infant (25–27). Fetuses of mothers using opioids and receiving no or inadequate prenatal care have an increased risk of having preterm birth, intrauterine growth restriction, and an increase incidence of fetal demise (26, 31, 35, 36). Several drugs have been identified to cause some signs of withdrawal in the infant, but the most common cause is *in utero* opioid exposure (3, 25–27, 37–40). The cutting of the umbilical cord causes an abrupt termination of the supply of opioids to the infant and increases the risk of developing NAS (25–27).

Clinically recognizable abstinence signs appear in 60–80% of opioid-exposed neonates. Their spectrum of signs is affected by variables such as the total fetal exposure, the amount and purity of the drugs taken by the mother, length of use, maternal and infant drug metabolism, and the individual kinetics of placental drug transfer (25–27, 38, 40). Genetic and epigenetic factors play an important role in the incidence and severity of NAS in neonates (41, 42). Timing of presentation of NAS is usually within the first 72 h of life but it can occur as late as 7 days of life (27, 38). Signs of NAS can be classified in: (a) neurologic manifestations due to increased excitability, including tremors, excessive and/or high pitch cry, hyperactive Moro, increased muscle tone, seizures; (b) gastrointestinal manifestations include diarrhea, vomiting, uncoordinated sucking, and swallowing; and (c) autonomic manifestations include fever, sweating, nasal stuffiness, and increased respiratory rate (25, 27, 37, 40, 43, 44). The most common signs are related to the neurologic and gastrointestinal manifestations, however, there is variability in the presentation of signs among neonates (27, 38, 40, 45).

Opioid excretion in breastmilk was recognized as early as 1901 (34). Breastfeeding was encouraged in these infants as it helped "calm them" and in some occasions ease their symptoms without pharmacological intervention (34, 46). Evidence has shown that mothers on methadone or buprenorphine have low, safe levels of the medication in their breast milk (47). Therefore, the current recommendation is to encourage breastfeeding in those infants whose mothers are on a supervised opioid maintenance program, are compliant and not using street drugs (27). Breastfeeding of these infants has demonstrated to be beneficial in decreasing their NAS signs and hospital stay and improving maternal–infant attachment most probably due to the soothing effect of breastfeeding (27, 47, 48).

AN OBJECTIVE EVALUATION OF INFANTS WITH NAS

Assessment and diagnosis of NAS starts with clinical suspicion based on maternal history (27, 32, 49). In order to provide an objective way to identify and categorize infants with NAS, several scoring systems were proposed (50), including the Finnegan Neonatal Abstinence Scoring System (FNASS) (25, 37), the Lipsitz tool (51), the Neonatal Narcotic Withdrawal Index (52), and the

Ostrea tool (35). Currently, the FNASS is the most commonly used scale (25, 27, 37, 53–56).

The FNASS was developed in 1975 using a clinimetric approach based on the most common signs of infants with NAS (25, 37, 57). The FNASS consists of 21 items, was analyzed for interuser reliability [mean interrater reliability coefficient of 0.82 (0.75–0.96)] and validated for the diagnosis of NAS (26, 58). The FNASS provides cutoff points (3 continuous scores ≥ 8 or 2 continuous scores ≥ 12) for the identification of the infants that may require pharmacological treatment and pharmacological guidelines for the treatment of infants with NAS (25, 37, 59). Subsequent analysis of the external factors that may influence the FNASS showed that it is an objective and reliable tool for the diagnoses and management of infants with NAS (60).

The Lipsitz tool was developed around the same time of the FNASS (1975) (51). It contains 11 items with scores from 0 to 3 and proposes to evaluate infants twice per day 90 min prior to feeds. It found sensitivity of 77% using scores ≥ 4 as an indication of withdrawal signs. Other scales like the Neonatal Narcotic Withdrawal Index (7 category scale that proposes a cutoff of 5 obtained twice during a 24-h period) (52) and the Ostrea tool (a six criteria scale) (35) were proposed; however, these scales did not gain much popularity in clinical settings.

The FNASS is a comprehensive and lengthy tool so there have been several attempts to modify it (40). The Neonatal Withdrawal Inventory (61) proposed an 8–point checklist that was derived from the FNASS. The investigators reported on inter-rater reliability, sensitivity and specificity using the same cutoff points as the FNASS; however, the scoring system was not validated. The FNASS-short form developed by Maguire et al (62) proposed a 7-point checklist using the same cutoff value as the FNASS. The simplified FNASS (sFNASS) is a 10-point scale that was statistically derived using the FNASS scores of a database of 185 patients (63). This was subsequently validated with a different database of 182 infants from another NICU with excellent correlation to the original FNASS. The sFNASS proposes cutoff values of 6 and 10 to identify scores of 8 and 12 on the original FNASS. However, a prospective evaluation of the sFNASS is needed (63).

The Maternal Opioid Treatment: Human Experimental Research (MOTHER) project developed a score by modifying the FNASS (64). The MOTHER NAS scale is a 19 item scoring system developed by adding (failure to thrive and irritability), removing (myoclonic jerks, mottling, nasal flaring and excessive sucking), or modifying the scores in the items of the original FNASS (65). Infants were evaluated twice daily and pharmacological treatment was offered by their proposed guidelines (64). Short screening tools based on the MOTHER NAS scale have also been proposed (66); however, its validation awaits further studies.

At present, there is no national consensus as to which tool to use, the cutoff points for treatment, and the interval between assessments (27, 50, 53, 56). A 2006 review by Sarkar et al. found that the FNASS was used by 65% of the 75 units that were analyzed, which is consistent with other reports (25, 27, 37, 53–56, 67). Conversely, most of the concerns were based in the length of the tool and the fact that it was designed for opioid withdrawal not for polydrug users. However, the majority of signs that infants experience from *in utero* polydrug exposure concomitantly with opioids (benzodiazepines, SSRI, sedatives) are similar and additive to those seen with opioids alone (27).

An evaluation of the validity of the cutoff points was made by Zimmermann et al. in 2010 (68). They applied the FNASS to infants without a history of opioid exposure and found that scores ≥8 should be considered pathological (68). Infants without opioid exposure commonly have scores <8 and, if a score of 8 is found in one evaluation, they subsequently became normal (68). This finding validates the suggestion of requiring 3 continuous abnormal scores to consider pharmacological treatment. To consider, in the development of the FNASS, 200 babies born without drug exposure (including no analgesics for labor and delivery) never received a score of ≥8 or higher. Most were in the 1–5 range. Current recommendations encourage each nursery to develop a protocol that includes a standard assessment tool for infants with NAS (27, 32).

MANAGEMENT OF NAS

Management of infants suffering from NAS has two main components, non-pharmacological and pharmacological interventions that are used as needed. Non-pharmacological options have been used since the early identification of congenital morphinism (34, 46). The main components of a non-pharmacological approach are recommended to decrease the environmental stimuli that the infant is exposed to (minimize environmental stimuli, swaddling, rocking) and minimize hunger (demand feedings) (32, 69–71). These interventions have demonstrated a decrease in the severity of symptoms (32, 50, 69) and new evidence is showing that they may be a key component in the management of infants with NAS (67, 69).

Recently, a new approach called the Eat, Sleep, Console (ESC) model was reported (69). This model is based on intensive non-pharmacological therapy of infants with NAS, specifically those from mothers on methadone. Over a period of 8 years, the investigators showed a decreased need for pharmacological therapy with a shortened hospital stay with the ESC method (when comparing a historical comparison group to the post intervention group) (69). However, it seems that constant maternal rooming-in is key to the model, making it difficult to apply nationwide and the model is based on a subjective evaluation (without a report of other signs of NAS), making it difficult to replicate (69).

Another concern is at which point the non-pharmacological interventions alone will have deleterious effects on the neurological development of infants with NAS. Infants with antenatal opiate exposure will have an increased noradrenergic activity, among other neurological abnormalities, once they are born and the opiate supply ceases (32, 43, 72, 73). Therefore, we question that not offering pharmacological treatment is enough for these infants and could be detrimental in the long term. Further research is necessary to evaluate the long term effects of the ESC model.

At present, infants diagnosed with NAS that are in need of a pharmacological treatment are managed in a neonatal intensive care unit (NICU) (28, 74). An infant with NAS admitted to the NICU will have difficult access to rooming-in and a low stimuli environment. Holmes et al. reported a program in which 207 infants were treated in a pediatric unit instead of a NICU (74).

This allowed constant rooming-in and resulted in decreased of use of pharmacological treatments, length of stay and overall health cost in an overall safe environment to the infant (74).

Initially, when NAS (Congenital Morphinism) was identified, no pharmacological treatment was given and these infants died, not only from the lack of treatment for NAS but, in some, from prematurity (3, 33, 34). Once the condition was recognized to be caused by the interruption of the placental supply of opiates, pharmacological interventions were provided resulting in improvement of the survival rates (33, 34). Initially, infants were treated with opium, paregoric and oral diluted morphine (34). Currently, the most common medications used in US nurseries include morphine and methadone with phenobarbital, clonidine, and buprenorphine being used alone or as adjuvant therapy. However, pharmacological management is not standardized; therefore, medication dosing and weaning varies center to center (27, 53, 55, 56, 75, 76). The threshold as to when an infant needs pharmacological intervention is questioned by some clinicians and, if treated, the choice of which medication to use remains controversial (27, 50, 53, 56, 67, 77–79).

Morphine and methadone have been the drugs of choice to treat infants where maternal opioid exposure is demonstrated and NAS is established (50, 53, 64). Morphine is given orally, typically every 3–4 h at 0.05–0.2 mg/kg/dose (50, 80). If the infant does not improve with the initial dose of medication, it can be increased to obtain the desired effect. Morphine can be weaned every 24–48 h (50, 80).

Methadone is also given orally every 4–12 h, titrated in a range of 0.05–0.1 mg/kg/dose, and then weaned over time (50). However, the use of methadone is controversial due to its long half-life and prolonged excretion rate that could require longer hospitalization (65, 79).

Clonidine, a non-opioid α2-adrenergic receptor agonist, has been recommended as a safe alternative for single-drug therapy of infants with NAS (72). Clonidine eases the signs and does not include the narcotic effects of opioids (80). Clonidine can be initially started at 0.5–1 μg/kg, followed by 0.5–1.25 μg/kg per dose q 3 h with proposed increments of 25% of the initial dose and weaning by 10% of the maximum dose every 48 h (50). A potential side effect is blood pressure fluctuations; however, no studies have reported this side effect at the doses used for the treatment of infants with NAS (72, 80). Clonidine also can be used as adjuvant medication when opioids were initially used.

Phenobarbital, a γ-amino butyric acid agonist with sedative and anticonvulsant properties, has been used for years for the treatment of NAS (70). However, phenobarbital is most commonly used as an adjuvant therapy and not as a single-drug medication (32, 80). Phenobarbital requires a loading dose of 5 mg/kg IV, IM, or PO and a maintenance dose of 3–5 mg/kg divided into three doses (every 8 h). Another approach is a loading dose of 20 mg/kg with a maintenance dose of 2–6 mg/kg day to achieve plasma level concentrations between 20 and 30 μg/mL (80, 81). Once the infant is controlled, phenobarbital can be weaned by 15% of the daily dose until the medication is discontinued (80).

Treatment of NAS must take into consideration factors that have been shown to enhance the expression of NAS and effect the response of infants to non-pharmacological and pharmacological interventions. These include neonatal (41, 42) (gestational age, neonatal metabolism, genetic predisposition, and epigenetics), maternal (12, 30, 38) (smoking, type, length, quality, and quantity of the used drug, SSRI use and the enrollment in Medication Assisted Therapy), and external factors (69) (decision to breast-feed and rooming-in possibility). Significant variability still persists regarding pharmacological treatment of infants with NAS (dose initiation, increments, and weaning). Therefore, each unit needs to develop a protocol to provide consistent treatment to the affected infants (27, 50).

NAS AS A NATIONAL EPIDEMIC

The incidence of NAS has steadily increased since the 1970s and has now become a significant public health problem (30, 37). NICU admissions due to NAS have increased from 7 cases/1,000 admissions in 2004 to 27 cases/1,000 admissions in 2013 with an increase in the median length of stay for infants with NAS from 13 to 19 days (28). The proportion of infants who received pharmacotherapy also increased, 74% in 2004–2005 to 87% in 2012–2013, resulting in a 35% increase in hospital costs (28).

Prescribed opioids are prevalent among mothers of infants with NAS. A cohort of more than 10,000 infants with NAS showed that methadone, opioid pain relievers and buprenorphine was used in 31, 24, and 15% of the mothers, respectively (28). Pregnant women tend to use less illicit drugs and smoking compared to non-pregnant women, excluding the group age between 15 and 17 years old (27). However, heroin use in developed countries is increasing (32); during the period of 2000–2012, NAS associated with use of heroin or an opioid prescription increased by fivefold (18). Mothers who abuse heroin are usually unmarried, unemployed, less educated and less insured; which usually implies that the pregnancies are unplanned and have minimal, if any, prenatal care (32).

State differences are noted when analyzing the incidence of NAS. A CDC report that included 28 states found an overall incidence of 6 cases per 1,000 hospital births in 2013 (82). This report also shows a wide difference among states (0.7 per 1,000 vs. 33.4 per 1,000 in Hawaii and West Virginia, respectively) (82). These differences can be attributed to opioid prescribing policies, prevalence of illicit opioid use, or using a different diagnostic code to classify the disease. Variations among states must be taken into consideration when designing public health policies for the prevention of NAS.

Strategies to decrease the incidence of NAS must start during the preconception period with an adequate planning and an honest discussion of how long-term opioid use affects the pregnancy and the infant (31, 82). Prescribed opioids during pregnancy is still one of the most common causes of NAS and should be carefully considered for each case (30). All states should have a prescription drug monitoring program, which has been demonstrated to be satisfactory in reducing inadequate prescribing and overdose deaths (82). Early identification of pregnant women who use illicit drugs is vital in order to improve outcomes (31), however, pregnant women need to have access to comprehensive, fear free treatment options that include medication-assisted treatment, prenatal care and psychosocial support with an honest discussion about the infant's future (31).

CONCLUSION

A national effort is needed to improve maternal health in the prenatal period and to standardize the assessment and management of their infants with NAS (3, 27, 32, 65). Research strategies need to take into consideration that a large number of mothers are not in a supervised maintenance program and are polydrug users (27, 39). Assessment of infants with NAS needs to be standardized (32) and the chosen assessment tool needs to have strong clinimetrics (57) rather than psychometric properties (83). Non-pharmacological approaches need to be offered to all mothers and infants that are affected (32, 69, 84). Infants diagnosed with NAS should be managed, when possible, in a setting where constant rooming-in is available (69, 74).

Recommendations for the pharmacological treatment of NAS, when indicated, need to come from randomized clinical trials with regard to which treatment to initially provide, how to escalate the dose and how to wean the medication(s), taking into consideration the safest time to be discharged home (32, 49). The current lack of education of the many disciplines involved in the assessment and treatment of drug dependence during pregnancy and NAS, makes it difficult for clinicians and researchers to approach this epidemic and to avoid the potential detrimental consequences to this maternal/infant dyad (27, 31, 69, 85). Clinicians, researches, and government funding agencies need to combine their expertise to provide adequate education and treatment protocols for drug dependent pregnant women and their infants with NAS. Without this, they will not live a full and healthy life due to this chronic relapsing disease with the potential to increase the intergenerational transmission of drug dependence and potentiate the epidemic.

AUTHOR CONTRIBUTIONS

EG-P conceived the work, drafted and revised the manuscript, approved the final manuscript as submitted, and agreed to be accountable for all aspects of the work. LF assisted in the conception of the work, made critical input and assisted in revisions, approved the final manuscript as submitted, and agreed to be accountable for all aspects of the work.

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REVIEW
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Opiate Drugs with Abuse Liability Hijack the Endogenous Opioid System to Disrupt Neuronal and Glial Maturation in the Central Nervous System

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The endogenous opioid system, comprised of multiple opioid neuropeptide and receptor gene families, is highly expressed by developing neural cells and can significantly influence neuronal and glial maturation. In many central nervous system (CNS) regions, the expression of opioid peptides and receptors occurs only transiently during development, effectively disappearing with subsequent maturation only to reemerge under pathologic conditions, such as with inflammation or injury. Opiate drugs with abuse liability act to modify growth and development by mimicking the actions of endogenous opioids. Although typically mediated by μ-opioid receptors, opiate drugs can also act through δ- and κ-opioid receptors to modulate growth in a cell-type, region-specific, and developmentally regulated manner. Opioids act as biological response modifiers and their actions are highly contextual, plastic, modifiable, and influenced by other physiological processes or pathophysiological conditions, such as neuro-acquired immunodeficiency syndrome. To date, most studies have considered the acute effects of opiates on cellular maturation. For example, activating opioid receptors typically results in acute growth inhibition in both neurons and glia. However, with sustained opioid exposure, compensatory factors become operative, a concept that has been largely overlooked during CNS maturation. Accordingly, this article surveys prior studies on the effects of opiates on CNS maturation, and also suggests new directions for future research in this area. Identifying the cellular and molecular mechanisms underlying the adaptive responses to chronic opiate exposure (e.g., tolerance) during maturation is crucial toward understanding the consequences of perinatal opiate exposure on the CNS.

Keywords: opioid drug abuse, glial maturation, neuronal maturation, perinatal development, human immunodeficiency virus, pediatric acquired immunodeficiency syndrome, neural stem cells, fetal abstinence syndrome

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INTRODUCTION

Maternal opiate abuse and neonatal abstinence syndrome are increasing at an alarming rate and this is in large part fueled by increases in the illicit use of prescription opiates (1–4). A 2015 press release from the National Institute on Drug Abuse noted that "Delivering mothers using or dependent on opiates rose nearly fivefold from 2000 to 2009" (5). Opiate drugs with abuse liability alter brain

development through direct and indirect actions on neuronal and glial maturation. The goal of this review is to examine our current understanding of the direct cellular and molecular effects of opiates on central nervous system (CNS) maturation. Although it was well established in the 1970s that *in utero* and perinatal exposure to opiate drugs would hinder brain maturation, not until the early 1990s was it realized that opiates *per se,* through direct actions on opioid receptor-expressing immature neurons and glia, could intrinsically affect the maturation of the CNS. This realization by no means discounts or understates the importance of the myriad psychosocial and psychiatric (e.g., anxiety and depression) problems, comorbid bacterial (e.g., pneumonia and sexually transmitted diseases) and viral [e.g., human immunodeficiency virus (HIV)] infections (bacterial and viral), endocrine (6), pulmonary, and cardiovascular complications (7, 8) resulting from opiate abuse that also influence brain development. Rather, by elucidating the direct developmental consequences of opiate exposure that are unavoidable even, if all the other comorbid psychosocial and medical problems associated with opiate addiction are treated, should provide insight into how to better manage the CNS consequences of perinatal opiate exposure and neonatal abstinence syndrome. Accordingly, a major goal of our studies has been to determine the direct cellular consequences of opioid exposure on brain maturation in children; rather than to study addiction *per se*. Many of the brain regions that are most dramatically affected by perinatal opioids are thought to be unrelated to addiction. Although understanding the underlying neural substrates of addiction is a critically important problem, and some speculative discussion on how developmental exposure to opioids might contribute to addiction in adults is provided in this review, many of the effects of opioid exposure presumably unrelated to addiction are essential for understanding the pathophysiological consequences of perinatal opioid exposure. Finally, the clinical effects of opiates on perinatal development have been reviewed elsewhere in the literature (7–13) and in this special issue of *Frontiers in Pediatrics*.

Opiates (derivatives of the opium poppy), such as morphine, which is a major bioactive metabolite of heroin in the brain, can have tremendous therapeutic value for alleviating chronic pain, but can also have significant abuse liability. Opiate drugs act by affecting and interfering with endogenous opioid receptors and peptides (14–20), which are collectively referred to as the endogenous opioid system (20–22). To understand the actions of opiate drugs during development, it is important to appreciate how endogenous opioid neuropeptides and receptors modulate growth and maturation. It is also important to specifically identify how opiate drugs interfere with the endogenous opioid system. Much work remains to be done.

OPIOIDS ARE MODULATORY

Unlike trophic factors, which can turn cellular processes on or off, e.g., neurotrophins that activate tyrosine receptor kinases (23) or wingless-type mouse mammary tumor virus integration site family (Wnt)/β-catenin signaling (24), that can switch cellular processes on or off, opioids are modulatory, meaning that they adjust biological responses quantitatively rather than qualitatively. This is especially evident during development. While opioids modify rates of ongoing cellular proliferation, differentiation, or death, and can modulate the effects of trophic factors, they do not trigger or discontinue maturational events. Instead, opioids act later in the developmental process, coordinating the timing and numerical matching of neurons and glia within a particular brain region and following the more dramatic actions of trophic factors. In fact, some brain regions that express endogenous opioid peptides and receptors during maturation—do so only transiently and no longer express them in the adult, which supports the notion that opioids are playing a unique role in development. Moreover, endogenous opioid peptides and receptors can be transiently expressed by adult neuronal progenitors in the subgranular zone (SGZ) of the dentate gyrus (discussed below). Thus, the effects of opioids on cellular growth are not restricted to embryonic and early postnatal development but continue to affect cellular maturation throughout ontogeny and are important mediators of adult neuroplasticity.

Another concept for understanding the actions of opioids in maturation is that their actions are largely dependent on context. The effects of opioids can vary depending on the cell-type, duration of exposure or stage of development, and may also depend on pre- or co-exposure to other factors (25). In neurons, for example, morphine-induced μ-opioid receptor (MOR) signaling typically activates extracellular-signal regulated kinase (ERK) 1/2 (26), while prolonged morphine exposure negatively *via* MOR regulates ERK 1/2 signaling in astrocytes (27). Coupling of MOR, δ-opioid receptors (DOR), κ-opioid receptors (KOR), and opioid related nociceptin receptor 1 (also known as the nociceptin or orphanin FQ receptor) to downstream signaling events may be similar or can differ among cell types (28). Despite an abundance of MOR binding early during development, MOR-dependent activation of $G\alpha_{i/o}$, as assessed by D-Ala^2-MePhe^4, Gly-ol^5-enkephalin (DAMGO)-stimulated [^{35}S]guanosine-5′-O-(3-thio)triphosphate ([^{35}S]GTPγS) binding, can increase as much as 19-fold from postnatal day 5 compared with some adult brain regions (29). This suggests that MOR receptor-effector coupling may be highly dynamic and vary at different times during maturation (29). In addition to differences in receptor-effector coupling, a highly speculative notion is that the molecular structure of MOR may differ among cell types (30). Multiple MOR polymorphisms and 19 splice variants have been reported (31, 32). MOR-1, MOR-1A, MOR-1X, and MOR-1K splicing variants of the human *OPRM1* gene have been reported to be differentially expressed by neurons, astroglia, microglia, vascular endothelial cells, and pericytes (30).

DEVELOPING NEURONS AND GLIA CAN EXPRESS OPIOID NEUROPEPTIDES AND RECEPTORS

Opioid receptors are expressed by the neural progenitor cells (NPCs) that are the common precursors of all CNS neurons and macroglia, inferring that opioids *per se* might directly influence very early lineage and fate decisions *via* paracrine or autocrine feedback loops. The occurrence of opioid peptides and receptors is not restricted to a particular stage of development, as opioids

can be expressed by developing neural cells throughout ontogeny. For example, radioligand binding (33–35), *in situ* hybridization (36, 37), and immunocytochemical (38–40) approaches have all been used to identify MOR, DOR, and/or KOR expression on immature neural cells in the ventricular zone (VZ) and subventricular zone (SVZ) (**Figure 1**). MOR and KOR transcripts are expressed in murine blastocyst-derived embryonic stem cells (41) and are also present in neural progenitors in SGZ of the adult hippocampus (**Figure 1**).

Endogenous opioid peptide genes can be transiently expressed during proliferation or differentiation, but not in the mature phenotype, suggesting that the expression is solely related to growth and development. Developing neural cells that temporarily express opioid peptides are particularly intriguing, since transient expression is not associated with the onset of the expression of an adult opioidergic phenotype, but presumably involved in some aspect of cellular maturation, which includes the proliferation, differentiation, and/or programmed cell death of immature neurons and glia or their progenitors. The proteases necessary for cleavage of opioid peptides to bioactive forms, such as those involved in proenkephalin [proprotein or prohormone convertases 1 (PC1) and 2 (PC2) and furin (43)], prodynorphin (*Pdyn*) [PC2 (44)] and processing of proopiomelanocortin (*POMC*)-derived precursors into β-endorphin [PC2 (45–48)], are expressed very early during maturation within germinal zones in the CNS (49). The endogenous opioid peptide gene most frequently expressed by immature neurons and glia is preproenkephalin (*Penk*), whose products can be expressed as partially or fully cleaved peptide products. The expression of proenkephalin peptides can be short-lived and can temporally coincide with cell division in neuroblasts (50–52) and glia (53). The cerebellar external granular layer (EGL) is a transient germinative zone comprised with granule neuron precursors (54, 55). The EGL forms from an outcropping of the SVZ at the rostral portion of the rhombic lips (56, 57). Enkephalin immunoreactivity is transiently expressed by neuroblasts in the EGL (50). EGL neuroblasts express *Penk* mRNA (58, 59), as well as partially processed proenkephalin peptide fragments and the fully processed enkephalin pentapeptide, Met-enkephalin (58, 59). However, the expression of *Penk* mRNA and enkephalin peptides largely disappears as the immature neurons differentiate into adult granule neurons (50, 58), suggesting the expression of *Penk* is unrelated to a mature, terminally differentiated phenotype. This represents a departure from the transcriptional maintenance programs typical of most developing neurons (60) that never switch off and in which continued activity is necessary to maintain the adult transmitter phenotype (61). *Penk* expression by developing astroglia (53, 62–64) and a variety of non-neuronal types during maturation infers that *Penk* expression is important for maturation. Finally, because neural progenitors express opioid receptors it is reasonable to speculate that opioids *per se* might directly influence neural cell maturation *via* paracrine or autocrine feedback. Although there is evidence that applying opioid antagonists alone can enhance growth *in vivo* (discussed below), this is rarely supported by findings in isolated opioid-expressing neural cells *in vitro* where opioid antagonists by themselves only infrequently effect cellular maturation.

Unlike the temporary *Penk* expression patterns that can be present in neuroblasts and immature neurons, *Pdyn* gene products do not appear to be expressed transiently by neuroblasts or immature neurons. Thus, subsets of immature neurons that express *Pdyn* mRNA or Pdyn-derived peptides in the cerebral cortex and striatum (65, 66), hippocampus (66, 67), and hypothalamus (68–70) of rats, retain their dynorphinergic phenotype in the adult. By contrast to neurons, Pdyn-derived peptides are transiently expressed by differentiating immature oligodendrocytes *in vitro* and disappears with maturation (39). *Pdyn* also differs from *Penk* in that *Pdyn* expression is not found in immature or mature astrocytes (53, 62).

The *POMC* gene is less widely expressed in the CNS compared with *Penk* or *Pdyn* genes, and typically in association with areas, such as the hypothalamus (71) and pituitary, that are involved in neuroendocrine function and stress (72, 73). Importantly, however, *POMC* is expressed and post-translationally processed into β-endorphin by adult hippocampal progenitors in the SGZ of the hippocampal dentate gyrus (74, 75) (discussed below).

ENDOGENOUS OPIOIDS TEND TO INHIBIT GROWTH

In the context of cellular maturation, endogenous opioids tend to inhibit growth (**Figure 2**). For example, artificially increasing

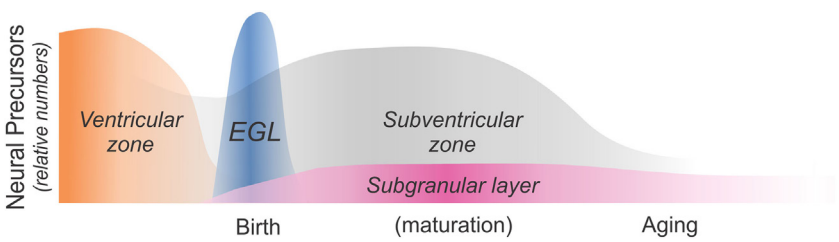

FIGURE 1 | Schematic diagram showing sites of neural precursor production throughout ontogeny. Neural cells are initially produced in the ventricular zone (VZ) and the subventricular zone (SVZ). The cerebellar external granular (or germinal) layer (EGL) is a secondary proliferative zone that arises from the brainstem and exclusively generates neurons (42). The SVZ becomes a major source of macroglia relatively early during maturation (approximately at birth in rodents and during the third trimester in humans), while the subgranular zone (SGZ) of the dentate gyrus is a major site of adult neurogenesis. As discussed in this review article, opiates affect the production and maturation of neurons and/or glia in each of these four regions and at different times throughout ontogeny.

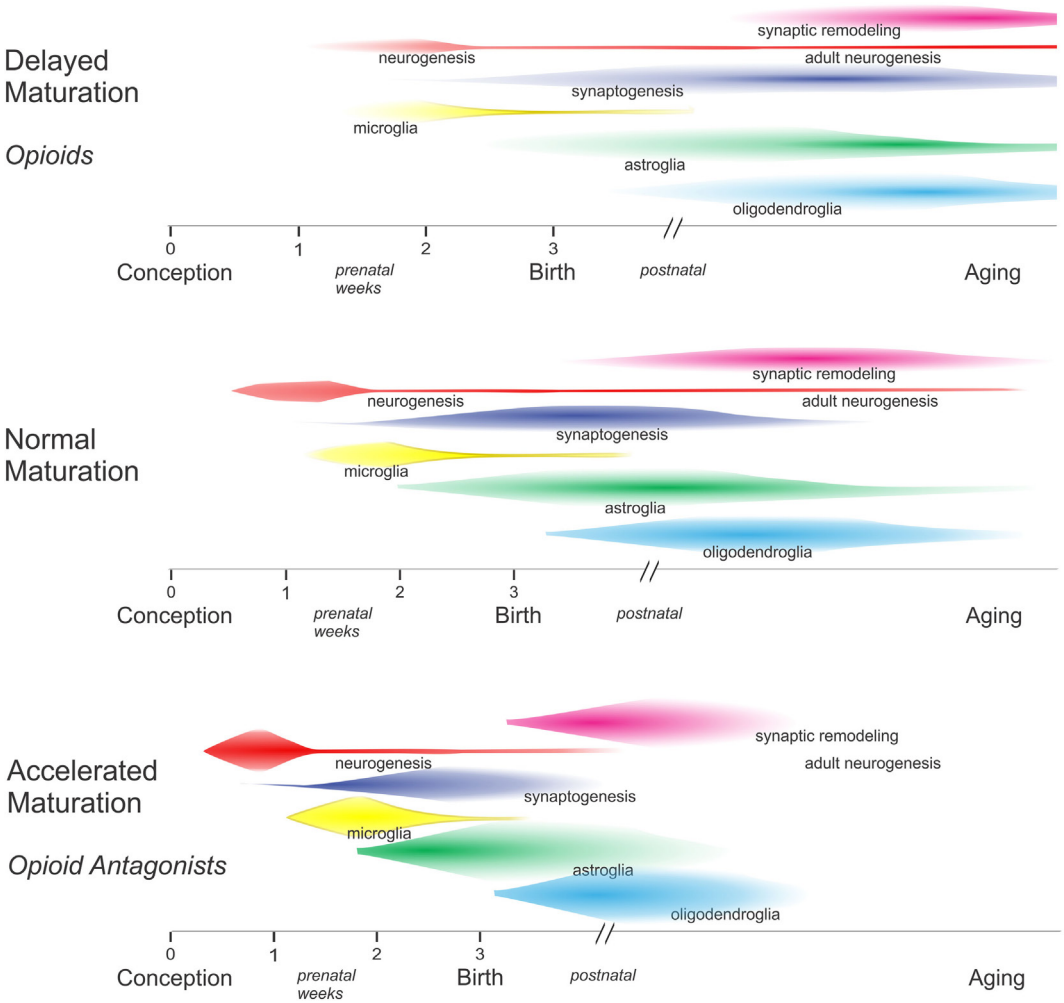

FIGURE 2 | In general, excess opioids can inhibit and delay growth, while opioid receptor blockade can transiently accelerate growth. By inhibiting maturation, opioids decelerate the pace of development among overlapping populations of neurons and glia within specific brain regions. In the absence of opioids, there is some evidence that critical periods may become abbreviated and some developmental events may have insufficient time for completion—resulting in an increased likelihood of errors.

endogenous opioid levels can reportedly inhibit the proliferation of EGL neuroblasts, while opioid receptor blockade using widely selective opioid receptor antagonists, such as naloxone and naltrexone, increase EGL neuroblast proliferation (39). There are exceptions to the notion that opioids inhibit growth, highlighting the concept that their actions are contextual and cell-specific. For example, acute opioid exposure is mitogenic to immature oligodendrocytes. Both effects on proliferation are discussed in more detail later.

That endogenous opioids inhibit growth is inferred by reports showing that chronic administration of high-dose opioid antagonists to mice and rats can accelerate growth (51, 76, 77) (**Figure 2**). The long-acting opioid antagonist naltrexone was particularly useful in this regard as a single daily dose of 50 mg/kg in rats or 10 mg/kg in mice was sufficient to continuously block MOR, DOR, and KOR for 24 h (51, 76, 77). Paradoxically, low dosages of naltrexone were found to inhibit growth, presumably because transient exposure to a low dose of naltrexone results in a compensatory upregulation of opioid peptides and receptors (78–80). The experimental evidence for this has not been consistent; the extent to which *Penk* mRNA and enkephalin levels are increased appears to differ among brain regions (78). Importantly, findings from studies with sustained exposure to high-dose opioid antagonists provide circumstantial evidence that the endogenous opioid system is tonically active and can modulate development (51, 77).

Opioid peptides are broadly associated with cellular maturation both within and outside of the CNS, and can affect cellular growth through canonical pathways involving the activation of guanine nucleotide-binding protein (G-protein)-coupled receptors (GPCRs) or by less well-characterized actions such as transcriptional regulation that involve non-canonical mechanisms. Much of our later discussions will be limited to opioid actions at GPCRs, which are the mode of action of abused opiate drugs. However, it is important to appreciate that proenkephalin contains DNA binding domains (81). Proenkephalin transcripts lacking exon 2

have been identified in rat forebrain neurons (82). Experimentally, the exon 2 deletion protein is redirected to the nucleus, presumably due to loss of a normal cytoplasmic-signaling sequence (83). Opioid peptides trafficked in this manner can act as transcription factors (84) to affect cells both within and outside the nervous system. By virtue of its actions as a transcriptional regulator, proenkephalin can regulate the proliferation and direct the fate of T-lymphocytes (84, 85), as well as modulate the production of cytokines such as IL-6 (84). In addition, proenkephalin-derived peptides (86) and the POMC-derived peptide, β-endorphin (87), are expressed by a large variety of cancers. This includes lung cancer cells (88, 89) and neoplastic cells of neural and non-neural origin (90). Opioid receptor activation can suppress the division of cancer cells in culture, while the blockade of opioid receptors can enhance cell proliferation (88, 89) or differentiation, mirroring the observations made in most non-neoplastic cells. Opioid-induced immune suppression, including diminished natural killer cell activity, phagocytosis, and antibody production may also promote tumor growth (91). Few studies have examined the effects of opiate tolerance on cancer cells and whether biological adaptation occurs following sustained exposure. The role of the endogenous opioid system and opiate drugs in neoplasia is inconclusive and has been reviewed elsewhere (92–94).

A key initial step toward understanding the mechanisms by which opioids modulate neuronal and glial maturation is to determine the extent to which opioids act directly or indirectly to alter proliferation, migration, differentiation, or survival of these cells and their progenitors. Since opioids have pronounced effects on a wide variety of physiological systems outside the CNS, such as respiration, gastrointestinal, and endocrine functions, and metabolism, that can in turn affect cell growth and development, determining the direct cellular effects of opioids is critical, and the direct effects of opioid signaling on the major types of CNS cells are considered individually in the following sections.

NEUROBLAST PROLIFERATION

To determine whether opioids might be directly affecting the growth of cerebellar EGL neuroblasts, we used a procedure developed by Hatten and coworkers (95, 96) in which enriched populations of EGL neurons are isolated using a two-step Percoll gradient and reaggregated into neurosphere cultures (95, 96). The isolated murine EGL neuroblasts and their granule neuron progeny possessed MOR and DOR, but not KOR, immunoreactivity *in vitro*, as well as proenkephalin immunoreactivity (97). In the EGL cultures, morphine exposure (1 μM) significantly reduced DNA synthesis at 24 h and the number of EGL cells and their granule neuron progeny at 48 h (97) (**Figure 3**). The inhibitory effects of morphine on DNA synthesis and cell numbers were prevented by naloxone (3 μM) suggesting that the anti-proliferative effects of morphine are due to the activation of specific opioid receptors. By contrast, the DOR agonists Met-enkephalin, [D-Pen^{2,5}]-enkephalin (DPDPE) and [D-Ala^{2}]-deltorphin II (Delt) (both were added at a 1 μM concentration) had no effect on DNA synthesis at 24 h (**Figure 3**) or on EGL cell numbers at 48 h (97).

While experiments *in vivo* have not yet fully assessed the direct effects of opioids on individual cell types, many of the *in vitro* findings are at least partly mirrored *in vivo*. Acute opiate exposure typically inhibits the proliferation of neuroblasts (51, 52, 98–105) *in vivo*. Initial studies showed that exposure to morphine or methadone markedly reduced cell numbers in the forebrain during the time when cells are generated from VZ/SVZ (99, 106, 107). Some caution is warranted when interpreting *in vivo* studies that largely rely on measuring the incorporation of thymidine analogs [e.g., [^{3}H]thymidine or 5-bromo-2′-deoxyuridine (BrdU)] using biochemical and/or morphologic (counting the number or proportion of [^{3}H]thymidine- or BrdU-labeled cells) to assess cell proliferation. Although there is typically a high degree of correlation between increased DNA synthesis and whether a cell subsequently divides, examining DNA synthesis alone can yield an inaccurate picture of cell division (108). Measuring DNA synthesis alone assumes (i) that cells entering the DNA synthesis (S) phase of the cell cycle will subsequently undergo mitosis, (ii) that the duration of the other phases of the cell cycle, or (iii) the incorporation of thymidine itself is not affected by the experimental conditions. Exploring other parameters such as cell death is paramount since neurons in the developing nervous system can be frequently overproduced only to be later culled through programmed cell death (discussed later).

Growth inhibition is not restricted to a particular brain region, stage of development, or opioid receptor type. For example, enkephalinamide reduces [^{3}H]thymidine incorporation in the forebrain, hypothalamus, and cerebellum of 11-day-old rats (104). Administering Met-enkephalin (52, 109) or other proenkephalin A derivatives (109) similarly attenuated DNA synthesis brain within the cerebellum and medullary area in 6-day-old rats. In a more detailed study, administering MOR (DAMGO), DOR [(D-Ser^{8})-leucine enkephalin-Thr (DSLET)], or KOR (bremazocine) agonists caused time-dependent (2.5, 4.5, and 8.5 h post-injection) alterations in [^{3}H]thymidine labeling and mitotic indices in germinal cells of the cortical VZ of embryonic day 16 rats exposed *in utero* that differed among MOR, DOR, or KOR agonists and that displayed subtle differences between right and left hemispheres (38). In general, the MOR agonist DAMGO was mitogenic at 8.5 h and to a greater extent at 4.5 h post-injection, while DOR activation with DSLET decreased [^{3}H]thymidine labeling and mitotic indices at 4.5 h post-injection suggesting the duration of the S and the mitotic (M) phases of the cell cycle are reduced (38). This study demonstrated the widespread and coordinated actions of the endogenous opioid system in modulating the generation of new neurons in the VZ or SVZ. Furthermore, the results provided novel evidence that KOR activation may regulate the development of circuitry involved in the lateralization of the brain (38).

A speculative notion is that the actions of KOR agonists during maturation may partially underlie the neurochemical and functional (right versus left)-asymmetry seen in the enkephalinergic and dynorphinergic systems in the adult CNS of rodents (110–113) and humans (114). For example, the lateralization of enkephalinergic and dynorphinergic processing in the anterior cingulate cortex is hypothesized to be involved in the differential regulation of positive and negative emotions and in the perception

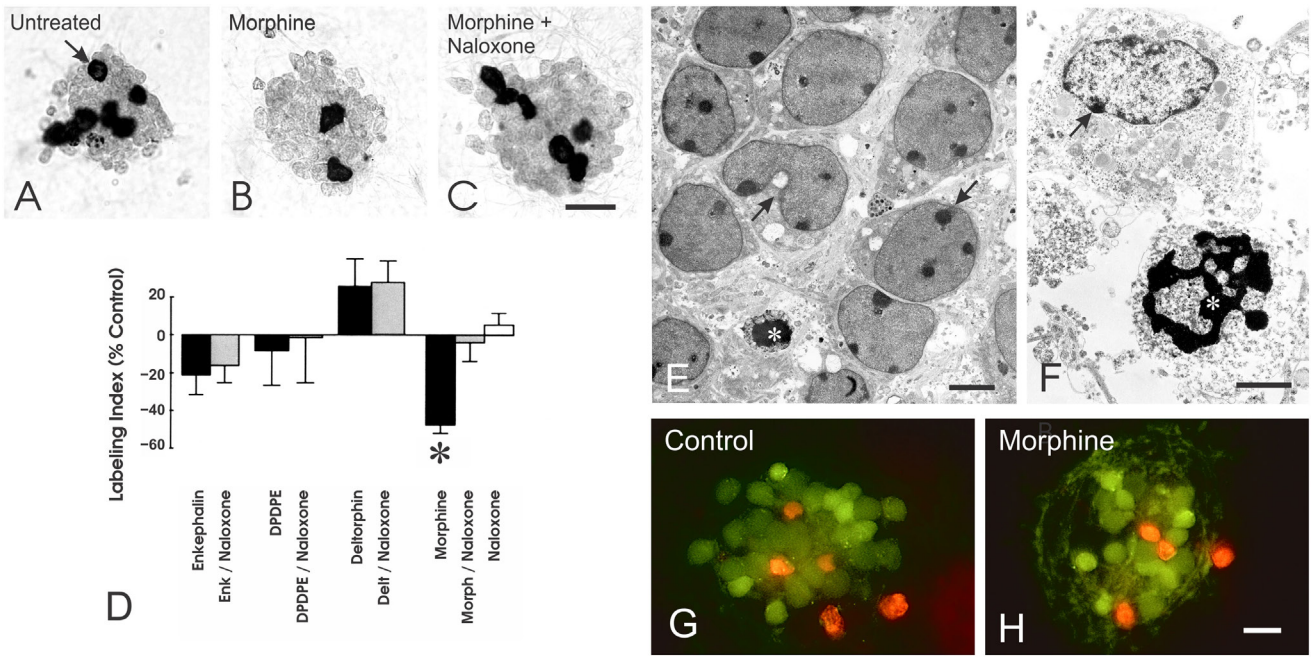

FIGURE 3 | Opioids can directly inhibit the proliferation, but not the survival, of neuronal progenitors in the cerebellar external granular layer (EGL) the cerebellar external granular layer (EGL) *in vitro*. **(A–C)** Neuroblasts from the EGL were isolated using Percoll density gradient centrifugation and reaggregated into neurospheres *in vitro*. Exposure (24 h) to the morphine-induced μ-opioid receptor agonist morphine (Morph), but not the δ-opioid receptor agonists Met-enkephalin (Enk), [D-Pen^{2,5}]-enkephalin (DPDPE), or [D-Ala^{2}]-deltorphin II (Delt), significantly decreased incorporation of the thymidine analog 5-bromo-2′-deoxyuridine (BrdU) by granule neuron precursors (*$p > 0.025$ versus untreated controls) **(D)**. Compared with untreated controls **(A)**, continuous morphine treatment (1 μM) for 24 h reduced the proportion of neuroblasts incorporating BrdU [**(B)**; arrow]. The inhibitory effects of morphine were prevented by concurrent administration of naloxone (3 μM) **(C,D)**. Neuroblasts incorporating BrdU were densely labeled (arrow); daughter cells appear near one another after dividing. Data represent the mean ± SEM of $n = 4$–6 experiments. Scale bar = 25 μm **(A–C)**. Effect of opioids on neuronal precursor survival. **(E,F)** Electron micrographs showing large numbers of viable and some degenerating EGL neuronal precursors in untreated cultures. **(E,F)** Compromised neurons displayed cytoplasmic vacuoles and swollen mitochondria [arrows in **(E)**] or accumulated cytoplasmic vacuoles and marginal heterochromatin [arrow in **(F)**], while some dying cells were evident and displayed pyknotic nuclei with shrunken and densely packed heterochromatin [* in **(F)**]. Scale bars = 5 μm **(E)** and 10 μm **(F)**. **(G,H)** Fluorescent images of living (*green fluorescence*) and non-viable (*red fluorescence*) immature neurons from the EGL in untreated control **(G)** and morphine-treated **(H)** neurospheres. Exposure to morphine (1 μM) for 24 h had no effect on EGL cell death (scale bar = 20 μm) **(H)**. Reprinted from Ref. (97); Copyright (2000), with permission from John Wiley and Sons.

of pain in the right- versus left-hand sides of the brain (114). Alterations in Pdyn-derived peptide processing, dynorphin levels, and KOR signaling in forebrain areas including the prefrontal cortex and striatum have been implicated in substance abuse and pro-addictive behaviors (115–118). Enduring changes in these systems during maturation are likely to cause lasting alterations in neural circuitry underlying addiction, as well as other CNS functions unrelated to addiction.

The developmental effects of opioids on the cerebellar EGL have been explored in more detail than in many other regions. Opioid peptides and receptors are highly expressed by the immature EGL cells (consisting of neuroblasts and immature, postmitotic neurons), while the mature granule neurons derived from this germinative zone lose this expression (34, 50, 58). Thus, the opioid system in the EGL is positioned to be more involved in maturational processes and less related to the development/maintenance of adult neurochemical systems (42). Manipulation of the opioid system alters the proliferation of EGL neuroblasts and production of granule neurons derived from the EGL (51, 52, 77, 105). Morphine or methadone, which act preferentially at MOR, reduced thymidine incorporation and/or resulted in reductions in EGL cell numbers *in vivo* (119–121). Alternatively, despite findings that opioid receptor antagonists augment EGL cell proliferation *in vivo* (51) and that EGL cells express proenkephalin peptides *in vitro* (97), neither the broad-spectrum opioid receptor antagonist naloxone nor selective DOR antagonists alone affected the proliferation of isolated EGL cells (97). Findings in isolated EGL cells suggest that opioids do not directly affect EGL neuroblast growth through autocrine and/or paracrine feedback. Instead, opioids may affect another aspect of cerebellar maturation, such as Purkinje cell development, which in turn can influence EGL cell growth (122, 123).

NEURONAL DIFFERENTIATION

Unlike the effects of morphine on EGL proliferation, morphine had no effect on granule neuron differentiation (97). Alternatively, dendritic growth was stunted by 48 h exposure to the selective DOR agonists enkephalin (1 μM) or Delt (1 μM), but not the selective DOR agonist DPDPE (97). The fact that distinct DOR agonists act differently at the same receptor strongly infers that the effects of DOR agonists on neuronal differentiation are

functionally selective and displaying "biased agonism" (124–126), meaning that individual ligands selectively stimulate different signal transduction pathways *via* the same receptor. Finally, the findings also provide clear evidence that different opioid receptor types, MOR or DOR, can independently regulate different aspects of development in the same cell.

Unlike the effect of morphine in EGL neuroblasts, morphine can inhibit the differentiation of Purkinje cells in organotypic explants from 1-day-old mice (127). At 7–10 days *in vitro*, calbindin-D_{28k}-immunoreactive Purkinje cells in control, as well as morphine-treated explant cultures, possessed dendrites with growth cones and filopodial processes indicative of active growth (**Figure 4**). The presence of developing synapses was also confirmed by using electron microscopy (**Figure 4**). Continuous exposure to morphine (**Figure 4E**) for 7–10 days *in vitro* caused concentration-dependent reductions in the length of growing dendrites in cerebellar explants derived from 1-day-old mice. Exposure to morphine for 7–10 days resulted in a significant reduction in dendritic length (EC_{50} = 49 nM) and the effects of a high (10 μM) concentration of morphine were completely reversed

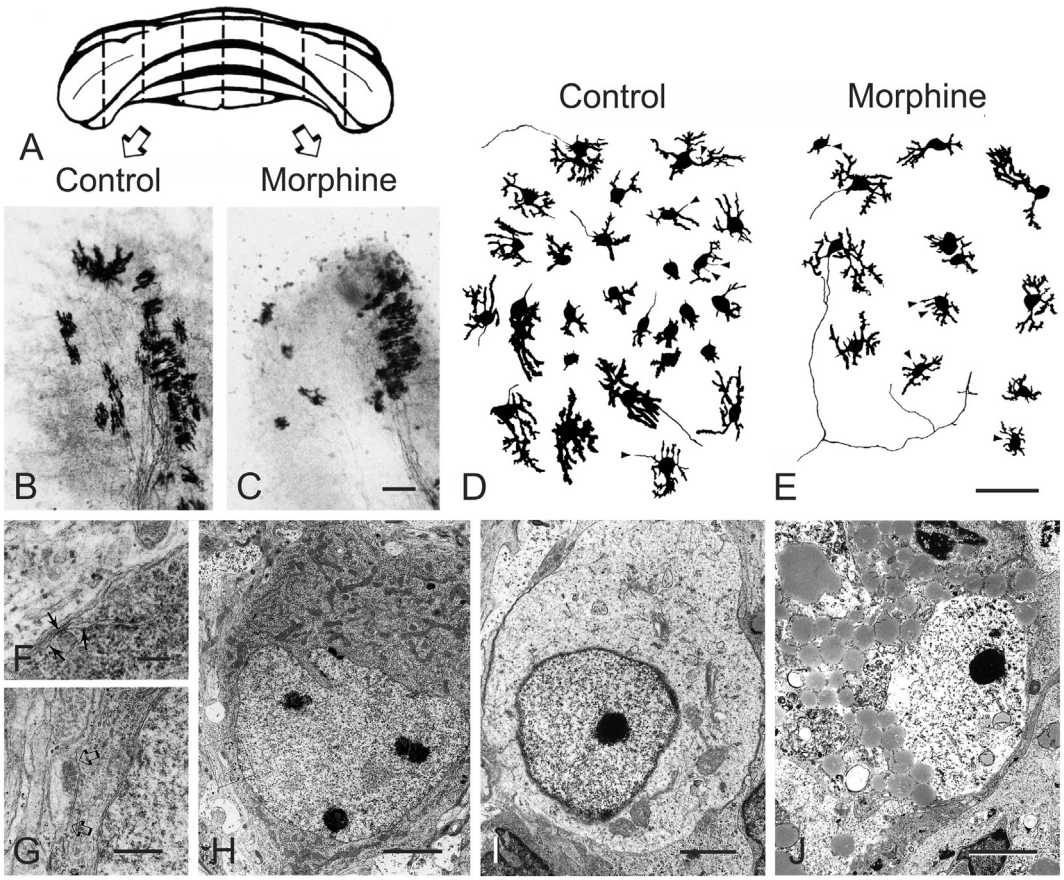

FIGURE 4 | Morphine inhibits Purkinje cell dendritic differentiation and increases their death in a concentration-dependent manner in organotypic cultures of the mouse cerebellum. **(A–C)** Bilaterally matched explant pairs were grown in the presence and absence of morphine. **(A)** Schematic drawing of the cerebellum from a 1-day-old mouse illustrating the "homologous- or mirror-pair" paradigm (129, 130) used to assess the experimental effects of opiates in this study (127). **(B,C)** Bilaterally matched explant pairs are matched in size, shape, and cytoarchitecture and this is maintained by organotypic culture conditions. Purkinje cells in the untreated control explants **(B)** had larger dendrites compared with their matched-pair counterparts continuously treated with morphine (1 μM) **(C)** for 7–10 days [scale bar = 25 μm; **(B,C)** are the same scale]. **(D,E)** Composite camera lucida drawings of calbindin-D_{28k}-immunoreactive Purkinje cells illustrating the effects of high concentrations (10 μM) of morphine on Purkinje cell numbers. Compared with untreated controls **(D)**, continuous exposure to morphine **(E)** for 7–10 days *in vitro* caused concentration-dependent reductions in dendritic length and in the number of calbindin-D_{28k}-immunoreactive Purkinje cells in cerebellar explants derived from 1-day-old mice. Filopodial processes (arrowheads) extend from the cell body or dendrites of developing calbindin-D_{28k}-immunoreactive Purkinje cells—some filopodia terminate in growth cones (arrowheads) [scale bar = 25 μm; **(D,E)** are the same scale]. **(F–J)** Electron micrographs of Purkinje cells in organotypic explants continuously exposed to morphine for 7–10 days *in vitro*. Many Purkinje cells in morphine-treated organotypic explants display normal ultrastructural features. Cerebellar Purkinje cells can be identified by unique features, such as subsurface or hypolemmal cisternae [arrows in **(F)**] (131–133) (scale bar = 1 μm). **(H)** Developing Purkinje cell (scale bar = 5 μm). **(G)** Higher magnification of the axosomatic synapse contacting the Purkinje cell in [**(H)**; arrows] (scale bar = 2 μm). **(I,J)** Degenerating cells in morphine-treated organotypic explants at 7–10 days *in vitro*. **(I)** A degenerating Purkinje cell with a deficit in cytoplasmic organelles and abnormally dense marginal heterochromatin (scale bar = 2 μm). **(J)** A large, dying neuron resembling a Purkinje cell with access lipid inclusions and glycogen in the cytoplasm and partial destruction of the nuclear membrane (scale bar = 3 μm). With progressive degeneration and loss of cellular morphology, it can be difficult to identify Purkinje cells with certainty. Reprinted from Ref. (127); Copyright (1994), with permission from Elsevier.

by concurrent naloxone administration. Importantly, the effects were shown to be concentration-dependent and preventable by naloxone suggesting that the effects of morphine are mediated by specific opioid receptors. Although the cellular site(s) of morphine's actions within the cerebellum are uncertain, the results clearly indicate that morphine, through direct actions on the cerebellum *per se*, can inhibit the differentiation of Purkinje cell dendrites. Morphine can directly reduce EGL cell proliferation and granule cell numbers (97), and given the well-known trophic ability of granule cells to direct Purkinje cell maturation (128) and known interdependence between these two cell types (122, 123), this is strong evidence that morphine modulates granule cell and Purkinje cell trophic interactions during development.

The cerebellum is becoming increasingly linked to neural circuitry involved in addictive behaviors including aversion, reward, motivational drive, and saliency (134, 135). Addiction is associated with "impaired response inhibition" and "salience attribution" (136–138). Emerging evidence suggests that the cerebellum maintains the homeostatic balance among these brain regions (134, 135), and is critical for habit formation, attention to novel stimuli, and behavioral inhibition—behavioral traits associated with addiction (135). Moreover, chronic exposure to a variety of substances in adults can result in structural deficits in the gray matter and/or white matter within cerebellar lobules VI, VIIb, Crus I, Crus II, and within the vermis (134, 139). Assuming greater sensitivity of developing versus the mature cerebellum, perinatal exposure to opiates is likely to have profound organizational effects on the neural circuits regulating drug taking behavior. Importantly, not only do Purkinje cells in the cerebellar vermis transiently express *Penk* mRNA and enkephalin immunoreactivity during development (58, 59), but also the dendritic complexity and/or the density of spines of Purkinje cells within the vermis of cerebellar lobule VIII in 10-day-old rats and lobules VI–VIII in 21-day-old rats is altered in response to prolonged opioid antagonist exposure during maturation (21). The extent to which the transient developmental increases in opioid expression in cerebellar neurons influence adult CNS functions is uncertain.

Exposure to morphine (100) or methadone (140) during development inhibits the neurochemical (140) and morphological (107, 120) maturation of the brain, including reductions in neuronal numbers (106). Hammer and coworkers demonstrated that sustained morphine (10 mg/kg/day) exposure *via* subcutaneous osmotic minipump from gestational day 12 until postnatal day 6 in rats results in significant reductions in neuronal density and in the absolute numbers of neurons in layers II–III, IV, and V of the primary somatosensory cortex, but not layer VI (103). The same study found no effect of morphine on the thickness of layers II through VI of the somatosensory cortex. Interestingly, sustained exposure to the opioid antagonist naltrexone (10 mg/kg/day) also decreased neuronal density in cortical layers II through V and the total number of neurons in layer V, while increasing the thickness of layers II–III and throughout the entire cortex (103). Possible explanations for similar actions of morphine and naltrexone might include: (i) Naltrexone may act by transiently enhancing dendritic differentiation in rat somatosensory cortex (21) and/or (ii) by increasing glial numbers (39). Either action would result in a decrease in the relative density and number of neurons per unit volume, while increasing the overall thickness of the cortex. Earlier work by Hammer and coworkers (141) established that exposure to morphine during perinatal development decreased the length of basilar dendrites of layers II–III pyramidal neurons in somatosensory cortex. Morphine-dependent reductions in basilar dendritic complexity were prevented by co-administering naltrexone (141). In confirmatory studies, the offspring of pregnant rats exposed to morphine (5 mg/kg first 3 days; 10 mg/kg thereafter) during gestational days 11–18, showed enduring reductions in dendritic complexity in layer II/III pyramidal neurons of the secondary visual cortex at postnatal day 25 (142). Decreases in pyramidal cell numbers in hippocampal areas CA1, CA2, and CA3 are also reported in postnatal day 18 or day 32 offspring of female mice chronically administered morphine (10 mg/kg/day) before mating, during gestation, and postnatally (143). More recently, postnatal morphine exposure (2 mg/kg, b.i.d.) from postnatal days 3 through 7 in rats was found to decrease incorporation of the thymidine analog BrdU by NPCs in the dentate gyrus of the hippocampus (144). In addition, the same study detected lower levels of glutamic acid decarboxylase (GAD), taurine, and *myo*-inositol by functional nuclear magnetic resonance spectroscopy, and decreased amounts of myelin basic protein (144) (important for myelinogenesis) in the hippocampus (144). Reductions in GAD, essential for the interconversion of glutamate to γ-aminobutyric acid, suggest marked imbalances in excitatory versus inhibitory neurotransmission.

The accelerated maturation seen with opioid receptor blockade can seem dramatic during peak periods of development in the cerebral cortex, hippocampus, and cerebellum. However, the effects are often transient and in many brain regions untreated controls appear to catch-up during later development (21).

NEURONAL CELL DEATH

The effects of opioids on cell death and survival in neural and non-neural cells, especially in relation to immune and endocrine function, chronic pain, and cancer in adults have been studied (145–147) and extensively reviewed previously (148).

Purkinje cell death is evident in organotypic explants from 1-day-old mice exposed to pharmacological concentrations of opiates (127). Continuous exposure to high concentrations of morphine (>1 μM) for 7–10 days *in vitro* caused reductions in the number of calbindin-D_{28k}-immunoreactive Purkinje cells in cerebellar explants derived from 1-day-old mice (127). The ability of morphine to decrease the number of calbindin-D_{28k}-immunopositive Purkinje cells was concentration-dependent (EC_{50} = 3.6 μM) and morphine-induced (10 μM) death in Purkinje neurons was antagonized by co-exposure to naloxone. To assess whether reductions in Purkinje cell numbers might potentially be due to a morphine-dependent decline in calbindin-D_{28k} expression, electron microscopy was used to confirm that Purkinje cell death was occurring especially in morphine-treated explant cultures (**Figure 4**). Although morphine was cytotoxic to Purkinje cells in organotypic explants from 1-day-old mouse cerebella, toxicity was not evident in Purkinje cells in explants taken from 7-day mouse cerebella (127). This suggests that, even at high concentrations, morphine is not inherently toxic to

Purkinje cells; rather, morphine is likely to be interfering with critical developmental events affecting survival during the first, but not second, week of postnatal development in mice. Although cell death is not seen with concentrations of morphine exceeding 1 μM in cultured mouse EGL cells (97) (**Figure 3**), prolonged (5-day) exposure to a range of morphine concentrations between 10^{-12} and 10^{-4} M induces classic apoptosis, including DNA fragmentation, in human fetal brain neurons (147). This suggests that the duration of exposure to opiates may also be important in triggering programmed cell death (147). The varied response among neuron types and conditions highlights that the mechanism(s) and pathways underlying opiate-induced cell death are far from being completely understood, and likely vary among cell types and context. Other investigators have demonstrated that opiates can modulate cell death *via* PI3K and ERK 1/2 signaling in Chinese hamster ovary or HEK293 cells stably transfected with the murine MOR (149). In some instances, opiates may affect cell survival in addition to their effects on proliferation, although this is not a consistent observation. Alternatively, surrounding glia, which can display extreme regional and developmentally diverse phenotypic patterns of MOR, DOR, and KOR expression (150, 151) (discussed below), may also contribute to varied developmental responses among different neuronal types to opioids.

The death of neuronal precursors is occasionally reported as a consequence of exposure to opiates (152) or endogenous opioids (153) by themselves. In addition, opioids can modulate the effects of existing apoptotic signals [especially in immune cells (154, 155)], and opiates may be cofactors in regulating neural cell death (156, 157) [see Ref. (148)]. Cell death is evident in cultured Purkinje cells with more chronic 7-day exposure to morphine (discussed above) (127), and Purkinje cell losses have been reported in chronic heroin abusers who are HIV-seronegative (158).

GLIAL MATURATION

The generation of neurons throughout the CNS is thought to be essentially complete at birth in rodents apart from the formation of interneurons of the olfactory bulbs, the dentate gyrus, and the cerebellar cortex (159–162). Nevertheless, in the 1980s, there were emerging reports demonstrating opioid-dependent alterations in DNA synthesis and cell numbers, without changes in cell survival, in neural cells throughout the brain long after peak periods of neurogenesis (98, 100, 104). In fact, in adult rats, increases in [^{3}H]thymidine labeling in the SVZ were evident 1 h after morphine injection (10 mg/kg, s.c.) (163) in brain regions that are not associated with adult neurogenesis. This discrepancy prompted us to question whether opioids might have widespread effects on the generation of glia throughout the CNS.

There were several indications that glial-restricted precursors (GRPs), as well as their astroglial and oligodendroglial progeny, could be a direct target of opioids during maturation. (i) As mentioned, opioid exposure has widespread effects on cell numbers during periods of peak gliogenesis, when most macroglial types in the forebrain are being generated from the SVZ and after the production of neurons is largely complete. The sustained mitogenic effects seen in the postnatal forebrain 8–10 weeks following exposure to naltrexone (98) could hardly be explained by alterations in neurogenesis—since the production of neurons from the SGZ and rostral migratory stream is relatively small compared with the observed alterations in cell numbers. In addition to timing, (ii) opioids affect sites of glial production in the SVZ. (iii) Opioid receptors are often expressed by GRPs and immature astroglia, including radial glia (40), and oligodendroglia, but less frequently by their adult counterparts (39) (**Figure 5**). (iv) Finally as discussed in detail below, opioids can directly affect the proliferation and differentiation of immature astrocytes and oligodendrocytes isolated *in vitro*. Thus, many of the effects of opioids on cell numbers in the postnatal CNS are largely attributable to enduring alterations in gliogenesis rather than neurogenesis.

ASTROCYTE PRODUCTION AND DEVELOPMENT

We and others have proposed that astroglia are direct targets for opioid drugs with abuse liability (25, 166–170). Astroglial growth has been shown to be inhibited by opioids *in vitro* and *in vivo* (25, 39, 109, 169, 171–174). Astroglia can express MOR, DOR, and/or KOR (39, 150, 168, 169, 171, 174–178). Opioid receptor expression by astrocytes in primary culture is developmentally regulated, differs among brain regions (150, 173, 175, 177, 179–181), and in the case of DOR, appears to be cell cycle-dependent (150, 179). Activation of MOR, KOR, or DOR causes reduced astroglial proliferation (169, 174, 177). In addition to inhibiting astroglial division, morphine also causes astrocytes to hypertrophy. MOR-dependent inhibition of astroglial proliferation and hypertrophy are mediated by increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (174, 177). In these studies, MOR-dependent increases in $[Ca^{2+}]_i$ could be elicited by morphine (100 nM) or the more selective MOR agonist H-Tyr-Pro-Phe (N-Me)-D-Pro-NH_2 (PL017) (10 nM, 100 nM, or 1 μM), and resulted from both extracellular Ca^{2+} influx *via* L-type Ca^{2+} channels and from Ca^{2+} mobilization from intracellular stores (174). The effects of morphine on astroglial proliferation and cellular hypertrophy can be mimicked by artificially increasing $[Ca^{2+}]_i$ (in the absence of morphine) or prevented by chelating intracellular Ca^{2+}. Nifedipine (1 μM) was used to assess whether influx through L-type Ca^{2+} channels might be operative. While blocking influx through L-type Ca^{2+} channels attenuated MOR-dependent $[Ca^{2+}]_i$ increases in astrocytes, this strategy had no effect on morphine's ability to inhibit proliferation or stimulate hypertrophy. Thapsigargin and dantrolene were used to assess the role of Ca^{2+} mobilization from intracellular sources. Sustained (24 h) exposure to thapsigargin (100 nM), which blocks sarco/endoplasmic reticulum (ER) Ca^{2+} ATPase (182) and prevents Ca^{2+} sequestration into ER thereby depleting the ER of Ca^{2+}, was intrinsically toxic to astrocytes confounding any interpretation of the results. Alternatively, blocking Ca^{2+}-induced Ca^{2+} release from intracellular stores using dantrolene (10 μM) selectively prevented morphine-induced decreases in astroglial proliferation and cellular hypertrophy (174). Dantrolene selectively blocks ryanodine receptors (RyR) by blocking Ca^{2+} channels associated with the RyR1 and RyR3, but not the RyR2, isoform (183). The results indicate that opiates regulate astroglial growth

and maturation through a pathway involving MOR-dependent mobilization of $[Ca^{2+}]_i$ *via* RyR1 and/or RyR3.

In flat-polyhedral (type I) astroglia isolated from the cerebral forebrain of postnatal day 1–4 ICR mice, KOR-mediated increases in $[Ca^{2+}]_i$ were initiated using the selective agonists *trans*-(+/−)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl] benzeneacetamide hydrochloride (U50,488H) (10 nM, 100 nM, or 1 μM) or (+)-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide (U69,593) (10 nM, 100 nM, or 1 μM) (184, 185). The effects of U69,593 on astroglial proliferation and hypertrophy could be mimicked by artificially increasing $[Ca^{2+}]_i$ in the absence of drug and could be prevented by the KOR antagonist nor-binaltorphimine (nor-BNI). Process-bearing (type II) astrocytes, which resemble radial glia, isolated from postnatal day 1–3 mouse spinal cord, widely expressed KOR. Unlike the results in type I astrocytes from the cerebral forebrain, exposing type II spinal cord astrocytes to the KOR agonist U50,488 (1 μM) for 3 DIV or 6 DIV increased DNA synthesis as assessed by BrdU incorporation *in vitro*, while concurrent administration of the KOR antagonist nor-BNI or the use of $KOR^{-/-}$ astrocytes negated the effects of U50,488 (186). A similar mitogenic effect was noted in serum-starved (28 h) astrocytes isolated from cerebral cortex of postnatal day 1 Sprague–Dawley rat pups or from immortalized rat cortical astrocytes (CTX TNA2; ATCC) exposed for 24 h to U69,593 (1 μM) or the KOR agonist derived from salvinorin A, MOM-Sal-B (1 μM) (187). The collective findings suggest that KOR activation can have both inhibitory and excitatory effects on astroglial proliferation and that this may differ among astroglial types, brain regions, and context. Inhibition of astrogliogenesis through MOR-and KOR-mediated events was

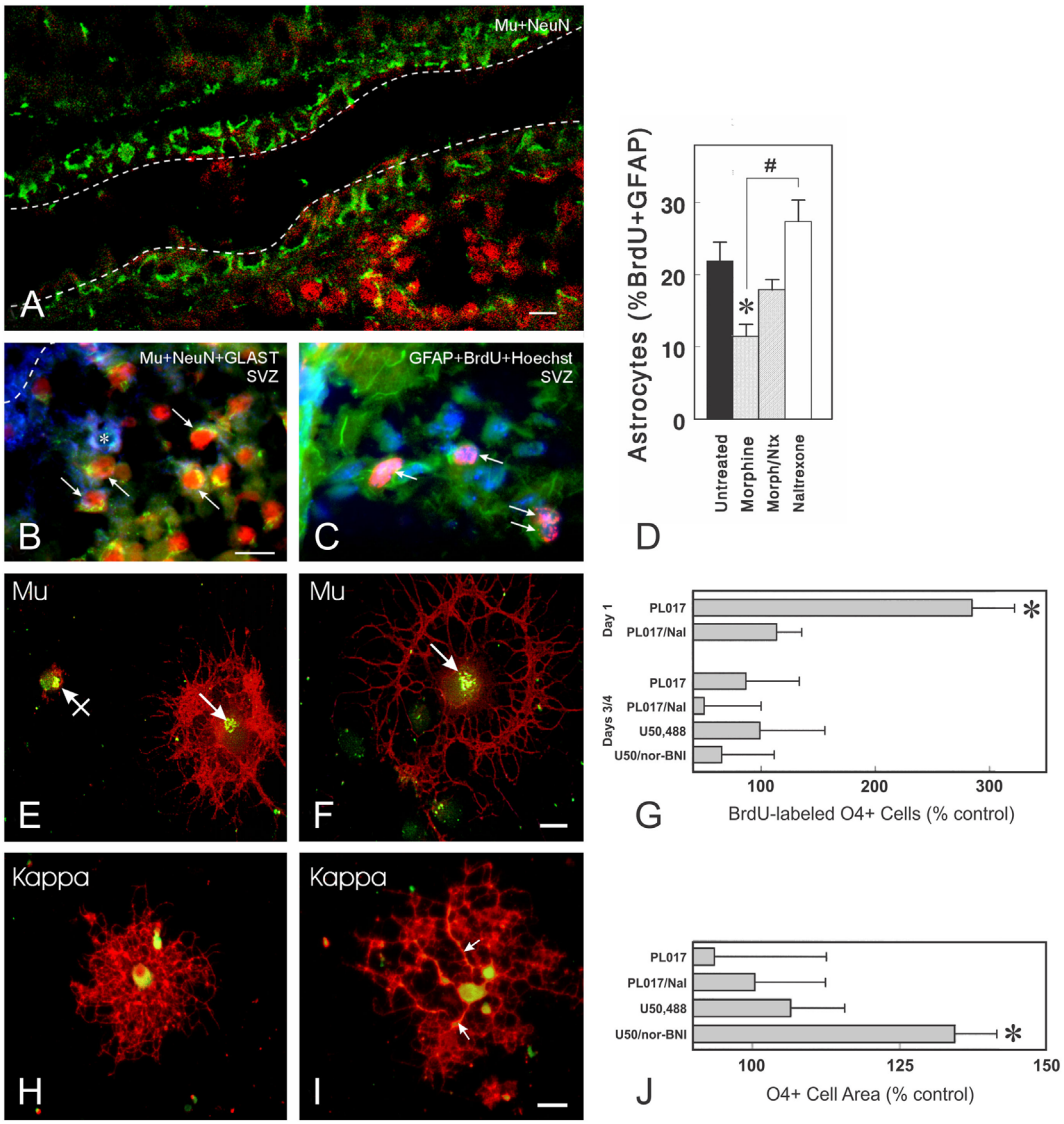

FIGURE 5 | Continued

FIGURE 5 | Opioid receptors can be expressed by immature neurons, astrocytes, and oligodendrocytes. **(A)** μ-Opioid receptor (MOR) (Mu; *green fluorescence*) immunoreactive cells can be present in some portions of the ventricular zone and/or subventricular zone (SVZ) of postnatal day 5 mice (scale bar = 20 μm). Many of the MOR+ cells entering the SVZ are NeuN-immunoreactive neurons (*red fluorescence*). The dashed lines represent the borders of the lateral ventricle. **(B)** Photomicrograph showing combined neuronal nuclear (NeuN; *red fluorescence*), glutamate transporter (GLAST, also known as excitatory amino acid transporter 1; *blue fluorescence*), and MOR (*green fluorescence*) immunofluorescence in cells from postnatal day 5 mice. Subpopulations of both neurons (arrows) and astroglia (*) express MOR. **(C)** Thymidine analog, 5-bromo-2′-deoxyuridine (BrdU) labeled (*red fluorescence*), and glial fibrillary acidic protein (GFAP+) astrocytes (*green fluorescence*) are indicated by the arrows **(C)**; cell nuclei are counterstained with Hoechst 33342 (*blue fluorescence*) [scale bar in **(B)** = 25 μm; **(B,C)** *are the same magnification*]. **(D)** Effect of morphine and/or naltrexone on BrdU incorporation in GFAP-expressing cells within the postnatal day 5-mouse brain. Morphine significantly decreased the proportion of BrdU+ astrocytes ($^{*}p < 0.05$) and there is a significant difference between morphine-treated and naltrexone-treated mice ($^{\#}p < 0.05$). **(E–J)** Oligodendrocytes express MOR and κ-opioid receptors (KOR) in a developmentally regulated manner *in vitro*. Oligodendrocytes are labeled using the specific anti-O4 antibody (*red fluorescence*) and colocalized with MOR **(E,F)** or KOR **(H,I)** immunoreactivity. **(E,F)** MOR are localized within discrete perinuclear regions of the cell body (arrows) (*green fluorescence*); areas where O4 and MOR or KOR are colocalized display *yellow fluorescence*. MORs can be associated with oligodendrocytes at different stages of development, including very immature oligodendrocytes with negligible O4 immunoreactivity [crossed arrow; **(E)**] and more mature oligodendrocytes with large process networks [arrow; **(F)**] [scale bar in **(F)** = 10 μm; **(E,F)** *are the same magnification*]. **(G)** The effect of opioid receptor agonists and antagonists on BrdU uptake in cultured O4+ oligodendrocytes of two different ages. At 1 day after enrichment, oligodendrocytes are still relatively immature and express only μ-receptors. A 27-h exposure to the MOR agonist PL017 increases BrdU uptake by nearly 300% compared with control values. In the presence of naloxone (PL017/Nal), this effect is not seen. At 3–4 days after enrichment, neither MOR nor a KOR agonist (U50,488) or antagonist [nor-binaltorphimine (nor-BNI)] affect BrdU uptake in a significant manner. Cells were counted on duplicate coverslips from 4 to 5 different cultures ($^{*}p < 0.01$; ANOVA with *post hoc* Newman-Keuls testing). **(H,I)** By contrast to MOR, KOR are distributed through the entire cell body cytoplasm. Occasional oligodendrocytes also have KOR-immunoreactivity within regions along cell processes [arrows; **(I)**] [scale bar in **(I)** = 20 μm; **(H,I)** *are the same magnification*]. **(J)** Effects of opioid receptor agonists and antagonists on oligodendrocyte cell area. After 48 h of exposure, the only significant effect is seen with the KOR antagonist, nor-BNI, which increased the average cell area compared with control cells ($^{*}p < 0.025$) **(J)**. This effect was not seen at 27 h (164). The finding that nor-BNI increases the elaboration of cytoplasmic processes provides circumstantial evidence that endogenous opioids are expressed and inhibit oligodendrocyte differentiation (164). Subsequent studies showed that differentiating oligodendrocytes produce dynorphins and these endogenous KOR peptide ligands appear to preferentially decrease the survival of oligodendrocytes during later maturation (165). Thus, an alternative explanation is that the loss of more mature oligodendrocytes results in a net increase in immature oligodendrocytes and the perception that dynorphin is restricting differentiation. Figures **(A–D)** are reprinted from Ref. (39); Copyright (2001), with permission from John Wiley and Sons. Figures **(E–J)** are reprinted with slight modifications from Ref. (164); Copyright (1998), with permission from John Wiley and Sons.

shown to be driven *via* ERK, by contrast to the implication of the p38 mitogen-activated protein kinase pathway in opioid-mediated attenuation of neurogenesis (41). Importantly, opioids can modulate basic fibroblast growth factor and/or epidermal growth factor activation of ERK in C6 glioma cells or in primary astrocytes (170, 188, 189). Whether DOR-dependent increases in astroglial $[Ca^{2+}]_i$ are also antimitotic have not been explored. The "reactive" cellular hypertrophy is accompanied by increased glial fibrillary acidic protein (GFAP) immunoreactivity and aspects may mimic reactive gliosis *in vivo* (174, 190).

In 4–5-day-old postnatal mice, 4.5 h of morphine (20 mg/kg s.c.) exposure significantly decreased BrdU incorporation by astrocytes in the SVZ of the lateral ventricle (39). A similar slowing of proliferation was suggested in a study where the G_2/M phase of cell cycle was lengthened in GFAP+ radial glia in the cerebral cortex when gestational day 15.5 (E 15.5) embryonic mice were exposed to morphine (E 15 pregnant dams received 3 × 10 mg/kg s.c. at 3 h intervals) (191). Based on findings that opioid receptor activation can regulate cellular development, it seems probable that the varied patterns of opioid receptor and peptide expression within diverse cell types permit the simultaneous and coordinated control of development within heterologous subpopulations of cells. Moreover, findings that many NPCs likely express MOR, and that morphine acutely alters astroglial development, prompt speculation that chronic opiate exposure might cause lasting changes in neural function by disrupting gliogenesis.

Morphine normally has little or no effect on astroglial viability during maturation after either acute or sustained exposure (192). Indeed, morphine can protect neonatal astrocytes against the cytotoxic effects of peroxynitrite (193). Similarly, astroglial apoptosis, as assessed by caspase-3 cleavage, was not evident in neonatal rat pups exposed to morphine (10 mg/kg; b.i.d.) on postnatal day 1 through 7 (194). Alternatively, morphine-induced death is evident in astrocytes in mixed-NPC cultures from 14-day mouse embryos (195). Although astrocyte death is not reported with exposure to opiates alone *in vitro*, sustained (96 h) exposure to morphine (500 nM) causes small, but nevertheless significant, increases in the death of immature astrocytes or glially restricted precursors that have been co-exposed to the HIV-1 Tat (transactivator of transcription) protein (100 nM) (156). This and other evidence suggests that the response of target cells to morphine can be "reprogrammed" by exposure to HIV-1 Tat. Morphine (and presumably other opiates) can act as biological response modifiers, imparting very different intracellular signals that instruct some astrocytes to die if they have been pre- or co-exposed to HIV-1 Tat (156), and perhaps other biological stressors. These findings illustrate the importance of context in shaping how developing neural cells interpret an opioid signal.

OLIGODENDROCYTES

Oligodendrocytes produce myelin in the CNS (196) and originate during a second wave of glial maturation following genesis of astroglia (197, 198). Slow generation of new oligodendrocytes and adaptive myelination are processes that continue throughout life (199). Importantly, chronic opiate exposure results in significant, selective disruption of white matter tracts throughout the CNS (200–203). While some of this injury may be due to indirect effects involving inflammatory changes or loss of support from

astroglia, it is also clear that significant effects of opiates occur directly on oligodendroglia. Our labs were the first to show that oligodendrocytes express MOR and KOR (164, 204). Unlike the situation in neuroblasts and astroglia, where MOR activation inhibits cell replication, MOR activation appears to be mitogenic in immature oligodendroglia since proliferation as assessed by BrdU was stimulated by the MOR-selective agonist PL017 (1 μM) and prevented by co-administration of naloxone (3 μM) (204). By contrast to MOR, KOR begins to be expressed in more mature, non-dividing oligodendroglia (164). KOR blockade with nor-BNI, in the absence of exogenously added opiates, increased the size of myelin-like membranes in cultured oligodendroglia (164), suggesting that the cells themselves might produce KOR agonists. This was later borne out by studies showing production and release of *Penk*- and *Pdyn*-derived peptide products by cultured oligodendroglia (165). Nor-BNI greatly enhanced glutamate-induced death of oligodendroglia while causing only modest effects on its own, again invoking the issue of context in opiate effects on survival as well as growth/differentiation (165). A relationship between KOR and oligodendrocyte function/myelin production was also suggested by our finding that KOR (but not MOR) was severely reduced on oligodendrocytes, but not neurons, in the jimpy mouse, a dysmyelinating mutant (205). More recent work has also shown that KOR agonists, including U50,488, can promote differentiation/myelination in human oligodendrocytes derived from immortalized pluripotent stem cells (206). Thus, similar to granule neurons, different aspects of oligodendroglial development appear to be independently regulated by opioid signaling. However, unlike granule neurons and their EGL precursors, which do not appear to express KOR *in vitro* (97), MOR activation in immature oligodendroglia is mitogenic (164).

More recently, chronic, 9 days MOR or KOR activation was shown to increase the production of young oligodendrocytes during the sequential differentiation of murine embryonic stem cells, seemingly at the expense of neurogenesis and astrogliogenesis (207). Effects of the MOR-selective agonist DAMGO (1 μM) or the KOR selective agonist U69,593 (1 μM) were similarly dependent on ERK and p38 mitogen-activated protein kinase (MAPK) signaling pathways.

Buprenorphine is a newer treatment for opiate addiction than methadone that was initially thought to have fewer side effects, less abuse liability, and greater safety because of its actions as a partial MOR agonist (208–211). In addition to its actions at MOR, buprenorphine can act as a partial antagonist at KOR (212) and as an agonist at nociceptin/orphanin FQ (NOP or OLR1) receptors (212, 213). Though beneficial in treating pregnant addicts (214–218), like methadone, buprenorphine can cross the placenta (219), has abuse liability (220–222), and at high doses it can have variable effects on neurogenesis (223, 224). Prenatal exposure to large dosages of buprenorphine can reduce myelin basic protein levels and the number of myelinated axons (213) and can trigger depressive-like behavior (225) in rats. The aberrant myelin patterns seen with high concentrations of buprenorphine are attributed to its actions as a partial KOR antagonist, although possible agonist actions at ORL1 receptors, which have an emerging role in CNS plasticity (226), cannot be overlooked.

Methadone is also beneficial as an addiction therapy during pregnancy. Unlike buprenorphine, methadone is a preferential MOR agonist with far fewer actions at KOR. Methadone crosses the placenta (227) and some methadone is present in milk during lactation (228). Recent evidence from the investigators that studied the effects of buprenorphine on white matter development described above, indicates that methadone also affects oligodendrocyte maturation and myelination. Gestational day 7 pregnant rats were continuously administered subcutaneous methadone (9 mg/kg/day) *via* osmotic minipumps throughout the pregnancy and during lactation until the day of sacrifice on postnatal days 11 or 19. This early methadone exposure significantly increased oligodendrocyte proliferation and differentiation, including the production of myelin basic protein, myelin proteolipid protein, and myelin-oligodendrocyte glycoprotein (229). Myelin ultrastructure was markedly affected. Methadone exposure (24 h) caused concentration-dependent increases in DNA synthesis in oligodendrocyte progenitors in cell culture (229), similar to what we had previously observed with the selective MOR agonist PL017 in immature oligodendrocytes *in vitro* (164, 204). Finally, these results suggest that increases in [^{3}H]thymidine labeling in the SVZ 1 h after morphine injection (10 mg/kg, s.c.) in adult rats mentioned earlier (163) may be due to a mitogenic effect of morphine on adult oligodendrocyte precursors, since immature astroglia show reduced proliferation following acute morphine exposure (39). The collective findings indicate that, unlike the response of neuroblasts and immature astroglia in which acute opiate exposure inhibits proliferation, MOR receptor activation can be mitogenic to young oligodendrocytes and their progenitors.

INHIBITION OF ADULT NEUROGENESIS AND CNS PLASTICITY

Although the intention of this review is to emphasize the role of opiates during development, opiate-dependent reductions in synaptodendritic complexity are not restricted to the pre- and post-natal brain maturation periods. The adult CNS remains highly plastic and modifiable with sustained opiate exposure; however, unlike the situation during development, the effects of opiates on dendritic culling in the mature brain tend to be less severe. Since several authoritative reviews on the effects of opioids (230–234), as well as other substances with abuse liability (235), on adult neurogenesis have been published, we will only minimally address the topic here.

Adult neurogenesis was first described in rodents (236, 237) and later described in nonhuman primates (238) and humans (239). In mammals, adult neurogenesis is thought to be restricted to two specialized "neurogenic" regions within the SGZ of the hippocampal dentate gyrus and within the SVZ of the lateral ventricle. Adult neurogenesis in both regions (the SGZ and the SVZ) declines with aging (240). Newly generated neurons within the SGZ become granule neurons within the dentate gyrus, while neurons produced within the SVZ of the lateral ventricle migrate through the rostral migratory stream to become interneurons in the olfactory bulb in rodents and nonhuman primates (241–243). In humans, new SVZ neurons do not appear to migrate into the

olfactory bulb, but instead remain as interneurons within the striatum (244). Adult hippocampal progenitors derived from the rat SGZ express the endogenous MOR peptide β-endorphin—a post-translational product of the *POMC* gene (245). In fact, POMC expression has been used as a marker for adult neural progenitors in the hippocampus (246). Adult hippocampal neural progenitors can be visualized in mice in which an enhanced-green fluorescent protein reporter is driven by *POMC* expression (246).

To what extent do opiate drugs directly affect the maturation and fate of adult neural progenitors? In the dentate gyrus, adult neurogenesis is sustained by a complex relationship between environmental factors imparted by adjacent neural cell types within the unique neurogenic niche of the SGZ (247–250). Although it may not be possible to preserve the complex spatial relationships [especially between the vasculature and the neurogenic niche (251, 252)] necessary to maintain the unique milieu of the neurogenic niche in cell culture (243, 251–253), acutely dissociated (in which physical cell-to-cell interrelationships are lost), reaggregate neurospheres (254), or organotypic (in which the vascular niche is no longer functional) cultures of adult SGZ neural progenitors continue to divide and retain key phenotypic characteristics permitting some experimentation *in vitro*.

The effects of morphine on adult hippocampal neurogenesis were first described by Eisch et al. (255). In these studies, adult rats received chronic morphine either subcutaneously (75 mg morphine time-release pelleted implant) for 5 days or *via* self-administration for 26 days. Importantly, morphine received *via* either delivery route had sustained reductions in BrdU-labeled neural progenitors in the dentate gyrus, granule cell layer, and/or hilus of the hippocampus, thus indicating reductions in adult neurogenesis (255). Among other outcomes, this effect may have consequences for drug-seeking behavior, as illustrated by the finding that radiation-induced destruction of adult neural progenitors increases morphine, but not sucrose, self-administration (256). This interesting result suggests that chronic opiate abuse, by limiting the pool of adult neural progenitors, may restrict the formation of new neural circuitry that is beneficial in limiting drug-seeking behavior (256).

Morphine-dependent decreases in neurogenesis have been more specifically attributed to increased numbers of neuroblasts being retained in the S phase of the cell cycle, as well as fewer young postmitotic neurons exiting the cell cycle (257). It was also suggested, based on cleaved caspase-3 immunoreactivity, that increased numbers of proliferating neuroblasts were dying (257); however, immunocytochemical evidence of caspase-3 cleavage is not definitive evidence of impending cell death since caspase-3 can be partially activated to intermediate levels during cellular remodeling without triggering downstream effectors, such as caspase activated DNase, necessary for cell death (258).

β-endorphin influences the growth of SGZ progenitors through autocrine and/or paracrine feedback (75, 245). The proliferative effects of β-endorphin in adult hippocampal neuronal progenitors are mediated through a signaling pathway involving phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3-kinase), $[Ca^{2+}]_i$, and MAPK activation (245). Importantly, in addition to neurons, the adult hippocampus contains multipotent progenitors that can also give rise to astrocytes and oligodendrocytes (259), so in addition to being mitogenic, β-endorphin can influence the balance of neuronal and glial types in the hippocampus. Adult neural progenitors in the hippocampus can express MOR and DOR, but not KOR (75). Broad-acting opioid receptor antagonists such as naloxone increase the proliferation of neuroblasts, but cause corresponding decreases in the production of astroglia and oligodendroglia *in vitro* (75, 245). Incubating adult rat hippocampal progenitors with the more selective MOR or DOR antagonists, β-funaltrexamine (10 μM) or naltrindole (1 or 10 μM), respectively, for 48 h increases the number of newly generated neurons, while reducing the number of astrocytes and oligodendrocytes in culture (75). This suggests the effects are selectively meditated by MOR or DOR or potentially MOR–DOR heteromeric complexes (260). Additional evidence that MOR activation inhibits adult neurogenesis is provided by findings that deleting MOR increases the generation of new neurons (261). The results intimate that in addition to modulating the proliferation and death of neuronal and glial precursors, opioids may additionally influence the cell fate decisions of bi- or multi-potential neural cell precursors in the SGZ.

OPIOIDS AND ADULT NEUROGENESIS OUTSIDE THE HIPPOCAMPAL SGZ

Recently, β-endorphin-expressing neurons in the hypothalamus were discovered to innervate and regulate the division of adult neural progenitors in the anterior ventral VZ–SVZ that will become deep granule interneurons within the olfactory bulb (262). In these studies, the selective KOR agonist ICI 204448 (263) was demonstrated to increase the proliferation of VZ–SVZ progenitors and this was prevented by the selective irreversible KOR antagonist 2-(3,4-dichlorophenyl)-*N*-methyl-*N*-[(1*S*)-1-(3-isothiocyanatophenyl)-2-(1-pyrrolidinyl)ethyl]acetamide hydrochloride (DIPPA) (264). While β-endorphin typically acts as a preferential agonist at MOR and to a lesser extent DOR, it is nevertheless modestly potent at KOR (265). It is interesting that the same *POMC* gene-derived neuropeptide, β-endorphin, affects adult neurogenesis in the hippocampal SGZ through actions at MOR and DOR (75, 245), and in the SVZ associated with the lateral ventricles *via* actions at KOR (262).

Opioids and Adult NPC Fate Decisions

If the selective MOR antagonist, β-funaltrexamine (10 μM) increases the number of newly generated neurons, while reducing the number of astrocytes (75) (as noted above), does exposure to opiate drugs, most of which are preferential MOR agonists, direct the fate of NPCs toward an astroglial lineage? Xu and coworkers have addressed this question and found that opiates can promote the differentiation of hippocampal NPCs toward an astroglial fate (266). Moreover, these investigators also demonstrate that although both morphine and fentanyl can promote NPCs toward an astroglial fate by activating MORs, they do so by activating different downstream signaling pathways (discussed below) with different consequences (266). By contrast, an escalating dose of methadone, also a preferential MOR agonist and frequently used in maintenance therapy for opiate addiction, failed to alter

adult hippocampal neurogenesis (267). The implications of these findings are important and indicate that functional selectivity or biased agonism [reviewed in Ref. (126, 268, 269)] is operative. As noted earlier, this implies that different opioid agonist ligands, acting through MORs, can differentially affect development by uniquely coupling to one or more signaling pathways downstream of MOR. For example, upon stimulation, GPCRs, such as MOR, can subsequently activate one or more downstream effectors including Gα and Gβγ (G-protein subunits), and β-arrestin—each of which can uniquely affect cell function. Therefore, each of the frequently abused opiate drugs (e.g., heroin, fentanyl, and oxycodone), newly synthesized designer drugs, and opiate addiction therapies (e.g., methadone and buprenorphine) may differentially effect the maturation and fate of adult hippocampal neural progenitors within the SGZ. Because the molecular mechanisms underlying how a particular agonist ligand selectively activates one or more signaling pathways downstream of MOR are not well understood, it is challenging to predict how a particular drug will affect development without empirical testing. Finally, because there is evidence that biased agonism similarly dictates the actions of opiates throughout maturation, then key aspects of our understanding of the effects of opiates on neuronal and glial development may need revisiting since a majority of studies to date have used morphine to study maturation.

Although the mechanisms by which opiates direct the maturation and fate of adult NPCs in the SGZ are not fully understood, morphine exposure directs NPCs toward an astroglial fate by increasing microRNA-181a (miR-181a) in NPCs *via* a notch1-dependent pathway (270). MicroRNAs such as miR-181a are short non-coding RNA sequences that bind specific classes of target mRNAs thereby interfering with translation. Since a single microRNA can coordinate the interference of a number of related mRNAs, microRNAs can act as master regulators of translation. Notch1 activation triggers a large number of specific transcriptional processes in a cell-specific manner (271), and in the case of adult NPCs, is necessary for morphine to direct NPCs toward an astroglial fate (266). The selective MOR agonist fentanyl (unlike morphine) acts *via* a pathway involving β-arrestin-dependent extracellular signal-regulated kinase (ERK) activation and inhibition of miR-190 production (266, 272), while morphine triggers protein kinase Cε (PKCε)-dependent activation of both ERK and trans-activation response element RNA-binding protein and increased levels of miR-181 (266); both morphine and fentanyl inhibit calcium/calmodulin-dependent protein kinase type IIα (CamKIIα) (266). Importantly, the actions of both morphine and fentanyl were prevented by the selective MOR antagonist Cys^2-Tyr^3-Orn^5-Pen^7-amide (CTOP) suggesting morphine's effects were mediated by specific MORs and that biased agonism is responsible for any differences between morphine and fentanyl (270).

CONCURRENT EFFECTS OF HIV

We have been particularly interested in the concurrent effects of HIV and opiates on CNS development, as HIV and opiate abuse are linked epidemics, highlighted by outbreaks of HIV in communities suffering from the recent surge in opiate addiction (4). In humans, the period of CNS development extends through adolescence, with full myelination in prefrontal cortex and attainment of executive function not achieved until early in the third decade of life (273–275). Individuals who are vertically infected at birth, or who become HIV+ during the period of CNS development, have a high degree of neuropathology and behavioral deficits (276, 277). We have hypothesized that this is due to specific effects of HIV, HIV proteins, and/or inflammation on developing NPCs, and that concurrent exposure to opiates worsens these outcomes. Our *in vitro* work has shown that the HIV-1 protein Tat, as well as supernatant from HIV-infected cells that contains virions, viral proteins, and inflammatory mediators, can reduce the proliferation of both murine and human neural progenitors, and that concurrent exposure to morphine exacerbates these anti-proliferative effects (278, 279). A similar outcome reported by others showed that Tat-morphine exposure prolonged the Gap 1 (G_1) phase of the cell cycle, and was dependent on the cyclin-dependent kinase inhibitor p21 (280). In neonatal mice exposed to HIV-1 Tat from embryonic day 17 to postnatal day 7, progenitor proliferation was also reduced, and 4 days of morphine co-exposure further reduced proliferation in SRY-Box 2-immunoreactive (SOX2+) cells (278). Although effects of morphine and Tat were not observed on overall cell populations in the striatum, this may require a longer period of morphine exposure.

Since HIV is a human-specific disease, we re-examined the findings in other models using a human primary neural progenitor system exposed to HIV to more closely mimic aspects of the disease. Human progenitors derived from 8 to 10 week *in utero* tissue samples were exposed to various HIV ± morphine treatment regimens. While morphine treatment over 48 h did not by itself affect human NPC proliferation, morphine did significantly enhance the anti-proliferative effect of HIV exposure acutely, and resulted in an altered doubling time (279). In the 2-week period after the removal of growth factors that sustain the progenitor phenotype, HIV alone accelerated the appearance of both astroglial and neuronal markers in human NPCs, and morphine significantly accelerated this premature differentiation process (**Figure 6**). As these results were obtained in 8–10-week human gestational tissue, they are especially relevant to developing systems exposed to opiates ± HIV. They mirror the findings discussed earlier in mice exposed perinatally and in murine cultures, which also showed profound, interactive reductions in progenitor proliferation due to morphine-HIV-1 Tat exposure. Overall, these outcomes suggest that chronic exposure of the developing CNS to morphine or HIV, but especially to the combination, is likely to alter the production of mature cells, as well as the differentiation choices of progenitors, biasing the balance of neuronal and glial populations produced as brain regions mature. Although the consequences of these effects have not yet been defined, they are potentially quite damaging to brain function. This study also confirmed that, at least under these conditions, NPCs could be infected and produce new virions. Of great importance, morphine enhanced the production of new HIV virions by naïve human progenitors in a serial dilution and passaging assay. Opiates may thus have

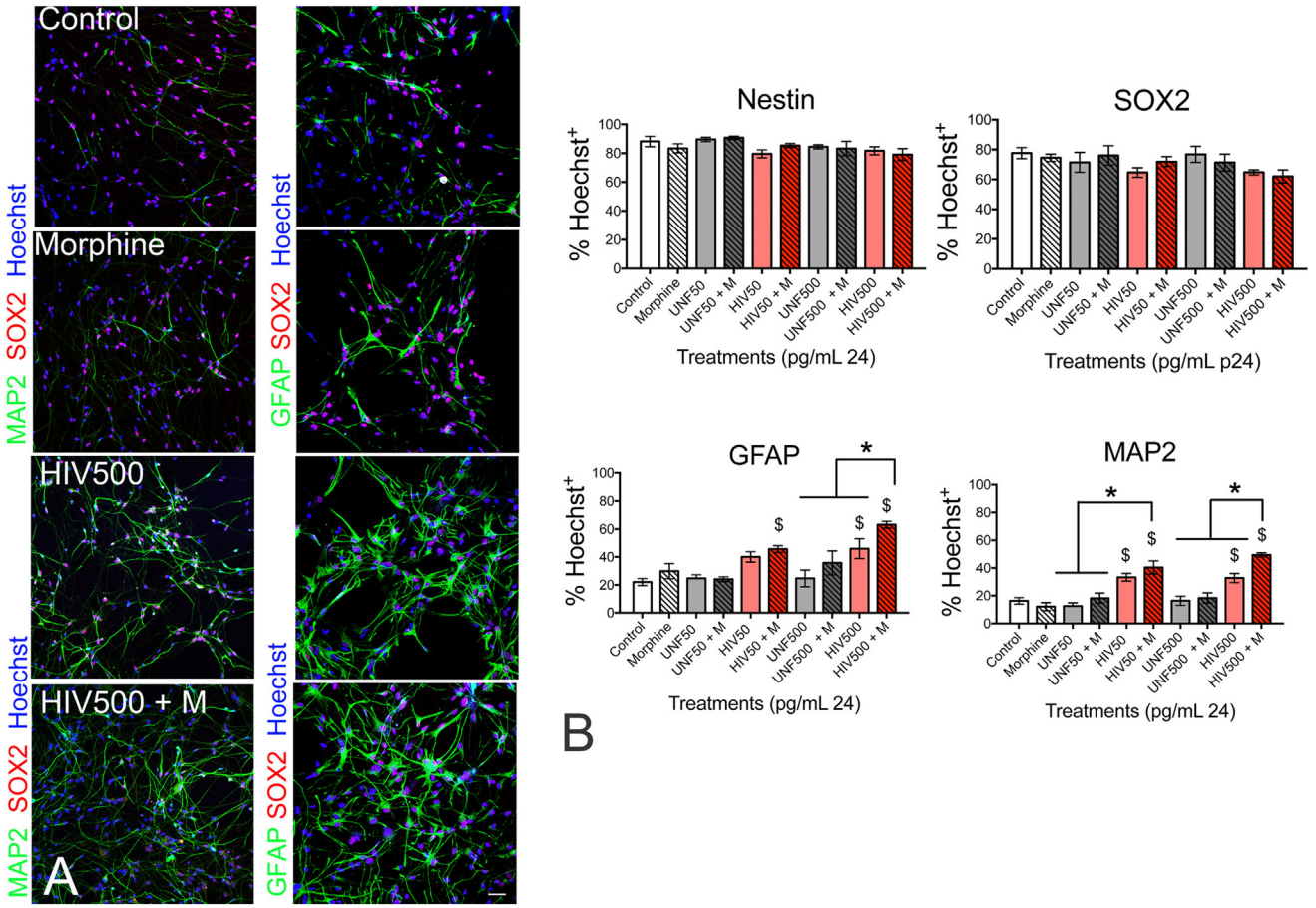

FIGURE 6 | HIV-1 ± morphine co-exposure promotes immature neural progenitor cells (NPCs) to become neurons and astrocytes. **(A)** Representative immunostaining of SRY-Box 2 (SOX2—a marker of undifferentiated NPCs; *red fluorescence*), microtubule-associated protein 2 (MAP2—a neuronal marker; *green fluorescence—left-hand panel*), and glial fibrillary acidic protein (GFAP—an astroglial marker; *green fluorescence—right-hand panel*) in human NPCs treated with morphine (500 nM), spent medium (supernatant) from HIV-1-infected (HIV_{sup}) peripheral blood mononuclear cells (PBMCs) containing 500 pg/ml of the HIV-1 p24 capsid protein alone (HIV 500) or with morphine (HIV 500 + M) for 12 days in cell culture. **(B)** Analysis of human NPCs continuously treated with morphine, HIV_{sup} at 50 pg/ml p24 (HIV 50), 500 pg/ml p24 (HIV 500) alone or with morphine (M), and equal dilutions of supernatant from uninfected PBMCs (UNF50 or UNF500) alone or with morphine (M). The percentage of NPCs expressing nestin (transiently expressed by immature neural cells), SOX2, MAP2, and GFAP from each treatment group was calculated from >200 cells per study. Error bars show mean ± SEM from $n = 5$ separate studies each using NPCs derived from independent tissue samples. $^{\$}p < 0.05$ versus control; $^{*}p < 0.05$. Reprinted from Ref. (279); Copyright (2017), with permission from Wolters Kluwer Health, Inc.

the effect of raising viral titers and worsening neurodegenerative outcomes in the developing brain.

OUTSTANDING QUESTIONS/ UNDERSTUDIED AREAS

Although the main purpose of this review has been to survey prior studies exploring the maturational effects of opiates on neurons and glia, it seems appropriate to briefly suggest new directions for future research in this area.

To What Extent Do Developing Cells Adapt to Chronic Opiate Exposure?

This review has emphasized the decline in cellular growth observed with relatively acute, constant, opiate exposure. However, outside of controlled, clinical settings opiate exposure is rarely acute, and blood levels of opiates often fluctuate widely within the same individual. Tolerance and dependence can be observed at the cellular level as evidenced by dichotomous regulation of adenylate cyclase responsiveness in NG108-15 neuroblastoma × glioma cells following 1–4 days exposure to morphine (10 μM) and after morphine withdrawal (281). However, limited studies have examined the cellular consequences of sustained opioid exposure in developing neurons or glia. Even fewer studies have examined the consequences of opioid tolerance or withdrawal on cellular maturation. Although the well-documented decline in growth after relatively short opiate exposure times has been emphasized in this review, a return to near normal numbers of immature astrocytes despite sustained exposure to the preferential DOR agonists Met-enkephalin (171) or DPDPE (169) has been observed and suggests that compensatory factors

can become operative. There is a paucity of literature examining the neurodevelopmental consequences of opiate tolerance or withdrawal on neuronal and glial maturation in the developing brain *in vivo*. An understanding of the cellular mechanisms underlying adaptive responses to chronic opiate exposure is crucial toward understanding the consequences of perinatal opiate exposure.

Modeling the Pharmacology of Self-Administration during Maturation

An even greater challenge is to better model the pharmacology of opiate drug exposure as seen with opiate self-administration in addicts during perinatal/postnatal development. The pharmacokinetics of "on-off" (intermittent, including contingent and non-contingent patterns of administration) versus "steady state" (continuous, e.g., as seen with an osmotic minipump or time-release drug implant) drug administration (282) and resultant pharmacodynamic differences are likely to have a significant impact on neuronal and glial maturation. Addicts inject opiates repeatedly, 3–4 times per day, to maximize the rewarding effects of the drug. Fluctuating levels of opiates are responsible for a relative "high" or "rush" and 3–4 periods of relative/mild withdrawal per day disrupt a wide variety of physiological systems and are through to be inherently more destabilizing than exposure to constant drug levels (282, 283). We speculate that fluctuating levels of opiates are more likely to have deleterious effects on CNS maturation than exposure to constant drug levels, which infers that the amount, frequency, and duration of drug exposure are important determinants of developmental outcome. Intermittent and fluctuating levels of opiates would be encountered during *in utero* exposure of offspring of opiate addicts; more constant levels of opiate exposure would be encountered when opiates were provided to neonates and newborns for pain management (284). Self-administration studies are particularly challenging to perform in pregnant or lactating dams, and may not be possible in young animals. Although the neuropharmacological and neurobehavioral consequences of intermittent versus continuous drug exposure, as well as contingent versus non-contingent opiate administration, have been examined in detail in adult animal models (285), the consequences of differing patterns of opiate administration on CNS maturation in children are unclear.

What Are the Long-Term Consequences of Opioids on Development?

A vast majority of studies in animals involve relatively acute exposure to opioids with durations lasting 1 week or less; with notable exceptions (255), studies that extend opiate exposure durations beyond 2 weeks are rare. In this regard, the clinical literature may provide more insight than studies examining acute bouts of opiate exposure in animals—especially in rodents in which development is relatively rapid. Moreover, a greater understanding of the pharmacokinetic consequences of opiate exposure (as noted in the preceding paragraph), as well as potential differences among opiate treatment options such as methadone versus buprenorphine (286, 287), are critical for designing strategies for treating mothers or offspring with opiates when medically necessary. Drug exposure causes long-term changes in neuroplasticity during the switch from casual drug use to an addicted state (17, 136, 288). Opiate-dependent alterations in neural circuitry may be greater during maturation when increased levels of MOR expression are evident in rodents (discussed earlier) and humans (289), and large-scale changes in CNS organization and synaptogenesis are occurring throughout the brain. For example, infant rats made briefly tolerant and dependent to fentanyl from P14 to P17, but not exposed thereafter, show lasting tolerance to morphine as juveniles and young adults 1 year later (290). By contrast, it cannot be assumed *a priori* that opiate exposure during development will only effect neural circuitry involved in addiction. In fact, this is unlikely the case since in many instances the cellular targets, and regional patterns of opioid peptide and receptor expression, during maturation are transient (and not present in adults) or thought to be unrelated to addiction (34, 50, 153, 291–293). It seems plausible that exposure to opiate drugs at critical periods during development results in lasting or permanent alterations in the structure and function of neural circuits directly related, as well as unrelated, to addiction.

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KH and PK both wrote the article and edited the submitted version.

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The Effects of Perinatal Oxycodone Exposure on Behavioral Outcome in a Rodent Model

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Opiate addiction is now a major public health problem. Perinatal insults and exposure to opiates such as morphine *in utero* are well known to affect development of the hypothalamic–pituitary–adrenal axis of the offspring adversely and are associated with a higher risk of developing neurobehavioral problems. Oxycodone is now one of the most frequently abused pain killers during pregnancy; however, limited data are available regarding whether and how perinatal oxycodone exposure (POE) alters neurobehavioral outcomes of the offspring. We demonstrated that exposure to 0.5 mg/kg/day oxycodone *in utero* was associated with hyperactivity in adult rats in an open field. No significant effects of POE were detected on isolation-induced ultrasonic vocalizations in the early postnatal period or on learning and memory in the water maze in adult offspring. Our findings are consistent with hyperactivity problems identified in children exposed to opiates *in utero*.

Keywords: prenatal opiate exposure, prenatal oxycodone exposure, behavior, hyperactivity, ultrasonic vocalization, learning and memory

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INTRODUCTION

The prevalence of opiates and prescription opioid abuse among pregnant women is a major public health concern. Non-prescription opioids are the second most abused illicit substance and one of the most commonly abused opiate pain relievers is oxycodone (1). In 2015, an estimated 10.4% of the population over 12 years of age used oxycodone products. Importantly, substance use in pregnant women and subsequent fetal exposure to drugs have been linked to adverse health effects for the maternal–fetal dyad. Opiates can affect the developing fetus directly or indirectly through various mechanisms. Opiates can cross the placenta (2–4) and act directly on fetal opioid receptors. Opiates can also enhance maternal secretion of cortisol in the mother or stimulate the secretion of stress hormones in the fetus (5), which can pose long-term effects to the developing fetus (6) and the hypothalamic–pituitary–adrenal (HPA) axis of the offspring (7, 8). In addition, because the HPA axis has important roles in programming neurobehavioral development (9) and dysregulation of the HPA axis has been linked to several neuropsychiatric disorders, such as anxiety (10), depression (11, 12), ADHD (13), and learning/memory deficits (14, 15, 16), any opioid-induced changes in the HPA-axis may also be associated with short- and long-term behavioral problems.

Both human and animal studies have shown that exposure to opiates has deleterious effects on neurodevelopmental outcomes. However, most of them used other opiates, including morphine

and heroin, which are mu-opioid receptor agonists, rather than oxycodone, a semisynthetic putative kappa opioid receptor (KOR) and partial mu opioid receptor agonist. Because illegal oxycodone use during pregnancy continue to rise, and the effects of opiates on the HPA axis differ depending on the type of opiate (17, 18, 19), it is important to determine the effects of perinatal oxycodone exposure (POE) on the neurobehavioral outcome of the offspring.

The current study was designed to examine the potential effects of POE on three diverse behaviors; isolation induced ultrasonic vocalizations (USVs) in neonatal pups followed by activity levels in adolescent rats and spatial learning in young adult rats. Isolation-induced USVs are the responses of young rat pups when separated from their mothers (20, 21). USVs are considered an adaptive response of the pup and these USVs correlate directly with distress and/or anxiety in the rat pup (22). USVs elicit maternal behavior and play an important role in the interaction between the pup and the dam (21). USV cues may be comparable to the crying sounds of human infants (23), which have been used to identify infants at risk for poor neurobehavioral outcomes (23, 24). Although existing data on the effects of prenatal opiate exposure on isolation-induced USVs remain limited, neonatal exposure to alcohol (25) or other illicit drugs, such as cocaine, can alter many USV characteristics (26). Therefore, we hypothesized that rat pups prenatally exposed to oxycodone would have an increased latency to the first USV and decreased USV numbers per minute.

The second testing paradigm used was an open field test, which has been widely used to study hyperactivity, anxiety, and stress in animals (27, 28, 29, 30). Although prenatal exposure to morphine was not associated with increased locomotor activity in the open field in rats (31, 32), human studies have shown that children exposed to opiates *in utero* manifest hyperactivity, impulsivity, and attention problems, whether the mothers were in opiate maintenance therapy or were polysubstance users (33, 34). Therefore, we hypothesized that POE would be associated with hyperactivity in the open field in rat offspring.

Learning and memory were assessed in a water maze. Children prenatally exposed to opiates have learning problems and lower scores on neurodevelopmental tests (35, 36, 37). In rats, prenatal exposure to morphine is associated with learning and memory deficits (38, 39). Thus, we hypothesized that POE would impair learning and memory of the offspring. To the best of our knowledge, these studies are the first to look at the effects of POE on these behavioral measures.

MATERIALS AND METHODS

Experimental Design: Animals and Prenatal Treatments

The study protocol was approved by the University of Kentucky Institutional Animal Care and Use Committee. Virgin female Sprague Dawley rats (Harlan, Indianapolis, IN, USA) weighing 194–223 g were individually housed at 22–25°C and maintained in a 14L:10D photoperiod (lights on at 0500 h) room with regulated 30–70% humidity. Rat chow and water were provided *ad libitum*.

Once released from quarantine, the females were fitted with a right atrial cannula connected to a subcutaneous (S.C.) access port implanted between the shoulder blades. The rats were allowed to recover for 1 week. To determine estrous cycles, vaginal lavages were obtained daily. Each female was group housed with a proven breeder male for breeding 1 week after cannulation. Gestational day (GD) 0 was designated as the day that sperms were detected in the vaginal smear, and the females were individually housed thereafter. Foster dams were bred at the same time and remained untreated throughout their gestation.

The cannulae were flushed daily *via* the S.C. port with sterile heparinized saline (0.4 cc, 100 IU/ml) until GD 8. From GD 8–21, the experimental dams were divided into three treatment groups that received either oxycodone (Mallinckrodt, St. Louis, MO, USA) at a low (OXY-L, 0.5 mg/kg/day, $n = 5$) or high dose (OXY-H, 2.0 mg/kg/day, $n = 12$) or an equivalent volume of vehicle [control (CON), normal saline solution (NSS, 1.0 ml/kg/day), $n = 12$] from GD 8–21. These solutions were slowly injected i.v. over 10 min *via* the S.C. access port manually.

On postnatal day (PD) 1, the pups were counted and weighed. Oxycodone or NSS was also administered on PD 1, 3, and 5 to the dams because brain development during early postnatal life overlaps the human "third trimester" brain growth spurt and to prevent maternal withdrawal symptoms that might affect maternal nursing behavior. On PD 2 all litters were adjusted to contain 10–11 pups with equal numbers of male and female pups when possible. At 1700 h on PD 5, all pups in each litter were transferred to an untreated foster dam to minimize exposure to altered maternal rearing behavior that has been described in rat dams after exposure to opiates (40). To preclude potential litter effects, only one male and one female from each litter were included in the behavioral studies (41). The pups were weighed daily and weaned at PD 25, when they were separated by sex.

Experimental Design: Behavioral Tests

Ultrasonic Vocalizations

Ultrasonic Vocalizations were determined according to published procedures (42). In brief, an ultrasonic bat detector (Ultra Sound Advice Model #S-25, UK—http://www.ultrasoundadvice.co.uk) set at 40 kHz with a condenser microphone (SM-1) set 21.5 cm above the test cage floor was used. The output was recorded on a SONY #WM-D8C Cassette Recorder using low noise cassette tapes. On PD 14, the pups in each litter were separated from the dams, remaining in their home cage and were brought to a neighboring test room one litter at a time. Their cages were placed halfway on a heating pad to provide warmth. During testing, the dam was placed in a new cage in the same test room. CON offspring ($n = 6$) and OXY-L ($n = 4$) and OXY-H ($n = 8$) rats underwent USV testing. The number of pups in the OXY-L group was smaller due to smaller numbers of dams in this group and limited numbers of pups available for all experiments that were performed. During USV testing, pups were isolated one at a time in a clean cage for 6 min during which USVs from the pup were recorded. A fan was used to provide white noise during testing. Upon completion of USV testing for the entire litter, the dam was returned to the home cage and the home cage was returned to their original animal room.

Assessment of USV: audio data for each 6-min test period were individually scored for latency to vocalization (seconds) and number of USVs per minute per test period. Scoring was performed independently by two experimenters who were blind to the treatment groups and used a stopwatch and clicker counter. Similarity of their scores was compared and indicated that the reliability between experimenters was greater than 90%.

Open Field Test

On PD 43, a group of offspring that had not undergone USV testing were transferred to another building, where they were housed for at least 7 days prior to subsequent behavioral testing. This was an independent group of subjects, i.e., not the same offspring that had previous USV testing. The open field test was preceded by 5 days of habituation to the new surroundings followed by 2 days of 3-min handling periods and weighing. CON offspring ($n = 30$, 19 males, 11 females) and OXY-L ($n = 8$, 4 males, 4 females) and OXY-H ($n = 32$, 17 males, 15 females) rats underwent open field tests on PD 50–51. The number of pups in the OXY-L group was smaller due to limited numbers available after other experiments (not described herein) were performed. The open field apparatus was a circular chamber 36 cm height and 58 cm diameter. This circular chamber was used to prevent thigmotaxis or excessive time in corners (43). In addition, the open field was divided into two zones, with the center zone representing 25% of the total area. All testing was conducted in a test room with white noise to reduce external distractions. On days 1 and 2 of testing, two rats were transferred in separate cages and brought into the test room for a 10 min habituation period. Each animal was then placed in a separate open field, and their activities were recorded with a Polytracker® (San Diego Instruments, San Diego, CA, USA) for 30 min. The animals were then returned to their home cages in the colony room upon completion of testing.

Activity was recorded in 5 min blocks across the 30 min test period for each day. Total distance traveled (cm), distance traveled in the center zone, and the ratio of distance traveled in the center zone to total distance traveled or to distance in the outer zone were determined.

Water Maze

On PD 55–56, two groups of offspring, CON ($n = 20$, 11 males, 9 females) and OXY-H ($n = 20$, 10 males, 10 females) were tested in the water maze. Rats from the OXY-L group were not tested due to the limited numbers of pups. This experiment was modified from a procedure previously described (43). The apparatus was a 130 cm × 90 cm × 40 cm black Plexiglas chamber, divided such that several divergent paths, each 18 cm wide, branched off from the central start area. The apparatus and methodology were modified from von Euler et al. (44). Water temperature was maintained at 76° ± 2°. In this test, rats must learn to swim and make three successive right/left choices to a platform that is submerged below the water level and invisible; the water was made black with the addition of non-toxic black tempera paint to obscure the submerged platform. A plastic sheet surrounded the maze, reducing extra-maze cues, including the experimenter. A major advantage of this maze is that control animals can learn the maze in a single day. Movement in the maze was recorded using a video tracking system (SMART program; Panlab, S.L.) (43).

On the first trial, the rat was placed in the maze and allowed to swim freely. If the animal did not reach the platform after 1 min, it was guided to the platform. After 5 s on the platform, the animal was transferred to a cage warmed by a heat lamp (25 W) for 30 s. Trials were repeated until the animal completed two consecutive trials without errors (wrong turns) or a total of three successful trials. The number of trials required to reach either criterion was recorded as the outcome measure. The next day the rats were tested identically for 24 h to determine retention.

Statistical Analyses

Statistical analyses were considered significant if $p < 0.05$. Multilevel linear regression models or linear mixed effects models were used to analyze USVs, open field and water maze data. Such models accounted for potential litter effects and correlation among outcomes from the same pup over time. The models were fitted using restricted maximum likelihood to test for the impact of treatment group, gender, time, and any potential interactions these variables have on the mean values for the data. Due to skewness and outliers of the data, a natural log transformation was applied when appropriate. The Kenward and Roger (45) approximation was used to estimate standard error and degrees of freedom. Variables (trial days, sex, and treatment groups) were treated as categorical and backward elimination at the 5% significance level was utilized. Kruskal–Wallis tests were applied to compare litter sizes and number of males per litter between treatment groups. A linear mixed-effects model was applied to compare differences in body weight between groups with treatment group, gender, PD, and the interaction of gender and PD included as predictors of weight. Tests were two-sided and were conducted in SAS version 9.4 (SAS Institute, Cary, NC, USA).

RESULTS

Litter Size and Body Weight

Perinatal oxycodone exposure did not affect litter size or number of males and females per litter (**Table 1**).

Although the average maternal weight gain during pregnancy was different between groups ($p = 0.005$), perinatal oxycodone treatment did not affect mean birth weight of male or female pups weighed within 24 h after birth (**Table 1**). Dams in the OXY-H group gained less weight than CON dams ($p = 0.01$) and OXY-L dams ($p = 0.023$) (**Table 1**). Oxycodone treatment did not affect weight gain of the pups; body weights up to PD 50 were not different between groups ($p = 0.61$). However, there was an interaction between gender and postnatal age ($p < 0.0001$); males and females had different growth trajectories over time, as expected (**Figure 1**).

Body weights of both male and females were similar across treatment groups in the rats tested in the open field and water maze ($p = 0.43$), the females weighing less than the males as expected ($p < 0.0001$) (**Table 1**). The body weight of the pups at the time of USV testing (PD14) (g ± SEM) were 30.9 ± 1.8 in male and 29.1 ± 1.6 in female CON group; 30.2 ± 1.9 in male and

TABLE 1 | Litter size, the number of male and female rat pups per litter, maternal weight gain during pregnancy and birth weight of the pups and weight of the young adult offspring at the neurobehavioral tests (g ± SEM).

Variable (number of litter)	CON (N = 11)	OXY-H (N = 13)	OXY-L (N = 5)	p-Value
Total number of pups per litter	13.3 (14) ± 2.9	12.3 (13) ± 4.4	14.4 (14) ± 1.5	n.s.
Number of females per litter	5.9 (6) ± 2.1	6.5 (7) ± 3.1	6.0 (6) ± 1.0	n.s.
Number of males per litter	7.4 (8) ± 3.0	5.8 (6) ± 2.5	8.4 (8) ± 0.5	n.s.
Maternal weight gain during pregnancy, mean ± SEM (g)	153 ± 7.3*	113.4 ± 10.0*#	156.1 ± 9.0#	*p = 0.01, CON vs OXY-H, diff 39.7 g #p = 0.023, OXY-H vs OXY-L, diff 42.8 g
Birth weight, mean ± SEM (g)				
Male (M)	M:6.4 ± 0.2	M: 6.2 ± 0.3	M: 6.1 ± 0.4	n.s.
Female (F)	F: 6.0 ± 0.2	F: 5.8 ± 0.3	F: 5.6 ± 0.5	n.s.
Body weight during neurobehavioral tests (adult)	M: 223 ± 6.7	M: 250 ± 1.5	M: 210 ± 8.0	n.s.
	F: 169 ± 5.4	F: 177 ± 3.5	F: 175 ± 4.9	

CON, control; OXY-H, oxycodone high dose; OXY-L, oxycodone low dose.

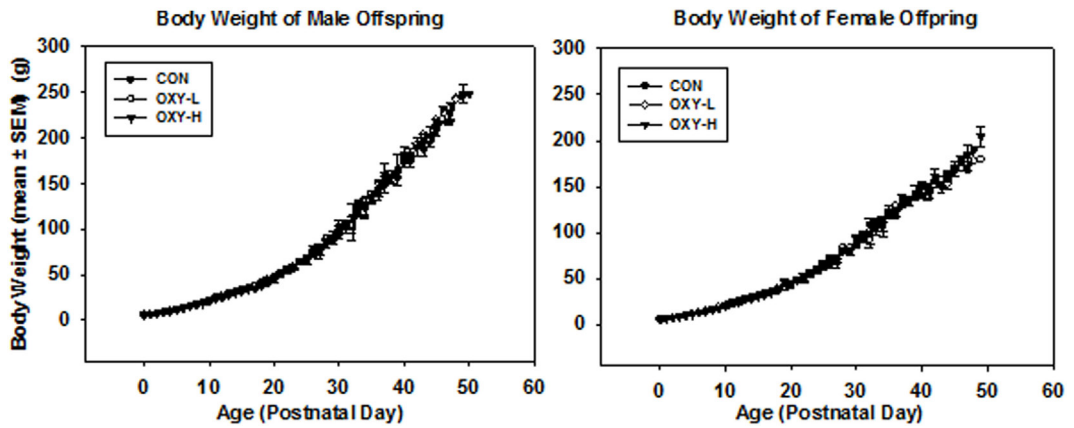

FIGURE 1 | Body weight of male and female offspring: left panel: body weight of male pups from birth through postnatal day (PD) 50 in control (CON, closed circles, n = 81), oxycodone low dose (OXY-L, open circles, n = 42) and oxycodone high dose (OXY-H, closed triangles, n = 76) groups. Right panel: body weight of female pups from birth through PD 50 in control (CON, closed circles, n = 65), OXY-L (open circles, n = 30) and OXY-H (closed triangles, n = 84) groups.

28.1 ± 1.9 in female OXY-L group; and 30.1 ± 1.4 in male and 28.7 ± 1.1 in female OXY-H group.

Ultrasonic Vocalizations

There were no significant sex differences so results were collapsed across sex for subsequent USV analyses. There was no significant difference in the latency to the first vocalization between treatment groups; however, there was a trend for longer latencies in OXY-exposed rats, **Figure 2A**. Latency to the first USV as plotted in Kaplan–Meier Plots is shown in **Figure 2B**, a point on the plot denotes the estimated probability of having a first USV after the given time point. Although there was a trend for prenatal oxycodone-exposed pups to display longer latencies to their first USV after isolation, there were no significant differences between treatment groups (p = 0.25), likely due to small sample sizes, **Figure 2B**.

Total USVs across time also did not differ among the three treatment groups (p = 0.85), **Figure 2C**. The number of USVs increased across time in all treatment groups (p = 0.0004); however, there was no significant effect of perinatal OXY exposure (p = 0.32), **Figure 2D**.

Open Field Test

Analysis of Total Distance Traveled in 30 Min

Analysis of the raw data indicated that there were no statistically significant differences in total distance traveled among rats in CON, OXY-L, or OXY-H groups (p = 0.26). There was also no effect of test day on the total distance traveled (p = 0.48). No interactions were significant. Female rats traveled a greater total distance compared to males [traveled 1,654 cm further than males, 95% CI: (539, 2,768), p = 0.004]. After a natural log transformation of the data, because of outliers and skewness, it was similarly determined that females traveled a greater total distance than males (p < 0.0001). In addition, this transformation of the data revealed that the OXY-L rats traveled farther than rats in both other treatment groups (vs OXY-H, p = 0.011; vs CON, p = 0.0004, **Figure 3**). This difference was likely largely driven by OXY-L-treated males on day 2.

There was no main effect of perinatal treatment or test day (p = 0.10 and p = 0.07, respectively) on the total distance per 5 min block. However, females travel 241 cm further than males in any given 5 min period (p = 0.003). The mean total per 5 min time block decreased over time (p < 0.0001), **Figure 3**.

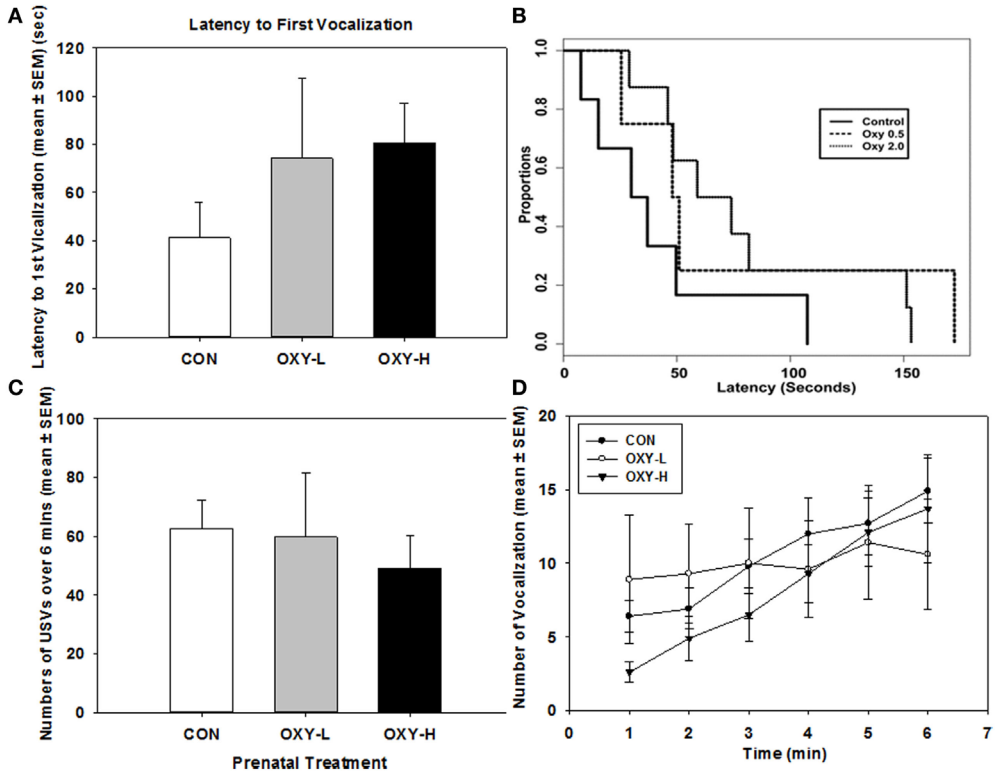

FIGURE 2 | **(A)** Latency to the first vocalization of rat pups; **(B)** Kaplan–Meier plot of latency to the first ultrasonic vocalizations (USVs) in rat pups in control (CON, solid line), oxycodone low dose (OXY-L, long dashed line), and oxycodone high dose (OXY-H, dotted line) groups; **(C)** total number of vocalizations of rat pups during USV test in CON (white bar; $n = 6$), OXY-L (gray bar; $n = 4$), and OXY-H (black bar; $n = 8$) groups; and **(D)** number of USVs per minute across the testing period in CON (closed circles), OXY-L (open circles), and OXY-H (closed triangles) groups.

Analysis of Total Distance Traveled in the Inner Zone, Outer Zone, and Mean Ratio of Distance Traveled in the Inner/Outer Zone

The Inner Zone

Rats exposed to OXY-L traveled more in the inner zone than rats in the other two treatment groups (OXY-L vs OXY-H, $p = 0.011$; vs CON, $p = 0.003$), likely due to the travel of the OXY-L-treated males on day 2, **Figure 4**. The distance traveled in the inner zone did not differ between days ($p = 0.61$), and no interactions were significant.

Oxycodone treatment did not affect the distance traveled in the inner zone per 5 min block, **Figure 4**. The large standard errors were likely due to the small sample size.

Outer Zone

There were no significant interactions. Neither oxycodone treatment nor testing day impacted the mean total outer zone distance ($p = 0.35$ and $p = 0.29$, respectively), **Figure 5**. After natural log transformation and removal of two outliers, there were main effects of gender ($p < 0.0001$) and treatment group ($p = 0.005$). Namely, males traveled to the outer zone less than females ($p = 0.003$) and OXY-L rats traveled more in the outer zone than the CON group ($p = 0.002$, **Figure 5**). There was no difference in outer zone distance traveled between either CON or OXY-L compared to OXY-H, **Figure 5**.

There were no interactions in outer zone distance per 5 min and no effect of oxycodone treatment ($p = 0.15$). Females travel 194 cm further than males in the outer zone in any given 5 min period ($p = 0.01$), **Figure 5**. In addition, rats tended to travel 113 cm further on the first test day in any given 5 min time period ($p = 0.032$), **Figure 5**. The mean total distance traveled in the outer zone per 5 min period decreased over time as expected ($p < 0.0001$), **Figure 5**.

The Ratio of the Distance Traveled in the Inner Zone to Outer Zone

The ratio of activity in the center zone vs outer zone of the field is a potential marker of motor impulsivity and/or anxiety (46). There were no differences in mean ratios across test days ($p = 0.18$) but there was a treatment group × sex interaction ($p = 0.003$; sex × treatment group interaction). Specifically, the estimated mean ratio of the distance traveled in the inner to outer zone of male OXY-L rats which was 0.245, was 0.095 larger than that of the CON, $p = 0.008$, and 0.112 larger than for OXY-H, $p = 0.002$ (**Table 2**). This pattern differed across sex; no differences in the inner:outer ratio were observed in the females (estimated mean ± SEM for days 1 and 2 in CON: 0.13 ± 0.01, OXY-L: 0.10 ± 0.05, and OXY-H: 0.17 ± 0.02) (**Table 2**). The differences in the males persisted when the ratios of distance traveled in the inner to outer zone for each 5 min time block were

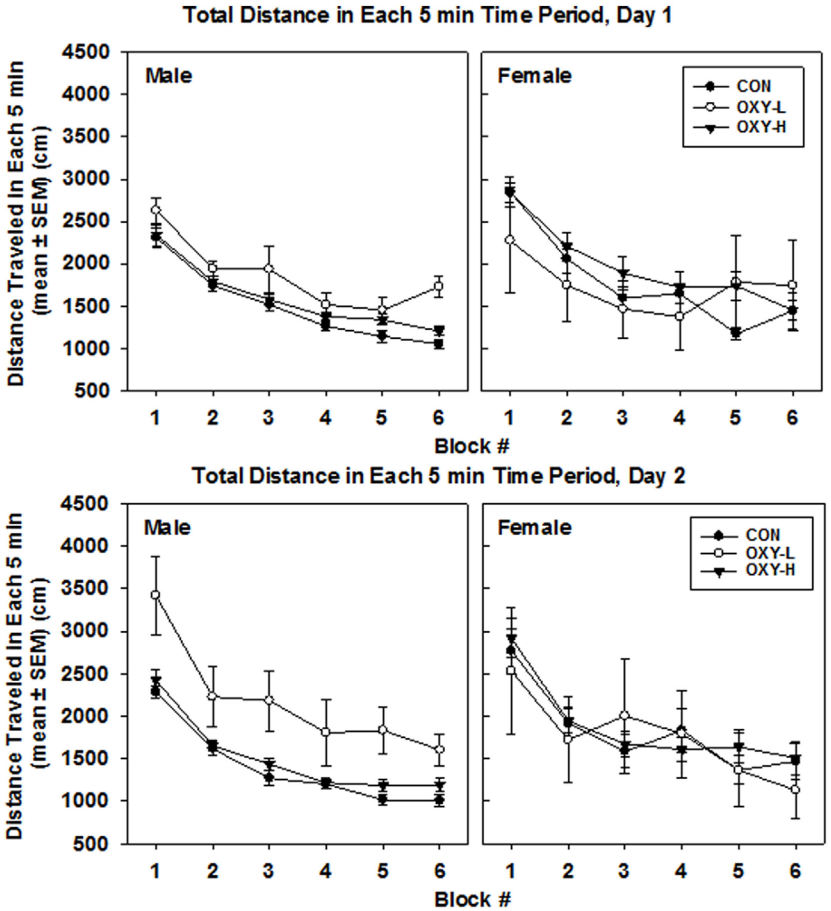

FIGURE 3 | Total distance traveled in each 5 min time block in the open field on test day 1 (upper panel) and test day 2 (lower panel), by males (left panel) and females (right panel) in control (CON, closed circles; male $n = 19$, female $n = 11$), oxycodone low dose (OXY-L, open circles; male $n = 4$, female $n = 4$), and oxycodone high dose (OXY-H, closed triangles; male $n = 17$, female $n = 15$) groups.

analyzed (OXY-L vs CON: $p = 0.04$; OXY-L vs OXY-H = 0.007), **Figure 6**.

Water Maze

Using a multivariate repeated measures Gaussian linear model to analyze the data with trial days, treatment groups and sex as predictor categories, there was no effect of oxycodone or sex on the mean values of the average number of trials until criterion performance was reached on both days ($p = 0.62$). The expected decrease in the mean values of the average number of trials on day 2 was observed in all groups ($p < 0.0001$) (**Figure 7**).

DISCUSSION

We explored the effects of POE on behavioral outcomes using three behavioral tests (USV, open field, and water maze) in juvenile, adolescent, and young adult rats. POE increased locomotion and preference for the center in the open field, consistent with hyperactivity. However, we did not find any effects of POE on USVs of rat pups when separated from their mothers or on learning and memory in the water maze.

Open Field Test

Oxycodone low dose rats were hyperactive and traveled more in the inner zone of the open field compared to CON and OXY-H rats, resulting in a higher mean ratio of inner:outer zone distance traveled by the OXY-L males. Thus, perinatal exposure to oxycodone was associated with hyperactivity behavior in adolescence. In addition, the increase in the ratio of inner zone:total distance traveled suggests that perinatal oxycodone reduced anxiety-like behavior and decreased normal species-typical thigmotaxic behavior in a new environment. Alternatively, these differences might reflect overall hyperactivity with a failure to inhibit entries to the center. It remains to be determined whether the increased travel in the center is due to deficits in hyperactivity, impulse control, or reduction in anxiety.

Our finding that perinatal exposure to oxycodone was associated with hyperactivity in the offspring is in agreement with human studies that identify hyperactivity, impulsivity, and attention problems in children exposed to opiates *in utero* (33, 34). In animal models, however, the results are more conflicting due to differences in studied drugs, which were mainly morphine, as well as in paradigms, ages, instruments, and end

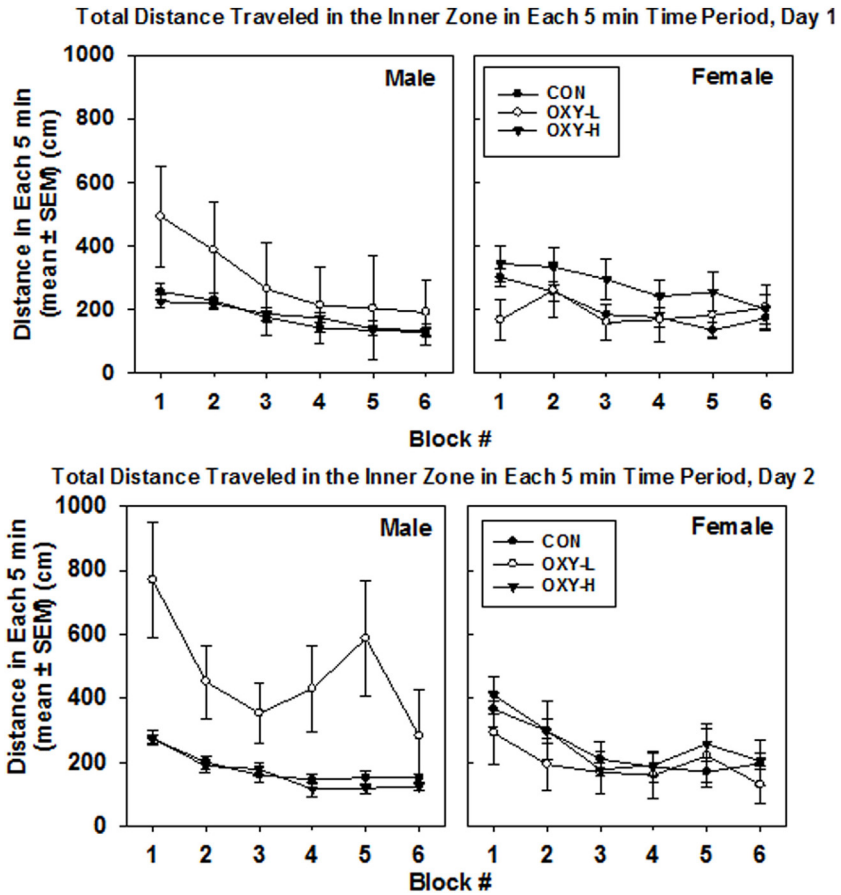

FIGURE 4 | Total distance traveled in inner zone in each 5 min time block in the open field on test day 1 (upper panel) and test day 2 (lower panel), in males (left panel) and females (right panel) in control (CON, closed circles), oxycodone low dose (OXY-L, open circles), and oxycodone high dose (OXY-H, closed triangles) groups.

points. For example, in one study, postnatal handling but not prenatal morphine exposure increased locomotor activities in the open field in adult male and female rats (31). In contrast, others reported that postnatal stress but not prenatal exposure to morphine increased locomotor activity in the open field (32). Thus, morphine yields different results than oxycodone in the open field. However, it should be noted that the effects of morphine are contradictory among studies and differ among tests. Thus, prenatal exposure to morphine was associated with increased anxiety-like behavior in an elevated plus maze (EPM) and reduction in time spent in the lit side of a light/dark box (L/D box) (47). Others reported that rats prenatally exposed to morphine exhibited decreased anxiety-like behavior in the EPM and L/D box with no differences in distance traveled over 30 min in the open field (48). Another study reported no significant anxiogenic effect of prenatal morphine exposure determined in the EPM (49). Finally, prenatal exposure to several opiates, such as methadone, buprenorphine, and most effectively morphine, increased anxiety-like behaviors in the light–dark transition test, with no effect on locomotor activity in an open field (50).

A possible mechanism by which prenatal exposure to opiates could result in hyperactivity may involve changes in multiple neurotransmitter signaling pathways such as dopaminergic pathways and the HPA system. It is well described that opioids have a significant role in controlling the release of dopamine and acetylcholine in the key reward regions of the brain including the ventral tegmental area (VTA) and the nucleus accumbens (51, 52, 53, 54). Long-term exposure to opiates leads to both structural and biochemical changes in the mesolimbic dopaminergic system; for example, a reduction in the cell size of dopaminergic neurons in the VTA (55), and increased levels of tyrosine hydroxylase, which is the rate-limiting enzyme in the synthesis of dopamine in the VTA (56). In addition, convincing evidence suggests that the impairment of dopamine-mediated development and the monitoring of motivated behavior and reward-related memory formation might be associated with ADHD symptoms (57, 58). Thus, taken together, it is possible that perinatal exposure to opiates may disrupt the normal development of dopaminergic reward-related circuits leading to hyperactive behavior. However, this speculation remains to be further elucidated.

Changes in the HPA system have also been linked to ADHD symptoms. In children, both reduced basal cortisol secretion and cortisol hyporeactivity have been associated with

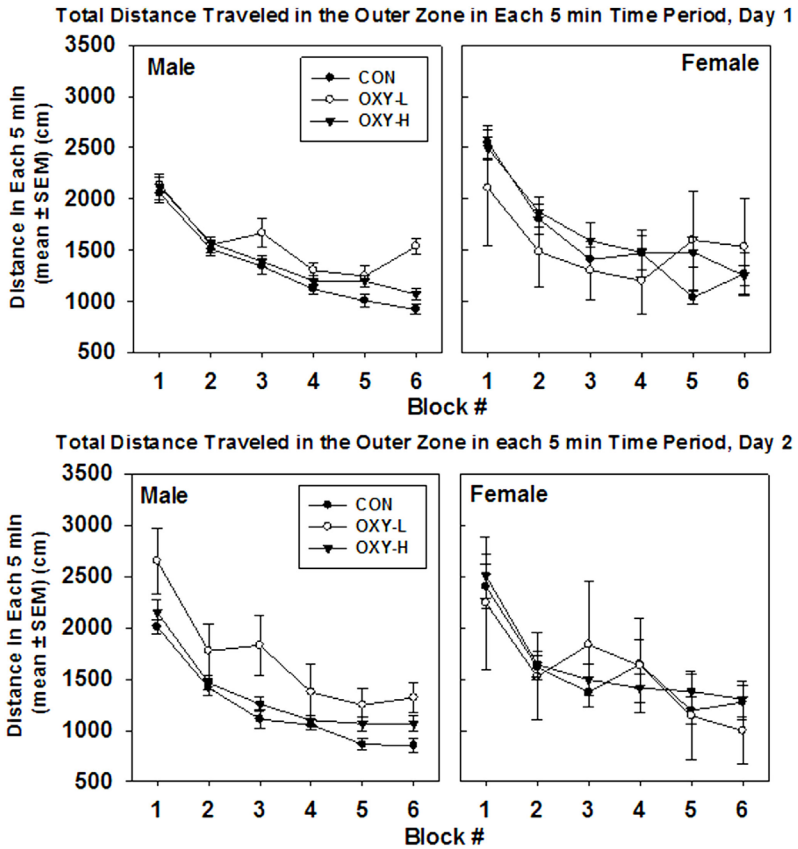

FIGURE 5 | Total distance traveled in outer zone in each 5 min time block in the open field on test day 1 (upper panel) and test day 2 (lower panel), by male (left panel) and female (right panel) rats in control (CON, closed circles), oxycodone low dose (OXY-L, open circles), and oxycodone high dose (OXY-H, closed triangles) groups.

TABLE 2 | The mean ratios of the distance traveled in the inner to outer zone in male and female rats in 30 min testing period.

Test day	CON (SEM)	OXY-L (SEM)	OXY-H (SEM)
Male			
1	0.13 (0.01)	0.19 (0.09)	0.13 (0.01)
2	0.15 (0.01)	0.27 (0.07)	0.13 (0.01)
Estimated mean day 1 and 2	0.14 (0.01)	0.24 (0.07)*	0.12 (0.01)
Female			
1	0.13 (0.01)	0.10 (0.04)	0.17 (0.03)
2	0.14 (0.02)	0.11 (0.04)	0.17 (0.02)
Estimated mean day 1 and 2	0.13 (0.01)	0.10 (0.05)	0.17 (0.02)

*$p < 0.05$ {the estimated mean ratio of distance traveled in the inner to outer zone of male OXY-L rats which was 0.245, was estimated to be 0.095 larger than that of the CON [95% CI: (0.027, 0.162), $p = 0.008$], and 0.112 larger than for OXY-H [95% CI: (0.042, 0.1830, $p = 0.002$)]}.

CON, control; OXY-H, oxycodone high dose; OXY-L, oxycodone low dose.

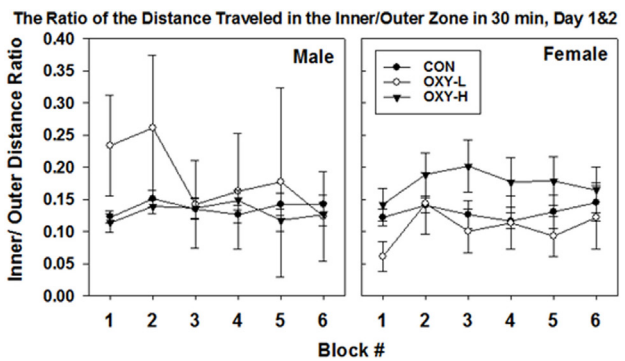

FIGURE 6 | The ratio of the distance traveled in the inner/outer zone in 30 min in the open field (mean ± SEM) by male (left panel) and female (right panel) rats in control (CON, closed circles), oxycodone low dose (OXY-L, open circles), and oxycodone high dose (OXY-H, closed triangles) groups. Male OXY-L rats had overall higher ratios of distance traveled in the inner to outer zone for each 5 min time block across time compared to other treatment groups (OXY-L vs CON: $p = 0.04$; OXY-L vs OXY-H = 0.007).

hyperactivity/impulsivity or a combined type ADHD (59). An abnormal diurnal rhythm and less effective negative feedback mechanisms after a dexamethasone suppression test were also identified more frequently in the children with ADHD that were severely hyperactive compared to those with milder symptoms (60). In agreement with many community-based studies, in which ADHD is more prevalent in males (61), in our study hyperactivity was more notable in the male OXY-L group. Interestingly, in adults with ADHD, although the basal salivary

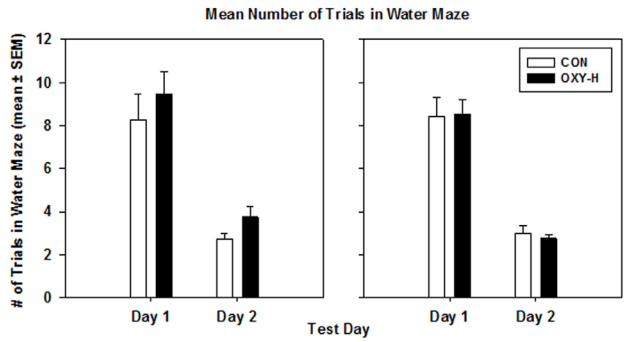

FIGURE 7 | The mean number of trials in the water maze in male (left panel) and female (right panel) rats in control (CON, white bars, $n = 20$, 11 males, 9 females) and oxycodone high dose (OXY-H, black bars, $n = 20$, 10 males, 10 females) group.

cortisol levels were not different, cortisol levels 20-min after a mental cognitive stress test were higher than those in healthy adult controls (62). These findings are similar to our observations that POE is associated with increased CRH-induced ACTH release (8) and with increased corticosterone reactivity to restraint stress in adult female rats (unpublished). However, we did not measure corticosterone, the major circulating glucocorticoid in rats, during the behavioral tests in the current studies. The discrepancy between hyperactivity in males and increased levels of corticosteroids in females could be due to the methods used to produce the stress in each study, relatively small sample sizes, and/or the gender of the participants. Referral biases and methodological difficulties were present in other human studies (63, 64). Thus, the relationship between POE, hyperactive behaviors, and abnormal HPA axis function that may interact with gender warrants further investigations, which should include the measurement of corticosterone during and after neurobehavioral testing. The abnormal HPA axis response to stress may be a promising candidate for use as a biomarker for early detection and treatment of ADHD in infants prenatally exposed to oxycodone/opiates.

Data from both animal and human studies suggest that early nutritional stress and malnourishment are associated with anxiety, high impulsivity, and attention problems (65). Although the weight gain of the OXY-H dams was significantly lower than that in the other groups, the weight gain of the OXY-L dams was actually comparable to that in the CON group. In addition, birth weights of the pups were comparable in all groups. Therefore, the hyperactivity in the open field of the OXY-L group could not solely be explained by poor maternal nutritional status.

Ultrasonic Vocalizations

Although there was a trend toward increased latency to first vocalization, oxycodone treatment had no effect on this end point or on the number of vocalizations per minute. The lack of differences in these USVs parameters may be due in part to the relatively low sample size in the OXY-L group. To our knowledge, there are no reports thus far on the effects of perinatal oxycodone on USVs In contrast to our results, however, perinatal cocaine treatment decreased the number of USVs on PD 1 and on PD 21, but this effect was not observed on PD 14 (66). Therefore, it is possible that the lack of an effect of oxycodone in our study occurred because testing was limited to PD 14. In fact, there are other types of USVs based on frequency and duration that are elicited as the rat becomes more mature (67). These include a 22 kHz USV emitted by juvenile and adult rats in response to predators and pain indicating a negative affective state (68, 69), and a 50 kHz USV emitted in adult rats in response to rewarding stimuli, expressing a positive affective state (70, 71). So it is also possible that POE may affect these other types of USVs that were not tested at an older age.

Water Maze

We did not find any effects of POE on spatial learning and/or memory in the water maze test as hypothesized; this may have resulted from not including rats perinatally exposed to a lower dose of oxycodone in this experiment due to the small sample size of this group. Previous studies using prenatal morphine exposure models report conflicting results; memory and learning in rodents are either impaired or enhanced. Namely, juvenile rats prenatally exposed to morphine had impaired spatial memory in the Morris water maze or Y-maze test (38, 72, 73), but aberrant memories such as morphine reward memory in the conditioned place preference or forced swim tests were enhanced (49, 74–76). In contrast to our study, Davis et al. (39) found that prenatal exposure to oxycodone impaired spatial learning and memory in a battery of spatial tasks; in the Morris water maze, rats prenatally exposed to OXY had increased latency and greater distance traveled to find the platform when the intertrial interval was long, not short. Rats prenatally exposed to oxycodone also had a decreased use of spatial strategies and more use of non-spatial strategies such as wall-hugging. In addition, the retention of learning memory in the T-maze, assessed 5 days after acquisition of the training, was impaired. This finding is actually consistent with our report that although POE male rats were able to discriminate between the stress and non-stress cues during a classical conditioning paradigm on PD 40, they had impaired discrimination ability when retested on PD 75 (77). Moreover, rats prenatally exposed to oxycodone have more reference memory errors in the radial arm maze (39). These differences in results could be due to many reasons. In their study, the dose and route of administration of OXY were different than those used in the current experiment with escalating doses of OXY (10 mg/kg/day up to 15 mg/kg/day) *via* gavage for 28 days prior to breeding. In addition, the pups were reared by their biological mothers in their study, while surrogate fostering was used in the current study. A number of previous studies have shown that opiate administration negatively alters maternal rearing behavior toward the pups including measures such as cleaning of the pups, delay of maternal behaviors, and maternal aversion to pup odor (78, 79, 80). Neonatal rearing condition and neonatal maternal interaction such as maternal separation had long-term effects on the stress response of the offspring including an increase in restraint stress-induced norepinephrine release in the PVN in adult rats (81) and changes in the HPA axis at multiple levels that could be linked

to epigenetic modification (82). Variations in maternal care also influence learning and behaviors of the offspring (83, 84). The adverse effects of neonatal maternal separation on the HPA axis were lessened by fostering the litters (85). Thus, fostering the pups when the dams were exposed to opiates could alter the HPA axis and possibly the neurobehavioral outcomes of the offspring. In addition, even though signs of withdrawal in the dams were monitored in the study by Davis et al., body weights of the OXY pups were approximately 10% lower than those of the controls, indicating possible neonatal opiate withdrawal and poorer nutritional status that can also affect long-term outcomes. In contrast, the body weights of the pups in our study were comparable in all groups.

In humans, prenatal exposure to opiates results in impairments in cognitive function and learning. Bunikowski et al. also reported that when evaluated at 1 year of age, children prenatally exposed to opiates had a mild psychomotor developmental impairment compared to the control group; these included impairments in "hearing and speech" and "intellectual performance" subscales (36). Guo et al. found that *in utero* opiate exposure was associated with impairments in the Auditory Rare Event Monitoring task and the Sternberg Memory task in children 7–12 years of age (35). More recently, Hunt et al. reported from their case–control study that infants prenatally exposed to opiates are more likely to experience neurodevelopmental impairments compared to healthy control infants, when assessed at 18 months and 3 years of age (37). The deleterious effects of prenatal opiate exposure on cognitive function persisted and did not decrease over time after controlling for permanent home placement and heroin use in the mother when children prenatally exposed to opiates were retested on the Wechsler Intelligence Scale for Children from 1 year old up to 8.5 years of age (86). These data suggest that prenatal exposure to opiates is associated with impaired cognitive functions. Therefore, although we did not detect any effects of POE on cognitive function, learning, and memory using a water maze as a paradigm, further investigation is warranted with different testing paradigms, testing at different ages, and with a lower dose of oxycodone.

Interestingly, we found that exposure to the lower dose of oxycodone of 0.5 mg/kg/day but not the higher dose of 2.0 mg/kg/day was associated with hyperactivity in the offspring. This difference could be due to the development of tolerance to the higher dose of oxycodone. Opiate tolerance is characterized to be pharmacodynamic, time and dose-dependent, and opioid receptor specific (87). Tolerance can develop after exposure to a KOR agonist (88) even for as short as 5 days (17). Whether or not tolerance to the stimulatory effects on the HPA axis by KOR agonists is dose-dependent has not been well studied, but tolerance to MOR agonists was dose-related (89). It is possible that in our study, rat dams that were exposed to the higher dose of oxycodone (2 mg/kg/day) may have developed opioid tolerance leading to a decreased fetal CORT exposure, thus there were fewer effects on the developing HPA axis and the neurodevelopmental outcomes of the offspring.

In summary, POE was associated with hyperactivity in young adult rats. In these studies, perinatal oxycodone treatment had no effect on the responses of pups isolated from their dams, or in learning and memory deficits, which could be due to the small sample sizes, testing paradigms, or the doses of oxycodone tested. These issues remain to be resolved.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the University of Kentucky Institutional Animal Care and Use Committee. The protocol was approved by the University of Kentucky Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

TS: designed and conducted the studies, analyzed the data, wrote the manuscript, PI of the funding. SL and MW: assisted in designing, conducting, and analyzing the data and edited the manuscript. PW: statistician, responsible for all data analysis. KW: conducted and analyzed the data. HB: assisted in designing and analyzing the data. SB: main assistant in designing, conducting, and analyzing the data and edited the manuscript.

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CASE REPORT
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Neonatal Abstinence Syndrome: Twins Case Series

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Background: Use or abuse of opioids and related drugs during pregnancy increases the risk of maternal and neonatal morbidities, including increased susceptibility to Neonatal Abstinence Syndrome (NAS). Severity of NAS is determined by both environmental and genetic factors, but the level of influence each one of them has in determining the severity of NAS is not fully understood. Since the incidence and severity of NAS vary a lot among susceptible infants, twin studies might give us valuable insights into understanding the relative roles of environmental and genetic factors at the onset of the disease and during its progression. Higher concordance of occurrence and severity of NAS in monozygotic twins compared to dizygotic twins would suggest a genetic role in the pathogenesis of NAS. However, comparable concordance suggests a non-genetic or an environmental basis. In this case series, we report neonatal outcomes including severity of NAS among monozygotic and dizygotic twins.

Methods: A retrospective chart review was performed for all newborn twins who were at risk of developing NAS, were born in our institution between January 2006 and December 2014, and had a gestational age of 30 weeks or greater.

Results: During the study period, we identified seven sets (total of 14 infants) of eligible twins, comprising six dizygotic and one monozygotic twins, from a total of 550 infants who were at risk of developing NAS. Among the seven sets of twins, two sets were concordant for severe NAS and required pharmacological management, three sets of twins were concordant in not having severe NAS and did not require pharmacological management, and the remaining two sets were discordant, where one of the twins required pharmacological treatment.

Conclusion: Five of the seven sets of twins in our study exhibited concordance and two sets showed discordance in withdrawal severity. Larger studies may help in understanding the roles of genetic and environmental factors in determining the severity of NAS.

Keywords: neonatal abstinence syndrome, intrauterine opioid exposure, severity of neonatal abstinence syndrome, dizygotic twins, monozygotic twins, newborn, genetic, determinants of neonatal abstinence syndrome

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INTRODUCTION

Maternal use, misuse, and abuse of prescription and nonprescription opioids and other illicit drugs, including heroin and cocaine, are increasing in the United States (1–3). With increasing drug use, risks of various maternal and neonatal morbidities such as preterm birth, low birth weight, possible congenital anomalies (4), neonatal abstinence syndrome (NAS), and adverse neurodevelopmental outcomes have increased (5, 6).

The incidence and severity of NAS have been variably reported to occur in 50–90% of infants following intrauterine exposure to drugs that are known to cause NAS (5, 6). Among the infants with severe NAS, there is a wide variation in the length of their pharmacological treatment, duration of hospital stay, need for intensive care, and other morbidities associated with NAS (5, 6), but what determines the variations in outcome is not completely known.

Twin studies are a powerful tool in understanding the roles of genetic and environmental factors in determining the incidence and severity of diseases. Incidence of twins is about one in eighty in spontaneous pregnancy, and incidence of NAS is estimated to be 3.4–5.6 per 1,000 births, resulting in about 21,000 infants being susceptible to NAS (2). Based on these data, we calculated that, annually, about 260 pairs of twins born in USA are susceptible to NAS. Twins can be dizygotic (non-identical) or monozygotic (identical). Dizygotic twins share about 50% of the genetic material while monozygotic twins share almost 100%. The composition of placenta and membrane—amnion and chorion—helps in determining whether twins are monozygotic or dizygotic. All dizygotic twins have a dichorionic-diamniotic membrane. Monozygotic twins can be dichorionic-diamniotic (about a third of monozygotic twins), monochorionic-diamniotic, or monochorionic-monoamniotic, depending on the time of splitting of the fertilized egg. If dichorionic-diamniotic twins are of the same gender, have the same blood group, and appear identical, they are usually designated as monozygotic twins in twin studies. Higher concordance of incidence of disease in monozygotic twins compared to dizygotic twins suggests a genetic basis of the disease (7).

We hypothesized that twin studies might give us valuable information in understanding the relative roles of environmental and genetic factors in the onset and progression of NAS where incidence and severity of disease vary widely among susceptible infants. If concordance of occurrence of NAS or severity of NAS is greater in monozygotic twins than in dizygotic twins, it would suggest a genetic basis of pathogenesis of NAS. However, lack of higher concordance among monozygotic twins compared to dizygotic twins would suggest non-genetic or environmental basis in pathogenesis of NAS. In our study, we have defined "infants at risk of NAS" as infants with intrauterine exposure to opioids and related substances. We have defined "severe NAS" as NAS requiring pharmacological management. Also, we have classified twins having concordance pattern of NAS if both or neither of the twins showed severe NAS, and discordance pattern of NAS if one of the twins, but not the other, exhibited severe NAS.

MATERIALS AND METHODS

We mined the database of MetroHealth Medical Center's Mother and Child Dependency Program—a multidisciplinary treatment program for drug-dependent mothers and their infants—to identify all twins born at a gestational age (GA) of 30 weeks or greater, who were at risk of developing NAS, and were born between January 2006 and December 2015. This study was approved by the Institutional Review Board. We collected the maternal and infants' demographics and other factors related to severity of NAS, including need and duration of pharmacological treatment, duration of hospitalization, mean NAS scores (modified Finnegan score) in the first, second, and third week of life, maximum NAS score, and cumulative dose of Morphine: the first-line medicine for treatment of NAS at MetroHealth. In demographics, we collected information including gender, birth weight, GA at birth, and types of twins: monozygotic or dizygotic. We also collected details about intrauterine exposure of the infants to methadone, buprenorphine, heroin, cocaine, marijuana, tobacco, and drugs used for treatment of underlying psychiatric conditions in the mother. Additionally, we collected information on the type of feeding: breast feeding or formula feeding. Amnionicity and chorionicity was determined based on obstetric ultrasound and, when necessary and available, infant's blood group and gender were also used to designate zygosity of twins.

CHARACTERISTICS AND OUTCOME OF SEVEN SETS OF TWINS

First Set of Twins

This set of diamniotic-dichorionic (dizygotic) twins was born at 38 weeks of GA to a 30-year-old Caucasian woman through emergency cesarean section for non-reassuring fetal heart tracing. She had a history of heroin use (both snorting and intravenous routes), cocaine use, and tobacco smoking, as well as a history of anxiety and depression, which were not treated. She was in the Methadone maintenance program, taking 45 mg methadone daily at the time of delivery. Her last reported illicit drug use was more than 4 months prior to delivery. She tested negative for Hepatitis B, Hepatitis C, and human immunodeficiency virus (HIV). Both twin A and B were male, with a birth weight of 2,775 g (blood group, A positive) and 2,830 g (blood group, O positive), respectively. Both infants were predominantly formula fed. Their median NAS scores in the first week were 3 and 2, respectively. Neither of the twins required pharmacological management for NAS, and both were discharged home on day 8.

Second Set of Twins

Diamniotic-dichorionic (dizygotic) twins were born at 38 weeks of GA to a 34-year-old Caucasian woman by scheduled repeat cesarean section. The mother had a history of intravenous heroin and cocaine use, tobacco smoking, as well as a history of anxiety and depression, which were not treated. She was in the Methadone maintenance program, taking 45 mg methadone daily at the time of delivery. Her last reported illicit drug use was more than 4 weeks prior to delivery. She was tested positive for Hepatitis C but negative for HIV and Hepatitis B. Both twin A and B were male with a birth weight of 2,365 g and 2,025 g, respectively. The infants' blood type was not available in their medical records. Both infants were predominantly formula fed. Their median NAS scores in the first week were 3 and 4, respectively. Neither of the twins required pharmacological management of NAS and both were discharged home on day 8.

Third Set of Twins

This set of diamniotic-monochorionic (monozygotic) twins was born at 38 weeks of GA by vaginal delivery to a 21-year-old Caucasian woman. She had a history of intravenous heroin and cocaine use, tobacco smoking, as well as a history of anxiety and post-traumatic stress disorders, which were not treated. She was in the Buprenorphine maintenance program, taking 8 mg buprenorphine daily at the time of delivery. Her last reported use of illicit drugs was more than 3 weeks prior to delivery. She was Hepatitis B, Hepatitis C, and HIV negative. Both twin A and B were female, with a birth weight of 2,861 g and 2,658 g, respectively. Both infants had O positive blood group and were exclusively formula fed. Their median NAS scores in the first week were 5 and 5, with the highest scores of 10 and 13 for twin A and B, respectively; both required NICU admission. Both infants required pharmacological treatment for NAS for 12 and 26 days and were discharged on day 26 and 36, respectively.

Fourth Set of Twins

This set of dichorionic-diamniotic (dizygotic) twins was born at 36 weeks of GA by vaginal delivery to a 29-year-old Caucasian woman. She had a history of heroin use (both snorting and intravenous routes), along with cocaine and marijuana, tobacco smoking, as well as a history of anxiety and post-traumatic stress disorder, which were not treated. She was in the Methadone maintenance program, taking 60 mg methadone daily at the time of delivery. Her last reported illicit drug use was more than 4 months prior to delivery. She was Hepatitis B, Hepatitis C, and HIV negative. Twin A was a female, with a birth weight of 2,121 g, and Twin B was a male, with a birth weight of 1,823 g. Both infants were exclusively formula fed. Twin A required pharmacological treatment for 9 days and twin B did not require any pharmacological treatment. Both infants were discharged home on day 19.

Fifth Set of Twins

This set of dichorionic-diamniotic (dizygotic) twins was born at 37 weeks of GA by vaginal delivery to a 32-year-old Caucasian woman. She had a history of using heroin, cocaine, marijuana and tobacco smoking as well as a history of unspecified mental health illness, which was not treated. She was not in any opioids assisted program. Her last reported illicit drug use was 4 weeks prior to delivery. She was Hepatitis C infected, but tested negative for HIV and Hepatitis B. Twin A was a female, with a birth weight of 2,410 g, and twin B was a male, with a birth weight of 2,505 g. Both infants were exclusively formula fed. Their median NAS scores in the first week were 2 and 3 for twin A and B, respectively, and they stayed in the normal newborn nursery (NBS). Neither of the infants required any pharmacological treatment and both were discharged home on day 8.

Sixth Set of Twins

This set of dichorionic-diamniotic (dizygotic) twins was born at 34 weeks of GA by vaginal delivery to a 27-year-old Caucasian woman. She had a history of using heroin (by snorting and intravenous route), cocaine, marijuana as well as tobacco smoking, but no history of mental health illnesses. She was in the Methadone maintenance program and was taking 120 mg methadone daily at the time of delivery. She tested negative for Hepatitis B, Hepatitis C, and HIV. Twin A was a female, with birth weight of 2,121 g, and twin B was a male, with birth weight of 2,105 g. Both infants were exclusively formula fed and both required pharmacological treatment for withdrawals for 32 days. Twin A was discharged on day 43, while twin B was discharged on day 41.

Seventh Set of Twins

This set of dichorionic-diamniotic (dizygotic) twins was born at 31 weeks of GA by vaginal delivery to a 31-year-old Caucasian woman. She had a history of heroin, cocaine, other opioids' (not specified in chart) abuse and tobacco smoking, as well as a history of anxiety and depression, which were not treated. She was in the Methadone maintenance program and was taking 50 mg methadone daily at the time of delivery. She was tested negative for Hepatitis B, Hepatitis C, and HIV. Twin A was a female, with a birth weight of 1,440 g (blood group, A positive) and twin B was also a female, with a birth weight of 1,570 g (blood group, O positive). Both infants were exclusively formula fed. Twin A required 14 days of pharmacological treatment, whereas no pharmacological treatment was required for twin B. Both infants were discharged home on day 49. Severe NAS is not very common among preterm infants born at 31 weeks, with a birth weight of about 1,400 g. We thus looked for alternative diagnosis that could explain the need of morphine but could not find anything other than NAS.

DISCUSSION

Of the seven sets of twins, there was concordance of severity of NAS in five sets of twin siblings, which included a set of monozygotic twins, and discordance in two sets of twin siblings. Among the five pairs of twins with concordance of withdrawal severity, two pairs of twins developed severe NAS and three pairs did not develop severe NAS. Although the pair of identical twin siblings developed severe NAS and required pharmacological treatment, the length of treatment and hospital stay varied greatly among the twin siblings. Twin A was treated pharmacologically for 12 days and stayed in hospital for 26 days while B was treated for 26 days and stayed in hospital for 36 days. In the two pairs of dizygotic twins with discordance of NAS severity, one pair required medical treatment while the other did not. A total of 6 out of the 14 (42%) infants required treatment.

To the best of our knowledge, this is the first case series of twins with NAS. Among all twins, we found 70% concordance of severity for withdrawal as measured by requirement of pharmacological treatment and 30% discordance of severity. Adequately powered twin studies may help in delineating the influence of genetic and environmental factors in determining the severity of NAS.

AUTHOR CONTRIBUTIONS

All authors were involved in conceptualizing and designing the work, RP prepared the initial draft and all authors revised the initial draft and agreed on the final version as submitted.

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Prenatal Opioid Exposure and Intermittent Hypoxemia in Preterm Infants: A Retrospective Assessment

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Introduction: Intermittent hypoxemia (IH) is defined as episodic drops in oxygen saturation (SpO_2). Preterm infants are at increased risk for IH due to their immature respiratory control/apnea of prematurity. The clinical relevance of IH is a relatively new observation with rising evidence linking IH to neonatal morbidities and long-term impairment. Hence, assessing factors that influence IH in preterm infants is imperative. Given the epidemic of opioid misuse in the USA, there is an urgent need to understand the impact of prenatal opioid exposure on neonatal outcomes. Hence, we wanted to assess the relationship between isolated prenatal opioid exposure and IH in preterm infants.

Methods: In order to accurately calculate IH, SpO_2 data were prospectively collected using high-resolution pulse oximeters during the first 8 weeks of life in preterm infants less than 30 weeks gestational age. Data related to prenatal opioid misuse were retrospectively collected from medical charts. Infants with tobacco or poly-drug exposure were excluded. The primary outcome measure is percent time spent with SpO_2 below 80% (%time-SpO_2 < 80). The secondary outcome measure is the number of severe IH events/week with SpO_2 less than 80% (IH-SpO_2 < 80).

Results: A total of 82 infants with isolated opioid exposure (n = 14) or who were unexposed (n = 68) were included. There were no significant differences in baseline characteristics between opioid exposed and unexposed groups. There was a statistically significant increase of 0.23 (95% CI: 0.03, 0.43, p = 0.03) in mean of the square root of %time-SpO_2 < 80. The number of IH-SpO_2 < 80 events was higher in the opioid exposed group (mean difference = 2.95, 95% CI: −0.35, 6.25, p-value = 0.08), although statistical significance was not quite attained.

Conclusion: This study shows that preterm infants prenatally exposed to opioids have increased IH measures compared to unexposed infants. Interestingly, the increased IH in the opioid exposed group persists beyond the immediate postnatal period.

Keywords: prenatal, opioid exposure, opiates, intermittent hypoxemia, apnea, preterm infants

Abbreviations: GA, gestational age; IH, intermittent hypoxemia; NICU, neonatal intensive care unit; SIDS, sudden infant death syndrome; SpO_2, oxygen saturation; THC, tetrahydrocannabinol.

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INTRODUCTION

Intermittent hypoxemia (IH) is defined as brief, episodic drops in oxygen saturation (SpO_2) (1, 2). Preterm infants are at increased risk for IH due to their respiratory control instability/apnea of prematurity superimposed on immature lung structure/function. IH in preterm infants can persist beyond discharge from the neonatal intensive care unit (NICU) (3). Brief episodes of oxygen desaturations may seem clinically insignificant, but these IH episodes, occurring up to hundreds of times per day, have a cumulative effect on neonatal morbidity and mortality. There is ample evidence showing a significant effect of IH on neurocognitive handicap, decreased neuronal integrity, increased inflammation and oxidative stress, and impaired growth (4, 5). Furthermore, IH has been linked to severe retinopathy of prematurity and long-term neurodevelopmental impairment such as worse language and motor outcomes (2, 6–9). The clinical relevance of IH is a relatively new observation with the advent of high-resolution pulse oximeters and assessing factors that influence IH is imperative.

There is a rise in substance misuse in the USA reaching a nationwide epidemic (10–15). There is an urgent need to understand the impact of prenatal opioid exposure on neonatal outcomes (5). Opioid exposure is associated with long-term neurobehavioral and developmental impairment in infants (16–23). Opioids are known to suppress breathing and respiratory effort especially in neonates (24). Since most mothers who misuse opioids have also been found to smoke and use poly-drugs that affect breathing pattern, it has been challenging to assess the isolated effect of prenatal opioid exposure on respiratory outcomes. Prenatal tobacco exposure alters respiratory control and worsens lung function (25–29). Prenatal exposure to other illicit drugs such as cocaine perturbs maturation of respiratory control, resulting in disruption of postnatal respiration (30). Only few studies were able to assess the effect of isolated opioid exposure on neonatal respiratory outcomes. However, these studies included mostly later preterm and term infants or were limited to short monitoring times and small sample sizes (31, 32). In this study, we utilize continuous high-resolution pulse oximeters to assess the relationship between isolated prenatal opioid exposure and IH in preterm infants during the first 2 months of life.

MATERIALS AND METHODS

Study Design and Data Collection

Oxygen saturation data were prospectively collected from 130 preterm infants less than 30 weeks gestational age (GA) admitted to our level 4 NICU between November 2014 and April 2017. We used high-resolution pulse oximeters (Radical 7: Masimo, Irvine, CA, USA) set at 2 s averaging time and 1 Hz sampling rate to continuously monitor patients during the first 8 weeks of life. In order to differentiate intermittent from sustained hypoxemia, we included events between 4 and 180 s (1). The exact threshold below which IH is clinically significant is controversial. A drop in SpO_2 to less than 80% is widely considered to be clinically relevant (1, 2, 6). Therefore, the primary outcome measure was defined as percent time spent with SpO_2 below 80% (%time-$SpO_2 < 80$). The secondary outcome measure was defined as the number of severe IH events with SpO_2 less than 80% (IH-$SpO_2 < 80$). Other outcome measures such as length of stay and neonatal morbidities were collected.

Pulse oximeters were equipped with serial data recorders (Acumen Instruments Corp.) for continuous data collection. Novel programs were utilized to filter and analyze data (Matlab, Natick, MA, USA) (1, 33). Data with artifacts were excluded. Only SpO_2 data with good signal were included in the analyses. Preterm infants less than 30 weeks GA were included. Infants with major congenital malformations were excluded.

Data related to substance misuse and tobacco use were retrospectively collected from medical charts. If a mother chronically used prenatal opioids and/or the maternal/neonatal drug screens were positive for opioids, then the infant was considered for screening. Infants were then excluded from the study if the mother used tobacco, alcohol, or other drugs (such as cannabis); i.e., in order to assess for isolated opioid exposure, patients with any other exposure were excluded. Infants in our cohort who were not exposed to opioids, tobacco, or other drugs served as controls. Neonatal meconium or urine drug screens are performed in the immediate newborn period. Positive drug screens due to opioids and other medications used for pain or sedation during delivery were excluded, as they do not represent prenatal misuse. Tobacco and alcohol use were collected from mothers' medical records, as the toxicology screens at our hospital do not test for alcohol or tobacco exposure. The study was approved by the University of Kentucky Institutional Review Board, and informed consent was obtained prior to SpO_2 data acquisition.

Statistical Analysis

Descriptive statistics for continuous variables are presented as either the mean with SD or median with interquartile range, and frequencies and percentages are given for categorical variables. Two-sample *t*-tests and Wilcoxon two-sample tests were used to compare opioid exposure to non-exposure with respect to continuous variables, and chi-square or Fisher's exact tests were used for categorical variables. To compare opioid exposure to non-exposure with respect to IH measures over time, we utilized multivariate Gaussian linear modeling in order to account for repeated measurements from subjects, and to adjust for the potential confounders of GA, birth weight, APGAR score at 5 min of life, gender, and the use of prenatal steroids. In order to meet statistical assumptions in these models, the square root of the IH measures was taken. Furthermore, weekly observations were weighted by the percentage of time IH was tracked during the given week. Analyses were conducted in SAS version 9.4 (SAS Institute, Cary, NC, USA), and all tests were two-sided with a 5% significance level.

RESULTS

Of the 127 infants in our database with complete data sets, 19.7, 29.1, and 4.7% were prenatally exposed to opioids, tobacco, and cannabis, respectively. None were exposed to alcohol, cocaine, and other illicit drugs. Opioid exposed infants were positive for

buprenorphine metabolites (64%), oxycodone (16%), and other opioids such as heroin and fentanyl (20%). A total of 82 infants qualified for analysis as they were either unexposed to any illicit drug/tobacco ($n = 68$) or exposed to opioids only ($n = 14$). **Figure 1** presents the flow diagram for patient eligibility and exclusion.

There were no significant differences in baseline characteristics as presented in **Table 1**. The mean GA was 27 weeks in both groups. There were no significant differences in birth weight, gender, and Apgar scores at 5 min of life. The vast majority of infants received prenatal steroids with no difference between groups. There were no significant differences in respiratory outcomes and neonatal morbidities between groups as presented in **Table 2**. Our cohort included preterm infants less than 30 weeks GA. Essentially all infants had respiratory distress syndrome and received surfactant. Severe bronchopulmonary dysplasia, postnatal steroids use for lung disease, and oxygen need at 28 days, 36 weeks postmenstrual age, and at discharge did not differ between opioid exposed and unexposed groups (all p = NS). Other neonatal morbidities such as patent ductus arteriosus, late onset sepsis, and necrotizing enterocolitis did not differ between groups (all p = NS). None of the exposed infants died versus nine deaths in the unexposed group ($p = 0.35$). The median length of stay was 17 days longer in the opioid group (85 days) compared to unexposed group (68 days); however, the results were not statistically significant ($p = 0.32$).

There was a statistically significant increase in our primary outcome measure, %time-$SpO_2 < 80$, as represented in **Figure 2**. The estimated difference in the means of the square root of %time-$SpO_2 < 80$ was 0.23 (95% CI: 0.03, 0.43, $p = 0.03$). The mean number of IH events was estimated to be 2.95 (95% CI: −0.35, 6.25, p-value = 0.08) higher in the opioid exposed group, as represented in **Figure 3**; however, this did not reach statistical significance. Note that these results represent the square root of means in order to meet statistical assumptions in these models; estimated medians for IH measures are calculated using our model results and are presented in **Figures 2B** and **3B**. Given increased death in the unexposed group, we then analyzed data excluding deaths, and results were similar. Specifically, there was a statistically significant increase in our primary outcome measure (%time-$SpO_2 < 80$) in the opioid exposed compared to the unexposed group, with an estimated mean difference (square root) of 0.24 (95% CI: 0.05, 0.44, p-value = 0.02). Furthermore, the mean number of IH events was estimated to be 2.98 (95% CI: −0.20, 6.16, p-value = 0.07) higher in the opioid exposed group, not quite reaching statistical significance.

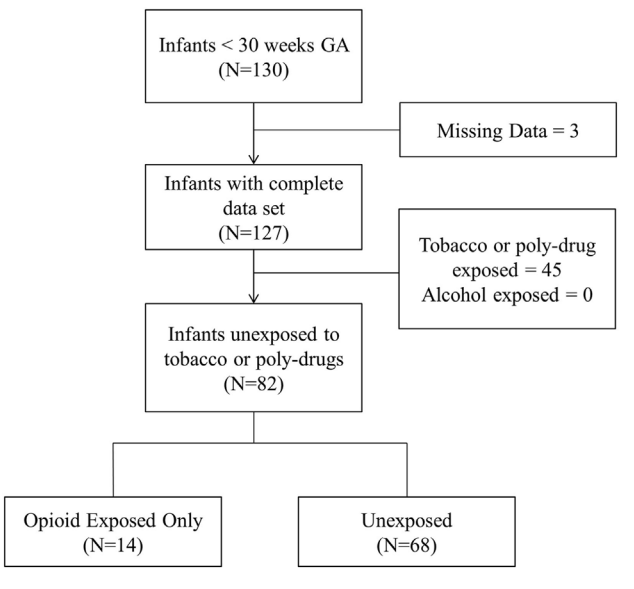

FIGURE 1 | Flow diagram for patient eligibility.

TABLE 1 | Baseline characteristics.

	Opioid exposed	Unexposed	*p*-Value
	***N* = 14**	***N* = 68**	
Gestational age (weeks)	27.0 ± 2.1	27.0 ± 1.6	0.97
Birth weight (g)	948 ± 263	928 ± 247	0.79
Male	6 (43%)	23 (34%)	0.54
Apgar 5 min	7 (6, 7.5)	6 (5, 7)	0.21
Prenatal steroids	12 (86%)	61 (91%)	0.62

Mean ± SD, median (interquartile range).

DISCUSSION

These results suggest that prenatal opioid exposure is associated with increased IH measures compared to unexposed preterm infants. This study has two main findings. First, interestingly, the increased IH measures in opioid exposed infants persisted beyond the early postnatal period. Preterm infants were continuously monitored with high-resolution pulse oximeters during the first 2 months of life. Second, we had the unique opportunity to assess the relationship between isolated opioid exposure and respiratory instability in preterm infants. It was challenging in the past to assess the relationship between isolated prenatal opioid

TABLE 2 | Neonatal morbidities and outcomes.

	Opioid exposed	Unexposed	*p*-Value
	***N* = 14**	***N* = 68**	
Received surfactant	14 (100%)	62 (91%)	0.58
Respiratory distress syndrome	14 (100%)	67 (99%)	1
Oxygen at 28 days of life	10 (71%)	39 (57%)	1
Oxygen at 36 weeks corrected age	7 (50%)	19 (28%)	0.26
Oxygen at discharge	9 (64%)	30 (44%)	0.18
Severe bronchopulmonary dysplasia	9 (64%)	27 (46%)	0.21
Postnatal steroids use for lung disease	6 (43%)	19 (29%)	0.35
Pneumothorax	1 (7%)	2 (3%)	0.43
Patent ductus arteriosus	8 (57%)	24 (35%)	0.13
Necrotizing enterocolitis	0 (0%)	2 (3%)	1
Late onset sepsis	3 (21%)	9 (13%)	0.43
Mortality	0 (0%)	9 (13%)	0.35
Length of stay (days)	85 (59, 101)	68 (56, 91)	0.32

Frequency (%), median (interquartile range).

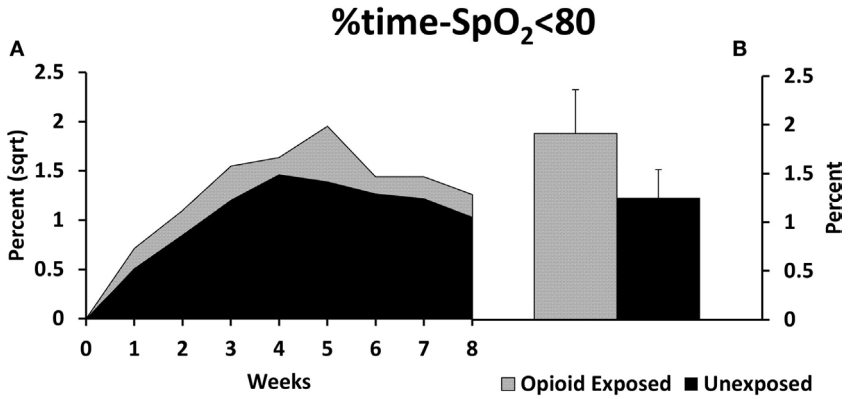

FIGURE 2 | **(A)** Preterm infants exposed to prenatal opioids had increased time spent with oxygen saturation less than 80% (%time-SpO_2 < 80) compared to unexposed infants ($p = 0.03$). The model adjusted for gestational age, birth weight, gender, prenatal steroids, and Apgar scores at 5 min of life. **(B)** This figure demonstrates the estimated average %time-SpO_2 < 80 medians in both groups calculated using the adjusted model results. Sqrt, square root.

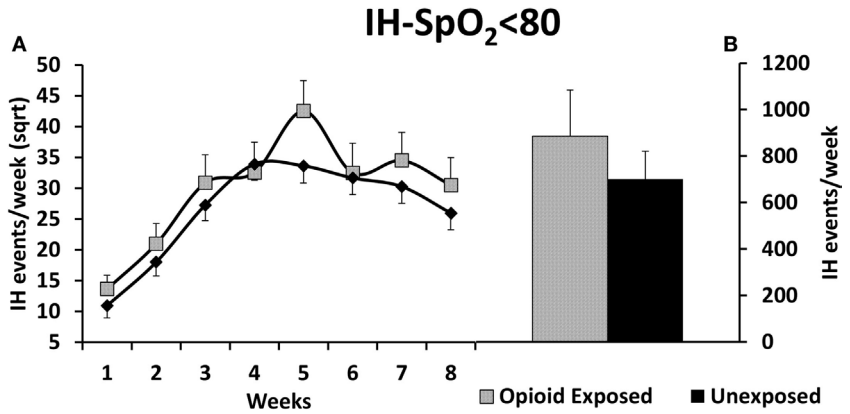

FIGURE 3 | **(A)** Preterm infants exposed to prenatal opioids did not have a significant increase in number of intermittent hypoxemia (IH) events per week (IH-SpO_2 < 80) compared to unexposed infants ($p = 0.08$). The model adjusted for gestational age, birth weight, gender, prenatal steroids, and Apgar scores at 5 min of life. **(B)** This figure demonstrates the estimated average IH-SpO_2 < 80 medians of opioid exposed versus unexposed preterm infants calculated using the adjusted model results. Sqrt, square root.

exposure and respiratory outcomes/IH, as the majority of women who use opioids also smoke or misuse poly-drugs. Given our cohort demographics, we had the ability to report this association in infants exposed to opioids only.

Another interesting secondary finding in our study is the steady increase in IH in the first month of life before plateauing and then decreasing. This natural progression of IH has been described before from another cohort of preterm infants less than 28 weeks GA (1, 2). Our study replicates this finding from a new cohort of preterm infants less than 30 weeks GA. The rise in IH may be related to peripheral chemoreceptor dysregulation and development of lung disease (34).

Patients in our opioid exposed and unexposed groups did not significantly vary in terms of baseline characteristics (such as age, weight, gender) and neonatal morbidities (such as lung disease, patent ductus arteriosus, late onset sepsis, and necrotizing enterocolitis). In addition, we adjusted in the model for factors that may influence oxygenation in preterm infants such as GA and prenatal steroids. The finding of nine deaths in the unexposed group compared to no deaths in the opioid exposed group may be due to chance. Secondary analyses excluding deaths showed similar results with increased IH in the opioid exposed group. A significant secondary finding in this study is the high prevalence of tobacco and drug exposure in our cohort of preterm infants. The frequency of opioid exposure in our preterm population is higher than previously reported, thus creating urgency toward addressing this significant problem in this vulnerable patient population (10, 12–15).

There are multiple proposed mechanisms by which prenatal opioid exposure may affect breathing patterns and subsequent persistent IH in preterm infants. Prenatal opioid exposure alters the response to carbon dioxide and depresses central respiratory control centers (31, 35–38); a main driver for respiratory output. Olsen and Lees demonstrated a blunted response to carbon

dioxide in methadone exposed infants compared to controls (31). Ali et al. compared the response to hypercarbia among three groups of term patients who were exposed to tobacco/substance misuse, tobacco alone, and unexposed controls. The authors showed a lower increase in central respiratory drive in response to hypercarbia in infants exposed to substance misuse as compared to tobacco alone and unexposed controls (35). Another mechanism that explains our results may be related to *in utero* hypoxia related to opioids. Prenatal opioids, especially street heroin, cause chronic intrauterine hypoxia leading to brainstem gliosis, resulting in injury to the central respiratory network. This may lead to respiratory instability and subsequent IH (36). Finally, data from animal models showed that exposure to opioid agonists caused downregulation of placental neurotransmitter receptors (39). Abnormalities or depletion of receptor sites, especially if the same process occurs in the fetal brain, could impair the function of the normal neonatal respiratory control network leading to frequent or prolonged apnea and subsequent IH.

Many studies have assessed the impact of prenatal opioid exposure on sudden infant death syndrome (SIDS) in infants with controversial results. This study does not address SIDS; rather, it focuses on IH, the end result of apnea of prematurity. However, the mechanism by which prenatal opioid exposure is associated with increased SIDS and IH may be similar. Although our study period focused on the inpatient setting, it is plausible that opioid exposed infants continue to have increased cardiorespiratory events/IH after discharge. Interestingly, compared to unexposed infants, opioid exposed infants had a trend toward longer length of stay (68 versus 85 days, p = NS), which may be related, in part, to persistent cardiorespiratory events.

A major limitation of this study is that data related to exposure were retrospectively collected. Another limitation is a lack of reporting daily caffeine use and daily respiratory support settings. At our center, virtually all infants with GA less than 30 weeks are started on caffeine therapy. Furthermore, our study focused on IH events and lacked reporting of apnea and bradycardia events. Lack of addressing heart rate is a limitation since bradycardia events may be associated with poor long-term outcomes (6). Another limitation is the small sample size; however, our sample size of isolated opioid exposure is relatively large compared to existing literature. This is a single center study; hence, our results may not be generalizable. Finally, we did not compare the long-term neurodevelopmental outcomes for exposed versus unexposed infants.

CONCLUSION

There is rising evidence linking IH to neonatal morbidities and impairment. However, the exact threshold (frequency, duration, severity) by which IH leads to injury in preterm infants needs further investigation; i.e., any increase in IH may be associated with impairment in preterm infants. Furthermore, there is a need to understand factors, such as prenatal opioid exposure, that may influence IH and subsequently increase neonatal morbidities. In this study, we show an association between prenatal opioid exposure and increased IH measures in preterm infants. Studies to address the relationship between opioid exposures, IH, and long-term neurodevelopmental outcomes are imperative. Given the rising epidemic of opioid misuse in the USA, understanding the relationship between opioid exposure, IH and long-term impairment is imperative. A larger prospective study aimed at understanding these relationships may have a direct impact on short- and long-term management of preterm infants.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of University of Kentucky Institutional Review Board (IRB). A written informed consent was obtained from all subjects included in this study. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the University of Kentucky IRB.

AUTHOR CONTRIBUTIONS

EA designed and conceptualized the study and was actively involved in the enrollment, data collection, as well as analysis and interpretation of the results. He drafted the initial manuscript and wrote the final manuscript as submitted. PW was involved in conceptualization of the study and performed the statistical analyses. He also critically reviewed the manuscript and made the final approval manuscript as submitted. AP, AS, DM, and AG were involved in the conceptualization of the study, data collection, as well as analysis and interpretation of the results. They critically reviewed the manuscript and approved the final manuscript as submitted. AP was involved in the conceptualization of the study, the data acquisition software development, as well as in the analysis and interpretation of the results. He critically reviewed the manuscript and approved the final manuscript as submitted. HB and PG were involved in the conceptualization of the study as well as analysis and interpretation of the results. They critically reviewed the manuscript and approved the final manuscript as submitted.

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Swallow–Breath Interaction and Phase of Respiration with Swallow during Non-Nutritive Suck in Infants Affected by Neonatal Abstinence Syndrome

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Background: The development of suck–swallow–breath rhythms during non-nutritive suck (NNS) may be an indicator of neurologic integrity. We have described swallow–breath (SwBr) interaction and phase of respiration (POR) with swallow during NNS in low-risk preterm (LRP) infants. NNS in infants with neonatal abstinence syndrome (NAS) has not been described with our method.

Method: Suckle, swallow, thoracic motion, and nasal airflow were measured during NNS in 10 infants with NAS and 12 unaffected infants (control). Logistic regression models were fit to describe the three types of SwBr and five types of POR in terms of the independent variables (gender, gestational age, birth weight, postmenstrual age, weeks postfirst nipple feed and swallows per study). We also compared the NAS group to 16 LRP infants.

Results: In the NAS group, there were 94 swallows in 18 studies. In the control group, there were 94 swallows in 12 studies. There were statistical differences between groups for all three types of SwBr. The distribution of SwBr in NAS was similar to LRP infants with NAS having fewer swallows with attenuated respiration and more with central apnea. For POR, there were few differences. Over time, the distribution of SwBr in NAS infants approaches that of control infants.

Discussion: Variability in SwBr and POR during NNS may represent neurologic dysfunction in infants with NAS. Specifically, term infants with NAS display an immature pattern of SwBr making them more similar to preterm infants, rather than a unique pathology. The distribution of SwBr and POR in NAS infants becomes more like term infants, possibly representing catch-up development as the NAS symptoms resolve.

Conclusion: SwBr in babies with NAS is different from that of unaffected term infants, actually being similar to preterm infants. Infants with NAS exhibit a dysmature pattern of NNS development which resolves over time.

Keywords: neonatal abstinence syndrome, non-nutritive suck, suck–swallow–breath coordination, infant feeding, neonatology

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INTRODUCTION

Efficient suckle-feeding can be considered to be the most complex skill a newborn infant must master to attain independent survival. However, feeding problems are frequent in preterm infants (1) and can lead to prolonged hospital stays (2). Poor feeding in the neonatal period may be an early indicator of neurologic injury (3, 4) and has been linked to language delay later in life (5).

The development of efficient suckle-feeding is dependent on the maturation and coordination of neuronal central pattern generators controlling suck, swallow, and breath (SwBr) (6). These same central pattern generators are also activated during non-nutritive suck (NNS). Thus, NNS may provide an earlier marker of intact neurodevelopment than nutritive feeding does.

We have previously used our method to study NNS in low-risk preterm (LRP) infants (7) and have shown that the interaction of SwBr and the phase of respiration (POR) incident to swallow develop in a predictable pattern in these infants. The progression of SwBr is influenced by increasing opportunities to practice the skill, or what can be considered "learning." The progression of POR was more affected by measures of maturation, indicating a developmental progression. Our method has not yet been applied to pathologic conditions.

Neonatal abstinence syndrome (NAS) is a constellation of withdrawal-like symptoms experienced by infants born to mothers who have been chronically taking opiates or other drugs/medications during the pregnancy. Feeding difficulty and disruption of suck–swallow–breath rhythms have been reported in infants affected by NAS (8, 9). In general, abnormal sucking and feeding behaviors in infants affected by NAS include excessive sucking, inattention, fussiness, and decreased swallow efficacy. The specifics of suck–swallow–breath organization during NNS in infants affected by NAS have not been described.

TABLE 1 | Abbreviations and acronyms used in this study.

NAS	Neonatal abstinence syndrome	Group of symptoms experienced by infants as a consequence of opioid withdraw. The group of infants in this study who were diagnosed and treated for neonatal abstinence syndrome
NAS1st	Subgroup of NAS study group	Subgroup of NAS infants including only one study from each patient. Allows analysis without repeated measures
LRP	Low-risk preterm	Group of "healthy" preterm infants with no sepsis, no IVH and relative low-risk for the development of bronchopulmonary dysplasia
NNS	Non-nutritive suck	Act of infant sucking on a pacifier with no milk intake
SwBr	Swallow–breath interaction	How swallow interacts with breath. Can occur in 3 types (AR, OA, CA).
AR	Attenuated Respiration	Deflection of the slope of the nasal airflow tracing without interruption in the overall breathing rhythm
OA	Obstructive Apnea	Cessation of nasal airflow for the duration of a swallow with continued chest movement
CA	Central Apnea	Cessation of both nasal airflow and chest movement for the duration of a swallow
POR	Phase of Respiration Incident to Swallow	Where in the respiratory cycle a swallow occurs. Can occur in 5 types (BE, ME, EE, MI, AP)
BE	Beginning Expiration	Transition from inspiration to expiration
ME	Mid-Expiration	Point between beginning expiration and end expiration
EE	End Expiration	Transition from expiration to inspiration
MI	Mid-Inspiration	Point between end expiration and beginning expiration
AP	Apnea	Period of no discernable breathing for 1 s prior to the time of a swallow

MATERIALS AND METHODS

We performed a prospective observational study comparing suck–swallow–breath coordination in infants with NAS and unaffected infants. The study participants included 10 term infants with NAS and 12 healthy term (control) infants. We also included a group of 16 LRP infants who were concurrently enrolled in the study and described in a previous manuscript (7). **Table 1** is a list of the abbreviations provided to help the reader understand the presented information.

The 10 infants in the NAS group were diagnosed by a combination of *in utero* exposure to substances known to result in NAS and postnatal symptoms consistent with the diagnosis. *In utero* exposures included benzodiazipines, methadone, cocaine, oxycotin, klonipin, fentanyl, THC, oxycodone, and buprenorphine. Five infants had poly-drug exposure. Five infants had single drug exposures (four methadone and one oxycodone). These included four mothers who were compliant with a methadone treatment program and another mother who was taking chronic pain medication. Finnegan scores at the time of enrollment ranged from 9 to 20. In our NICU at the time, babies who were at risk for NAS were monitored for symptoms of NAS and scored with the standard Finnegan Score tool (10). Per institutional norm, infants who score 8 or more on 2 consecutive scores, or 12 or more once, were given a diagnosis of NAS and treated with opiate replacement therapy. At the time of this study, the treatment for NAS in our facility was not standardized. Infants were treated with morphine or methadone. The protocol for adjusting doses was not proscribed. Two infants required adjunctive therapy with phenobarbital. Infants were studied once per week from the time of enrollment until discharge from the hospital. There were 94 swallows collected from 18 studies in this group.

The control group included 12 babies born between 37 and 42 weeks of gestation, appropriate size for gestational age, 5-min Apgar score of 7 or more and with no congenital anomalies or metabolic disorders. These infants were studied once prior to discharge from the hospital. There were 94 individual swallow events collected over 12 studies from these infants.

We also compared the NAS group to a group of 16 LRP infants who were born at less than 36 weeks (mean gestational age at birth: 28 5/7, mean postmenstrual age at study: 35 3/7), appropriate size for gestational age (mean birth weight: 1,056), no congenital anomalies, no IVH of grade 3 or 4 and deemed to be "low risk" for bronchopulmonary dysplasia per the definition in our previous publication (7). There were 176 swallows in 35 studies in this group.

Because of potential statistical problems with repeated studies in the NAS group and not in control infants, we analyzed the data using only a single study for each NAS infant. This decreased the number of studies in this group (NAS1st) to 10 and the number of swallows to 49.

Informed consent was obtained from the parent(s) of each infant prior to the infant's participation in the study. The project complies with all applicable HIPAA standards and was approved by the Institutional Review Board of the University of Kentucky.

We have previously published the specific method and equipment for preparing the babies for the study and data collection (7). To summarize the salient portion of the study for this project, the infant study participants were prepared in the following manner:

- A 5 F nasopharyngeal catheter was placed and connected to a pressure transducer to measure swallow pressure.
- A second catheter was placed through a pacifier so that the catheter tip was flush with the nipple and connected to a transducer to measure suckle pressure.
- Respiratory effort was measured with a stretchable band placed around the infant's chest.
- Nasal airflow was measured with a thermistor bead placed at the opening of the nares.

The infants were offered a pacifier for 1-min of NNS just prior to a nutritive feeding. Data were collected and displayed as multichannel linear graphs, using the Windaq Waveform Browser (Dataq Industries, Akron, OH, USA). The entire 1-min sequence of NNS was canvassed for swallows, noted as deflections in the naso-pharyngeal pressure recording. The type of SwBr and POR were classified, as described below. During NNS, swallow occurs infrequently. When it does occur, the SwBr must abruptly interact for about 1–2 s, in what has been termed "deglutition apnea" (11), and should not be confused with apnea (AP) of prematurity which requires 15–20 s of no breathing to be defined as AP.

We can identify three types of SwBr interaction: central apnea (CA) (cessation of both nasal airflow and chest movement), obstructive apnea (OA) (cessation of nasal airflow but continued rhythmic chest movement), or attenuated respiration (AR) (a slight deflection of the slope of the respiratory line on the graph at the time of the swallow without disruption of the respiratory rhythm). Examples of each type of SwBr are shown in **Figure 1**.

The respiratory cycle can be divided into five phases (POR): beginning expiration (BE), mid-expiration (ME), end-expiration (EE), mid-inspiration (MI), and AP. Swallows occur at any of these PORs. It has been hypothesized that the most mature pattern exists when the swallow occurs at a point of minimal air movement (BE, EE, or AP) (11).

Independent variables for this analysis included gender, birth weight, gestational age, postmenstrual age, number of swallows in the study, and weeks postfirst nipple feed (time between first nipple feed and day of study). Day-of-life at the time of the study is used only for demographic descriptions and not for analysis because it would create a collinearity issue since postmenstrual age is a function of gestational age + day-of-life.

Infants were invited to return for developmental testing [Bayley-III and Preschool Language Scales version IV (PLS-IV)] at 12 and 24 months. 11 infants from the control group returned at 12 and 24 months. 7 infants in the NAS group returned at 12 months and 5 returned at 24 months. One of the NAS infants at 24 months could not complete all of the assessments.

We used Stata 13.1 (College Station, TX, USA) to calculate descriptive statistics on patient demographics and to construct logistic regression models relating the odds of each type of SwBr and POR to the independent variables defined above. Statistical inferences were made *via* generalized estimating equations (12) with an exchangeable structure to take into account the correlations inherent to repeated assessments on the same baby. The number of patients/studies came from a subset of a larger study of infant feeding. Power calculations were based on that study and these patients were selected because they had data that was useable for this analysis.

RESULTS

Demographics

Demographic characteristics of the infants in the study groups are shown in **Table 2**. Each group was approximately 60% female and 40% male. Mean birth weight and gestational age were similar for each group. Babies in the NAS group were studied weekly during their in-hospital treatment. Thus, day-of-life, postmenstrual age, and weeks postfirst nipple feed are greater in the NAS group. The number of swallows per study was not different between the two groups. Because of the bias caused by having repeated studies

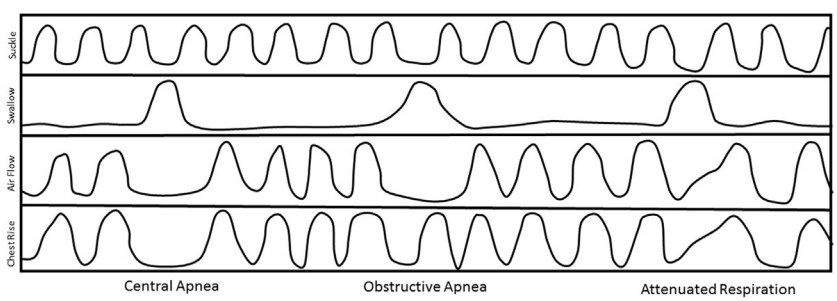

FIGURE 1 | Stylized examples of each type of swallow and breath (SwBr). Example of a four-channel recording of suckle, swallow, air flow, and chest movement. Three swallows are shown as deflections in the swallow channel tracing. SwBr is defined by the respiratory effort and airflow coincident to each swallow.

TABLE 2 | Patient population characteristics.

	Control	NAS	NAS1st
Swallows	94	94	49
Studies	12	18	10
Babies	12	10	10
Day-of-life	2.8 + 1*	17 + 11*	8.7 + 3.2*
Postmenstrual age	39.8 + 0.9*	41.7 + 2.4*	40.2 + 1.6*
Weeks postfirst nipple feed	0.3 + 0.2*	2.4 + 1.7*	1.2 + 0.4*
Swallows per study	8 + 7	5 + 2	5 + 2
Gestational age	39.5 + 1.0	39.5 + 1.8	
Birth weight	3,344 + 393	3,045 + 560	
Male/female	5/7 (42/58%)	4/6 (40/60%)	

There are significant differences between NAS and control for day-of-life ($p < 0.0001$), postmenstrual age ($p = 0.003$), and weeks postfirst nipple feed ($p < 0.0001$). There are significant differences between NAS1st and control for day-of-life ($p < 0.0001$) and weeks postfirst nipple feed ($p < 0.0001$).
**indicates statistical significance.*

in the NAS group and not in the control group, we performed the analysis using only a single study for each baby in the NAS group (NAS1st). The significant differences for day-of-life and weeks postfirst nipple feed remained. This is because babies in the NAS group were enrolled in the study after their symptoms had progressed to the point of being diagnosed with NAS, which can take over a week, depending on the drug of exposure, timing of the last dose and specific genetic polymorphisms which affect NAS timing and severity. The difference in Postmenstrual Age was eliminated.

Distribution of SwBr and POR in NAS

The percent of swallows occurring at each type of SwBr and POR for the study groups are shown in **Table 3**. Section A shows the results of univariate analysis comparing NAS and control with differences for SwBr as follows. AR is less common in NAS than control (OR = 4.05, $p = 0.0041$). There was no difference for OA. CA was more common in NAS than control (OR = 0.212, $p = 0.0353$). For POR, there were no differences between NAS and control infants for BE, ME, EE, or AP. MI was more common in NAS than control (OR = 0.28, $p = 0.0361$).

Using multivariate analysis (**Table 3**, B) to control for differences in the independent variables, CA was predicted to be more common in NAS then control (OR = 0.1117, $p = 0.0075$). OA was more common in NAS then control (OR = 0.351, $p = 0.0241$). AR was less common in NAS than control (OR = 8.1, $p = 0.0004$). OA was more common in females (OR = 0.387, $p = 0.0011$) and AR was more common with advancing weeks postfirst nipple feed (OR = 1.64 per week, $p = 0.0210$). There were no predicted differences between NAS and control for ME, MI, or EE. BE was less common in NAS than control (OR = 4.60, $p = 0.0026$). AP was more likely in NAS than control (OR = 0.099, $p = 0.0092$). AP was less common with advancing weeks postfirst nipple feed (OR = 0.456 per week, $p = 0.0115$).

SwBr and POR with a Single NAS Study

Babies in the control group underwent one study prior to discharge from the hospital, while babies in the NAS group were studied weekly during their hospital stay. In order to evaluate the data without the inherent bias introduced by repeated measures on the same baby, the analysis was performed using only a single study from each baby in the NAS group (NAS1st). This decreased the number of swallows for analysis to 49. **Table 3** shows the results of univariate analysis of NAS1st vs. control. The statistical difference between NAS1st and control for CA and AR remained. CA was more common in NAS1st than control infants (OR = 0.146, $p = 0.0042$). AR was less common in NAS1st than control infants (OR = 4.401, $p = 0.0022$). For POR, the significant difference initially noted between NAS1st and control for MI was no longer present. However, BE was less common in NAS1st than control infants (OR = 2.565, $p = 0.0451$).

TABLE 3 | Distributions of SwBr and POR for each group.

	SwBr			POR				
	CA	OA	AR	BE	ME	EE	MI	AP
A								
Control	5	20	74	41	19	24	5	10
NAS	22 *	36	41 *	28	12	28	19 *	14
B								
Control	3	11	91	68	24	21	18	0
NAS	19 *	27 *	57 *	32 *	17	26	32	3 *
C								
Control	5	20	74	41	19	24	5	10
NAS1S	35 *	27	39 *	22 *	14	39	4	20
D								
NAS	22	36	41	28	12	28	19	14
LRP	25	32	43	21	9	48 *	7	15

A: distribution of SwBr and POR for control vs. NAS. There are significant differences for CA and AR in SwBr and for MI in POR.
B: predicted distribution of SwBr and POR with multivariate analysis. There are significant differences for all three types of SwBr and BE and AP in POR.
C: distribution of SwBr and POR for control vs. NAS1st. There are significant differences for CA and AR in SwBr and for BE in POR.
D: distribution of SwBr and POR for NAS vs. LRP. There are no significant differences for SwBr. There is a significant difference for EE in POR.
**indicates statistical significance.*

SwBr and POR in NAS and LRP

Having established that the distribution of SwBr is different between NAS and control, we were interested to determine if the NAS infants were similar to preterm infants. Thus, we compared the SwBr in NAS to a group of LRP infants. As shown on **Table 3**, the percentages of each type of SwBr were similar to that of the LRP babies, with no statistically significant differences for any SwBr. There were some differences noted for POR.

Developmental Follow-up

Study participants returned for developmental follow-up assessments with the Bayley Scales of Infant Development version III (Bayley-III) and the PLS-IV. **Table 4** shows the average scores for infants in the control and NAS groups. At 12-month follow-up, the average scores for Bayley-III and PLS-IV assessments were not different between the control and NAS infants. At 24 months,

Bayley-III scores were not different between control and NAS infants. PLS-IV scores at 24 months were statistically lower in the NAS group, but the number lost to follow-up limits the ability draw conclusions from this data.

DISCUSSION

Successful newborn feeding requires coordination of the rhythms of suck, SwBr (13–16). Feeding problems have been identified as a sequelae of hypoxic–ischemic injury (17) and later neurologic injury such as cerebral palsy (16). Mild disruptions of the rhythmicity of suckle feeding can be linked to less severe injury (18) and may predict subsequent feeding and neurologic problems (19, 20). Abnormal newborn feeding behaviors have been linked to language delay at 18 months (5). The progression of normal rhythmic suckle feeding is dependent on the interaction of brainstem central pattern generators for suck, SwBr (6). These central pattern generators are also activated during NNS. Therefore, we hypothesize that the organization of suck, SwBr during NNS may be an early indication of the integrity of the neonatal CNS.

Babies affected by NAS are known to have disorders of the central nervous system which can manifest as excessive suck, abnormal breathing and feeding difficulty. Kamal et al. has shown that infants affected by NAS have an abnormal ventilatory response to hypercarbia in the newborn period and at 6–12 weeks of life that may increase their risk for SIDS (21, 22). Finnegan et al. included excessive suck, respiratory distress and other gastrointestinal disturbance among the symptoms of NAS included in their tool to track the severity of NAS disease (10). Most studies of abnormal feeding related to NAS have focused on behaviors of the infant or mother. Maguire et al. (23) found the infants with NAS exhibit characteristic behaviors such as tremors, hyperextension, altered muscle tone, and multiple transitions between behavioral states that contribute to difficult feeding. Furthermore, these infants spend an excessive amount of time fussing, including averting the face from the mother, pulling or turning away or otherwise resisting, hyperextension, flailing arms or vocal objections to feeding, "but not a robust cry." LaGasse et al. (8) found that opiate-exposed infants had prolonged sucking with fewer pauses, more feeding problems such as spitting up and refusal, and increased arousal. None of these studies looked at the development of suck–swallow–breath rhythms. Gewolb et al. (9) evaluated the integration of these rhythms during feeding in infants born to mothers with drug-abuse problems. They found subtle abnormalities of respiratory control and swallow rhythmicity in drug-exposed infants. Drug-exposed infants were less efficient feeders than control infants (decreased volume/swallow), but this decreased efficiency was offset by a faster swallow rate. The differences they noted at 3 days of life between drug-exposed and unaffected control infants were not present when the infants were studied at 1 month of life.

Very few studies of NAS have focused on NNS and none have looked at the integration of SwBr during NNS. Maone et al. (24) offered cocaine-exposed infants, and control babies, a standard pacifier and a sucrose flavored pacifier. The drug-exposed cohort had about the same suck rate as control infants when offered the unflavored pacifier. However, when offered a sucrose-flavored pacifier, the cocaine-exposed cohort increased their suckle rate in a statistically significant manner.

Our previous work evaluating NNS in LRP infants supports the idea that there is an interaction between SwBr that occurs during NNS, similar to deglutition AP during nutritive feeding and that this interaction, which we identified as SwBr and POR incident to swallow, or POR, progresses in a predictable and measurable fashion (7). We hypothesized that disruptions of this progression may correlate with abnormalities of central nervous system development. Now, we turn our attention to term infants and a disease state that is known to cause feeding difficulty, suck irregularities and neurologic abnormalities.

In our primary analysis, we compared a group of term infants affected by NAS to a group of healthy infants delivered at term gestation. We found particularly pronounced differences between the infants with NAS and control infants, especially related to SwBr. The differences were less pronounced for POR. Babies with NAS had significantly more swallows occurring with CA and fewer occurring with AR than control babies. There was a nonsignificant trend toward more swallows with OA in the NAS group as well. When controlling for identified confounders with multivariate analysis, the differences in predicted distributions for CA and AR were still present. Additionally, the difference in predicted percentage of swallows at OA became statistically significant.

The only significant difference for POR was for more swallows occurring at MI. It may be more difficult to identify statistically significant differences in POR since there are more categories in which swallows are grouped, thus requiring more statistical power. Furthermore, in our previous study of LRP infants, we found that the progression of POR was affected mostly by postmenstrual age, suggesting a developmental process that was not affected by practice or learning (7). Since there is very little

TABLE 4 | Developmental follow-up of infants in control and NAS groups.

		Control	NAS	*p*-Value
12 months				
	Enrolled	12	10	
	Follow-up	11	7	
Bayley-III	Cognitive	102.5 ± 10.1	102.9 ± 12.2	0.9524
	Motor	96.2 ± 5.5	97.4 ± 15.3	0.8512
	AC	99.6 ± 9.5	102.7 ± 20.8	0.7367
PLS-IV	EC	103.8 ± 18.4	104.4 ± 16.1	0.9458
	TLS	102.3 ± 14.4	103.3 ± 18.5	0.9079
24 months				
	Follow-up	11	5[a]	
Bayley-III	Cognitive	95.3 ± 6.3	90.7 ± 5.2	0.1477
	Motor	99.7 ± 9.1	97 ± 5.7	0.4899
	AC	101.8 ± 12.6	82.2 ± 11.2	**0.0205**
PLS-IV	EC	105.5 ± 12.6	79.8 ± 12.1	**0.0081**
	TLS	104 ± 13.7	82.2 ± 12.1	**0.0086**

There were statistical differences for PLS scores at 24 months.

[a]*One infant in the NAS group at 24 months did not complete all of the assessments.*

AC, auditory comprehension; EC, expressive communication; TLS, total language score.

Boldface indicates statistical significance.

difference in the postmenstrual age for babies across this study, it is not surprising that we find only minor differences in POR.

Given that bias can be introduced into the analysis by the fact that some babies in the NAS group were studied weekly while in the hospital but control babies only had a single study, we sought to limit the effect of repeated measures by performing the analysis with a single study for each NAS baby. This decreased the number of swallows available for analysis and thus decreased the statistical power. However, the statistically significant differences for SwBr remained with more swallows from NAS infants occurring at CA and fewer occurring at AR. The difference noted in POR for MI was no longer present, but a statistical difference for BE was noted. It is possible that either, or both, of these associations may occur by chance, given the number of comparisons we are analyzing in this work. Even if these differences in the distribution of POR are real, it is easy to conclude that any variability in POR introduced by NAS is quite small compared to the differences in SwBr.

Having established that the distribution of SwBr is different for infants with NAS versus control infants, we were interested to determine if these differences were a reflection of immaturity of the suck–swallow–breath reflexes or a completely different pathological process. Thus, we compared the NAS infants to a group of LRP infants. These infants were relatively healthy aside from being born preterm, with no sepsis, no severe intraventricular hemorrhage and at low-risk for developing bronchopulmonary dysplasia. These babies were studied once per week from the onset of nipple feeding until discharge from the NICU. Interestingly, there were no statistically significant differences between the NAS and LRP groups for any of the SwBr types. There was a single significant difference in POR noted, with more swallows occurring at EE in the LRP group. Thus, it appears that the coordination of suck–swallow–breath rhythms in infants with NAS is immature, more like preterm infants, when compared to term infants.

Our previous work in preterm infants suggested that the distribution of SwBr was heavily influenced by the number of attempts the baby had to try nipple feeding, regardless of Gestational Age at delivery or Postmenstrual Age at the time of the study (7). Thus, it seems that the coordination of SwBr may be a learnable skill. Since babies with NAS do experience improved feeding over the course of their treatment, it is logical to assume that there will be an improvement in their SwBr distribution. In fact, comparing the distribution of SwBr in the total NAS group and the single-study NAS group, one can appreciate that the additional NAS studies are changing the SwBr distribution toward that of term infants. That is to say, the percentage of CA in NAS1st is 35%. With the added weekly studies, the percentage of CA is 22% in the NAS group. For control infants it is 5%. Similarly, AR changes from 39% in NAS1st to 57% in whole NAS group and 74% in control infants. Thus, we hypothesize that the distribution of SwBr in these NAS infants is approaching that of the control infants in a manner similar to that described by Gewolb et al. (9) for nutritive feeding. This also suggests that the differences noted in day-of-life, postmenstrual age, and weeks postfirst nipple feed does not account for the statistical differences in our results. Since NAS babies are older (day-of-life, postmenstrual age) and have been feeding longer (weeks postfirst nipple feed) we would predict that they would be more mature than the control group, rather than the immature pattern we have described.

Previous studies of feeding in infants with NAS have described excessive fussiness, inattention, spitting, and decreased efficiency. It is possible that the pathologic immaturity of suck–swallow–breath reflexes is contributing to the infants' overall state of dysfunction. It is unclear from this data if the immature suck–swallow–breath coordination we are describing is a function of *in utero* exposure to various toxins or occurs as a consequence of the treatment for NAS. This dataset does not allow us to answer these questions because there were variations in the specific exposures and the choice of treatment as well as the fact that all of the NAS babies were receiving medical treatment at the time of the study. If we will see a similar dysfunction with studies of nutritive feeding is also unclear.

CONCLUSION

Infants affected by NAS have an immature pattern of SwBr during NNS when compared with healthy term infants, making them more like preterm infants in this respect. There is evidence to support the idea of "catch-up" development as the distribution of SwBr approaches that of healthy term infants as the affected infants are studied subsequently over time. This is interesting because it shows that infants with NAS are not affected by a unique pathology, but rather are showing an immature pattern which improves over time. It is unclear if the dysfunction of SwBr is a consequence of the initial exposure or due to the treatment. Likewise, the improvement in SwBr may be simply related to maturation or could be due to receiving appropriate treatment. The immature pattern of suck–swallow–breath reflexes may be a contributing factor to the overall feeding difficulty experienced by babies with NAS. Future studies can focus on improving the development of suck–swallow–breath integration in infants with NAS.

ETHICS STATEMENT

Informed consent was obtained from the parent(s) of each infant prior to the infant's participation in the study. The project complies with all applicable HIPAA standards and was approved by the Institutional Review Board of the University of Kentucky.

AUTHOR CONTRIBUTIONS

ER was responsible for the project design, data collection and interpretation, and manuscript preparation. DG was responsible for subject identification and enrollment and data collection. CB was responsible for statistical methods, data analysis, and manuscript preparation.

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Plasma Brain-Derived Neurotrophic Factor Levels in Newborn Infants with Neonatal Abstinence Syndrome

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Background: Brain-derived neurotrophic factor (BDNF) is a type of growth factor that promotes growth and survival of neurons. Fetal exposure to opiates can lead to postnatal withdrawal syndrome, which is referred as neonatal abstinence syndrome (NAS). Preclinical and clinical studies have shown an association between opiates exposure and alteration in BDNF expression in the brain and serum levels in adult. However, to date, there are no data available on the effects of opiate exposure on BDNF levels in infant who are exposed to opiates *in utero* and whether BDNF level may correlate with the severity of NAS.

Objective: To compare plasma BDNF levels among NAS and non-NAS infants and to determine the correlation of BDNF levels and the severity of NAS.

Methods: This is a prospective cohort study with no intervention involved. Infants ≥35 weeks of gestation were enrolled. BDNF level was measured using enzyme-linked immunosorbent assay technique from blood samples drawn within 48 h of life. The severity of NAS was determined by the length of hospital stay, number of medications required to treat NAS.

Results: 67 infants were enrolled, 34 NAS and 33 non-NAS. Mean gestational age did not differ between the two groups. Mean birth weight of NAS infants was significantly lower than the non-NAS infants (3,070 ± 523 vs. 3,340 ± 459 g, $p = 0.028$). Mean BDNF level in NAS group was 252.2 ± 91.6 ng/ml, significantly higher than 211.3 ± 66.3 ng/ml in the non-NAS group ($p = 0.04$). There were no differences in BDNF levels between NAS infants that required one medication vs. more than one medication (254 ± 91 vs. 218 ± 106 ng/ml, $p = 0.47$). There was no correlation between the BDNF levels and length of hospital stay ($p = 0.68$) among NAS infants. Overall, there were no significant correlations between BDNF levels and NAS scores except at around 15 h after admission (correlation 0.35, $p = 0.045$).

Conclusion: Plasma BDNF level was significantly increased in NAS infants during the first 48 h when compared to non-NAS infants. The correlations between plasma BDNF levels and the severity of NAS warrant further study. These results suggest that BDNF may play a neuromodulatory role during withdrawal after *in utero* opiate exposure.

Keywords: intrauterine opiate exposure, effect of opiate exposure, neonatal abstinence syndrome, neurobehavioral outcome, brain derived neurotrophic factor

Abbreviations: BDNF, brain-derived neurotrophic factor; NAS, neonatal abstinence syndrome; CREB, cAMP response element binding; ADHD, attention-deficit hyperactive disorder; PGi, nucleus paragigantocellularis; LC, locus coeruleus; VTA, ventral tegmental area; THC, tetrahydrocannabinol.

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INTRODUCTION

Brain-derived neurotrophic factor (BDNF) is a member of neurotrophin family that is highly expressed in central and peripheral nervous system. The functional maintenance and survival of neurons depends on the availability of BDNF (1, 2). BDNF regulates neuronal survival, promotes neurite outgrowth, and maintains synaptic connectivity in the nervous system (3). BDNF has a neuromodulatory effect on learning and memory (4) and drug addiction (5, 6). BDNF plays significant role in brain synaptic plasticity (7) and locomotor sensitization (8) after opiate withdrawal. Furthermore, several studies demonstrated alterations of serum or plasma BDNF levels in drug abuser (9, 10) and implicated BDNF in the development of addiction (11).

Brain-derived neurotrophic factor is synthesized by neuronal and glial cell populations (12); however, BDNF is also expressed in several non-neuronal tissues such as immune cells and vascular endothelium (13–15). Altered levels and expression of BDNF may lead to abnormal fetal growth and brain development (16). Preclinical study reported increasing peripheral BDNF levels that were positively correlating with the cortical BDNF levels as the animal maturating from early postnatal period to young adults (17). Although there are no normative data for BDNF levels during infancy, study showed that plasma BDNF levels also increased during the early perinatal period in healthy full term infants (18). There was a wide range of peripheral BDNF levels in healthy adult human; however, BDNF levels decreased significantly with increasing age or weight and was affected by gender (19, 20). Expression of BDNF is influenced by many conditions including stress, cigarette smoking (21), alcoholic consumption (22), and depression (23). Decreased serum BDNF levels were found in adults with attention-deficit hyperactive disorder (24) and other neuropsychiatric disorders (23).

In the past decade, neonatal abstinence syndrome (NAS) has been a major health problem. There has been substantial increase in incidence of maternal opiate use and NAS leading to increased health related costs (25). NAS signs include poor sleep, high pitch cry, increased muscle tone, jitteriness, loose stool, poor feeding, etc. Prenatal opiate exposure also results in long-term deleterious consequences including behavioral problems, speech and cognitive deficits, poor social skills, anxiety, aggression, and poor fine and gross motor coordination (26, 27). To date, no reliable biomarker has been identified to predict the severity of NAS and long-term outcomes of children exposed to opiates *in utero*. However, BDNF may be a good candidate biomarker. Opiate exposure is known to induce apoptosis, downregulate cAMP response element binding expression, and decrease in dendritic branching and spine density, and BDNF protects neurons against these effects (28). Opiate withdrawal can affect serum BDNF level (29) and BDNF expression in the brain (30). Decreased BDNF expression and protein in the brain is associated with behavioral, learning, and memory problems (12, 24, 31). Previous study using a rat model found that opiate exposure compromised memory, increased anxiety levels, and decreased BDNF precursors in the hippocampus (31). Although these studies suggested the likely effects of *in utero* opiate exposure on the BDNF level and its role in long-term neurobehavioral outcome, to date, there has been no published study on the correlation of NAS/opiate exposure on the levels of BDNF in human infants. Therefore, in the present study, we aimed to assess possible changes in serum BDNF levels in opiates exposed infants compared to unexposed infants and to determine the correlation of BDNF levels with the severity of NAS.

MATERIALS AND METHODS

This prospective cohort study was performed on 68 infants born at ≥35 weeks of gestation age (GA) and admitted to Kentucky Children's Hospital Neonatal Intensive Care Unit (NICU) and Newborn Nursery between 2015 and 2016 in Lexington, KY, USA. Inclusion criteria for NAS infants were infants born to mother with history of opiate intake or urine drug screen (UDS) test positive during pregnancy and were admitted in NICU for withdrawal symptoms. These infants were born in University of Kentucky or transferred from outside hospital within 1 week of life. Infants unexposed to opiates by history and/or UDS negative were enrolled in non-NAS group. The exclusion criteria were neonates with major congenital anomalies, infants of mothers <18 years of age, infant transferred from outside hospital after 1 week of life, infant born at GA < 35 weeks, infant who are critically ill, parental refusal to consent, and parents unavailable to consent. The study was approved by the University of Kentucky Institutional Review Board.

Parents of infants who met the inclusion criterion were identified and approached. The informed consent was obtained from the parents. Blood samples, 1.2 ml, were collected from all the subjects at 48 h of life in the non-NAS group and within the first 48 h after the admission to the NICU in the NAS group during the regular blood draw for lab work. Plasma was separated by centrifugation and stored in −80°C till all samples were collected. Measurement of plasma level of BDNF was performed by an enzyme-linked immunosorbent assay (ELISA) method using the human BDNF kit (RayBio Human BDNF ELISA, RayBiotech Inc., GA, USA), according to the manufacturer's instructions. Plasma samples were diluted 1:100 for BDNF measurement. All BDNF measurement was performed in duplicate. Both NAS and non-NAS were run together in the same plate. The BDNF content was expressed as nanogram (ng) of human recombinant BDNF protein per milliliter of plasma.

We followed the clinical practice guideline for NAS treatment for all NAS infants. NAS scoring using the Finnegan Scale was performed every 3 h after admission, equaled to total of 16 time points in the first 48 h after admission. Opiate replacement therapy with morphine sulfate was started at 0.05 mg/kg, q3 h was started when met the criteria: 3 consecutive scores each ≥9 or 2 consecutive scores ≥13. Morphine dose was increased by 25% of the prior dose if the consecutive scores still met the above criteria and symptoms not captured until reached the maximum dose of 0.12 mg/kg/dose. Second medications were added if the symptoms were still not under controlled with scores met the criteria despite being on the maximum morphine dose based on the history; clonidine for opiate use, phenobarbital for barbiturate use, and diazepam for benzodiazepine use. Morphine weaning was started if the scores remained at desired limits for at least

48 h by 0.02 mg per dose q 24–48 h. The infants were monitored for at least 48 h after morphine was discontinued before discharge. If the infants were also on second medications, they were discharged home on tapering doses with close follow-up at the NICU graduate clinic.

Statistical Analysis

An independent sample *t*-test was conducted to compare the BDNF levels between NAS group and non-NAS group. A value of $p \leq 0.05$ was considered to indicate significance. We also studied the correlation between BDNF levels and the severity of NAS among infants exposed to opiates *in utero*. The severity of NAS was defined by the length of stay in hospital and whether more than one medicine was needed to treat withdrawal symptoms. Pearson correlation test was performed to study the possible association between BDNF level and length of stay. SAS version 9.4 (SAS Institute, Cary, NC, USA) was used for analyses.

RESULTS

Total of 67 infants were enrolled, 34 in NAS group and 33 in non-NAS group. One infant from NAS group withdrew from study by the parents.

Mean gestational age did not differ between NAS (38.5 ± 1.3 weeks) and non-NAS groups (39 ± 1.2 weeks), $p = 0.84$ (**Table 1**). Mean birth weight of the NAS infants was significantly lower than the non-NAS infants, 3,070 ± 523 vs. 3,340 ± 459 g, $p = 0.028$ (**Table 1**). Mean BDNF level was significantly higher in NAS infants compared to non-NAS infants (252.2 ± 91.6 vs. 211.3 ± 66.3 ng/ml, difference 41 (2, 80) $p = 0.04$) (**Figure 1**).

The area under the receiver operating characteristic curve (ROC) curve is estimated to be 0.641. Based on the minimum Euclidean distance using the ROC curve, the estimated optimal cutoff BDNF level for predicting groups is 240. Using this cutoff value, estimated sensitivity is 61.7%, specificity is 60.6%, positive predictive value is 61.7%, and negative predictive value is 60.6%.

Among the NAS group, 29 infants required one medication whereas 4 infants required two medication for the treatment of NAS, 1 infant did not required any medication for the treatment. There were no differences in BDNF levels between NAS infants who required one medication vs. more than one medication [254 ± 91 vs. 218 ± 106 ng/ml, difference 36 (−64, 137), $p = 0.47$]. The correlation between the BDNF levels and length of hospital stay among NAS infants was not detected using Pearson Correlation (correlation 0.07, $p = 0.68$) (**Figure 2**).

In the first 48 h after admission for NAS, there were no significant correlations between the BDNF levels and NAS scores at 15 of the 16 time points (Spearman Correlation 0.31 to −0.21, $p = 0.07$–0.95). However, there was a marginally significant correlation between BDNF levels and NAS scores at time point 5 (around 15 h after admission) (Spearman correlation 0.35, $p = 0.045$). There were no significant correlations between BDNF levels and maximum scores ($p > 0.05$), and the means or the total scores in the first 48 h of admission ($p = 0.43$). The distribution of the NAS scores significantly changed over time based on Friedman's test ($p < 0.0001$) as depicted in **Figure 3**.

Infants in the NAS group were also exposed to other substances, which include cocaine, benzodiazepines, tetrahydrocannabinol (THC), tobacco, amphetamine, methamphetamine, and gabapentin. When BDNF levels among infants with NAS were compared across levels in the given variables, there was no significant difference (**Table 2**).

TABLE 1 | Baseline characteristics.

	Neonatal abstinence syndrome (NAS)	Non-NAS	*p*-Value
	***n* = 34**	***n* = 33**	
Maternal age (years), mean (SD)	27 (5)	29 (6)	0.1
Gestational age (weeks), mean (SD)	38.5 (1.3)	39 (1.2)	0.84
Gender: Male (%)	21 (62%)	18 (54.5%)	
Female (%)	13 (38%)	15 (45.5%)	
Birth weight (g), mean (SD)	3,070 (523)	3,340 (459)	**0.028**
APGAR: 1 min, median (range)	9 (8–9)	8 (6–9)	NS
5 min, median (range)	9 (8–9)	9 (8–9)	NS

Mean birth weight of the NAS infants was significantly lower than the non-NAS infants, 3,070 ± 523 vs. 3,340 ± 459 g, p = 0.028.
NS, not significant.

DISCUSSION

To our knowledge, this is the first study on plasma BDNF level in infants exposed to opiates *in utero*. Our results showed that plasma BDNF level was significantly increased in NAS infants in early withdrawal phase compared to non-NAS infants. There were no statistically significant correlations between plasma BDNF levels and the severity of NAS based on the length of hospital stay or the number of medications needed for treatment. The BDNF levels did not correlate with NAS scores at any given time point except at around 15 h after admission for NAS, at which point, most of the infants had been started on morphine. The

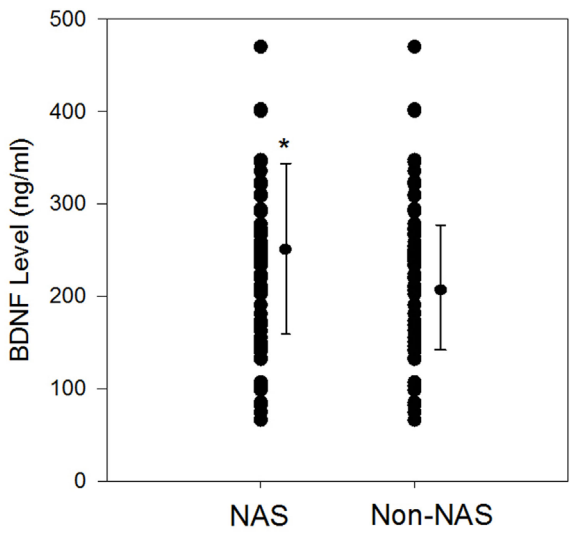

FIGURE 1 | Scatter plots and mean plasma brain-derived neurotrophic factor (BDNF) (±SD) levels (ng/ml) among neonatal abstinence syndrome (NAS) infants during withdrawal phase compared to non-NAS; *$p = 0.04$.

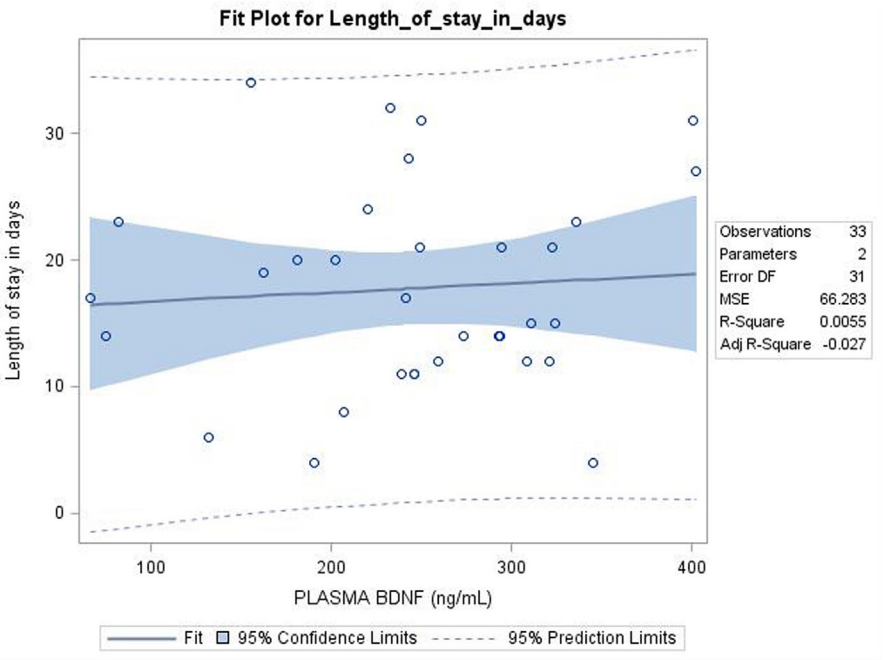

FIGURE 2 | Pearson correlation showed no relation between Plasma Brain derived neurotrophic factor (BDNF) level (ng/ml) and length of stay among neonatal abstinence syndrome infants; $p = 0.68$.

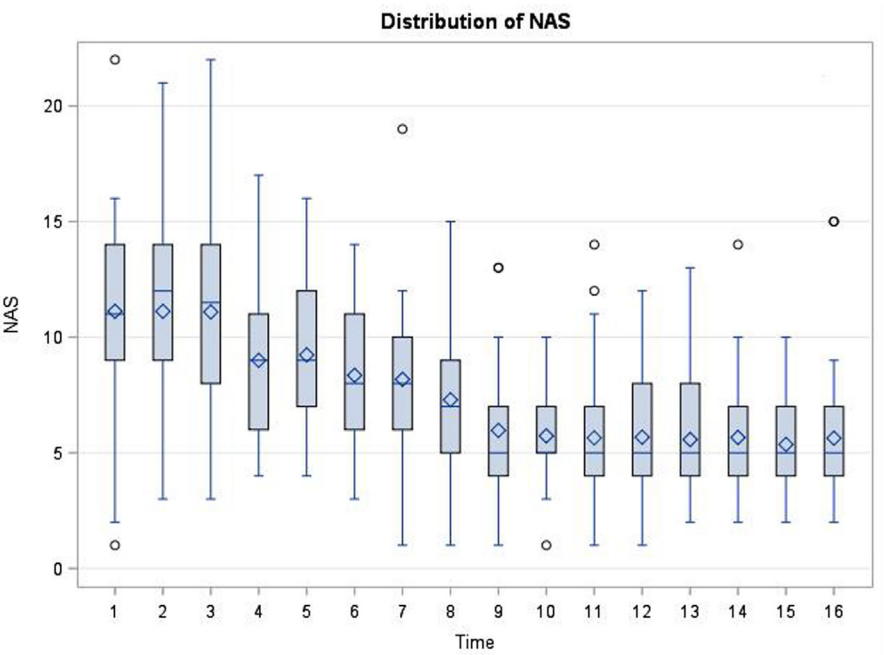

FIGURE 3 | Distribution of neonatal abstinence syndrome (NAS) scores at each time point (every 3 h, total of 16 time points) in the first 48 h after admission for NAS. The scores significantly changed over time based on Friedman Test ($p < 0.0001$).

NAS Scores distribution became less dispersed over time after being treated as could be expected. Based on the data, we did not find the cutoff BDNF level that will provide good sensitivity or specificity to predict NAS.

The lower birth weight in NAS infants was consistent with the effect of opiates on the birth weight previously described in the literature (32), but whether this factor may contribute to the higher BDNF levels in this group remains to be further

TABLE 2 | Brain-derived neurotrophic factor levels among infants with neonatal abstinence syndrome and comparisons across levels in the given variables.

Variable	Yes	No	Difference
Tobacco	244 ± 80 (n = 28)	289 ± 138 (n = 6)	44 (–39, 128), p = 0.29
Alcohol	No use	No use	
Cocaine	223 ± 101 (n = 4)	256 ± 91 (n = 30)	33 (–67, 133), p = 0.51
Benzodiazepine	292 ± 79 (n = 7)	242 ± 93 (n = 27)	–51 (–129, 28), p = 0.20
Tetrahydrocannabinol	248 ± 93 (n = 10)	254 ± 93 (n = 24)	6 (–66, 77), p = 0.88
Other medications	218 ± 93 (n = 15)	279 ± 83 (n = 19)	61 (–1, 123), p = 0.053
Hepatitis C	269 ± 82 (n = 19)	230 ± 101 (n = 15)	–39 (–103, 25), p = 0.06

Mean ± SD (n).

elucidated. The existing data regarding the effects of low birth weight/intrauterine growth restriction on the BDNF levels are still conflicting; at term, the BDNF levels of infants with IUGR were not different from those born appropriate for gestational age (AGA) (33) while the BDNF levels were lower in very preterm infants with severe growth restriction (34).

Our results regarding the increased BDNF levels were in line with previous preclinical and clinical studies. Chronic opiate exposure and acute withdrawal induced upregulation of BDNF genes expression in the nucleus paragigantocellularis in rats (30). Similarly, serum BDNF levels in heroin addicts were higher at the baseline and remained higher than in control subjects after 1 month of heroin cessation (29). Serum BDNF levels correlate well with changes in cortical BDNF levels (17) and measurement of the peripheral BDNF levels may reflect BDNF concentrations in the central nervous system (CNS) and processes in the CNS in opiate-use disorders (35). We, therefore, may postulate that the increase in plasma BDNF level during this early phase of NAS could indicate the upregulation of the BDNF gene expression in the CNS. Together, these support the concept that BDNF might play a critical role in NAS. BDNF plays an important role in the opiate-induced plasticity of noradrenergic locus coeruleus neurons (36), which is implicated in pathogenesis of addiction and withdrawal in adult (37). Additionally, increased BDNF expression may counteract the effect of chronic opiate exposure on the neurons; chronic opiate administration contributed to biochemical and morphological changes in ventral tegmental area (VTA), and some of these changes in VTA were prevented or reversed by the infusion of BDNF into this brain region in rat (38, 39). Taken together, the increased plasma BDNF level in our study is perhaps reflective of the increased BDNF expression in the CNS as a compensatory response to neuronal insult.

Brain-derived neurotrophic factor levels can be influenced by various factors commonly found among substance users such as smoking (21), alcoholic consumption (22), and various neuropsychiatry disorder, which includes depression, anxiety, bipolar disorder, and schizophrenia (23). Recent study by Ghassabian et al. reported that smoking and drinking during pregnancy was associated with lower neonatal BDNF levels (40). In attempt to control for these factors, infants in the control group were not exposed to cigarette smoking during pregnancy. Infants in both groups were not exposed to alcohol during pregnancy. In addition, we compared the BDNF levels among the infants in NAS group with the given variables including maternal smoking, maternal use of other substances including cocaine, benzodiazepine, THC and other neuropsychiatric medications, maternal hepatitis C infection; we found no differences in the BDNF levels in these small subgroups.

Besides the CNS and peripheral nervous system, BDNF is synthesized in other tissues including vascular endothelium and immune cells (13–15). In addition to fetal and neonatal synthesis, maternal passage and placenta synthesis can contribute to the difference in BDNF levels in the newborn (41–43). Thus, it is possible that these factors also contributed to the increased BDNF level in our study.

Our study had certain limitations including maternal polysubstance use as mentioned, inconsistent timing of the blood draw depending on when the infants were admitted for NAS management, and some infants received initiation of treatment with morphine before blood draws; these factors could affect the BDNF levels. Larger sample size is warranted for the study in the future to be able to control for these confounders. Our plasma BDNF levels had a wide distribution consistent with the literature (18); however, the means were higher than previously reported by others. This could be due to different assays used and perhaps the samples had clotted, therefore, became serum samples, which reported to give much higher BDNF levels (44).

In summary, we observed that serum BDNF levels were increased in NAS infants during early withdrawal phase when compared to non-NAS infants. These results suggested that increased serum BDNF levels might be associated with the pathophysiology of opiate exposure and withdrawal in the neonates. Serial measurement of plasma BDNF levels during the withdrawal phase in infants with NAS and during developmental follow-up of these infants would be vital to further understand the role of BDNF in NAS and the outcomes of these infants.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of University of Kentucky, Institutional Review Board with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Institutional Review Board.

AUTHOR CONTRIBUTIONS

LS: designed, conducted the study, analyzed data, and wrote the manuscript. HH: performed the ELISA and helped with analysis of the data. AP: helped collecting data, edited the manuscript. PW: biostatistician, analyzed the data. HB: helped with the design and analysis of the study, edited the manuscript. JB and PG: helped in the design of the study and lab. TS: main adviser for LS, helped with the design, conduction and analysis of the study, and wrote and edited the manuscript.

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EEG Findings in Infants With Neonatal Abstinence Syndrome Presenting With Clinical Seizures

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Neonatal abstinence syndrome (NAS) refers to a constellation of signs occurring in newborn infants who were exposed to opioids or opiates *in utero*. These manifestations include poor feeding, gastrointestinal disorders, abnormal sleep patterns, and neurological signs such as jitteriness, tremors, and seizures (1, 2). Myoclonus, jitteriness, and tremors often may be interpreted as seizures and therefore treated as epileptic seizures.

Objective: To determine whether seizure like activity observed in infants with NAS correlate with electroencephalogram (EEG) findings.

Design/ Method: We reviewed the standard EEG or video electroencephalogram (VEEG) of infants with NAS who were admitted because of seizure-like clinical activity. The exclusion criteria were major neurological anomalies, hypoxic ischemic encephalopathy, metabolic disorders, or with clinical diagnosis other than NAS.

Results: Forty neonates met study criteria; 28 had standard EEG recordings and 18 had VEEG. Mean gestational age was 38.5 weeks. The onset of seizure-like clinical activity was as early as day 1 and as late as day 16 of life. The clinical seizure-like activity described at the referring hospital were jerking, rhythmic movement of the extremities, or tremors. Only three (7.5%) neonates had epileptic seizures. There were increased sharp transients in frontal, central, temporal, and or occipital regions. VEEG showed disturbed non-rapid eye movement (REM) sleep with frequent arousal, jittery movements, or sleep myoclonus.

Conclusion: Clinical seizure-like activity correlates poorly with epileptic seizures in infants with NAS. In neonates with NAS, a VEEG would be useful to determine if the clinical seizure-like activity is of epileptic origin or not, prior to initiation of anti-seizure medications.

Keywords: seizures, neonatal abstinence syndrome, electroencephalography, prenatal opiate exposure, sleep myoclonus

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INTRODUCTION

Neonatal abstinence syndrome (NAS) is a clinical diagnosis, and a consequence of the abrupt discontinuation of chronic fetal exposure to substances, specifically opiates or opioids used by the mother during pregnancy. The prolonged half-life of opioids in the infants and the ease with which opioids can cross the fetal blood-brain barrier of the fetus may increase the severity of

withdrawal manifestations in infants with NAS (3). Furthermore, accumulation of the drug in the fetus may result in the increased production of various neurotransmitters through a cascade of enzymatic activities (4). Locus coeruleus is the most important center of activity in opioid withdrawal. This is the principal noradrenergic nucleus of the brain and is extremely sensitive to opioid status (5), i.e., withdrawal of the inhibitory effect of opiate on the adrenergic neurons results in the hyperactivity of these neurons (6). Most infant with NAS manifests with central nervous system irritability, autonomic over reactivity, and gastrointestinal tract dysfunction. Central nervous system manifestations reported with NAS are tremors, exaggerated Moro reflex, hypertonia, and myoclonic jerks (7). These can mimic seizures and therefore, an EEG may be required for confirmation prior to treatment.

Of neonates with NAS, 2–11% manifest with seizures (8). The underlying cause of seizures in NAS is not known, although the threshold for seizure activity may be decreased due to upregulation of sodium channels as a result of receptor instability (9). NAS may involve a short intense initial phase, consisting of tremors, seizures, irritability, feeding problems, vomiting, diarrhea, hyperthermia, and other systemic signs which lasts for upto 1–2 weeks. Later initial phase may be followed by a long chronic and relapsing course that includes hyperirritability, sleep disturbances, hyperphagia, and other neurologic and autonomic signs (lasting for a few weeks to a few months) (10). NAS symptoms depend on the type of drug exposure. In intrauterine opioids exposure, such as heroin is associated with earlier onset and shorter duration of withdrawal, compared to methadone or buprenorphine, which is associated with late onset and prolonged withdrawal. In the nonopioids class, methamphetamine exposure is associated with early withdrawal symptoms (11).

The jerking movement in NAS may be interpreted as seizures, thus an indication for anti-convulsant therapy. However, these jerking movements may represent benign neonatal sleep myoclonus (BNSM), which has been reported in neonates with NAS (1). Benign neonatal sleep myoclonus (BNSM) is a phenomenon typically observed in newborns during the first 4 weeks of life. This condition is not associated with abnormal EEG. BNSM does not need treatment and does not affect neurodevelopmental outcome (12–14).

Since clinical observation of myoclonus, jitteriness, or tremors can be misinterpreted as epileptic seizures, it prompted us to explore the association between myoclonus, jitteriness, tremors, and or seizure-like activity and EEG findings in babies with NAS.

METHODS

This retrospective study had approval by the Institutional Review Board (IRB) and met compliance with the Health Insurance Portability and Accountability Act. The IRB waived informed consent procedure for this study. Inclusion criteria included admission to the neonatal intensive care unit, history of intrauterine opioid exposure with or without other substances, seizure-like activity reported as a clinical manifestation, and the infant having had routine EEG or VEEG. Opioid exposure refers to a mother admitting use during pregnancy or had a positive urine test for opioids or the baby had positive urine or meconium drug test. Seizure-like manifestation was described as jerking or rhythmic movement of extremities, myoclonus during sleep, apnea, and/or tremors. The exclusion criteria were major neurological anomalies, hypoxic ischemic encephalopathy, metabolic disorders, or with clinical diagnosis other than NAS. Interval assessment or monitoring of these infants used the Finnegan Neonatal Abstinence Scoring System (2). Oxygen saturation monitored by pulse oximetry, blood glucose; sodium, potassium, and calcium were monitored. All infants were monitored with Finnegan scoring system. If 3 consecutive scores were $\geq$8 or 2 consecutive scores of $\geq$12, infants were treated with morphine escalated and weaned according to the unit protocol. If infants had electrographic seizures, they were treated as per our neonatal intensive care unit (NICU) guidelines.

The EEG and VEEG were reviewed by one of the investigators (GK), a pediatric epileptologist. Routine EEG was done for 1 h and VEEG was done for 48 h. The Xltek EEG/PSG System (Natus Neuro Inc., Pleasanton, CA) was used for both EEG and VEEG; it has components needed for EEG and VEEG. The EEG and VEEG waveforms were assessed for the background activity, sharp transients, and electrographic seizures. Background activity is considered normal if there is no focal slowing and no prolonged "trace alternant" (in term neonates 6–8 s is normal). Sharp transients are normally present in newborn infants but disappear by 50 weeks of post conceptional age. Sharp transients that are repetitive in same area with slow background and increased frequency are considered abnormal (15). Neonatal electrographic seizure refers to a sudden, repetitive, evolving, and stereotyped event of abnormal electrographic pattern with amplitude of at least 2 μV and a minimum duration of 10 s (16). Descriptive statistics were used and no EEG scoring system was used.

RESULTS

Forty neonates with NAS were admitted to our level IV neonatal intensive care unit (NICU) for seizure-like manifestation during January 2010- November 2015. The patient characteristics are shown in **Table 1**. There were 23 males and 17 females. The mean (SD) gestational age was 37.5 (1.9) weeks and mean (SD) birth weight was 2,843 (620) grams. All infants were exposed to tobacco during pregnancy and of whom 29 infants were exposed to opioids only. The remaining 11 infants were exposed to more than one drug (marijuana, benzodiazepines, methamphetamines, and/or gabapentin).The onset of seizure-like manifestation was as early as day 1 and as late as day 16 of life, mean (SD) of 4.1 (4) days. Twenty-seven neonates were treated for seizure-like clinical sign at the referring hospital before EEG was performed; 23 were treated with phenobarbital, two with morphine, and one with levetiracetam. In these

TABLE 1 | Patient characteristics.

Total number of subjects	40
Males	23 (57.5%)
Females	17 (42.5%)
Mean gestational age (SD)	37.5 weeks (1.9)
Mean birth weight (SD)	2,843 g (620)
Intrauterine exposure to single drug	29 (72.5%)
Intrauterine exposure to poly drugs	11 (27.5%)
Day of life onset of reported seizure, mean (SD)	4.1 (4)
Day of life onset of reported seizure, median (range)	2.5 (1–16)
Number of Pts treated for clinical seizures with anti-seizure medications before admission/EEG	23 (57.5%)
Not treated for clinical seizures	17 (42.5%)

TABLE 2 | Electroencephalographic findings in infants with neonatal abstinence syndrome observed to manifest with clinical seizures.

Routine EEG and VEEG ($n = 40$)	***n*** (%)
BACKGROUND ACTIVITY	
Normal	29 (72.5)
Mildly slow	11 (27.5)
SHARP TRANSIENTS	
Fronto- centro-temporal (FCT)	21 (52.5)
Fronto centro temporal occipital (FCTO)	8 (20)
Centro temporal (CT)	7 (17.5)
Centro temporal occipital (CTO)	2 (5)
No transient spikes	2 (5)
SEIZURES	
No seizures	37 (92.5)
Seizures present	3 (7.5)
VEEG ($n = 18$)	
Benign neonatal sleep myoclonus (BNSM)	13 (72)
Increased arousal pattern	5 (28)

patients, seizure like clinical activity were described as jerking or rhythmic movement of extremities, myoclonus during sleep, apnea, and/or tremors. Based on review of the EEG or VEEG, only three neonates had epileptic seizure. Findings on EEG are shown in **Table 2**. Most of the neonates (72.5%) had normal background activity and 27.5% had mildly slow activity. Three neonates (7.5%) with epileptic seizures also had mildly slow background activity. There were sharp transients in frontal, central, temporal, and or occipital regions. Additionally, in the 18 (45%) neonates who had continuous video monitoring, detailed review showed disturbed non-REM sleep with frequent arousal pattern, jittery movements, and sleep myoclonus. BNSM was seen in 13/18 (72%) neonates and 5/18 (28%) had increased arousal pattern. Oxygen saturation monitored by pulse oximetry, blood glucose, sodium, potassium, and calcium were within normal limits (**Table 3**).

TABLE 3 | Oxygen saturations and biochemical parameters on admission.

Parameters	**Mean and standard deviation (SD)**
Oxygen saturations, %	96.65 (1.57)
Blood glucose, mg/dL	81 (16.69)
Sodium, mmol/L	139 (3.38)
Potassium, mmol/L	4.7 (0.72)
Calcium, mmol/L	9.4 (0.94)

DISCUSSION

We report EEG findings in neonates with NAS, who were admitted for clinical seizure like activity. The neurological examination was normal, except for the irritability, tremors, and increased tone, which are not unusual findings in babies with NAS.

Herzlinger and co-investigators reported an incidence of 5.9% of documented seizures in neonates with NAS (8). In our study, of infants with NAS who presented with seizure-like activity, majority (92.5%) had no electroencephalographic seizures; only three or 7.5% had seizures consistent with the EEG pattern. Our findings suggest that seizures if confirmed by EEG studies occur at a much lower incidence than in previous reports based on clinical findings. Our findings on EEG were consistent with disturbed non-REM sleep with frequent arousal, Jittery movements, or sleep myoclonus (1, 17). Benign neonatal sleep myoclonus or (BNSM) is a distinctive disorder characterized by rhythmic myoclonic jerks, which occur when the child is asleep and stop when the child is awakened. BNSM may appear on the first day of life and persist for several weeks to months. BNSM is often mistaken for epilepsy but the EEGs are always normal. The pathophysiological mechanism of BNSM is not fully elucidated (18). It may a result of a benign discharge in the reticular activating system, where the initiation and synchronization of normal sleep is controlled (19). Another possibility is that BNSM is related to transient immaturity or imbalance of the serotoninergic system (20). Because of the spontaneous resolution with age, BNSM it is likely related to brain maturation. Previously reported clinical seizures in NAS could at least in part have been myoclonic jerks (12). An earlier study showed around 67% of infants with NAS and had EEG procedure had BNSM (1). In our study of 18 neonates with VEEG, BNSM was seen in a comparable incidence (13/18 or 72%). Five or 28% had increased arousal pattern. In children and adults, the most specific interictal electrographic signs of the seizure activity are spikes and sharp waves. The neonatal situation is more complex, since some sharp transients are commonly seen in their EEG (21). Further, in healthy neonates, sharp EEG transients usually appear against a normal background and which do not signify the presence of encephalopathy nor support the presence of seizures. The clinical significance of sharp wave transients on neonatal electroencephalographic (EEG) recordings remains controversial (22). Rowe et al. stressed that EEG background abnormalities are a more accurate independent predictor of neurodevelopmental

outcome rather than sharp wave transients. In our study there were increased sharp transients in frontal, central, temporal, and occipital regions, but with normal background activity (23). EEG background activity was normal in 72.5% of our patients; none of them had electrical seizures. Eleven or 27.5% had mildly slow pattern background; the three with electrical seizures were among those with mildly slow background activity.

Few studies examined sleep in NAS. Opiate-exposed newborns have shown a significant increase in active sleep and a reciprocal decrease in quiet sleep than controls (23–26). O'Brien and Jeffery reported on a reduction of quiet sleep in infants with NAS compared to their control group (8). Also, adult and preclinical studies demonstrated that sleep is altered in opiate withdrawal (27–30). The mechanisms behind sleep disturbances during NAS remain unclear, but could be due to changes to the central nervous system (CNS) during opiate dependency *in utero*, and due to hyperactivity of the CNS during withdrawal (23, 25). In our study, the neonates who had VEEG, 28% did have increased arousal pattern.

LIMITATIONS

Our study merely focused on infants with NAS admitted for seizure-like clinical manifestation. VEEG was not performed in all study subjects. The small number of infants in our study precluded analysis of any correlation between the EEG findings and the type of opioids or other drugs to which the infants were exposed. The impact of medication used to treat NAS (or seizures) on the EEG was not analyzed.

CONCLUSION

Clinical manifestation of seizure-like activity correlates poorly with epileptic seizures in infants with NAS. In infants with a history of intrauterine drug exposure mainly opioids presenting with seizure like activity with no other medical conditions, obtaining a video EEG would be ideal to determine if the seizure-like manifestation is of epileptic origin, before starting anti-seizure medication. If available, a VEEG is a preferred modality to be able to correlate abnormal movements with electroencephalographic findings.

ETHICS STATEMENT

Study was approved by University of Kentucky Institutional Review Board.

AUTHOR CONTRIBUTIONS

MP reviewed the charts, EEG along with pediatric neurologists. ZH did the chart review and EEG review. GK did review of EEG's. HB guided through the project.

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Decreasing Total Medication Exposure and Length of Stay While Completing Withdrawal for Neonatal Abstinence Syndrome during the Neonatal Hospital Stay

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Introduction: Neonatal abstinence syndrome (NAS) is a rapidly growing public health concern that has considerably increased health-care utilization and health-care costs. In an effort to curtail costs, attempts have been made to complete withdrawal as an outpatient. Outpatient therapy has been shown to prolong exposure to medications, which may negatively impact neurodevelopmental and behavioral outcomes. We hypothesized that the implementation of a modified NAS protocol would decrease total drug exposure and length of stay while allowing for complete acute drug withdrawal during the neonatal hospital stay.

Methods: Data were derived retrospectively from medical records of term (≥37 0/7) infants with NAS who were treated with pharmacologic therapy in the University of Louisville Hospital Neonatal Intensive Care Unit from 2005 to 2015. The pharmacologic protocol (SP1) for infants treated between 2005 and March 2014 (n = 146) dosed oral morphine every 4 h and utilized phenobarbital as adjuvant therapy. Protocol 2 (SP2) initiated after March 2014 (n = 44) dosed morphine every 3 h and used clonidine as adjuvant therapy. Charts were reviewed for demographic information and maternal drug history. Maternal and infant toxicology screens were recorded. The length of morphine therapy and need for adjuvant drug therapy were noted. Length of stay was derived from admission and discharge dates.

Results: The length of morphine therapy was decreased by 8.5 days from 35 to 26.5 days (95% CI 4.5–12 days) for infants treated with SP2 vs. SP1 ($p < 0.001$). The need for adjuvant pharmacologic therapy was decreased by 24% in patients treated with SP2 vs. SP1 ($p = 0.004$). The length of stay was decreased by 9 days from 42 to 33 days (95% CI 5.1–13 days) for infants treated with SP2 vs. SP1 ($p < 0.001$). The decreased length of stay resulted in an average reduction of hospital charges by $27,090 per patient in adjusted 2015 US Dollars.

Conclusion: This study demonstrates that total drug exposure and length of stay can be reduced while successfully completing acute withdrawal during the neonatal hospital stay.

Keywords: neonatal abstinence syndrome, length of stay, neonatal, drug withdrawal, protocol, infant, newborn

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INTRODUCTION

Perinatal opiate abuse is a rapidly expanding public health concern that has markedly increased health-care costs and utilization. Neonatal abstinence syndrome (NAS) occurs in the exposed infant after the abrupt cessation of maternal opiates at birth and accounts for a substantial proportion of the health-care burden (1, 2). The national incidence of NAS increased from 3.4 to 5.8 per 1,000 hospital births between 2009 and 2012; Neonatal Intensive Care Unit (NICU) admissions for NAS increased four-fold between 2004 and 2013 (1, 3). Regional and state variation in the incidence of NAS has been found to cause disproportionate burden on highly affected areas such as the southeastern United States (3, 4).

Clinically significant symptoms appear in 55–94% of opiate exposed neonates (5). The onset and severity of symptoms are impacted by the sum total of fetal exposure, the type(s) and purity of drug(s) consumed during pregnancy, maternal and infant metabolism, genetic and epigenetic factors, and variability in the kinetics of placental drug transfer (6–11). Infants who require pharmacologic therapy to treat withdrawal symptoms are cared for in the NICU in many regions of the US (1). The percentage of NICU days attributed to NAS increased from 0.6% in 2004 to 4% in 2013 (1). The proportion of NAS infants who require pharmacotherapy has also increased from 74% in 2004–2005 to 87% in 2012–2013 (1). The severity of withdrawal is assessed with observer-rated scales. The most commonly used scale is the Finnegan Score for Neonatal Abstinence Syndrome (FNAS) (5). FNAS assesses 21 clinical symptoms of drug withdrawal, which allows for a thorough evaluation (6). However, it is a lengthy tool that requires extensive training and experience to ensure accurate scoring (12). Variability in the application of FNAS complicates care and prolongs hospitalization for infants with NAS (13–17).

The surge in the number of infants diagnosed with NAS, variability in the assessment of symptoms and increased need for pharmacologic treatment of affected infants has intensified the financial impact of the disease. National aggregate hospital charges reached $1.5 billion in 2012 (3). State Medicaid programs are financially responsible for 80% of the cost for the treatment of NAS (3). Implementation of measures to stunt the growing economic impact of NAS while ensuring the short- and long-term safety and development of affected infants is crucial. Primary prevention measures could markedly decrease the incidence of NAS (4). Unfortunately, such measures require diligence and compliance from the dependent mother in conjunction with her health-care providers, which are often difficult to attain. Secondary interventions including pharmacologic and non-pharmacologic therapy for the withdrawing neonate remain variable and best practice has yet to be established (13, 18).

In 2014, a group of neonatologists (including the primary author), nurse practitioners, and neonatal nurses from the regional referral centers in Kentucky were tasked with standardizing pharmacologic treatment for affected infants throughout the state. Subsequently, a new treatment protocol was developed based on the best-available evidence. In Kentucky, infants requiring pharmacologic care for acute opioid withdrawal are typically cared for in an NICU. We hypothesized that implementation of the state-vetted protocol would decrease postnatal drug therapy by decreasing the total days of morphine therapy and the need for adjuvant therapy. In addition, we expected that a decrease in pharmacologic therapy would improve the length of stay and reduce hospital costs. All infants evaluated in this study were treated in the NICU of a single inner-city hospital between 2005 and 2015, and all infants completed pharmacologic therapy before discharge.

MATERIALS AND METHODS

The University of Louisville Institutional Review Board approved this retrospective cohort study before study initiation. Data were derived from medical records of infants treated in the University of Louisville Hospital Neonatal Intensive Care Unit (Louisville, KY, USA) from 2005 to 2015. NICU log books were used to identify term infants (greater than or equal to 37.0/7 weeks gestation) with NAS. Charts were reviewed for demographic information including sex, gestational age, and birth weight. Maternal drug history and maternal and infant toxicology screens were recorded (Roche Cobas Integra 800). Pharmacologic intervention, including the length of morphine therapy and need for adjuvant drug therapy, was obtained. Length of stay was derived from admission and discharge dates for each infant.

Infants cared for in the NICU between 2005 and March 2014 ($n = 146$) were treated with a locally generated protocol (SP1—**Figure 1**), and infants treated after March 2014 ($n = 44$) were treated with the state-generated protocol (SP2—**Figure 2**). Both treatment protocols used morphine as the first pharmacologic agent; however, morphine dosing was every 4 h in SP1 and every 3 h in SP2. Weaning of the morphine was also more aggressive in SP2 vs. SP1. In addition, infants treated with SP1 received phenobarbital as adjuvant therapy if symptoms were not well controlled, on high dose oral morphine while infants treated with SP2 received clonidine.

Differences in birth weight and gestational age between treatment groups were assessed with a Student's *t*-test, and differences in sex between groups were assessed with a χ^2 test (goodness of fit) SPSS version 22. ANCOVA (R version 3.1.2) was used to evaluate the impact of each protocol on the length of morphine therapy and length of hospital stay. The model controlled for birth weight, gestational age, sex, and fetal exposure to heroin, methadone, buprenorphine, multiple opiates, and benzodiazepines. Exposure was determined by maternal history and/or maternal and infant toxicology screens. Interactions between *in utero* drug exposure and the protocol were evaluated with backwards elimination multiple regression. The need for adjunctive therapy in the infants treated with each protocol was assessed with a with a χ^2 test (goodness of fit) SPSS version 22. For all analyses, statistical significance was set at $p < 0.05$.

RESULTS

The primary outcome measure was length of morphine therapy. Secondary outcomes were need for adjunctive therapy and length of stay. Demographic data are presented in **Table 1**. No statistically

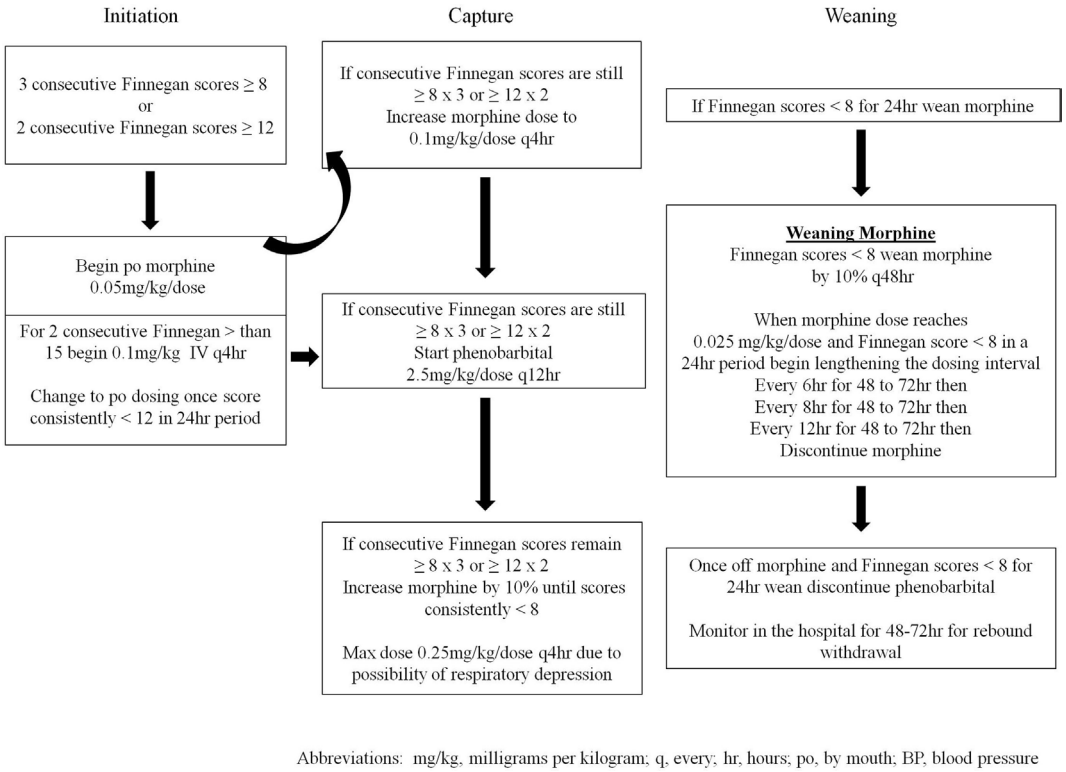

FIGURE 1 | Protocol 1 (SP1) for the initiation and treatment of neonatal abstinence syndrome.

significant differences were noted in the gestational age, weight, or sex between infants treated with SP1 or SP2.

The average length of morphine therapy was decreased for infants treated with SP2 by 8.5 days from 35 to 26.5 days (95% CI 4.5–12 days) when compared with infants treated with SP1 ($p < 0.001$) (**Figure 3**). The need for adjunctive pharmacologic therapy was decreased by 24% in patients treated with SP2 vs. SP1 ($p = 0.004$) (**Figure 4**). The average length of stay for infants treated with SP2 was decreased by 9 days from 42 to 33 days (95% CI 5.1–13 days) when compared with infants treated with SP1 ($p < 0.001$) (**Figure 3**). The decrease in length of stay was calculated to result in an average reduction of hospital charges by $27,090 per patient (adjusted 2015 US Dollars) using national averages for per day charges derived by Patrick et al. (3). The use of multiple opiates and benzodiazepines was not found to be statistically different between protocols (**Table 2**).

One relevant and significant interaction was noted within the backwards elimination multiple regression between methadone and protocol 2 (SP2). While the average difference in length of stay for all infants was 8.5 days when comparing SP2 vs. SP1, infants exposed to methadone *in utero* had a 1-day reduction in the benefit (e.g., an average difference of 7.5 vs. 8.5 days).

DISCUSSION

In this cohort of infants from a single inner-city hospital, we found that medication exposure and length of stay were decreased with the implementation of a state endorsed standardized pharmacologic treatment protocol. This finding lends additional support to the efficacy and cost effectiveness of standardized treatment for infants with NAS (5, 19–23). It also highlights the importance of statewide collaborative efforts in enhancing short-term outcomes and reducing hospital costs.

All of the infants in our cohort were exposed *in utero* to opioids; the type(s) of exposure was variable as depicted in **Table 2**. Total fetal exposure was wide ranging and difficult to quantify due to maternal misreporting and limitations in maternal and infant toxicology screens. Fetal exposure to multiple opiates and multiple drugs of abuse, specifically benzodiazepines and tobacco, is known to exacerbate the severity of withdrawal (24). In our data analyses, we controlled for exposure to multiple opiates and benzodiazepines and did not find a statistically significant impact on the effectiveness of the protocol. Previous studies have shown that infants exposed to methadone require a longer length of treatment than those exposed to buprenorphine and other short acting opiates such as heroin (14). We found that infants exposed to methadone *in utero* and then treated with SP2 vs. SP1 had an average length of morphine therapy and length of stay that was 1 day greater than infants exposed to other opiates during pregnancy ($p = 0.003$). In other words, infants exposed to methadone do not receive as much benefit from the new protocol as infants not exposed to methadone. Additional research is needed to evaluate the efficacy of tailoring treatment protocols to *in utero* exposure.

Prenatal exposure to opiates, especially long acting opiates such as methadone, has been shown to decrease myelination in the developing rat brain and in a small cohort of exposed infants (25, 26). The endogenous opioid system is crucial to

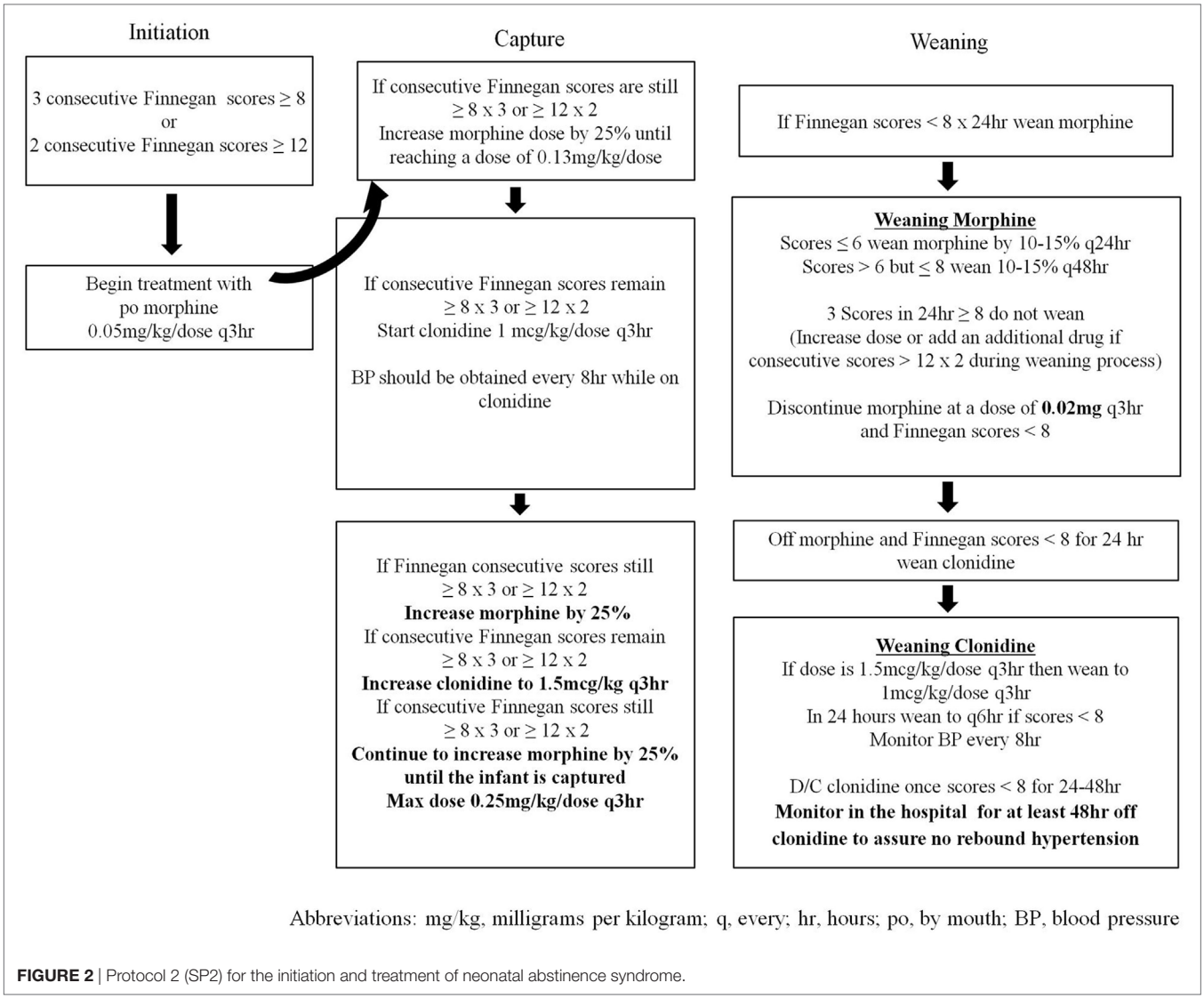

FIGURE 2 | Protocol 2 (SP2) for the initiation and treatment of neonatal abstinence syndrome.

TABLE 1 | Demographics of study infants.

	Protocol 1 (SP1) (n = 146)	Protocol 2 (SP2) (n = 44)	p-Value
Birth weight (kg)	3.01 ± 0.46	2.93 ± 0.39	0.07[a]
Gestational age (weeks)	39.0 ± 1.1	38.7 ± 1.3	0.21[a]
Male (%)	57.2	59.1	0.82[b]

[a]*p-Values calculated with t-test.*
[b]*p-Value calculated with χ^2 test of independence.*

ensure appropriate oligodendrocyte development and proper myelination. *In utero* exposure to exogenous opiates inhibits endogenous opioid production and may decrease myelination in the brain (26). *In utero* opioid exposure has also been correlated with decreased brain volumes and suboptimal long-term developmental outcomes (25, 27, 28). Little is known about the specific impact of postnatal opioids on the developing brain. Given the negative neurological outcomes of intrauterine exposure, every effort should be made to decrease the duration of postnatal opioid treatment. In our study infants treated with SP2 had an average of 8.5 fewer days of postnatal opiate therapy than infants treated with SP1. Although the impact was modest, we were able to demonstrate a decrease in total postnatal opioid exposure.

Adjunctive therapy has been used to decrease the duration of opioid treatment during acute neonatal withdrawal. Clonidine and phenobarbital are the most commonly used agents. Phenobarbital has been noted to decrease the severity of opioid withdrawal and reduce length of stay and cost of hospitalization (19, 29, 30). Of note, infants enrolled in these studies were discharged home on phenobarbital. Many infants remained on the medication for weeks to months after the initial hospital discharge (19, 24, 29). Phenobarbital acts on the gamma-aminobutyric acid receptors in the central nervous system inducing sedative effects. Extended treatment with phenobarbital may negatively impact neurodevelopmental and behavioral outcomes (31). Clonidine has been evaluated in several small studies and has been shown to be a safe and efficacious adjunctive therapy for infants with NAS. The use of clonidine in the pharmacologic management of

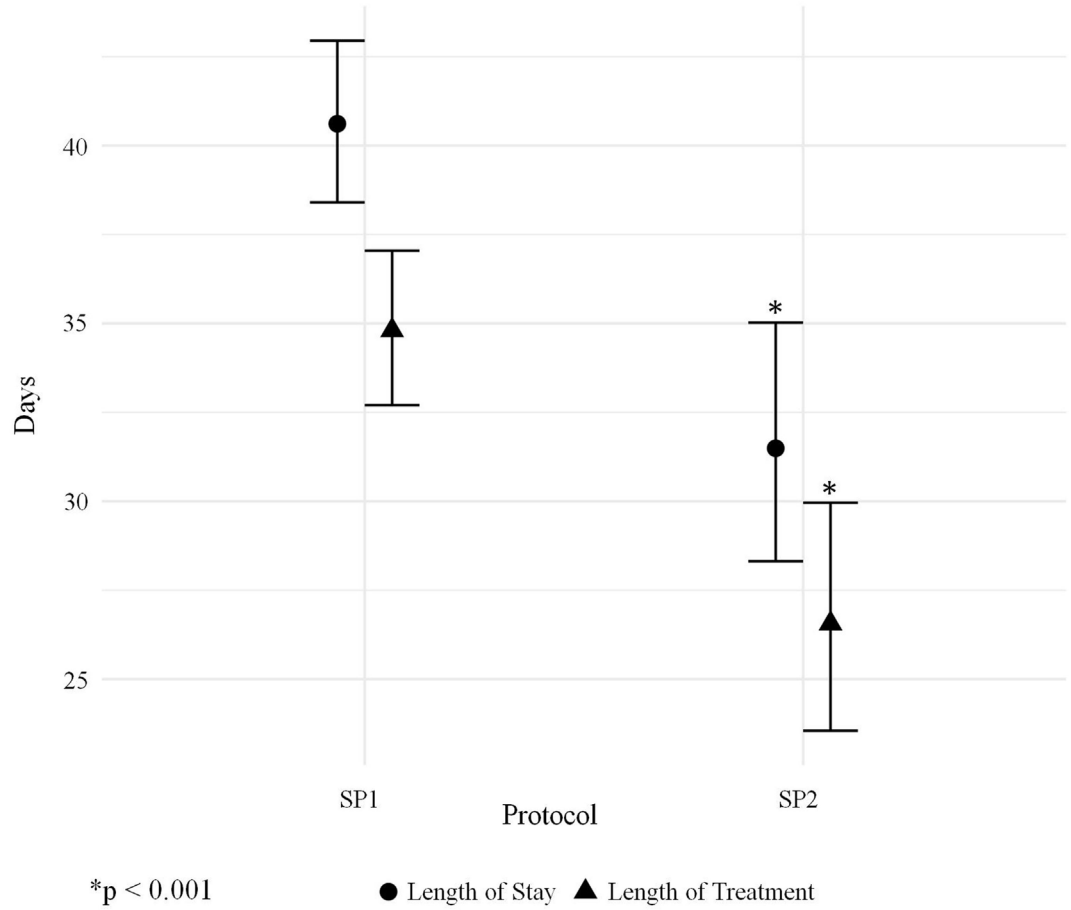

FIGURE 3 | Adjusted means for length of morphine therapy and length of stay.

infants undergoing acute opiate withdrawal has been associated with a decrease in the duration of treatment and length of stay (32–35). Yet, it should be noted that the impact of clonidine on developmental outcomes remains unknown. In view of the uncertain long-term impact of both commonly used adjuvant medications, treatment with a single opioid would likely prevent potential side effects and decrease the chance of harm. We found that simply transitioning morphine from every 4 to every 3 h led to a 24% reduction in the need for adjuvant therapy.

The rapid growth of *in utero* opiate exposure and NAS over the last decade has stretched health-care resources and escalated health-care costs. Between 2000 and 2009, national estimates for total hospital charges attributed to NAS increased from $190 million to $720 million (2). Inflation-adjusted mean hospital charges for infants requiring pharmacologic therapy for NAS in 2012 reached $93,400 per patient (3). State Medicaid programs bear the majority of the financial burden and marked variability exists between states (4). Using data from Patrick et al (3), a mean extrapolated daily charge for NAS pharmacologic treatment is approximately $3,010.00 (2015 adjusted dollars). When extrapolated costs were applied to our study population, the use of SP2 decreased hospital charges by $27,090 per patient. When applied to the 44 patients treated with SP2, the average decrease in hospital charges approached $1.19 million. Prolongation of the hospital stay due to child protective services (CPS) interventions may have affected the differences in length of stay. We did not collect data on incidence of CPS engagement and thus cannot determine the effect of this parameter on our outcomes.

In this study, the implementation of a pharmacologic treatment protocol developed by a statewide collaboration led to a decrease in total opioid exposure, need for adjunctive therapy, and length of stay while completing acute withdrawal during the neonatal hospitalization. Completing pharmacologic therapy before initial hospital discharge will decrease total medication exposure and has the potential to decrease harm in a population that is known to be at increased risk for adverse post-discharge outcomes (32, 36).

The results of this study focus exclusively on the impact of modifying the pharmacologic treatment protocol. It is well known that the severity of opioid withdrawal is dependent upon and affected by a multiplicity of factors. The impact of environment, breastfeeding, and parental engagement cannot be under estimated (33–35, 37–40). The development of a comprehensive evidence-based treatment protocol will require consideration of

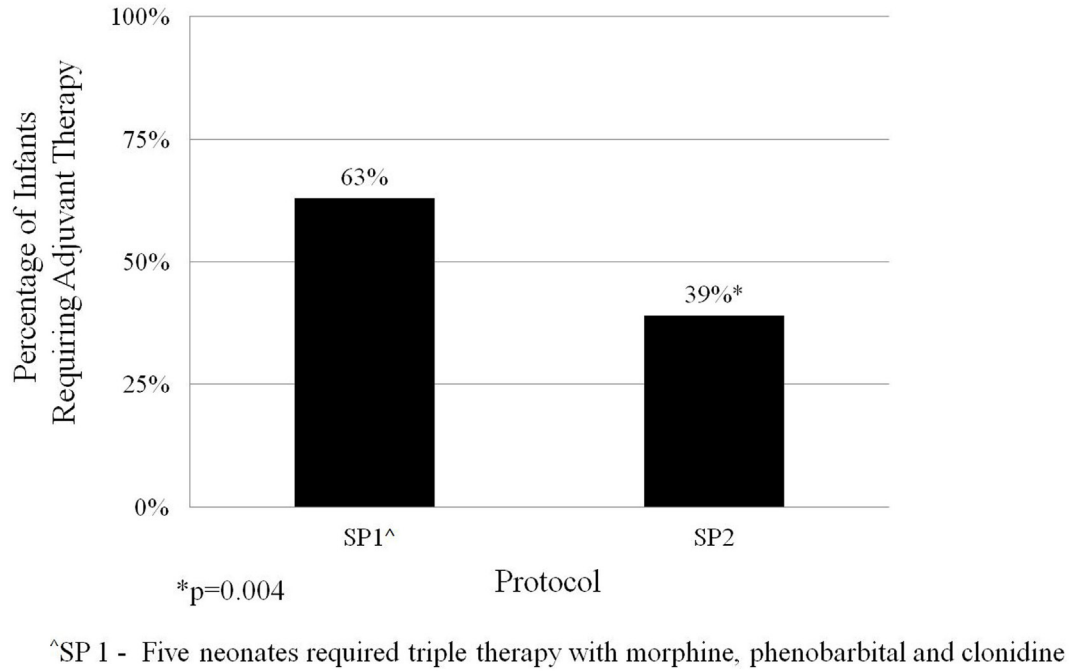

FIGURE 4 | Adjuvant therapy for neonatal abstinence syndrome by protocol.

TABLE 2 | Fetal drug exposure to opioids and other drugs of abuse.[a]

	Total	Protocol 1 (SP1)	Protocol 2 (SP2)
	Number (%) (*n* = 190)	Number (%) (*n* = 146)	Number (%) (*n* = 44)
Opioids			
Buprenorphine	18 (9)	9 (6)	9 (20)
Heroin	24 (12)	13 (8)	11 (25)
Hydrocodone	29 (15)	28 (19)	4 (9)
Hydromorphone	4 (2)	2 (1)	2 (5)
Methadone	74 (39)	61 (42)	13 (30)
Morphine	11 (6)	3 (2)	8 (18)
Oxycodone	47 (24)	43 (29)	4 (9)
Oxymorphone	5 (3)	4 (3)	1 (2)
Unspecified opiates	88 (46)	67 (46)	21 (48)
Other drugs of abuse			
Amphetamines	11 (6)	8 (5)	3 (7)
Barbiturates	2 (1)	2 (1)	0
Benzodiazepines	20 (10)	18 (10)	2 (5)
Cocaine	26 (14)	19 (13)	7 (15)
Gabapentin	2 (1)	2 (1)	0
Marijuana	18 (9)	14 (10)	4 (9)
Methamphetamine	4 (2)	2 (1)	2 (5)

[a]*Fetal exposure was determined through maternal history, and urine toxicology screens combined with neonatal urine and meconium toxicology screens.*

all impacting factors. We acknowledge that the study is limited in its retrospective design and thus is subject to bias and inaccuracies within the medical record. We also realize that only the impact of the entire treatment protocol can be assessed with this study design. In addition, patients treated with SP1 were collected over a 9-year period during which trends in maternal usage and drugs of abuse may have varied from current patterns of abuse during pregnancy. Subtle changes in neonatal care over the last 11 years could have impacted on the short-term outcomes for affected infants.

AUTHOR CONTRIBUTIONS

LD—principal investigator and study designer. She approved the final version to be published. TL—substantial contributions to the design of the work and was involved in the analysis and interpretation of the data for the work and in revising the work critically for intellectual content. He approved the final version to be published. PR—substantial contributions to the design of the work and was significantly involved in the revising the work critically for important intellectual content. She approved the final version to be published. All the authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

ETHICS STATEMENT

This study is a retrospective chart review of infants admitted to the Neonatal Intensive Care Unit at the University of Louisville Hospital. The study was approved by the IRB at the University of Louisville.

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MINI REVIEW
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The Genomics of Neonatal Abstinence Syndrome

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Significant variability has been observed in the development and severity of neonatal abstinence syndrome (NAS) among neonates exposed to prenatal opioids. Since maternal opioid dose does not appear to correlate directly with neonatal outcome, maternal, placental, and fetal genomic variants may play important roles in NAS. Previous studies in small cohorts have demonstrated associations of variants in maternal and infant genes that encode the μ-opioid receptor (*OPRM1*), catechol-*O*-methyltransferase (*COMT*), and prepronociceptin (*PNOC*) with a shorter length of hospital stay and less need for treatment in neonates exposed to opioids *in utero*. Consistently falling genomic sequencing costs and computational approaches to predict variant function will permit unbiased discovery of genomic variants and gene pathways associated with differences in maternal and fetal opioid pharmacokinetics and pharmacodynamics and with placental opioid transport and metabolism. Discovery of pathogenic variants should permit better delineation of the risk of developing more severe forms of NAS. This review provides a summary of the current role of genomic factors in the development of NAS and suggests strategies for further genomic discovery.

Keywords: genomics, opioids, neonatal abstinence syndrome, precision medicine, treatment

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INTRODUCTION

An epidemic of neonatal abstinence syndrome (NAS) currently exists due to dramatic increases in prenatal opioid exposure. Significant variability has been noted in the incidence and severity of NAS among neonates exposed to prenatal opioids. The promise of precision medicine has prompted increasing interest in discovery of genomic variants that could better predict the development and severity of NAS and suggest new therapeutic approaches for both prevention and treatment (1–3). Although the prevalence of NAS has remained stable between 1997 and 2011 in England and Australia (4), NAS has increased significantly in the United States over the past several years from 7 cases/1,000 neonatal intensive care unit admissions in 2004 to 27 cases/1,000 admissions in 2013 (5). The economic (now >$1.5 billion per year in the United States), emotional, and social burdens associated with NAS are substantial due to the frequent need for prolonged hospitalizations and the increased risk of adverse long-term neonatal neurodevelopmental outcomes (6, 7). While most neonates developing NAS respond well to treatment with opioids, a subset of exposed infants will require additional pharmacological therapy (e.g., phenobarbital, clonidine) and longer hospitalizations (1, 8–10). Multiple environmental factors such as maternal socioeconomic status and prenatal exposure to other illicit and prescription drugs contribute to neonatal outcome. Precision medicine initiatives involving genomic-based

prediction of NAS severity are particularly attractive due to previous associations between individual genomic variants and outcome, poor predictive accuracy of most clinical and demographic factors, and rapidly falling sequencing costs (2, 11, 12). Epigenetic regulation of genes associated with maternal and fetal drug metabolism and placental drug metabolism and transport may also contribute to disease severity and have been reviewed previously (13).

ASSOCIATIONS OF GENOMIC VARIANTS WITH NAS RISK AND SEVERITY

Based on twin studies, opioid addiction is heritable (14). This heritability has prompted case–control studies that have examined the association of several single-nucleotide polymorphisms (SNPs) in genes known to be involved in opioid metabolism and addiction with length of hospitalization, need for pharmacologic treatment, and total days of opioid treatment in neonates with NAS (12) (**Table 1**). Results suggest that SNPs in *both* mother and infant are associated with severity of NAS (2, 15–17). For example, in studies of opioid-exposed newborn infants and their mothers (2, 15), SNPs in maternal and infant genes that encode the μ-opioid receptor (*OPRM1*), catechol-*O*-methyltransferase (*COMT*), and prepronociceptin (*PNOC*) were associated with differences in neonatal outcomes (length of hospitalization and reduced need for treatment with two medications). While the SNPs chosen for study are known to have important physiologic roles in opioid drug response and withdrawal in adults, the effect sizes of these SNPs have been modest and have not had a significant impact on clinical practice (2, 16). In addition, differences in allele frequencies suggest that the use of a limited number of SNPs as biomarkers of disease severity will require race-/ethnicity-based stratification and assessment of admixture (**Table 1**). Functional annotation and prediction programs may permit improved risk evaluation of individual SNPs (18) and comparison of the contributions of maternal and infant drug metabolism to risk of NAS. Finally, the complexity of the genomic architecture, developmental regulation of gene expression, and the contributions of maternal medications and confounding diagnoses will require further refinement of SNP-based strategies before they can be used clinically.

FUTURE DIRECTIONS FOR GENOMICS OF NAS RESEARCH

Extension of current SNP-based studies of the genomics of NAS may benefit from leveraging available, diverse large exome and genome databases (e.g., http://gnomad.broadinstitute.org/) to evaluate the predicted function and frequency of both common and rare SNPs in genes known to be associated with heritable addiction or alterations in drug metabolism. Falling sequencing costs will permit complete genomic resolution for discovery of clinically actionable genomic variants in individual mother/infant dyads. This approach should avoid the need to rely on linkage disequilibrium or imputation. Unbiased whole exome or genome sequencing would permit the evaluation of the contributions of rare variants in both recognized and unrecognized genes to NAS severity. The statistical challenges associated with rare variant discovery may be partially addressed by using family study design (e.g., twin studies) to reduce the contributions of highly diverse genomic backgrounds to phenotype and multiple confounding co-variables present in case–control studies. Examining infants with the

TABLE 1 | Genes associated with NAS phenotype.

Gene	SNP	Genotype	Associated NAS phenotype	Annotation	CADD score[c]	Allele frequencies		
						EA	Afr	Latino
OPRM1	rs1799971	G allele[a]	Less likely to receive treatment; shortened length of stay	Missense Asn133Asp	24.1	0.13	0.03	0.21
COMT	rs4680	G allele[a]	Less likely to receive treatment with two or more medications; shortened length of stay	Missense Val158Met	11.4	0.48	0.69	0.6
	rs4680	G allele[b]	Less likely to receive treatment with two medications		11.4			
	rs740603	A allele[a]	Less likely to receive pharmacological treatment; shortened length of stay	Intronic	0.2	0.49	0.56	0.61
	rs740603	A allele[b]	Less likely to receive any pharmacological treatment		0.2			
PNOC	rs351776	A allele[a]	More likely to be treated with two medications; longer length of stay	Intronic	1.3	0.44	0.78	0.69
	rs351776	A allele[b]	More likely to receive treatment with two medications; longer length of stay		1.3			
	rs2614095	A allele[a]	More likely to be treated with two medications	Intronic	2.9	0.46	0.11	0.27
	rs4732636	A allele[a]	Less likely to receive medication treatment	Upstream	6.3	0.28	0.1	0.36
OPRK1	rs702764	C allele[a]	More likely to receive treatment with two medications	Synonymous	0.4	0.13	0.49	0.28
OPRD1	rs204076	A allele[b]	More likely to receive treatment with two medications; longer length of stay	Downstream	5.6	0.66	0.83	0.82
DRD2	rs1799732	Deletion	Less likely to require treatment with medication	Upstream	N/A	0.1	0.5	0.12

[a]*Infant allele.*

[b]*Maternal allele.*

[c]*Combined annotation-dependent depletion (17).*

EA, European–American; Afr, African; N/A, not applicable (frame shift).

extremes of NAS severity may also improve statistical power to identify relevant genomic variants (19). A review of copy number and structural variant databases to identify individuals who are haploinsufficient for specific genes might also provide a strategy to evaluate functional impact of loss of function alleles (20). Standard phenotyping that incorporates predictions of functional impact on the metabolism of opioids, as has been proposed for the cytochrome P450 genes, along with currently accepted measures of NAS severity may also be useful (21, 22). Developmental regulation of relevant drug metabolizing and placental transport genes will likely require model systems for testing gestational age-specific effects of individual variants (23, 24). Finally, development of statistical approaches that incorporate confounding variables like breastfeeding, smoking, and use of protocol-driven medication weaning strategies (25) will be important to compare the contributions of genomic variants with these kinds of clinical variables. Application of precision medicine to the problem of NAS could benefit the many adults and neonates exposed to opioids by providing individualized risk prediction and leveraging discovery of candidate genes for development of novel therapeutic strategies (3, 12). For example:

(A) If we can establish high-risk genetic profiles in young women, can we then limit opioid exposure?
(B) Can we establish low-risk genetic profiles in young women and safely wean them off their medication-assisted treatment without an increased risk of relapse?
(C) If high-risk profiles are found in pregnant women, can we intervene and modify risk factors such as limiting cigarette smoking and polypharmacy?
(D) With high-risk profiles identified in neonates, could we administer low doses of opioids immediately after birth and limit the development and severity of NAS?
(E) If lower risk profiles are identified in neonates, could we send them home earlier and carefully follow them up as an outpatient?
(F) Can pharmacogenomics identify optimal treatment strategies (i.e., best practices) for the mother and her neonate?
(G) If genetic risk factors can be combined with clinical and demographic variables, can we develop "risk assessment" models that can be applied to neonates, older children, and adults to determine the risk of addiction following exposure to opioids (e.g., low, medium, high)?

These are some of the potential benefits of a more precision medicine-guided approach that could be adopted with rapid development of novel genomics methodologies. What is most important is sufficient funding, and legislative efforts are currently driving this innovation and offer a path forward to address NAS in this highly vulnerable population.

AUTHOR CONTRIBUTIONS

FC and JD conceptualized and drafted the entire manuscript; DW contributed data analysis.

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REVIEW
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Judging the Neonatal Abstinence Syndrome Assessment Tools to Guide Future Tool Development: The use of Clinimetrics as Opposed to Psychometrics

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In the face of the current Neonatal Abstinence Syndrome (NAS) epidemic, there is considerable variability in the assessment and management of infants with NAS. In this manuscript, we particularly focus on NAS assessment, with special attention given to the popular Finnegan Neonatal Abstinence Score (FNAS). A major instigator of the problem of variable practices is that multiple modified versions of the FNAS exist and continue to be proposed, including shortened versions. Furthermore, the validity of such assessment tools has been questioned, and as a result, the need for better tools has been suggested. The ultimate purpose of this manuscript, therefore, is to increase researchers' and clinicians' understanding on how to judge the usefulness of NAS assessment tools in order to guide future tool development and to reduce variable practices. In short, we suggest that judgment of NAS assessment tools should be made on a clinimetrics viewpoint as opposed to psychometrically. We provide examples, address multiple issues that must be considered, and discuss future tool development. Furthermore, we urge researchers and clinicians to come together, utilizing their knowledge and experience, to assess the utility and practicality of existing assessment tools and to determine if one or more new or modified tools are needed with the goal of increased agreement on the assessment of NAS in practice.

Keywords: Finnegan Neonatal Abstinence Score, formative model, Neonatal Abstinence Syndrome Score, predictive accuracy, reflective model

INTRODUCTION

The number of infants developing Neonatal Abstinence Syndrome (NAS) is reaching epidemic proportions with increasing number of affected infants receiving pharmacotherapy and increasing length of stay in hospital (1–4). Therefore, there has been much research recently addressing the assessment and management of infants with NAS. Unfortunately, there is considerable variability in practice with respect to both of these aspects (2, 5–8). Inherently, this implies an apparent lack of agreement among researchers and clinicians in terms of best methods.

In this manuscript, we focus on the assessment of infants with NAS. Multiple assessment tools have been developed and are in use, with the Neonatal Abstinence Syndrome Score, also known as the Finnegan Neonatal Abstinence Score (FNAS), being the most commonly used (5, 7, 9–12). However, the FNAS has been modified multiple times and is not used in exactly the same manner at every institution (5, 13). For instance, the MOTHER NAS score (MNS) (14, 15) made considerable revisions to the FNAS and may be increasing in popularity.

Due to the existence of multiple assessment tools that are implemented in practice, it is apparent that researchers and clinicians have not come to a common agreement on how to best assess NAS. Therefore, we address how to view the theoretical modeling framework for NAS from which assessment tools arise. Specifically, we contrast viewpoints based on psychometric (16–21) and clinimetric (17–19, 22) principles, concluding that NAS assessment tools should be viewed clinimetrically. We hope that conveying this modeling framework will give researchers and clinicians a realistic construct on how to properly judge NAS assessment tools, as utilizing a psychometric viewpoint will only lead to the conclusion that existing tools are invalid (21, 22). Ultimately, we hope that with this increased understanding on how to view NAS assessment, future work on creating or modifying assessment tools will lead to improved and standardized NAS assessment and thus management.

In the following section, we give relevant details on theoretical models corresponding to psychometric and clinimetric viewpoints, respectively, with regards to NAS assessment. We argue that a clinimetric-based formative model (17–19, 22) should be preferred, and then discuss how to judge tools and give examples from the literature. Finally, we provide a discussion on future tool development, followed by concluding remarks.

PSYCHOMETRIC- AND CLINIMETRIC-BASED MODELS FOR NAS ASSESSMENT

Psychometrics—Reflective Model

Psychometric properties are regularly emphasized in practice (22), and hence, a reflective model (18, 19) is often used as the default to determine the validity of an assessment tool. **Figures 1** and **2** demonstrate a generic reflective model and an assumed reflective model for NAS, respectively. Applying this model to NAS, it is assumed that NAS causes each item to present, and therefore, items should be a reflection of NAS and thus should be highly correlated; i.e., items should have good internal consistency or reliability (17–19). As a result, equal weighting of items is preferred (18, 23).

Clinimetrics—Formative Model

Generic and NAS-based representations of formative models arising from clinimetrics-based reasoning are given in **Figures 3** and **4**, respectively. With this model, the items are not required to be correlated, and each item contributes, with potentially varying magnitudes, toward NAS severity (22). Specifically, the weights depicted in **Figures 3** and **4** can vary in value according to item impact, and the weighted items are summed to form a modeled severity of NAS. **Figure 4** specifically depicts the FNAS, which applies different numerical weighting for crying, general convulsions, mottling, etc. due to their perceived differing influences on NAS severity. In practice, the modeled severity may not be the true severity, implying a degree of discrepancy, also known as the disturbance, between the model and the truth. See, for instance (17–19), for specific details on formative models.

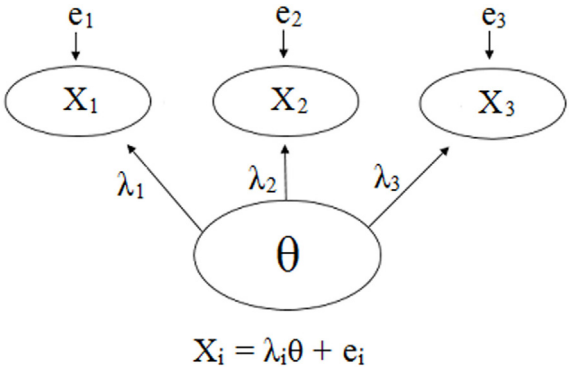

FIGURE 1 | Visual and mathematical representations of a generic psychometrics-based reflective model in which items X_1, X_2, and X_3 are caused by and hence are reflections of the hypothetical construct θ. Each item has its own equation in which the influence of θ is denoted by λ, and the items may have measurement error, *e*. The items are expected to be strongly correlated due to the influence θ has on each one. Note that the number of items is not restricted, and a scenario with three items was chosen for simplicity.

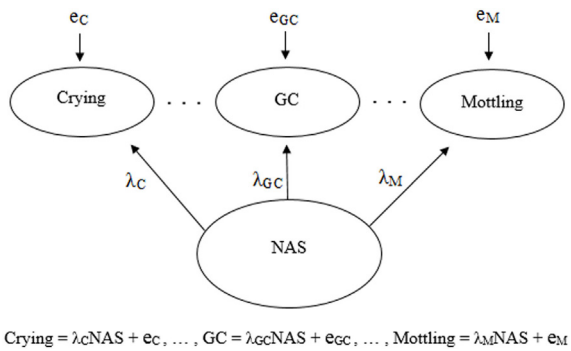

FIGURE 2 | Visual and mathematical representations of a psychometrics-based reflective model in which crying, general convulsions (GC), mottling, etc. are caused by and hence are reflections of neonatal abstinence syndrome (NAS). Each symptom has its own equation in which the influence of NAS is denoted by λ, and the symptoms may have measurement error, *e*. The symptoms are expected to be strongly correlated due to the influence NAS has on each one.

Which Theoretical Modeling Framework Works Best for Judging NAS Assessment?

Table 1 provides a concise comparison of reflective and formative models. Evidence has shown that NAS assessment tools do not

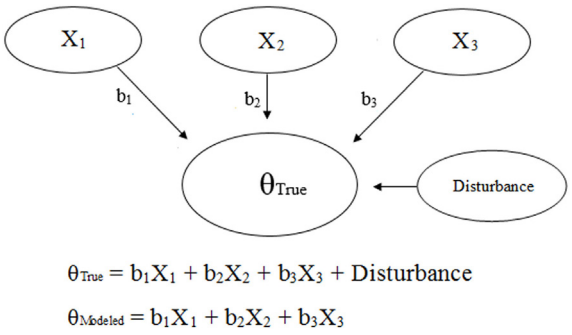

FIGURE 3 | Visual and mathematical representations of a generic clinimetrics-based formative model in which items X_1, X_2, and X_3 combine to form the true hypothetical construct θ_{True}. The disturbance term represents the influence of all other factors besides X_1, X_2, and X_3 on θ_{True}. The numerical weights applied to the items in the assessment tool are represented by b_1, b_2, and b_3. Note that the number of items is not restricted, and a scenario with three items was chosen for simplicity.

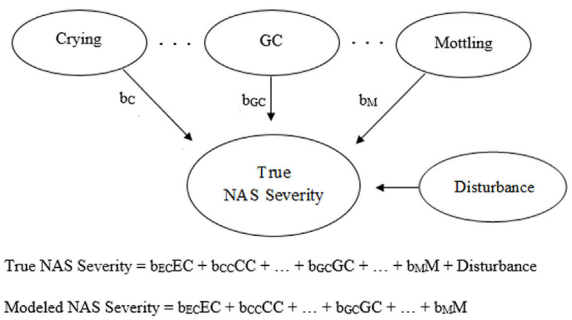

FIGURE 4 | Visual and mathematical representations of a clinimetrics-based formative model in which crying (EC, excessive crying; CC, continuous crying), general convulsions (GC), mottling (M), etc. combine to form Neonatal Abstinence Syndrome (NAS) severity. The disturbance term represents the influence of all factors not accounted for by the given assessment tool. The numerical weights applied to the corresponding items in the assessment tool are represented by b_{EC}, b_{CC}, b_{GC}, and b_M. In the visual representation, b_C is equivalent to either b_{EC} or b_{CC}, depending on the severity of crying.

TABLE 1 | A comparison of reflective (psychometrics) and formative (clinimetrics) models.

Aspect	Reflective model	Formative model
Item role	All items are influenced by and, therefore, reflect Neonatal Abstinence Syndrome (NAS)	All items build up NAS severity
Item correlation	All items are highly correlated (internal reliability)	Items do not need to be correlated
Item weighting	Equal weighting	Unequal weighting, depending on perceived impact the item has on NAS severity

coincide with a reflective model. Admittedly, NAS can initially be thought of in terms of a reflective model, as NAS causes symptoms, and thus items in the FNAS, for instance, to present. However, the occurrence, severity, and duration of symptoms are quite variable across and within infants (13, 14, 21, 24), and thus do not reflect NAS to the same degree. As a result, Jones et al. (20) found *via* low Cronbach's alphas on the FNAS and MOTHER NAS scale that not all items are highly correlated, and Bada et al. (25) also found *via* odds ratios that not all items are highly interrelated. We note that Cronbach's alpha is a measure of the degree of correlation among a group of items in order to assess internal consistency (26).

The signs of NAS, or symptoms if truly arising from NAS, build up NAS severity. Furthermore, clinical reality is that certain symptoms are more critical than others when determining the severity of NAS, and these symptoms and their severity may not highly correlate. Therefore, the formative modeling approach should be preferred as it provides a clinically meaningful basis on which NAS assessment tools can be judged. This approach also provides flexibility in that information other than symptom severity can be employed.

JUDGING NAS ASSESSMENT *VIA* A FORMATIVE MODELING FRAMEWORK

General Approach

An ideal approach to obtaining a NAS assessment tool based on a formative approach would be to conduct an empirical study utilizing a large amount of clean or robust data from the target population, and to use that data to determine which items should be incorporated within the resulting tool and how much weight to assign each item. The primary interest in using tools to assess NAS severity is to determine when pharmacologic treatment is needed. Therefore, if we were able to obtain in this hypothetical study accurate data on when pharmacologic treatment is needed, logistic regression modeling could be employed to obtain estimated weights (see mathematical models presented in **Figures 3** and **4**) which indicate the importance of each item. The resulting tool could then be used on a separate large dataset of clean or robust data in order to judge and (hopefully) validate the predictive accuracy of the tool. An example of such an approach is the derivation of the Score for Neonatal Acute Physiology—Perinatal Extension (27).

Unfortunately, there is no way to determine true NAS severity and thus the need to treat, and therefore, we cannot judge tools based on their true predictive accuracy. As a result, assessment tools will need to be judged based on existing knowledge about NAS severity; i.e., we need to use knowledge from up-to-date research and clinical experience to make an educated guess as to what the ideal model for severity should look like. Essentially, judgments will need to be based on face validity (22), and we want a tool based on utility (e.g., good perceived predictive accuracy, or low disturbance based on **Figures 3** and **4**), practicality, and "clinical common sense" (22). Unfortunately, different people will have different judgments with respect to these considerations.

Specific judgments on a tool should be made with respect to three steps used to create an assessment tool based on a formative model (17). The first two steps consist of determining the potential variables to form the tool and ultimately deciding

which variables, or items, should actually be utilized. The third step is to determine how much weight should be assigned to each item. Furthermore, judgments must be made on the value of pharmacologic treatment cutoff scores, and how to use them. For instance, the decision to treat when using the FNAS is often based on whether or not three scores, or their average, of 8 in a row are observed (9–11), thus also implying two 12s in a row. A summary of basic considerations, as well as differences in opinion that are seen in practice, is given in **Table 2**. **Table 2** also summarizes issues in current practice as discussed in the remainder of this manuscript.

EXAMPLES

Although many people may not have realized the underlying theoretical framework, NAS assessment tools have been created *via* formative modeling approaches out of necessity. For instance, Finnegan et al. (9, 10) used the three steps as just discussed. In brief, common symptoms were chosen based on the literature and experience, and weights assigned to each item were chosen based on pathologic significance. Furthermore, the treatment cutoff was chosen based on experience.

The fact that multiple modified versions of the FNAS exist, and that not even treatment cutoff scores are used consistently across institutions (5, 6), demonstrates the fact that people have formatively judged the FNAS and have determined it could use change. Multiple reasons for this exist. As time goes on, our knowledge with respect to NAS improves, and therefore, the way true NAS severity is perceived may change, thus alternating the way we want to model the construct of NAS severity. For instance, the MNS was created by Jansson et al. (14) to improve upon the FNAS. Both irritability and failure to thrive, based on weight loss, were added whereas some items were removed or combined, for instance, due to overlap. Furthermore, the FNAS is lengthy and clinicians may desire a shorter tool (28) that is more practical and potentially more reliably scored, and therefore, shortened scores have been proposed (29). Even a short functional assessment approach has been developed which is based on feeding, sleeping, and ability to console when crying (30). We note that, unlike with a reflective model, heterogeneity of items in the assessment tool is clinically ideal as the use of correlated items unnecessarily adds time and complexity to scoring in practice.

An empirical approach that has been taken to formatively judge a NAS assessment tool, including its treatment cutoff value(s), is the use of data with the outcome of interest of whether or not the subject has NAS. Although this outcome is not ideal and will not provide definitive results because interest is actually in the unknown true need to treat, results can still be suggestive. For instance, Zimmerman-Baer et al. (31) studied 102 healthy neonates and found that the 95th percentile of their scores, from a version of the FNAS comprised of 28 items, never exceeded 8. Although this suggests that infants not in withdrawal will tend to have lower FNAS scores, and scores of 8 or above are mostly in infants who are withdrawing, this does not provide strong information with respect to the true need to treat infants in withdrawal. Specifically, interest is with respect to when NAS infants need to be treated, not if they actually have NAS. However, these particular results may be suggestive that, although a single score of 8 to deem treatment is needed is insufficient, an average of three 8s or two 12s in a row may suffice as adequate treatment cutoffs for this particular 28 item tool.

Although not a direct factor with respect to judging the formative nature of a tool, we feel it is important to note that the inter-rater reliability (IRR) of NAS assessment tools has received a notable amount of attention, as many items incorporated in NAS tools are based on subjective judgments. Although scorers should be trained, they may not always have complete agreement on the magnitude or occurrence of a clinical sign involved in a particular tool (23, 24). For instance, IRR coefficients have been shown, for example, to range from 0.70 (33) to 0.96 (9, 10) with the FNAS, 0.89 to 0.98 with the Neonatal Withdrawal Inventory (32), and greater than 0.94 with the MNS (5, 15). Furthermore, Gomez-Pomar et al. (33) showed that the degree of variance in

TABLE 2 | Considerations and obstacles in the evaluation and creation of Neonatal Abstinence Syndrome (NAS) assessment tools.

Basic considerations
Visualize what the ideal model would look like using knowledge from up-to-date research and clinical experience
– Consider face validity; i.e., perceived clinical utility – Consider practicality; e.g., scoring time – Consider pharmacologic treatment cutoff values based on the ideal model
Differences in opinion
People can have varying opinions on a perceived ideal model. For instance, opinions can vary with respect to:
– the utility of an item; i.e., is the item needed in the tool, and if so, how much weight should be assigned to the item? – the practicality of the item; e.g., does the amount of time it takes to score the item outweigh its added utility to the tool? – what treatment cutoff value(s) should be used, and how should they be used?
As a result of differences in opinion, perceived ideal assessment tools will also differ. This is one reason why new or modified assessment tools continue to be developed
Issues in current practice
Advances in research and differences in opinion continue to result in different or modified tools being proposed and used. Examples include:
– additions and reductions in the utilized items, as in the MOTHER NAS score (14, 15) – shortened tools
Empirical research on treatment cutoffs has used NAS as the outcome of interest, as opposed to the true need to treat, and can, therefore, only provide suggestive evidence with respect to the need for treatment
Judgments should not only consider inter-rater reliability, but also the utility of items when deciding whether or not to include the item in a tool
Future tools should be developed based on a formative modeling strategy and can be created by adding or reducing items from existing tools. Furthermore, other information can be used in conjunction with such tools
Assessment tools based on formative modeling can be developed to encompass a variety of exposure types
Tools tend to only be developed by a small group of people before being published and presumably used. Due to likely differences in opinion from other clinicians and researchers, implementation and ultimately standardization in practice is unlikely
To help standardize NAS assessment, experts should come together to decide on the best formative model(s) and ultimately assessment tool(s) to use

FNAS scores attributable to unreliable scoring is small (≤9.8% in the studied institutions). Although when viewed from a psychometric viewpoint, an interobserver reliability of 90% is desired (21, 34), there may still be enough utility in keeping any items in the tool that cause lack in IRR as opposed to removing them. Therefore, judgments should not only consider IRR, but also the utility of items. For instance, although it may be difficult to clearly distinguish the cutoffs between mild, moderate, and severe tremors, incorporation of this item with the chance of misclassification can still be more useful than not using this item simply because of this potential difficulty.

FUTURE TOOL DEVELOPMENT

As more is learned about NAS, assessment tools may need to evolve. Based on a formative modeling approach, new items and their perceived impacts, *via* appropriate weights, can be added to tools, and numerical treatment cutoff values modified accordingly. Alternatively, items deemed to have little utility or practicality in the presence of other items can be removed. We do note that newer information, such as knowledge on genetics and the mother's prenatal treatment (5), may be difficult to incorporate into a standard assessment tool. However, research could be conducted on how to best utilize such information in conjunction with the tool. For instance, Grossman et al. (30) recently demonstrated that a simple functional assessment can work well when combined with other novel strategies for NAS management.

Formative models can also conform to variations that exist in practice and that should be considered for future tool development. Presenting NAS symptoms may depend on the type of opioid causing withdrawal, among other possible factors (6, 13, 21, 35). Furthermore, many infants' withdrawal can be a result from exposures to multiple types of drugs, which would not correspond to a single opioid-based tool. This issue is amplified when there is also exposure to non-opioids (21, 36). Extending upon the issue of heterogeneous exposures, variations in the distributions of exposure types may also exist across different institutions due to the different populations they care for (33). All of these variations will contribute to a lack in correlation among items in as assessment tool, and thus the tool will be deemed inappropriate if based on a reflective modeling viewpoint. However, when based on a formative model, a tool is allowed to encompass the spectrum of opioids. Specifically, this modeling approach allows us to simultaneously assess symptoms from different types of exposures, thus building up a case for the need to treat. This is important because it is clinically irrelevant to restrict the applicability of a tool to a single exposure type when in real life we are faced with multidrug-exposed withdrawing infants (37). We do note, however, that this issue adds a notable degree of complexity in terms of judging the utility of any such tool, and multiple tools may be required; e.g., a unique tool for a single or specific mixture of exposures, or even a unique tool for institutions observing similar distributions in exposures. Unfortunately, no existing or future tools will be ideal, but we hope to obtain the best guidance as practically possible from these tools (28).

Major contributing factors to the existence of a variety of NAS assessment tools include the fact that researchers have been independently judging and creating these tools and differences in opinion exist. As a result, it is very difficult for any new tool to be widely assessed and ultimately implemented in practice, as other clinicians and researchers may not agree with the proposed tool in terms of its utility and practicality. Therefore, we urge that a large group of experts in the area of NAS assessment should come together and pool their knowledge and clinical experience to assess the utility and practicality of existing assessment tools and to determine if one or more new or modified tools are needed. If needed, such "best" tools should be formulated with consideration of both perceived utility and practicality based on a formative modeling strategy. We suspect there will never be complete agreement and, therefore, compromises and ultimately a consensus in terms of the included items and the assigned weights to each item is likely needed (17). Furthermore, randomized clinical trials may be warranted to confirm the superiority of any such tools to existing tools on outcomes of importance such as length of stay and duration of opioid treatment (13, 38).

CONCLUSION

There is push in the NAS literature on the need for a new assessment tool that is psychometrically valid (21). However, this inherent proposed use of a reflective model does not correspond to the real-world diversity of NAS manifestations observed in practice and the fact that numerical scores from an assessment tool do not completely dictate the decision to start pharmacological treatment. Therefore, in this manuscript, we argue that judgments on, and therefore future development of, NAS assessment tools should be based on a formative modeling approach. Such an approach can take into account the complexity of the presenting symptoms of NAS, their severity, and potentially the fact that heterogeneity of symptoms and their severity exists across different types and mixtures of opioid exposures. Finally, current assessment tools must be judged using current knowledge and experience, and judgment can vary in opinion. Therefore, one or more assessment tools based on a formative model and created based on the input of a large group of experts may be needed in order to lead to increased agreement on the assessment of NAS in practice.

AUTHOR CONTRIBUTIONS

PW conceived the work, drafted and revised the manuscript, approved the final manuscript as submitted, and agreed to be accountable for all aspects of the work. EG-P assisted in the conception of the work, made critical input and assisted in revisions, approved the final manuscript as submitted, and agreed to be accountable for all aspects of the work.

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