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Abstract

Coincident with the opioid epidemic in the United States has been a dramatic increase in the number of children born with neonatal abstinence syndrome (NAS), a form of withdrawal resulting from opioid exposure during pregnancy. Many research efforts on NAS have focused on short-term care, including acute symptom treatment and weaning of the infants off of their drug dependency prior to authorizing their release. However, investigations into the long-term effects of prenatal opioid exposure (POE) on brain development, from the cellular to the behavioral level, have not been as frequent. Given the importance of the perinatal period for human brain development, opioid-induced disturbances in the formation and function of nascent synaptic networks and glia have the potential to impact brain connectivity and cognition long after the drug supply is cut off shortly after birth. In this review, we will summarize the current state of NAS research, bringing together findings from human studies and preclinical animal models to highlight what is known about how POE can induce significant, prolonged deficits in brain structure and function. With rates of NAS continuing to rise, particularly in regions that already face substantial socioeconomic challenges, we speculate as to the most promising avenues for future research to alleviate this growing multigenerational threat.

Significance Statement

There has recently been a significant rise in the number of infants born with neonatal abstinence syndrome (NAS) after prenatal opioid exposure. Though little is known about the long-term effects of early life opioid exposure on brain connectivity, this review attempts to consolidate the existing literature from both preclinical models and humans. Elevated NAS

diagnosis rates suggest that this public health crisis will only worsen with time. Therefore, there is an immediate need to understand the impact of prenatal opioids on brain development to predict future healthcare concerns and develop strategies to improve long-term outcomes in children born with NAS.

Introduction

Since the 1990s, the rate of abuse of opioid drugs has risen dramatically in the United States (Scholl, Seth, Kariisa, Wilson, & Baldwin, 2018). One of the most significant outcomes of this opioid epidemic has been a sharp rise in the incidence of neonatal abstinence syndrome (NAS). NAS is the clinical diagnosis used to describe the collection of signs and symptoms commonly observed in the newborns of mothers who abused certain drugs, such as opioids, during pregnancy (NAS specifically resulting from opioids has also been termed NOWS, for neonatal opioid withdrawal syndrome). The fetus develops a physical dependence on these drugs and, after being separated from the supply of drug at birth, the infant soon displays the symptoms of withdrawal, typically within 72 hours. Signs and symptoms can include irritability, tremors, excessive crying, poor feeding, diarrhea, and, in some of the more severe cases, seizures (Kocherlakota, 2014). Scoring scales, such as the Finnegan Neonatal Abstinence Scoring System, are used to numerically evaluate infants displaying signs of NAS and help determine appropriate treatment plans (Finnegan, Connaughton, Kron, & Emich, 1975). Length of hospital stays for affected infants average 16 days but vary depending on the severity and duration of symptoms (Hudak & Tan, 2012). In addition, maternal drug use has also been correlated with increased risk of sudden infant death syndrome (SIDS), with some estimates at nearly a 4-fold

increase compared to the general population (Cohen, Morley, & Coombs, 2015; Kandall, Gaines, Habel, Davidson, & Jessop, 1993).

Incidence of NAS in the United States has rapidly increased in recent years (S. W. Patrick, Davis, Lehmann, & Cooper, 2015; S. W. Patrick et al., 2012). The prevalence of NAS rose to 6.5 cases per 1,000 neonatal hospitalizations reported in 2014 compared to 1.5 per 1,000 in 1999 (Haight, Ko, Tong, Bohm, & Callaghan, 2018). In West Virginia, where access to treatment facilities for opioid abuse is limited (Brown, Goodin, & Talbert, 2018), the rate of NAS from October 2016 to December 2017 was 52.6 cases per 1,000 live births (Umer et al., 2019). NAS represents a serious emotional toll for the families of these children and a significant financial burden for healthcare providers, with approximately $462 million in NAS treatment costs financed by Medicaid as recently as 2014 (Winkelman, Villapiano, Kozhimannil, Davis, & Patrick, 2018), prompting considerable research efforts into improving clinical care and treatment for NAS infants. However, it is known, though less well-documented, that *in utero* exposure to drugs, including opioids, can have significant effects on a child's neurological development long after the symptoms of NAS have dissipated (H. L. Johnson, Glassman, Fiks, & Rosen, 1990; Nygaard, Moe, Slinning, & Walhovd, 2015; van Baar, Soepatmi, Gunning, & Akkerhuis, 1994). Therefore, the purpose of this literature review is to summarize the findings of both clinical and basic research studies that have examined the long-term effects of prenatal opioid exposure (POE) within three primary categories: 1) Cellular and molecular development within the central nervous system (CNS); 2) size, structure, connectivity and function of different CNS regions and circuits; and 3) behavior and cognition. Combining current findings from bench to bedside within these areas will ideally help identify gaps in our current knowledge and direct future investigations into the longitudinal effects of opioids on neurological development.

Part 1: Cellular and Molecular Development Within the CNS

Opioid receptors and their ligands

Our discussion of the long-term effects of *in utero* opioid exposure begins by examining the effects opioids can have on critical cellular processes in the developing CNS. In mammals, opioid drugs function primarily through opioid receptors, the three most prominent of which are the μ, κ, and δ receptors. These G-protein coupled receptors are normally stimulated by endogenous opioids such as endorphins, enkephalins, and dynorphins (Benarroch, 2012). The endogenous opioid system is also differentially targeted by many prescription and illegal drugs. Morphine and its derivatives (e.g. heroin, methadone, oxycodone, etc.) are primarily active at μ receptors but are also κ and δ receptor agonists. In contrast, buprenorphine, a derivative of the opiate alkaloid thebaine, has been shown to be a partial agonist at μ receptors but an antagonist at κ and δ receptors (Welsh & Valadez-Meltzer, 2005). Furthermore, the major active metabolite of buprenorphine, norbuprenorphine, is an agonist of δ receptors while a partial agonist of μ and κ receptors (Huang, Kehner, Cowan, & Liu-Chen, 2001). A growing body of evidence indicates that endogenous opioids and their receptors are vital to healthy nervous system development and function, including analgesia, respiratory/cardiac function, intestinal transit, mood, hormone secretion (Barnes, Jen, & Dunbar, 2004; Bottcher, Seeber, Leyendecker, & Wildt, 2017; Bueno & Fioramonti, 1988; Callaghan, Rouine, & O'Mara, 2018; Dhawan et al., 1996; Irnaten et al., 2003; Kwok, Devonshire, Bennett, & Hathway, 2014; Lalley, 2008), and neuronal differentiation.

Neurons and neuronal progenitors

μ- and κ-opioid receptors have been detected on the surfaces of both rodent and human-derived embryonic stem cells and neural progenitor cells, while binding of endogenous opioids can induce embryonic stem cells to differentiate via the extracellular signal-regulated kinase (ERK)/mitogen-activated protein (MAP) kinase signaling pathway (E. Kim et al., 2006; Sheng et al., 2007; Tan, Cunningham, Joshi, Oei, & Ward, 2018). Preclinical studies suggest that the localization of opioid receptors to different regions of the brain may have a significant impact on development. Immature mice have been shown to have a higher percentage of μ-opioid receptor expression within the subventricular zone (SVZ), a known site of neurogenesis, compared to adult mice (Stiene-Martin et al., 2001). κ-opioid receptors similarly appear to be developmentally downregulated in rats within sites of cellular proliferation and neurogenesis (including the ventricular zone/SVZ and the dentate gyrus of the hippocampus) (Tan et al., 2018). Given the importance of endogenous opioids and their receptors in the developing CNS, an introduction of exogenous opioids, particularly in excess, could have a lasting, dramatic impact on brain maturation.

In the clinic, pregnant mothers who are addicted to opioids commonly undergo medication-assisted treatment (MAT) which combines less addictive prescription opioid drugs—namely methadone or buprenorphine—with behavioral counseling in order to treat addiction and achieve better health outcomes for the mother and child. However, emerging evidence asserts that any opioid use during pregnancy, even when part of a medical treatment plan, can have deleterious effects on the developing fetus that may vary depending on the opioid used (i.e. morphine compared to methadone) (Burke & Beckwith, 2017; Kayemba-Kay's & Laclyde, 2003). Preclinical investigations have shown opioids to stunt the proliferation and development of neurons within the CNS, with prenatal exposure to buprenorphine in rats leading to reduced

levels of neuronal specific protein markers (Neu-N and MAP-2) as well as stem/progenitor biochemical markers of the neural lineage (e.g. nestin, Sox2, KLF4, and doublecortin) at postnatal day 21 (Wu et al., 2014). These same rats showed decreased brain-derived neurotrophic factor (BDNF) expression and signaling, with greater than 30% decreased mRNA expression in both males and females as well as a nearly 20% reduction in phosphorylation of its high affinity receptor TrkB. Since BDNF signaling via TrkB is vital for neurogenesis, axon growth, and synaptic plasticity (Cohen-Cory, Kidane, Shirkey, & Marshak, 2010; Kramár et al., 2012), the relationship between BDNF and exogenous opioids is likely clinically significant given that plasma levels of BDNF were found to be increased in 2-day-old human infants diagnosed with NAS compared to healthy infants (Subedi et al., 2017).

Altered expression of growth factors and signaling molecules do not appear to be the only mechanism through which opioids affect neuronal development. In free-floating cultured rat cortical neurospheres, buprenorphine was shown to reduce proliferation of neural stem/progenitor cells in a dose-dependent manner (Wu et al., 2014). It has also been shown that opioids alter neuronal expression of apoptotic proteins (namely caspase-3, Bcl-2, and Bax) within rats, particularly within the hippocampus (Wang & Han, 2009). Dysregulation of apoptosis could lead to inappropriate cell death, offering another potential pathway through which these drugs may interfere with normal neuronal development. Not only is the number of neurons in the developing CNS affected by the presence of opioids, but the growth and maturation of those neurons also appears to be influenced. Excitatory pyramidal neurons isolated from the somatosensory cortices of heroin-exposed mouse pups at postnatal day 3 showed fewer and shorter dendrites compared to neurons from control pups (Lu, Liu, Long, & Ma, 2012), indicative of impaired synaptic connectivity and interneuronal communication. These findings

corresponded with a behavioral phenotype that showed reduced tendency to explore a familiar object moved to a novel location in heroin-exposed mice at postnatal day 120, suggesting impaired short-term spatial working memory (Lu et al., 2012); these and other behavioral findings will be discussed in greater detail in Part 3 of this review.

Glial cells: Astrocytes

While a great deal of attention has been given to the development of neurons and their precursors, glial cell development under the influence of opioids has been considerably less studied (Figure 1). Astrocytes are the most numerous glial cell type in the CNS and serve many vital functions, including the ability to regulate the development of synapses within the CNS (Chung, Allen, & Eroglu, 2015). In mice, μ-opioid receptors have been confirmed on the surface of astrocytes within the hippocampus, nucleus accumbens (NAc), and ventral tegmental area (VTA), three brain regions associated with reward and addiction (Nam et al., 2018). The presence of these receptors makes astrocytes susceptible to receptor-ligand interactions with both endogenous and exogenous opioids. Morphine and other μ-opioid receptor agonists have been shown to inhibit murine astrocyte growth and development by suppressing DNA synthesis, as demonstrated by decreased bromodeoxyuridine (BrdU) incorporation (Hauser et al., 1996). Evidence for the importance of astrocytes in the development of opioid addiction was provided with mice that showed enhanced morphine conditioned place preference (CPP) after administration of astrocyte-conditioned medium (ACM) into either NAc or cingulate cortex; the ACM-injected mice actively chose to spend more time in a chamber paired with morphine injections compared to either morphine alone or a chamber paired with saline injections (Narita et al., 2006). This experiment was performed in adult mice, so future investigations will be

necessary to determine whether prenatal opioid exposure would result in the same elicitation of enhanced CPP that was observed.

Opioids have the potential to significantly impact astrocyte-neuronal interactions at critical stages of early development, since cultured immortalized rat astrocytes subjected to prolonged treatment with μ-opioid receptor agonists, including morphine, showed decreased production of the glycoproteins thrombospondin-1 and -2 (TSP1 and 2) (Phamduong et al., 2014). In the past two decades, preclinical investigations have revealed that astrocytes secrete TSPs to initiate a signaling pathway that promotes synaptogenesis in developing neurons (Christopherson et al., 2005; Eroglu et al., 2009) in a process critical to the formation of intracortical connectivity and maturation of dendritic spines in the rodent brain (Risher et al., 2018). Neurite outgrowth and excitatory synaptic density were both inhibited by chronic morphine in rat astrocyte/neuron co-cultures (Ikeda et al., 2010), while the same study showed decreased astrocyte expression of TSP1 in rat pups with repeated morphine injections. These basic research findings take on heightened significance with the knowledge that astrocytes represent a particularly highly specialized cell type in humans compared to lower animals, including increased cortical TSP expression (Caceres, Suwyn, Maddox, Thomas, & Preuss, 2007; Oberheim, Wang, Goldman, & Nedergaard, 2006). Taken together, these results indicate that the effect of opioids on the modulation of astrocyte signaling, particularly astrocyte-mediated synaptogenesis, may underlie many of the developmental aberrations associated with POE in both animal models and humans.

Glial cells: Oligodendrocytes

Oligodendrocytes, another type of glial cell vital to the development and normal functioning of the CNS, are primarily known for their role in forming myelin sheaths to facilitate

saltatory conduction along the length of axons. Preclinical studies have shown that, as with other cells within the CNS, oligodendrocytes express opioid receptors and can synthesize endogenous opioids (Eschenroeder, Vestal-Laborde, Sanchez, Robinson, & Sato-Bigbee, 2012). Treatment of pregnant rats with opioids has been shown to increase the expression of myelin-associated proteins (Sanchez, Bigbee, Fobbs, Robinson, & Sato-Bigbee, 2008) and can lead to accelerated oligodendrocyte maturation and myelination within the corpus callosum of the exposed pups (Vestal-Laborde, Eschenroeder, Bigbee, Robinson, & Sato-Bigbee, 2014). In terms of clinical relevance, given that accelerated myelination in humans has been associated with epileptic seizures (Goldsberry, Mitra, MacDonald, & Patay, 2011) among other disorders, the interaction of opioids and these myelinating glial cells during prenatal development certainly warrants further investigation.

Glial cells: Microglia

Another glial cell type that expresses μ, κ, and δ opioid receptors are microglia (Chao et al., 1996; Maduna et al., 2018; Shrivastava et al., 2017), the primary innate immune cell of the CNS. Though not originally generated in the brain (they are derived from bone marrow prior to migrating into the CNS), microglia play critical roles in numerous brain processes including synaptic refinement and response to pathogens and injury (Schafer, Lehrman, & Stevens, 2013). Basic research investigations have also implicated microglia in the formation of behaviors associated with addiction. Schwarz and Bilbo (2013) demonstrated increased levels of the immune receptor TLR4 on microglia in the NAc, which correlated with robust reinstatement of morphine CPP in adulthood in rats that were exposed to morphine as adolescents. Interestingly, similar effects were not observed in rats whose first exposure to morphine occurred during adulthood, underscoring the importance of the timing of drug exposure in synaptic circuits that

are still maturing. Compared to other cell types, relatively little is known about how microglia respond to opioids during fetal development, but one recent study in rats exposed to prenatal methadone demonstrated altered cortical microglial morphology at postnatal day 10 in conjunction with upregulated inflammatory markers TLR4 and MyD88 (Jantzie et al., 2019). The condensed cellular structure and decreased branching observed were consistent with the "activated" microglial phenotype associated with neuroinflammation and disease (Bilbo & Stevens, 2017). Whether these microglial changes persist into later ages, what role these cells play in possible cognitive dysfunction after POE, and the impact of opioids on human microglia remain crucial open questions.

Opioid receptor expression and activity

The expression and binding properties of opioid receptors, whether located on neurons or glial cells, can be influenced by the presence of exogenous opioids. In a recent study, newborn rat pups prenatally exposed to oxycodone showed decreased midbrain expression of the μ-opioid receptor 1 (OPRM1) gene (Vassoler, Oranges, Toorie, & Byrnes, 2018), though this result was seen only in females and not males. Additionally, μ-opioid receptor binding potential to an enkephalin analog was shown to be significantly lower in the brains of one-day-old rat pups whose mothers had received daily injections of 0.3 or 0.6 mg/kg buprenorphine during pregnancy (Belcheva et al., 1998). The production and release of endogenous ligands that bind to these receptors can similarly be affected by POE, as demonstrated by the finding that levels of met-enkephalin were shown to be significantly lower in the striata of rats prenatally exposed to morphine (Tempel, Yang, & Basheer, 1995). These results indicate a general trend wherein hijacking of the endogenous opioid system by exogenous opioid exposure during early development elicits a downregulation of endogenous opioid signaling. Importantly, however,

since each of these studies was performed on 0- to 1-day-old pups, follow-up studies in older animals as well as in human tissue will be necessary to determine the persistence and clinical implications of the findings.

Part 2: Size, Structure, Connectivity and Function of CNS Regions and Circuits

POE stunts rates of growth and development

Widespread changes in cellular function and morphology associated with *in utero* opioid exposure are often accompanied by observable changes in the size, structure, and function of entire brain regions and the pathways that connect them. For decades, researchers have observed lower birth weights and smaller head circumferences in POE children (H. L. Johnson, Diano, & Rosen, 1984; Ornoy, Segal, Bar-Hamburger, & Greenbaum, 2001; Wilson, McCreary, Kean, & Baxter, 1979), a trend that holds in rats and mice as well (Ford & Rhines, 1979; Wu et al., 2014; Zagon & McLaughlin, 1977). In general, low birth weight in humans (regardless of cause) has been associated with higher rates of behavioral problems throughout childhood and early adolescence, often resulting in poor scholastic performance (McCormick, Gortmaker, & Sobol, 1990; Taylor, Margevicius, Schluchter, Andreias, & Hack, 2015). Additionally, low birth weight in infants with prenatal drug exposure is significantly correlated with higher rates of mortality in the first two years of life (Ostrea, Ostrea, & Simpson, 1997), further compounding the issues involved in caring for these children.

Other structural deficits associated with POE in humans are not outwardly apparent and can only be observed through medical imaging (Table 1). As early as 18-22 weeks gestation, ultrasound studies have found that fetuses whose mothers were undergoing methadone maintenance therapy had significantly larger thalamic diameter-to-head circumference ratios

compared to a control group (0.90 ± 0.1 versus 0.87 ± 0.1 mm; p = 0.01) (Schulson et al., 2014). One ultrasound study observed abnormally small, slit-like ventricles in 13 out of 16 NAS-affected 1-month-old infants compared to 0 out of 11 control infants (E Pasto et al., 1985). Magnetic resonance imaging (MRI) studies, which have previously shown reduced white matter and grey matter within the brains of habitual opioid-using adults (Upadhyay et al., 2010; Y. Yuan et al., 2009), revealed certain regions in the brains of prenatal opioid-exposed children (including the basal ganglia, thalamus, and cerebellar white matter) to be significantly smaller compared to age-matched control children after adjusting for intracranial volume and birthweight. This result was consistent in both infants (Q. Yuan et al., 2014) and children 10-14 years of age (Sirnes et al., 2017). More widespread deficits were observed in an MRI study examining 17-22-year-olds whose mothers had abused multiple drugs, including heroin, during pregnancy; these individuals had significantly smaller whole brain, cerebral cortex, cerebral white matter, basal ganglia, pallidum, thalamus, and cerebellar white matter volumes compared to controls (Nygaard et al., 2018). Taken together, these results show that the smaller brain region sizes observed in POE infants are wide-ranging and can persist into adulthood.

Potential impact of POE on the mesolimbic dopamine pathway

In studies of opioid addiction and the CNS, much attention has been focused on brain regions associated with the mesolimbic dopamine pathway. Also known as the dopamine reward pathway, this pathway works to encode experiences as pleasurable and reinforce future behavior to seek out those experiences again (Figure 2). This process begins in the VTA, where the cell bodies of dopaminergic neurons extend axonal processes to other regions of the brain including the NAc, hippocampus, and prefrontal cortex (PFC) (Adinoff, 2004; J. E. Lisman & Grace, 2005). Opioids affect the reward pathway by binding to opioid receptors on the surface of

interneurons within the VTA, thus inhibiting release of the inhibitory neurotransmitter GABA and subsequently disinhibiting the release of dopamine by nearby dopaminergic neurons (S. W. Johnson & North, 1992). In the NAc, increased phasic release of dopamine in response to external stimuli has been shown to reinforce pleasurable behaviors including eating food, engaging in sexual activity, or taking drugs of abuse (Lingford-Hughes & Kalk, 2012). The PFC, the brain region most associated with decision-making and impulse control, projects to the NAc and modulates its activation (Pierce & Kumaresan, 2006). The hippocampus appears to form a functional loop with the VTA to promote seeking out novel experiences and expecting reward when engaging in behaviors that were pleasurable in the past (J. E. Lisman & Grace, 2005). Currently, little is known about how POE affects the formation and strengthening of the mesolimbic dopamine circuit, with the majority of the findings being speculative based on changes in reward seeking or depressive-like behaviors. Since μ-opioid receptors in the VTA have been shown to regulate dopaminergic and serotonergic outputs to PFC, NAc and other brain regions (K. L. Smith et al., 2019), one can envision a scenario in which prenatal opioids would hamper multiple neurotransmitter systems during periods for neurodevelopment. Therefore, future studies examining changes in the size, structure, and function of these brain regions will be important in understanding how reward processing and motivation might be altered in affected children as they age.

Sex differences with POE

Sex hormones produced by either the ovaries or testes are vital to the normal fetal development. Within the CNS, the presence of male or female specific hormones is necessary for sex-specific differences in the size and structure of certain brain regions across species, including humans (A A Ehrhardt & Meyer-Bahlburg, 1979; Xin, Zhang, Tang, & Yang, 2019). Sex

hormones may also modify the effects of POE on neurological development and function. The expression of opioid receptors within the brains of rats has been shown to be influenced by the presence of estrogen and progesterone (Cruz et al., 2015), but the exact role of ovarian hormones in the development of the endogenous opioid system remains unclear. Additional evidence for an interaction between sex hormones and opioids during development can be observed in the presentation of human infants with NAS. In one retrospective study, it was observed that male infants were more likely than females to be diagnosed with NAS (1.18 odds ratio after adjusting for maternal age, race, and education level) and were more likely to require pharmacologic treatment after being diagnosed (1.24 adjusted odds ratio) (Charles et al., 2017). Sex differences in head circumference (35.78 ± 0.27 cm in males compared to 33.40 ± 1.43 cm in females; p=0.035) and types of symptoms (e.g. females showed greater instances of diarrhea, excessive sucking, tremors, sleep disturbances, and crying; males were more likely to have hyperactive Moro reflex and nasal stuffiness) have also been observed (Stevens et al., 2018). Further investigation is required to identify the cellular and molecular mechanisms that underlie these sex-biased observations.

Insight from functional studies

Electrophysiology is commonly used in preclinical studies to investigate synaptic conduction within and between brain regions, providing insight into neuronal function and network plasticity. One frequently studied plasticity phenomenon is long-term potentiation (LTP), wherein a sustained increase in excitatory postsynaptic potential (EPSP) is observed after repeated stimulation of a presynaptic neuron; this result is often interpreted as a strengthening of synaptic transmission in that circuit (Bliss & Lomo, 1973). With regard to the hippocampus, LTP is generally thought to represent the process whereby long-term, episodic memories are formed

(J. Lisman, Cooper, Sehgal, & Silva, 2018). In adult rats prenatally exposed to morphine, maintenance and longevity of LTP within their hippocampi were shown to be attenuated compared to rats treated with saline (Villarreal, Derrick, & Vathy, 2008). This finding may offer mechanistic insight into the observation that POE is associated with deficits in learning and memory (Chen et al., 2015; Slamberova et al., 2001; Wang & Han, 2009). Intriguingly, rats prenatally exposed to morphine showed significant improvements in LTP and the Morris water maze (spatial learning task) with exercise and enriched environment housing (Ahmadalipour, Ghodrati-Jaldbakhan, Samaei, & Rashidy-Pour, 2018), suggesting a possible therapeutic intervention to alleviate some of the long-term cognitive deficits associated with early life drug exposure.

Given the invasive nature of electrophysiological techniques, certain imaging modalities, such as functional magnetic resonance imaging (fMRI), are currently the predominant methodology for clinical investigations into neuronal activity. Specifically, fMRI uses changes in blood flow to approximate the level of activity within specific brain regions while a patient performs certain tasks. An fMRI study of POE children between the ages of 9 and 12 years showed significantly greater activity within their prefrontal cortices (involved in inhibitory control and executive function) during working memory-selective attention tasks compared to unexposed children (Sirnes et al., 2018). Another fMRI study investigated adolescents (12-15 years of age) prenatally exposed to heroin and other drugs while performing a visuospatial working memory task when compared to a control group (Schweitzer et al., 2015). Though overall performance on the task was similar between groups, the drug-exposed children showed aberrant circuit activation that resulted in less efficient signaling; the authors indicated that this type of function was reminiscent of developmentally immature brains. Taken together, these

findings suggest that the brains of POE children have impaired interneuronal connectivity and therefore must compensate through other suboptimal mechanisms.

Part 3: Behavior and Cognition

Findings from rodent behavioral studies

A variety of behavioral and cognitive deficits have been documented in POE animal models (Table 2) and humans, which may represent consequences of the anatomical and physiological changes discussed thus far. Tests of anxiety-type behaviors in animal models have shown significant differences between drug-exposed and control subjects. Adult rats whose mothers were injected with morphine during pregnancy were shown to spend less time both in the light compartment of a light/dark box and while exploring the open arms of an elevated plus maze compared to rats whose mothers received saline (Ahmadalipour et al., 2015; Klausz et al., 2011). These test results are suggestive of more pronounced anxious phenotypes among prenatal morphine-exposed rats; similar behaviors were observed in rats prenatally exposed to buprenorphine and methadone as well (Chen et al., 2015). Whether this anxious behavioral profile reflects changes within the mesolimbic dopamine pathway and altered sensitivity to dopamine has yet to be determined.

Given the importance of opioids in reward processing, it is not surprising that reward-seeking and depressive behaviors are affected in POE animals. Adult rats exposed to morphine *in utero* showed a greater preference for a saccharin/water solution over pure water compared to control rats (with females showing an increased hedonic effect compared to males), indicating a generally elevated response to pleasurable stimuli (Gagin, Cohen, & Shavit, 1996). Differences are also seen in exposed animals when faced with unpleasant stimuli, as when rats prenatally

exposed to buprenorphine showed reduced escape-oriented behaviors in the forced swimming and tail suspension tests; this result was interpreted as POE rats exhibiting greater depressive behavior than control rats (Hung et al., 2013; Klausz et al., 2011; Wu et al., 2014). Though merely speculative at this point, POE-induced changes in connectivity or strength of the mesolimbic dopamine reward pathway could certainly contribute to these trends of elevated response to rewarding stimuli and greater depressive behavior in response to unpleasant stimuli.

Studies utilizing memory tests have consistently shown worse performance by POE mice and rats compared to control rodents. Rats prenatally exposed to morphine, methadone, or buprenorphine subjected to tests of novel object recognition on postnatal day 44 were shown to have impaired recognition memory compared to control rats (Chen et al., 2015). When tested with the Morris water maze on postnatal day 30, rats who underwent prenatal heroin exposure took significantly longer than unexposed rats to follow visual cues and swim to a platform they had previously located, indicating deficits in learning and memory (Wang & Han, 2009). Another investigation into learning and memory deficits used a symmetrical maze and a radial arm maze (RAM) to show significant sex-related differences in the ability of prenatal morphine-exposed rats to solve the mazes (Slamberova et al., 2001). Male rats required less time to complete the symmetrical maze, while both sexes showed deficits in completing the RAM in a similar amount of time to sex-matched controls. The presence or absence of ovarian hormones was implicated as the reason for these sex-related differences, since ovariectomized POE females had poor performance on RAM delay trials (measuring working spatial memory) that could be rescued by hormone replacement injections.

Effects of POE on human cognition

Studies of behavior and cognitive ability in animals can yield valuable insight into possible mechanisms of opioids affecting normal CNS development, but ultimately these evaluations must performed on humans. Studies use a variety of scales and tests to evaluate human cognitive function at different developmental stages. A recent meta-analysis by Yeoh et al. (2019) of 26 human POE studies provides an excellent overview of the current knowledge, so we will only highlight a select few in the current discussion. Male infants with a history of prenatal opioid exposure have been shown to score higher than females on tests of habituation, which measure the ability of the infant to ignore extraneous environmental stimuli (Jones, O'Grady, Johnson, Velez, & Jansson, 2010). One of the earliest published human POE studies showed that children aged 3 to 6 years old who were prenatally exposed to heroin performed worse on the General Cognitive Index and on the perceptual performance, quantitative, and memory subtests of the McCarthy Scales of Children's Abilities (Wilson et al., 1979). Several years later, a study compared a group of 12-month-old children that had been prenatally exposed to methadone to an unexposed, age-matched control group by utilizing the Bayley Scales of Infant Development. It was found that 20% of the methadone exposed group had Mental Development Index scores indicative of developmental delay, while only 4% of the unexposed children had such low scores (H. L. Johnson et al., 1984). A more recent study used the second edition of the Bayley Scales of Infant Development (Bayley-II) to evaluate a group of boys whose mothers had abused multiple drugs, including heroin, during pregnancy. The average scores for these children at years 1, 2, and 3 were, respectively, 9.7, 12.9, and 10.3 points lower than the average scores for an unexposed control group (Nygaard et al., 2015). The same cohort scored 15.4 points lower on the McCarthy Scales of Children's Abilities at age 4.5 and 14.8 points lower on the Wechsler Intelligence Scale for Children at age 8.5, suggesting that

diminished cognitive ability among prenatally exposed children does not improve over time. An analysis of standardized test scores in New South Wales, Australia also provides evidence that prenatally exposed children do not catch up with non-exposed peers in terms of cognitive ability. A cohort of children who had been diagnosed with NAS as infants scored lower on the National Assessment Program: Literacy and Numeracy (NAPLAN) than the statewide average and were more likely to not meet a minimum standard score in grades 3, 5, and 7 (ages 8–9, 10–11, and 12–13 years respectively) (Oei et al., 2017). These persistent trends imply that opioid exposure in early stages of development lead to lasting, detrimental cognitive changes.

While standardized tests can be used to reveal deficits in cognitive function associated with POE, in some cases this exposure appears to negatively impact cognition to the point where the affected child develops a diagnosable disability. A study in Tennessee found that a group of school aged children (ages 3-8 years) with a history of NAS were more likely to have been evaluated for, diagnosed with, or treated for an educational disability compared to an age-matched control group of children (Fill et al., 2018). The authors of this study also noted that children in the NAS group required specialized classroom therapies or services at the rate of 15.6% compared to 11.7% in the control group ($p<0.0001$). Another study used the Truven Health Analytics' Multi-State Medicaid Database to follow the psychiatric outcomes of over 1,000 children who had been diagnosed with NAS as infants. Within their first 5 years of life, these children were more than twice as likely to have been diagnosed with either disturbance of conduct, hyperkinetic syndrome of childhood, adjustment reaction disorder, or an intellectual disability compared to children with no history of NAS (Laura J. Sherman, Mir M. Ali, Ryan Mutter, & Justine Larson, 2019). Given the increasing rates of NAS nationwide, observations

such as these highlight the importance of POE research to an educational system that will have to accommodate the specific needs of the affected children.

Longitudinal studies: Tracking the effects of POE into adulthood

The vast majority of studies comparing cognitive function and mental health between POE individuals to non-exposed controls are focused on children and adolescents. Given that the rise in opioid addiction and overdose is a relatively recent phenomenon (Kolodny et al., 2015), there are only limited longitudinal studies following subjects into adulthood. One study that included adult subjects examined a group of individuals 17-21 years of age whose mothers had abused heroin and other drugs during pregnancy. It was found that members of this drug-exposed group had significantly higher rates of attention deficit disorder/attention hyperactivity disorder (ADD)/ADHD) and substance abuse disorder compared to a non-exposed age-matched control group (Nygaard, Slinning, Moe, & Walhovd, 2017). In addition, the exposed group performed significantly poorer on the Wechsler Abbreviated Scale of Intelligence (WASI) test of general mental abilities and on multiple tests of memory compared to the non-exposed control group (Nygaard et al., 2017).

To date, few studies have investigated whether POE predisposes an individual towards developing addiction later in life. Although it is well-accepted that many forms of addiction carry some level of heritability (Ducci & Goldman, 2012), only a handful of published basic research studies have attempted to isolate POE as the sole potential contributing factor to increased risk of developing an opioid use disorder. One study showed that rats prenatally exposed to morphine demonstrated increased morphine self-administration as adults compared to non-exposed rats (Hovious & Peters, 1985). In another study, rats prenatally exposed to morphine showed enhanced conditioned place preference when treated with morphine as adults

(Gagin, Kook, Cohen, & Shavit, 1997). Given the involvement of opioids in the development of the reward pathway, it is possible that prenatal opioids may predispose the brain towards addiction to rewarding stimuli other than opioids. Rats prenatally exposed to morphine demonstrated increased self-administration of both the opioid drug heroin and also cocaine, a stimulant drug with an entirely different mechanism of action than opioids (Ramsey, Niesink, & Van Ree, 1993). Considering the lack of longitudinal data on humans, the field would greatly benefit from animal studies that subject prenatally-exposed pups to rigorous behavioral testing specifically designed to measure their impulsivity and drug seeking behavior at various stages of development and maturity. Such studies may better illuminate the role of POE in the development of addictive behaviors later in life, thereby informing treatment and prevention strategies for human children born with NAS.

Confounds to consider when interpreting the impact of POE on development

Potentially, there are many confounding variables to consider in studies of cognitive development among children born to parents with substance abuse disorders. Socioeconomic status is perhaps the most relevant of these confounds, as it is highly correlated with rates of substance abuse, quality of perinatal care, and success in education (M. K. Kim et al., 2018; LaGasse, Seifer, & Lester, 1999; M. E. Patrick, Wightman, Schoeni, & Schulenberg, 2012; Thomson, 2018). It is understandably impossible to remove all variables except for opioid exposure when conducting a study on human patients. Theoretically then, studies of children born with NAS who went on to be raised by someone other than the birth mother may yield valuable insight. However, even when they are adopted at an early age (e.g. younger than 1 year) and raised in a nurturing environment, children born to heroin-addicted mothers have been shown to have a higher rate of inattention and behavioral problems from school-age to late

adolescence than matched non-drug-exposed controls (Nygaard et al., 2017; Ornoy, 2003). Further compounding the issue of NAS heterogeneity is the use of other drugs in conjunction with opioids during pregnancy. Polysubstance use is a growing concern in the field of opioid abuse and is positively correlated with the diagnosis of NAS (Roth, Loudin, Andrews, Evans, & Davies, 2020). One drug in particular, the anticonvulsant gabapentin, has been increasingly co-abused along with opioids (R. V. Smith, Havens, & Walsh, 2016), resulting in Schedule V controlled substance classification in several states (including several located in the Appalachian region of the Eastern United States). Though gabapentin was initially believed to have no potential for abuse or addiction (Vickers Smith et al., 2018), mounting evidence suggests that this is not the case. One survey of opioid use disorder patients found that about 1 in 4 also used gabapentin for nonmedical reasons (Bastiaens, Galus, & Mazur, 2016). Some patients self-report that the co-abuse serves to potentiate the experienced opioid high (Baird, Fox, & Colvin, 2014). While this may be due to the activity of gabapentin at the α2δ-1 subunit of the L-type voltage-regulated calcium channels to inhibit neurotransmitter release and/or reduce neuronal hyperexcitability (Patel & Dickenson, 2016), this explanation requires further investigation and is subject to debate. Regardless, behaviors not commonly observed in NAS, such as tongue thrusting, back arching, and eye wandering, have been observed in children born to mothers who co-abused both opioids and gabapentin (Loudin et al., 2017). These infants also required gabapentin weaning in addition to being treated for opioids, resulting in significantly increased hospital lengths-of-stay. Drugs taken in addition to opioids may lead to either diminished, enhanced, or significantly altered changes in developmental outcomes in prenatally exposed children, making it difficult to parse out the exact role or mechanism of opioids in these changes. In the case of gabapentin, prenatal exposure to this drug alone would likely impact the formation

of neuronal networks in the developing fetus by inhibiting the synaptogenic TSP pathway (discussed in Part 1) via their shared receptor, α2δ-1 (Walker, Risher, & Risher, 2020); the combination of prenatal opioids and gabapentin would therefore represent a critical threat to long-term brain connectivity and function.

Conclusion

In summary, this review has summarized what is known and what important questions still remain about the effects of opioids on the developing and maturing nervous system. In our attempt to maximize the breadth of the review, we have collated findings from both preclinical and human studies. However, when considering the animal models of POE, a critical caveat should be kept in mind: despite the widespread use of rodents as *in vitro* and *in vivo* models for human development and the key insights gained from these models, the timeline for rodent neurodevelopment does not correlate directly with that of humans. A newborn mouse, for example, is approximately neurodevelopmentally equivalent to a human fetus in the late second trimester (Ross, Graham, Money, & Stanwood, 2015). The significant differences between rodent and human fetal development warrant the need for noninvasive, ethical methods of studying human tissues at early developmental stages. Tissue from post-mortem humans has been effectively and reproducibly used to study the expression of opioid-receptor-encoding mRNA molecules, demonstrating their presence in the brain, spinal cord, and peripheral organs such as the intestines (Peng, Sarkar, & Chang, 2012). In addition, recent advances in three-dimensional *in vitro* culture techniques have led to brain organoids derived from induced human pluripotent stem cells that have been used to study changes in human protein expression and cell maturation. This model system is non-invasive and allows for the study of functional, living, human tissue (Di Lullo & Kriegstein, 2017), making it a potentially invaluable tool in the study

of the effects of opioid exposure on the developing human brain. These recent models could lead to a more translational understanding of the natural processes disrupted by exogenous opioids and elucidate the role of glial cells in these processes. Going forward, it would be constructive to pursue the opportunities that are now available to explore the effects of opioid exposure on human CNS development at the cellular and molecular level. In addition, further studies into how interactions between neurons and glial cells are affected by prenatal drugs and what role these interactions play in the pathology of NAS could provide key insights into potential future treatments and neurodevelopment as a generalized process. Finally, while many studies have investigated the effects of opioids in isolation on neurodevelopment, substance users rarely abuse only one drug. The interactions of multiple drugs may have unique effects on the development of exposed children that deserve additional investigation.

One of the primary goals of studying the effects of POE on human neurodevelopment is to raise awareness in the parents of the affected children as well as the clinicians and other caregivers responsible for the medical, psychiatric and psychological care of those children. Improved understanding of the effects of POE on behavioral and cognitive development, particularly during school age and adolescence, may inform parents and educators looking to meet the unique developmental and educational needs of impacted children. Behavioral studies targeting POE as an adverse early life event could explore whether or not the changes in development caused by this exposure predispose a child to develop addiction later in life. In regions of the United States that are struggling in terms of education and economic development, such as Central Appalachia where the rates of NAS can exceed 10% of all live births (Umer et al., 2019), the information gained from such behavioral studies could be vital to improving quality of life for those affected by opioid abuse. Therefore, further studies examining NAS, the

underlying molecular and cellular mechanisms involved, and its short and long-term effects on neurological development are critical to successfully combat the ongoing societal effects of opioid addiction.

Conflict of Interest Statement

The authors declare no conflict of interest.

Author Contributions

Both authors take responsibility for the accuracy of citations contained in the review manuscript. *Conceptualization*, T.B. and W.C.R.; *Writing – Original Draft*, T.B.; *Writing – Review & Editing*, T.B. and W.C.R.; *Supervision*, W.C.R.; *Funding Acquisition*, W.C.R.

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Table 1: Summary of the effects of prenatal opioids observed in different brain regions in either humans or rodents

Effects of Prenatal Opioids on Regions of the Brain		
Brain Region	**Functions**	**POE Effect**
Basal Ganglia	Motor control, motor learning, executive function, emotions	Humans: Decreased size in infants, 10-14-year-olds, and 17-22-year-olds (Yuan et al., 2014; Sirnes et al., 2017; Nygaard et al., 2018)
Thalamus	Integrate and relay sensory information, sleep and wakefulness	Humans: Decreased size in 10-14-year-olds and 17-22-year-olds (Sirnes et al., 2017; Nygaard et al., 2018)
Cerebellum	Coordination of voluntary motor function, balance, posture	Humans: Reduced white matter volume in 10-14-year-olds and 17-22-year-olds (Sirnes et al., 2017; Nygaard et al., 2018)
Prefrontal cortex	Inhibitory control, executive function, logical reasoning, planning, working memory	Humans: Greater activity during working memory-selective attention tasks in 9-12-year-olds (Sirnes et al., 2018)
Hippocampus	Long-term memory	Rats: Altered expression of apoptotic proteins (Wang et al., 2009); Attenuated maintenance and longevity of LTP (Villarreal et al., 2008)
Cerebral Cortex	Speech, language, decision making, learning, memory, vision, motor control, somatosensory processing, etc.	Humans: Decreased size in 17-22-year-olds (Nygaard et al., 2018)
Corpus Callosum	Connection and communication between hemispheres	Rats: Accelerated oligodendrocyte maturation and myelination (Vestal-Laborde et al., 2014)
Striatum	Goal oriented voluntary movement, reward and reinforcement	Rats: Decreased levels of met-enkephalin (Tempel et al., 1995)
Ventricles	Production and circulation of cerebrospinal fluid	Humans: Decreased size and slit-like shape in infants (Pasto et al., 1985)

Table 2: Observed effects of prenatal opioid exposure on rodent behavior

Behavioral Effects of Prenatal Opioids	
Aspect of Behavior	**POE Effect**
Anxiety	Decreased time in open arms of Elevated Plus Maze; Decreased time in light compartment of Light/Dark Box (Chen et al., 2015; Ahmadalipour et al., 2015; Klausz et al., 2011)
Depression	Longer immobility time in tail suspension and forced swim test (Wu et al., 2014; Klausz et al., 2011; Hung et al., 2013)
Reward Seeking	Greater preference for saccharin water (Gagin et al., 1996); Increased morphine self-administration (Hovious and Peters, 1985); Enhanced conditioned place preference (Gagin et al., 1997)
Memory	Longer escape latency in Morris Water Maze (Wang and Han, 2009); Reduced retention in novel object recognition test (Chen et al., 2015); Longer completion time in radial arm maze (Slamberova et al., 2001)

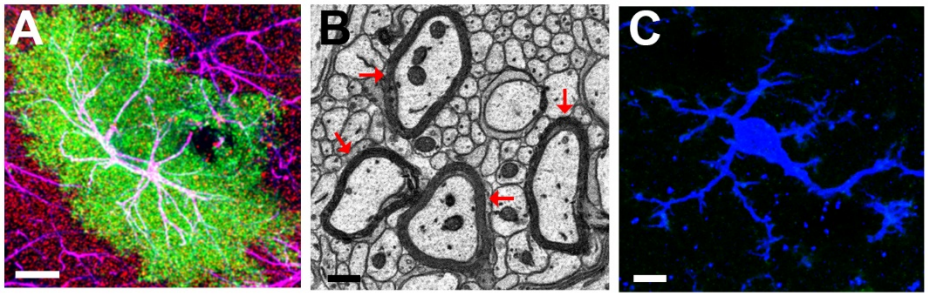

Figure 1: Glial cell function in development. A) Confocal reconstruction of green fluorescent protein (GFP)-tagged astrocyte (green) co-labeled with glial acidic fibrillary protein (GFAP; magenta; scale bar, 10 µm). Red punctate staining indicates locations of excitatory synapses, which are ensheathed by astrocytic processes (figure courtesy of Dr. Louise Risher, Marshall University). Astrocytic support of synapses is proposed to be disrupted by chronic opioid exposure. B) Electron micrograph showing cortical axons (arrows) previously myelinated by oligodendrocytes (scale bar, 0.5 µm). Accelerated myelination has previously been reported with POE. C) Confocal image of microglial cell body and processes labeled with Iba1 (scale bar, 5 µm). Microglial activation may represent a primary pathogenic mechanism following fetal exposure to opioids (Figures in B and C courtesy of Dr. W. Christopher Risher).

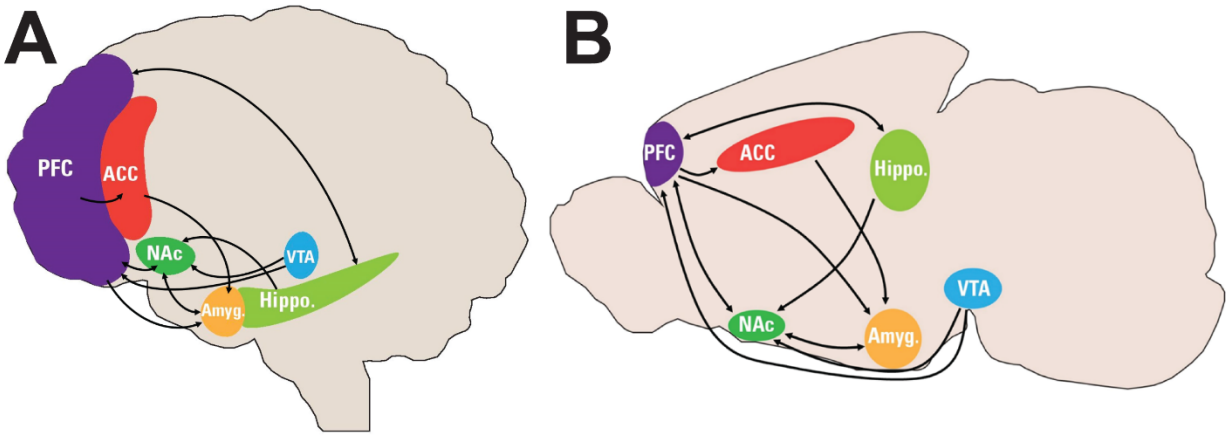

Figure 2: The mesolimbic dopamine pathway. Map of A) human and B) rodent brain highlighting areas associated with addiction/reward/withdrawal and the connections between

them: ventral tegmental area (VTA), hippocampus (Hippo.), amygdala (Amyg.), anterior cingulate cortex (ACC), nucleus accumbens (NAc), and prefrontal cortex (PFC).