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NLRP3 inflammasome: a key player in neonatal brain injury

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Among neonates, hypoxic-ischemic encephalopathy is the most significant cause of mortality and hypoxia-ischemia is among the leading causes of brain damage. The microglia are primary mediators of neuroinflammation. NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome activation is the first line of defense in the central nervous system. Numerous studies have shown that the NLRP3 inflammasome is activated and proinflammatory cytokines are upregulated upon hypoxia-ischemia–induced brain damage. However, aberrant activation of the NLRP3 inflammasome results in cell death and brain tissue damage. Given that neonates are particularly vulnerable to neuroinflammation, which may cause lifelong disabilities, it is important to target the pathways involved in its complex nature to improve their prognosis. The potential use of compounds or drugs that target inflammasome activation to relieve hypoxia-induced brain damage has become significant. This review describes the NLRP3 inflammasome in neonates to contribute to the development of therapeutic approaches.

Key words: NLRP3 inflammasome, Neonatal, Brain injury, Hypoxia

Key message

Hypoxic-ischemic encephalopathy is the major cause of neonatal brain injury. NOD-like receptor family pyrin domain containing 3 inflammasome activation leads to neuroinflammation, which significantly affects newborn mortality. The establishment of preventive and therapeutic strategies against brain damage requires a thorough understanding of the mechanisms underlying neuroinflammation and inflammasome activation in the neonatal brain.

Introduction

NOD-like receptor family pyrin domain containing 3 (NLRP3) protein, a cytosolic pattern recognition receptor,[1] constitutes a multiprotein complex referred to as the NLRP3 inflammasome, which acts as the first line of defense in response to stimuli from invading pathogens or intracellular danger signals.[2] The NLRP3 inflammasome maintains homeostasis as the primary function of innate immunity.[3] The broad range of stimuli sensed by NLRP3 originates from microorganisms including bacteria, fungi, and viruses in the form of conserved structures collectively referred to as pathogen-associated molecular patterns (PAMPs). On the other hand, damage-associated molecular patterns (DAMPs) are found within or around cells under physiological conditions; however, once released upon cellular damage, disruption, or death, DAMPs act as molecular patterns,[4] namely uric acid crystals, extracellular adenosine triphosphate (ATP), and beta-amyloid plaques.[5] The recognition of PAMPs and DAMPs by cellular receptors activates intracellular signaling pathways and triggers NLRP3 inflammasome assembly.[6]

The NLRP3 inflammasome, the most extensively studied inflammasome, comprises 3 main components: NLRP3 receptor, a sensor protein; apoptosis-associated speck-like protein containing a Caspase recruitment domain (ASC); and effector protein pro-Caspase-1.[7] The central protein of the inflammasome complex, NLRP3, has 3 domains: N-terminal pyrin domain (PYD), central NACHT domain as an oligomerization domain, and C-terminal leucine-rich repeat (LRR) domain.[2] Recognition of danger signals by the LRR domain results in the oligomerization of NLRP3 monomers through interactions with the NACHT domain.[5] The N-terminal PYD of NLRP3 is involved in ASC recruitment through PYD-PYD interactions, as PYD is one of the 2 protein-binding domains of ASC. The ASC speck is formed by multiple ASC filaments, which recruit pro-

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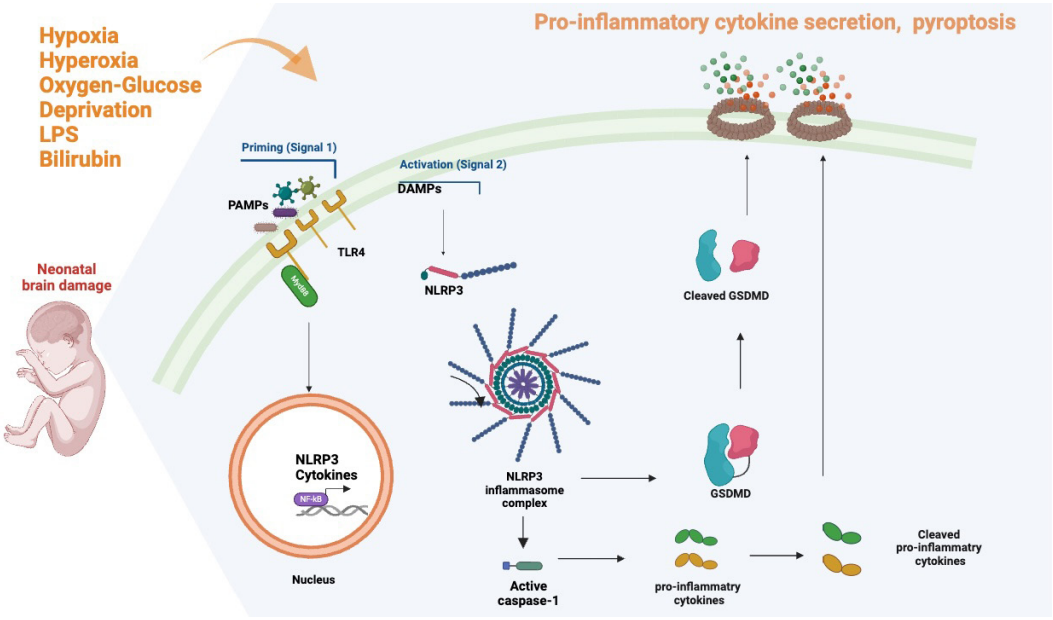

Graphical abstract. NOD-like receptor family pyrin domain containing 3 inflammasome activation in neonatal brain injury. LPS, lipopolysaccharides; PAMP, pathogen-associated molecular pattern; DAMP, damage-associated molecular pattern; TLR4, Toll-like receptor 4; NLRP3, NOD-like receptor family pyrin domain containing 3; GSDMD, Caspase-1 also cleaves Gasdermin D.

Caspase-1 through the second domain of ASC, C-terminal CARD.[8]

NLRP3 inflammasome activation requires 2 separate signaling steps: priming and activation. NLRP3 and pro-interleukin (IL)-1β protein expressions are available at the basal level but low and insufficient, necessitating a priming signal (signal 1) to induce their transcription.[5] Priming stimuli can include ligands for receptors IL-1 receptor, Toll-like receptors (TLRs), and NOD-like receptors (NLRs) as well as cytokine receptors such as tumor necrosis factor (TNF) receptor, leading to the activation of the transcription factor nuclear factor kappa B (NF-κB).[3,9] In the activation step (signal 2), the NLRP3 receptor is activated and the inflammasome complex subsequently formed through the recruitment of ASC and pro-Caspase-1. Intracellular ATP and K+ efflux, lysosomal destabilization, and mitochondrial reactive oxygen species (ROS) are among the molecular mechanisms underlying NLRP3 activation and complex formation.[10] Upon inflammasome complex formation, the effector protein Caspase-1 is activated; consequently, active Caspase-1 cleaves pro-IL-1β and pro-IL-18 cytokines and maturates each, leading to their secretion. Caspase-1 also cleaves Gasdermin D (GSDMD), which forms pores within the cellular membrane and eventually results in pyroptosis, a form of cell death.[11]

NLRP3 inflammasome activation is involved in distinctive pathways in different cell types, namely, canonical, noncanonical, and alternative.[12] The subsequent activation of Caspase-1 and GSDMD follows the recruitment of ASC and pro-Caspase-1[13]; thus, the priming and activation steps are generally referred to as the canonical pathways of NLRP3 inflammasome activation.[14] In contrast, human Caspase-4,5 or murine Caspase-11 can detect cytosolic lipopolysaccharides (LPS) originating from pathogens, including gram-negative bacteria.[12] In the noncanonical pathway, GSMDM is cleaved by the aforementioned caspases, resulting in pores within the cell membranes and leading to pyroptosis without necessitating inflammasome assembly.[15] In the noncanonical pathway, pyroptosis is directly induced, which leads to canonical cleavage and activation of the proinflammatory cytokines pro-IL-1β and pro-IL-18.[14] Moreover, in the alternative activation pathway, NLRP3 and Caspase are involved in pro-IL-1β cleavage, maturation, and secretion; however, pyroptosis is not observed in this particular type of activation.[16,17] Alternative activation has been observed in human monocytes via Caspase-8 activation.[18,19]

NLRP3 inflammasome activation has been implicated in various pathogenic diseases, particularly neuroinflammation, which is closely linked to neonatal brain injury. Several factors contribute to brain injuries in newborns. Hypoxic-ischemic encephalopathy (HIE) is a leading cause of brain injury, severe neurologic disability, and death in infants worldwide.[20,21] Neuroinflammation following hypoxia-ischemia (HI) causes neuronal injury and brain cell death in HIE.[22] HIE is characterized by immune response activation and ROS and nitrogen species generation, mitochondrial damage, and interrupted vascular flow. Microglial cells play a primary role in neuroinflammation during HIE.[23,24] Aberrant microglial activation causes

widespread neuroinflammation, ultimately leading to cellular death.[25] Infections such as chorioamnionitis and neonatal sepsis can also initiate inflammatory responses that harm the brain. Prematurity is another significant risk factor since preterm infants are especially susceptible to hyperoxia and other forms of brain injury owing to their immature neurological development. One critical aspect of this injury is periventricular leukomalacia (PVL), a condition characterized by death of the white matter near the ventricles that is frequently associated with preterm birth. Neuroinflammation plays a crucial role in PVL, as the activation of glial cells and release of proinflammatory cytokines can exacerbate neuronal damage and disrupt normal brain development.[26,27]

Hyperbilirubinemia can also increase a newborn's risk of brain injury. The fat-soluble nature of bilirubin enables it to pass through the blood–brain barrier and aggregate within the brain cells.[28] Understanding the underlying mechanisms is essential to the development of effective prevention and early intervention strategies.[29] Collectively, these neonatal diseases disrupt the developing brain and contribute to neuroinflammation. Although *in vitro* modeling of neonatal brain injuries is challenging, a few *in vitro* and *in vivo* disease models and clinical studies have investigated brain injury and NLRP3 inflammasome activation in neonates. This review investigates the role of the NLRP3 inflammasome in conditions that lead to neonatal brain injury (Fig. 1) and discusses possible therapeutic approaches.

Evidence of NLRP3 inflammasome activation in *in vitro* model of neonatal brain injury

1. Microglia

Microglia, the resident immune cells that act as the first line of defense in the central nervous system (CNS), have numerous functions including immune surveillance, synaptic pruning, phagocytosis, and CNS development.[30] Given that *in vitro* modeling of neonatal brain injury is

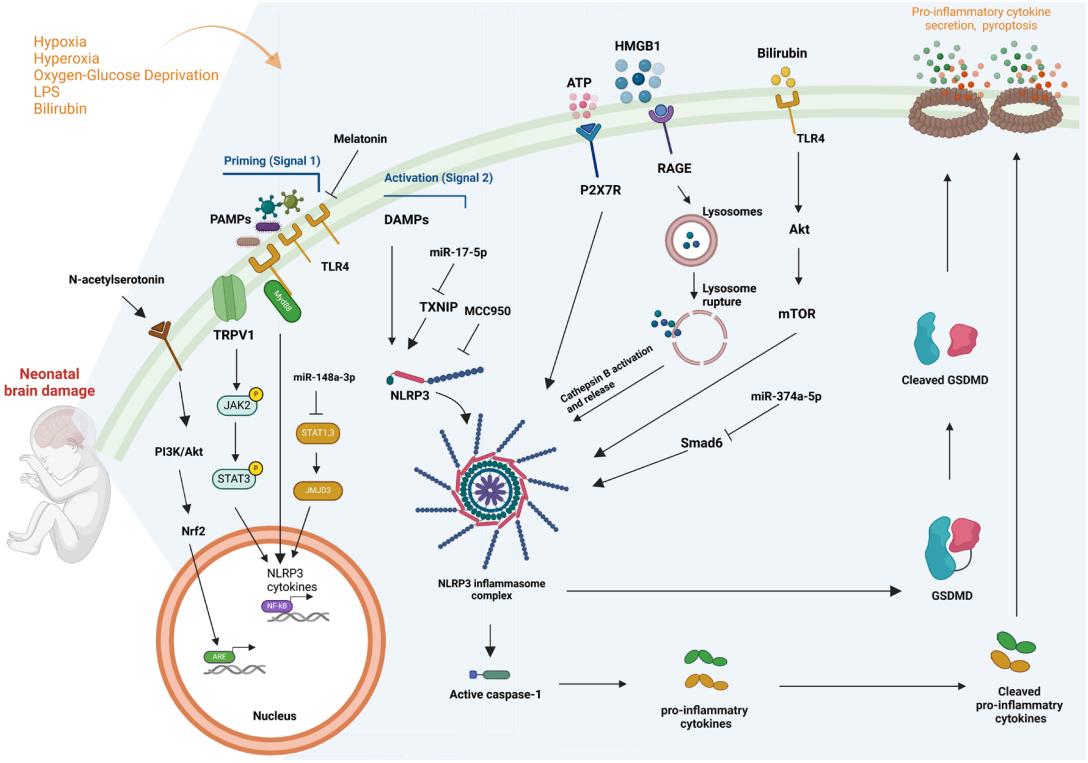

Fig. 1. Summary of neonatal brain damage-induced NLRP3 inflammasome activation and related pathways. The NLRP3 inflammasome is activated upon neonatal brain damage and plays an important role in neuroinflammation. Once PAMPs activate TLR4 signaling and DAMPs are sensed by NLRP3 within the cell, the NLRP3 inflammasome complex is formed, and the proinflammatory cytokines IL-1β and IL-18 are cleaved by active Caspase-1 and secreted. Several pathways are associated with NLRP3 inflammasome activation in neonatal brain damage, namely PI3K/Akt, JAK2/STAT3, mTOR, the Smad6 axis, and miRNAs. DAMPs, damage-associated molecular patterns; IL, interleukin; mTOR, mammalian target of rapamycin; NLRP3, NOD-like receptor family pyrin domain containing 3; PAMPs, pathogen-associated molecular patterns; PI3K, phosphoinositide 3-kinase; TLR4, Toll-like receptor 4; LPS, lipopolysaccharides; ATP, adenosine triphosphate; P2X7R, P2X7 receptor; HMGB1, high mobility group box 1; TRPV1, transient receptor potential vanilloid 1; TXNIP, thioredoxin-interacting protein; JAK2, Janus kinase 2; STAT3, signal transducer and activator of transcription 3; JMJD3, Jumonji domain-containing protein 3; GSDMD, Gasdermin D; NF-κB, nuclear factor κB. Graphic created using Biorender.

Table 1. Mechanisms of action of *in vitro* models of hypoxia-induced NLRP3 inflammasome activation

Cell line	Model/inducer	Outcome	Mechanism	References
BV2 microglia cell line	OGD	Caspase-1 ↑, GSDMD ↑	HMGB1/RAGE/Catepsin	24)
Primary microglia from 1- to 2-day-old CD1 mice	Free bilirubin for 24 hr	HMGB1↑, TNF-α↑, IL-1β↑, IL-6↑, IL-10↑, Arg1↑	-	35)
Primary microglia from cerebral cortex of neonatal rats	Unconjugated bilirubin for 24 hr	NLRP3 ↑, ASC ↑, IL-1β ↑	Akt–mTOR	36)
N9 microglia cell line	Bilirubin and LPS for 6 hr	NLRP3 ↑, Caspase-1 ↑, IL-1β ↑	-	37)
Primary microglia postnatal day-1 rat	LPS for 24 hr	NLRP3 ↑, ASC↑, Caspase-1↑, TNF-α↑, IL-1β↑, IL-6↑	miR-374a-5p – Smad6	20)
Primary microglia culture from 1/3-day-old rats	LPS for 24 hr	NLRP3↑, ASC↑, Caspase-1↑, IL-1β↑, IL-6↑, TNF-α↑	-	39)
Primary microglia from postnatal day-1 rat	LPS and serum amyloid A protein for 1–24 hr	NLRP3 ↑, IL-1β ↑	P2X7R	40)
A microglial cell line derived from C57BL/6 mice (NR-9460)	NMEC RS218	IL-1β↑, IL-1α↑	TLR4/Myd88	41)
Primary mouse cortical astrocytes from P0 mice	OGD	NLRP3↑, ASC↑, Caspase-1↑, TNF-α↑, IL-1β↑, IL-6↑, IL-10↑	JAK2-STAT3	42)
Rat hippocampal astrocytes from 1-day-old rats	OGD	NLRP3↑, ASC↑, Caspase-1↑, GSDMD↑, IL-1β↑, IL-18↑	miRNA-148a-3p STAT/JMJD3 axis	43)
Primary cortical astrocytes from 3-day-old rats	Unconjugated bilirubin for 6 and 12 hr	Caspase-1 ↑, NLRP3 ↑, IL-1β ↑, IL-18 ↑	-	44)
PC12 rat pheochromocytoma cells	OGD	NLRP3↑, ASC↑, GSDMD↑, IL-1β↑, IL-18↑	-	46)
PC12 rat pheochromocytoma cells	CoCl2 for 24 hr	NLRP3↑, ASC↑, Caspase-1↑, TNF-α↑ , IL-1β↑, IL-6↑, IL-18↑, NfκB↑	-	22)
PC12 rat pheochromocytoma cells	OGD	NLRP3↑, ASC↑, Caspase-1↑	-	47)
Primary neuron culture from D18 rat fetus brain	OGD	Caspase-1 ↑, NLRP3 ↑, IL-1β ↑	-	48)

ASC, apoptosis-associated speck-like protein containing a Caspase recruitment domain; GSDMD, Gasdermin D; HMGB1, high mobility group box 1; IL, interleukin; LPS, lipopolysaccharide; NF-κB, nuclear factor κB; NLRP3, NOD-like receptor family pyrin domain containing 3; NMEC, neonatal meningitis-associated *Escherichia coli*; OGD, oxygen/glucose deprivation; TNF-α, tumor necrosis factor-alpha.

complicated, only a limited number of studies have been conducted (Table 1). For the *in vitro* HI model, an oxygen/glucose deprivation (OGD) method was used under hypoxic conditions. Previous studies demonstrated that the pro-inflammatory parameters TNF-α, IL-1β, inducible nitric oxide synthase, and NF-κB were upregulated upon OGD in primary microglia of 3-day-old rats and a BV2 microglia cell line.[31-33] In a recent study of microglial inflammation in neonatal HIE, Caspase-1 and GSDMD expression levels were elevated in BV2 microglia-like cells upon OGD. The authors employed recombinant high mobility group box 1 (HMGB1) to show that Caspase-1 and GSDMD levels were further increased, whereas use of the HMGB1 inhibitor glycyrrhizin significantly alleviated Caspase-1 and GSDMD expression levels. Cell viability significantly decreased in HT22 hippocampal neuron-like cells incubated with conditioned medium from BV2 microglia subjected to OGD, suggesting that its effect on neurons occurs through microglial inflammasome activation.[24]

Bilirubin, an inducer of neuroinflammation, causes pyroptosis.[34] Upon free bilirubin treatment, HMGB1, TNF-α, IL-1β, IL-6, IL-10, and Arg1 levels were upregulated in microglia isolated from neonatal mice.[35] Furthermore, in primary microglia isolated from the cerebral cortex of neonatal rats, treatment raised NLRP3 and ASC protein levels, ASC speck formation, and IL-1β cytokine mRNA and protein levels. Bilirubin-induced increases in NLRP3 and ASC levels are mitigated by rapamycin, a mammalian target of rapamycin inhibitor.[36] We recently demonstrated that bilirubin-induced neurological dysfunction in newborns is mediated by NLRP3 inflammasome activation. We also demonstrated that active Caspase-1 and IL-1β release, as well as final cell death, are significantly connected with the upregulation of NLRP3 expression in N9 microglial cells exposed to bilirubin. To ascertain whether NLRP3 was necessary for bilirubin-induced inflammation, we performed functional *in vitro* studies using NLRP3 small interfering RNA. Our results suggested that the NLRP3 inflammasome is most likely responsible for bilirubin-induced inflammasome activation and cellular death.[37]

LPS are often used to introduce neuroinflammation.[38] Other studies investigating NLRP3 inflammasome activation in the microglia of neonates demonstrated that LPS treatment in primary microglia derived from neonatal rats led to upregulation of the inflammasome markers NLRP3, ASC, and Caspase-1 and increased expression levels of the proinflammatory cytokines TNF-α, IL-1b, IL-6, and IL-8.[20,39] We also showed that LPS-induced NLRP3 and ASC levels and -cleaved Caspase-1 protein levels were reduced by miR-374a-5p, which targets Smad6.[20] Additionally, oste-

opontin, a secretory extracellular matrix glycoprotein, reduces elevated levels of inflammasome parameters and cytokines.[39]

Studies have shown that other factors induce inflammasome activation. The TLR4 agonist serum amyloid A protein (SAA) has an effect similar to that of LPS in NLRP3 inflammasome activation. In this study, LPS and SAA treatment increased NLRP3 levels and IL-1β cytokine secretion. Use of the P2X7R antagonist alleviated the IL-1β levels in primary rat microglia.[40] In addition, limited studies investigated NLRP3 inflammasome activation in neonates against bacterial infections. Neonatal meningitis-associated *Escherichia coli* (NMEC) causes sepsis and meningitis in newborns. In a recent study, the levels of proinflammatory cytokines IL-1b and IL-1a increased upon NMEC infection. When microglia were treated with an inhibitor of NLRP3-dependent Caspase-1 activation (MCC950), IL-1b and IL-1a secretions were attenuated, suggesting that cytokine secretion in response to infection was dependent on the NLRP3 inflammasome. The evidence indicates that neonatal neuroinflammation depends on microglial NLRP3 inflammasome activation via the TLR4/Myd88 axis.[41] Together, these findings suggest that NLRP3 inflammasome activation occurs through numerous factors and that subsequent proinflammatory cytokine secretion is involved in microglia-induced neuroinflammation in neonates.

2. Astrocytes

Astrocytes are glial cells in the CNS involved in numerous processes, including neurodegeneration and hypoxia-induced brain death (HIBD). Studies have shown that NLRP3, ASC, and Caspase-1 inflammasome parameters as well as the proinflammatory cytokines TNF-α, IL-1β, IL-10, and IL-6 are increased in primary cultured hippocampal astrocytes upon OGD.[42] In another study of primary rat astrocytes, when the cells were treated with OGD and subjected to hypoxia, NLRP3 and cleaved Caspase-1 were significantly upregulated, whereas treatment with the ZJU37 inhibitor receptor-interacting protein kinase-1/-3 attenuated this increase. NLRP3, ASC, Caspase-1, GSDMD, IL-1β, and IL-18 levels were elevated in primary hippocampal astrocytes after OGD. Dexmedetomidine relieved the expressions of inflammasome parameters and proinflammatory cytokines.[43] NLRP3 inflammasome activation was also observed in cultured rat astrocytes treated with unconjugated bilirubin. NLRP3 protein level, Caspase-1 activity, and IL-1β and IL-18 cytokine levels and pyroptosis were increased upon bilirubin treatment. These increased levels were attenuated by VX-765 treatment.[44] Altogether, these results suggest that hypoxia activates the NLRP3 inflammasome in the microglia and astrocytes of the CNS. Evidence also indicates that hypoxic microglia are involved in pyroptotic cell death and proinflammatory mediator production. Targeting microglial NLRP3 inflammasome activation would be a promising way to attenuate cell death and neuronal injury as well as improve HIBD pathophysiology. Given the current lack of effective treatment for HIBD, it is important to establish a target to relieve damage progression.

3. Neurons

Furthermore, NLRP3 inflammasome activation and microglial inflammation during neonatal brain damage have been investigated in neuronal cells. Neurons are the cells of the brain that are involved in information exchange within the CNS and have unique roles in the CNS.[45] Following HIBD, neuronal cell death occurs in the neonatal brain.[46]

A neonatal HIBD model was established using $CoCl_2$-induced PC12 pheochromocytoma cells, which are widely used in HIBD studies. The authors provided evidence that $CoCl_2$ decreases neuronal viability following HIBD. Moreover, expression levels of TNF-α, IL-1β, IL-18, and IL-6 proinflammatory cytokines as well as NF-κB, Iκb, NLRP3, ASC, and Caspase-1 were significantly upregulated.[22,46] In another study, the OGD-induced increases in NLRP3, ASC, and Caspase-1 were suppressed by HIBD-associated peptide treatment in PC12 rat cells.[47] Treatment with diallyl disulfide reverses this increase in NLRP3 inflammasome parameters and cellular death.[46] Moreover, NLRP3, Caspase-1, and IL-1β levels were significantly increased in OGD-treated primary neurons isolated from D18 rat fetuses.[48] Together, these findings suggest that the microglial NLRP3 inflammasome plays a significant pathological role in HIBD and that its activation could rescue HIBD-induced neuronal damage.

In vivo NLRP3 inflammasome activation in neonatal brain injury

1. Hypoxia

In vitro NLRP3 inflammasome activation upon hypoxia initiated further investigations of NLRP3 inflammasome activation *in vivo* to elucidate its physiological validity (Table 2). In an *in vivo* study, NLRP3 was upregulated upon neonatal HI in mice. Within 24 hours of HI, NLRP3 expression was significantly increased in the brain.[49] In this study, we investigated whether NLRP3 or ASC deficiency protected against neonatal HI brain injury. RNA sequencing data revealed impaired the $NLRP3^{-/-}$ murine hippocampal transcriptional response to inflammation. TNF-α levels were also decreased in $NLRP3^{-/-}$ and $ASC^{-/-}$

Table 2. Mechanisms of action of *in vivo* models of hypoxia-induced NLRP3 inflammasome activation

Model	Outcome	Pathway	References
Neonatal HIBD in mice	NLRP3 ↑		49)
Neonatal HIBD in NLRP3$^{-/-}$ and ASC$^{-/-}$ mice	NLRP3$^{-/-}$ mice had more activated microglia than Wt mice, but ASC$^{-/-}$ animals had less active microglia	-	50)
Neonatal HIBD in a rat model with, NLRP3 inhibitor	MCC950, NLRP3 inhibitor, significantly reduced pyroptosis in a rat model of neonatal HIBD	-	21)
Neonatal rat model of inflammation-sensitized HIBD	IL-1β, IL-6, iNOS, and TNF-α, Arg1, CCL11, IL-1, and TGF-β and NLRP3↑ in sorted CD11b/c microglia of brain samples	-	51, 52)
Neonatal HIBD in a rat model	Caspase-1, IL-1β, IL-18↑ NLRP3 expression ↑ at 6 hr to 24 hr after HI, which declined 48 h after HI.	-	51, 52)
Neonatal HIBD model with Gingko Biloba	IL-1β, IL-18↑ NLRP3↑ Caspase-1 and the nuclear translocation of NFκB P65↑	-	56)
Neonatal HIBD in a rat model with IRE1α inhibitor	TXNIP/NLRP3 inflammasome activation	TXNIP/NLRP3 - miR-17-5p	57)
White matter damage (WMD) caused by endotoxin and ischemic hypoxia in neonatal rats	NLRP3, IL-1β and IL-18 ↑	TLR4/NFκ-B pathway	58)
Neonatal HIBD in a rat model with N-acetylserotonin derivative	NLRP3↑	PI3K/Akt/Nrf2 pathway	59)
Neonatal HIBD in a rat model with neferine	Caspase-1, ASC, GSDMD, TNFα, IL-β, IL-18, IL-6 ↑	-	22)
HI-induced model of cerebral white matter damage (WMD)	NLRP3, Caspase-1, IL-1β, TNFα, IL10 ↑	-	48, 51-55)
HI-induced model of cerebral white matter damage (WMD) with caffeine	NLRP3 inflammasome activation in neonatal rats	-	54)
Neonatal rat model of inflammation-sensitized HIBD	NLRP3, CXCL1 and CXCR2↑	CXCL1/CXCR2 pathway	43, 60)
Neonatal HIBD in a rat model with Dexmedetomidine	IL-1β and IL-18 cytokine levels and NLRP3, ASC, Caspase-1, GSDMD ↑	-	43)
Rat model of neonatal sepsis with rCC16 (recombinant club cell protein)	NLRP3, Caspase-1, TNFα, IL-1β and IL-6 levels, phosphorylated p38-MAPK and ERK ↑	-	63)
HI in Wt and Nrf2$^{-/-}$ mice	NFκB and NLRP3↑	-	61)
Neonatal HIBD in a rat model with maternal treadmill exercise	NLRP3, Caspase-1 ↑	-	53)
Neonatal hyperoxia in mice	NLRP3, IL-1β, Caspase-1 ↑	-	37)
Neonatal bilirubin-induced neuronal damage (BIND) model in rats with rapamycin	NLRP3 and ASC protein ↑	-	36)
Neonatal BIND model in Nlrp3$^{-/-}$, Caspase-1$^{-/-}$ and WT mice	NLRP3, Caspase-1 and IL-1β↑ Diminished in knockouts	-	37)
Neonatal meningitis-associated *Escherichia coli* (NMEC). NLRP3$^{-/-}$and Caspase-1$^{-/-}$ mice	IL-1b and IL-1a ↑ Diminished in knockouts	-	41)
Neonatal encephalopathy (NE) patients	IL-1ra, IL-18, NLRP3 and IL-1 ↑	-	64)
Mice model of preterm brain injury	NLRP3 and IL-1β ↑	-	62)

ASC, apoptosis-associated speck-like protein containing a Caspase recruitment domain; CXCL1, chemokine (C-X-C motif) ligand 1; CXCR2, C-X-C chemokine receptor 2; ERK, extracellular signal-related kinase; GSDMD, Gasdermin D; HI, hypoxia-ischemia; HIBD, hypoxia-induced brain death; IL, interleukin; iNOS, inducible nitric oxide synthase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κB; NLRP3, NOD-like receptor family pyrin domain containing 3; TGF-β, transforming growth factor-beta; TNF-α, tumor necrosis factor-alpha, TXNIP, thioredoxin-interacting protein; Wt, wild-type.

versus wild-type mice, whereas plasma levels of IL-1 and IL-18 were unaffected. The authors concluded that ASC deficiency is neuroprotective but that NLRP3 deficiency exacerbates brain damage later in the course of neonatal HIBD.[50] Thus, here we confirmed the importance of NLRP3 inflammasome activation in neonatal HIBD.

A number of studies have shown activation of the NLRP3 inflammasome and increased levels of the proinflammatory cytokines IL-1β, IL-18, and IL-10 as well as increased mRNA and protein expressions of NLRP3 and Caspase-1 in rat models of neonatal HIBD.[48,51-55] These findings reveal that NLRP3 inflammasome activation further activates pathways that contribute to neuroinflammation in HIBD. The identification of the underlying mechanisms and pathways could contribute to the development of potential treatments by targeting these pathways.

In animal models of neonatal HIBD, brain injury is reduced by several medications that block NLRP3 activation. These studies also investigated downstream processes. For example, the administration of *Gingko biloba* prior to ischemia induction prevents activation of the NLRP3 inflammasome and reduces brain damage.[56] Moreover, in a neonatal HIBD model in rats, inositol-requiring transmembrane kinase/endoribonuclease 1α suppression reduced TXNIP/NLRP3 inflammasome activation through miR-17-5p.[57] Another study found that the administration of MCC950, an NLRP3 inhibitor, dramatically reduced pyroptosis in a rat model of neonatal HIBD.[21] The increased

expression levels of NLRP3 and downstream inflammatory factors including IL-1β and IL-18 were reduced by melatonin therapy via mitochondrial autophagy and TLR4/NF-κB pathway activity reduction.[58]

Furthermore, in a rat model of HIBD, an N-acetylserotonin derivative exerted neuroprotective effects by activating the PI3K/Akt/Nrf2 pathway and inhibiting the NLRP3 inflammasome.[59] Neferine, an alkaloid extracted from lotus seed embryo, was also used as a rescue therapy in a rat model of HI. Authors showed that, compared to the control group, newborn HI model rats had significantly higher levels of Caspase-1, ASC, GSDMD, TNF-α, IL-β, IL-18, and IL-6. Treatment with neferine considerably decreased these variables.[22] In another study, caffeine treatment alleviated NLRP3 inflammasome activation in neonatal rats.[54] IL-1β, IL-18 NLRP3, ASC, Caspase-1, and GSDMD levels were elevated upon HIBD in hippocampal astrocytes of neonatal rats. Dexmedetomidine treatment mitigates these elevated levels.[43,60] In another study, an HIBD model was established in wild-type and $Nrf2^{-/-}$ mice. Hydrogen treatment alleviated cellular injury and the inflammatory response following HI via the Nrf2-mediated NLRP3 and NF-κB pathways. The absence of Nrf2 abolishes the suppressive effect of hydrogen on NLRP3 pathway expression.[61] Interestingly, another study showed that maternal treadmill exercise attenuated alterations in Caspase-1 and NLRP3 gene expression and provided better neurological outcomes in a rat model of neonatal HI.[53] These results suggest that addressing NLRP3 inflammasome activation is important for attenuating HIBD, although the long-term effects of these agents at the cellular and behavioral levels were not investigated in the aforementioned studies.

2. Hyperoxia and preterm brain injury

In addition to hypoxia-induced brain injury, hyperoxic brain damage occurs via NLRP3 inflammasome activation in newborns. Our group demonstrated for the first time that exposure to O_2 in the early periods of life elevated NLRP3 expression in a mouse model of preterm brain injury. The neonatal murine brain displayed a global increase in immunopositive cells for NLRP3 and IL-1β after 7 days of hyperoxic exposure. Hyperoxia-treated mice demonstrated a significant increase in the number of Caspase-1 positive cells in the prefrontal and parietal areas.[62]

3. Infections

Sepsis and meningitis are intertwined conditions in neonates that occur in response to bacterial infections. One study found that postnatal day 10 rats with sepsis had considerably higher levels of NLRP3, Caspase-1, and the proinflammatory cytokines TNFα, IL-1β, and IL-6. They also discovered that sepsis elevated the levels of phosphorylated p38-MAPK and extracellular signal-regulated kinase (ERK) in the cortex. Treatment with recombinant club cell protein 16 treatment inhibited the increased levels of phospo-p38-MAPK and phospho-ERK as well as the expression levels of NLRP3, Caspase-1, and proinflammatory cytokines.[63] In another study, IL-1b and IL-1a were secreted from microglia upon induction with neonatal meningitis-associated *Escherichia coli*. To further elucidate whether cytokine secretion is dependent on NLRP3 inflammasome activation, we used NLRP3 ad Caspase-1 knockout mice and observed diminished cytokine secretion.[41]

4. Neonatal jaundice

Unconjugated bilirubin is another inducer of the NLRP3 inflammasome in the newborn brain. Bilirubin-induced encephalopathy impairs multiple brain regions, including the hippocampus and the basal ganglia. NLRP3 and ASC protein levels increased in the hippocampal tissue obtained from rats after a bilirubin injection, while rapamycin treatment reduced bilirubin-induced neuroinflammation.[36] Our group revealed, for the first time, that bilirubin activates microglial NLRP3 inflammasomes *in vitro* and *in vivo* in newborn brains.[37] Bilirubin treatment noticeably increased neuronal loss in wild-type versus $Nlrp3^{-/-}$ and $Caspase\text{-}1^{-/-}$ mice. Taken together, our findings suggest that the NLRP3 inflammasome is essential for microglial activation and bilirubin-induced neuronal damage.[37]

Clinical studies in NLRP3 inflammasome activation

Numerous *in vitro* and *in vivo* studies have investigated NLRP3 inflammasome activation and its potential drug targets for clinical studies. A few studies have examined the NLRP3 inflammasome in neonates. For example, NLRP3 inflammasome activation was examined in serum samples from patients with neonatal encephalopathy (NE) in the clinical setting. Serum samples were collected on a serial basis during the early weeks after birth and at school age from 40 babies with NE and 19 healthy babies. No difference was observed in NLRP3 expression in the peripheral blood of neonates with NE versus healthy controls at baseline. However, when the serum samples were stimulated with LPS in neonates with NE, NLRP3 expression was upregulated on day 3 versus baseline. ASC expression was comparable between controls and patients with NE and remained unchanged after LPS stimulation compared to nonstimulation in newborns. Compared with LPS-stimulated controls, school-aged children with NE had

higher NLRP3 levels after LPS stimulation.[64]

NLRP3 inflammasome activation is also crucial in the pathogenesis of other neonatal conditions, including Zika virus infection, that leads to significant CNS damage, particularly among those with microcephaly.[65] An association between NLRP3 and neuroinflammation was also observed in cryopyrin-associated periodic syndromes (CAPS), a group of rare autoinflammatory diseases caused by *NLRP3* gene mutations. Neonatal-onset multisystem inflammatory disease (NOMID), a severe form of CAPS, is characterized by systemic inflammation, neurological impairment, skin rashes, and arthritic manifestations. In these patients, *NLRP3* mutations lead to dysregulated inflammatory responses that severely affect multiple organ systems including the CNS.[66] Anakinra, an IL-1 receptor antagonist, demonstrated promising long-term efficacy at controlling systemic inflammation and ameliorating neurological symptoms in patients with NOMID. However, its effectiveness depends on treatment timing, i.e., early intervention prevents irreversible damage, while delays in diagnosis and treatment may lead to persistent symptoms, such as CNS inflammation, hearing loss, and secondary amyloidosis.[67] In summary, these findings underscore the importance of NLRP3 inflammasome activation in the progression of neonatal diseases and highlight the therapeutic potential of targeting this pathway in inflammatory conditions.

Table 3. Potential preventive or therapeutic reagents used in *in vitro*, *in vivo* and clinical studies mentioned in this article

Study type	Reagents	Mechanism of action	References
In vitro	Glycyrrhizin	HMG1 inhibitor	24)
	Rapamycin	mTOR inhibitor	36)
	NLRP3 siRNA	siRNA	37)
	miR-374a-5p mimic	Targeting Smad6	20)
	Osteopontin	Secretory extracellular matrix glycoprotein	39)
	A740003	$P2X_7R$ antagonist	40)
	MCC950	NLRP3 inhibitor	41)
	ZJU37	RIP1/RIP3 inhibitor	68)
	Dexmedetomidine	α2-adrenoceptor agonist	43)
	VX-765	Caspase-1 inhibitor	44)
	HIBDAP	Pyroptosis suppressor	47)
	Diallyl disulfide	Pyroptosis suppressor	46)
In vivo	Gingko Biloba	NLRP3 supressor	56)
	miR-17-5p	IRE1α supressor	57)
	MCC950	NLRP3 inhibitor	21)
	Melatonin	TLR4/NF-κB pathway inhibitor	58)
	N-acetylserotonin	PI3K/Akt/Nrf2 pathway activator	59)
	Neferine	NLRP3 inflammasome activation supressor	22)
	Caffeine	A2aR antagonist	54)
	Dexmedetomidine	α2-adrenoceptor agonist	43, 60)
	Hydrogen	Activator of Nrf2-mediated NLRP3 and NF-κB pathways	61)
	rCC16	Pyroptosis suppressor	63)
Clinic	Anakinra	IL-1 receptor antagonist	67)

HMG1, high mobility group 1; IL, interleukin; IRE1, inositol-requiring transmembrane kinase/endoribonuclease 1α; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor κB; NLRP3, NOD-like receptor family pyrin domain containing 3; PI3K, phosphoinositide 3-kinase; RIP1, receptor-interacting protein 1; RIP3, receptor-interacting protein 3; siRNA, small interfering RNA; TLR4, Toll-like receptor 4.

Conclusion

Understanding the mechanisms driving newborn brain injury is critical to the development of preventive and therapeutic measures such as the reagents mentioned in this article (Table 3). We can better identify at-risk populations and design tailored therapies by understanding the complex processes involved in neonatal brain injury, such as neuroinflammation and the functions of specific molecular players such as the NLRP3 inflammasome. This understanding not only improves our capacity to minimize acute damage, but it may improve the long-term results for affected infants. As research on the complexity of neonatal brain injury progresses, collaboration across disciplines will be critical to converting the results into effective therapeutic practices, eventually protecting the health and development of vulnerable babies.

Footnotes

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