Chapter

The Role of Purinergic Signaling in the Pathophysiology of Perinatal Hypoxic-Ischemic Encephalopathy

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Abstract

Perinatal hypoxic-ischemic encephalopathy (HIE), known as birth asphyxia, remains a major contributor to poor neurodevelopmental outcomes including cerebral palsy and seizures. One striking feature of HIE injury is a delayed progression of neuronal degeneration that spreads over time from the most severely damaged areas outward into neighboring undamaged regions. There is increasing evidence that these lesions act as sites of origin for waves of spreading depression (SD), a wave of neuronal and glial depolarization, that progressively enlarge the brain lesions. While the pathophysiology of SD is still under debate, there is increasing evidence that purinergic receptors in conjunction with connexin and pannexin 1 channels are necessary for sustained propagation of the waves and neuroinflammation. This review intends to discuss the relative contribution of purinergic signaling and connexin and pannexin 1 channels to trigger and spread SD waves leading to the development of progressive brain lesions under conditions of perinatal HIE.

Keywords: spreading depression, gap junctions, hypoxic-ischemic encephalopathy, purinergic signaling

1. Introduction

Perinatal hypoxic-ischemic encephalopathy (HIE), also known as birth asphyxia, is a neurological syndrome that affects newborns worldwide, causing life lasting sequelae and not only impacting individual lives but also economy and public health systems [1, 2]. It is estimated that HIE occurs between 1 and 8 per 1000 newborns, although this number can be widely more expressive accordingly to the region, especially in low-income countries [3, 4]. Besides, HIE is considered to be the third major cause of neonatal mortality, accountable for 23% of newborn deaths and averaging about 1 million children annually [5, 6]. In consonance, the morbidity is not far behind: HIE accounts for 10% of all cerebral palsy cases,

compromising the quality of life of these patients and their families [7–9]. In this scenario, understanding the factors and mechanisms involved in HIE etiology is crucial to the development of new therapeutic strategies and improvement of mortality and morbidity rates.

Over the last years, several studies emerged intending to explain the pathophysiology of HIE [10–13]. The gaps in these topics are highlighted as particular priorities among the many critical areas that remain to be integrated. It is a consensus that the primary event for HIE burden is a global reduction of blood flow to the fetal brain during pregnancy or birth process [13]. This ischemic-hypoxic insult may result from various maternal or fetal conditions such as umbilical cord prolapse, rupture of the uterus and placental insufficiency [1, 14, 15]. One of the most intriguing features of HIE, however, is not even the ischemic injury per se, but the ability of the initial lesion to expand to previously undamaged areas (i.e., secondary lesions) [16]. Much of this process remains undefined and saving perilesional regions at risk, termed penumbra, challenges physicians and scientists. In this context, increasing evidence supports extracellular ATP and purinergic receptors as significant players in HIE pathophysiology and, consequently, a unique addition as interventional targets for the limited repertoire of drugs currently available as therapeutic approaches for HIE [17–19].

Purinergic receptors are a class of ligand-gated receptors divided into two groups P1 and P2, responsive to nucleosides and nucleotides, respectively. P1 receptors have four members described (A1, A2A, A2B e A3) [20–22]. On the other hand, P2 receptors are subdivided into two families P2Y and P2X. Both P2Y receptors and the P1 receptors are G coupled protein receptors (GPCRs), and the P2X receptors are ionotropic receptors. Those receptors are expressed in various systems in the organism, as the vascular system, the immune system, the gastrointestinal system, the renal system, and the central nervous system (CNS) [20–22]. Specifically, in the CNS, it is believed that some purinergic receptors could play essential roles in ischemia decreasing the symptoms and the extent of brain damage [23, 24].

Along with purinergic signaling, there are strong pieces of evidence in the literature correlating spreading depression (SD), a phenomenon characterized by self-sustained waves of depolarization of a sizeable population of cells, and hypoxic-ischemic insults [25, 26]. HIE-mediated SDs erupt in the brain, encumbering tissue structure and function, and raising fascinating—and still unanswered—questions concerning their initiation (i.e., genesis) and propagation. Even more ominously, erupting SDs accelerate tissue damage following HIE or traumatic brain injury (TBI) [27, 28]. Studies by many authors, including our group [29], suggest that lesions act as a site of origin to these waves, which slowly propagate through the brain surface [30]. So far, the role of glia (astrocytes and microglia) in initiation, propagation, and recovery of SD is poorly understood [31, 32]. Although there is evidence that SD has an impact on astrocytes and microglia [31, 32], the consequences of their activation need to be further explored. Considering SD as an electrochemical event, ionic changes in the extracellular medium are important to the genesis and maintenance of propagated SDs. In this regard, recent studies present pannexins and connexins paved the way for sustainable SD propagation [26]. Hence, HIE detains a multifactorial mechanism, compelling not only purinergic signaling but also spreading depression, connexins, pannexins and many other biological processes yet to be defined.

This article intends to review the aspects and empirical evidence made on HIE-mediated brain injury through the functional interaction between purinergic signaling, connexins, pannexins, and SDs. To the best of our knowledge, this is the first review to discuss the functional interplay among purinergic signaling and the above

mentioned "players" as key elements to trigger and to either support or to interrupt the propagation of waves of SD within the HIE's penumbra. Understanding the underlying mechanisms of HIE is expected to develop more effective neuroprotective approaches during the highly prevalent condition of perinatal hypoxic distress.

2. Purinergic signaling

Since the first description of the physiological effects of purines and pyrimidines, by Drury and Szent-Györgyi in 1929, several pieces of evidence increased the discussion on their activity in the context of the CNS [23, 24, 33–35]. This novel family of receptors is virtually expressed in all human cells types, including neurons and glial cells at central nervous system, hence raising the several hypotheses on their role in the context of a myriad of neurological disorders, such as, neurodegenerative diseases, traumatic brain injury, CNS tumors, epilepsy, psychiatric disorders, and ischemic encephalopathy [36, 37].

The early 1970s classification of the novel receptor family—then called purinoreceptors—proposed by Geoffrey Burnstock is considered to be one of the first steps to current comprehension and systematic research of purinergic receptors, which stratifies them into two groups, taking in consideration their molecular characteristics and downstream signaling [38].

The adenosine-activated receptors, or P1 receptors, present four subtypes (A1, A2A, A2B, and A3), each of these consisting of seven folds of transmembrane protein domains (TM1-TM7) linked to an N-terminal extracellular domain and a C-terminal intracellular domain. In contrast, P2 receptors are subdivided into two families: metabotropic P2Y receptors and ionotropic P2X receptors. Among several other mammal species, humans present eight members of the metabotropic P2Y, (described as P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14), and seven members of the ionotropic P2X receptors, numbered P2X1 to P2X7 [22, 39–42].

The P1A2A and P1A2B molecules are Gs-protein-coupled receptors, therefore stimulating adenylate cyclase and leading to downstream production of cyclic adenosine 3′,5′-monophosphate (cAMP) second signaling, once they are bound to their active ligand. On the other, hand P1A1 and P1A3 subtypes molecules are Gi protein-coupled receptors, leading to adenylate cyclase inhibition and cAMP degradation. In addition, some of the A2B receptor isoforms may also be associated with Gq domains, leading to a phospholipase C and inositol triphosphate second signaling pathways [42] (**Figure 1**).

Metabotropic P2Y receptors are G protein-coupled receptors activated by different nucleotide types, including ATP, ADP, UTP, UDP, and UDP-glucose. Similarly to P1 receptors, the seven transmembrane domain/ns, three extracellular and three intracellular loops, and C-terminal intracellular and extracellular N-terminal ends are found in their structure. P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11 receptors are associated with Gq protein, leading to an increase of the intracellular calcium through the internal stores culminating in a protein kinase C activation, whereas P2Y12, P2Y13, and P2Y14 are coupled to Gi protein, inhibiting adenylate cyclase and cyclic AMP [39]. Concerning the ionotropic P2X receptors, they are non-selective cation channels modulated by ATP [43]. Once activated these receptors act as transmembrane channels, allowing the flow of mono and divalent cations, such as, K^+, Ca^{2+}, and Na^+ accordingly to their gradient [44, 45]. Although some data in this context is found to be controversial, it is important to mention that some subtypes as P2X2, P2X4, and P2X7 are described to open a non-selective transmembrane pore, if submitted to high concentrations or prolonged exposure

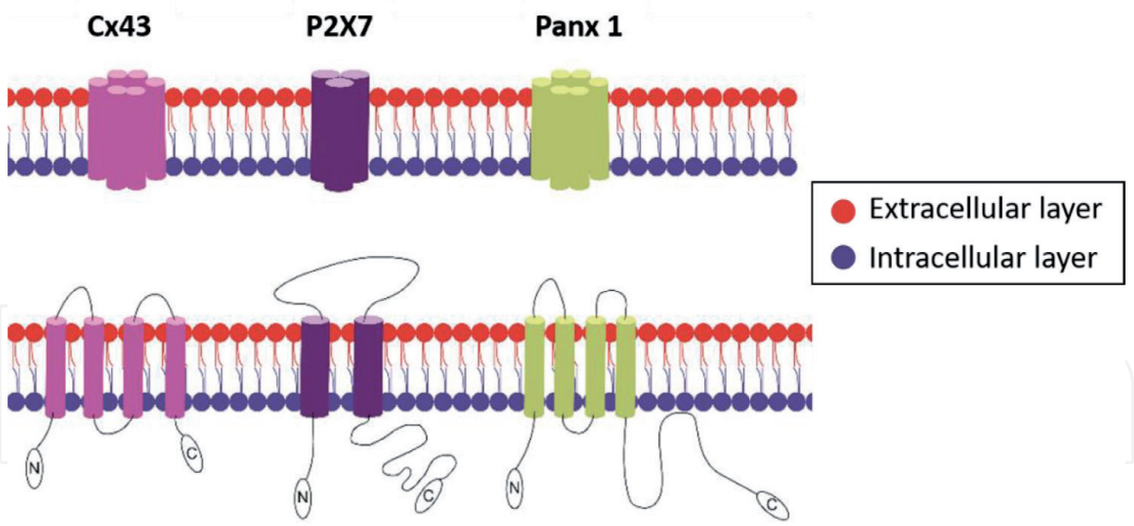

Figure 1.
Structural features of Cx43, P2X7 and Panx1. Each functional P2X7 receptor is a trimer, with the three protein subunits arranged around a cation-permeable channel pore. The subunits all share a common topology, possessing two plasma membrane spanning domains (TM1 and TM2), a large extracellular loop with the ATP binding site, and containing 10 similarly spaced cysteines and glycosylation sites, and intracellular carboxyl (C) and amino termini (N). (Left) Connexin 43 (Cx43) and pannexin 1 (Panx1) (right) share similar membrane topology, with four α-helical transmembrane domains (M1–M4) connected by two extracellular loops and one cytoplasmic loop, where both amino NH2 (N) and carboxy COOH-termini (C) are intracellular. However, different from connexins, the pannexins possess an extracellular glycosylation site which impedes gap junction formation by these channels. Upper panel—(left) a Cx hemichannel (HC) and pannexon (right) are formed by connexins and pannexins, respectively, that oligomerize laterally.

to their ligand. In this condition, these receptors allow the passage of molecules up to 900 Da, which includes organic ions and most of the frequently used fluorescent dyes [46–48] (**Table 1**). P2X receptors present two transmembrane domains connected by an extracellular loop, besides a C-terminal and an N-terminal domain in the intracellular milieu [56]. They are capable of homotrimeric and heterotrimeric assemble [57, 58] (**Figure 1**).

As suggested before, the ischemic CNS context, purinergic receptors might represent an important molecular target to restrain the condition's irreversible

Channel	Blockers	Dye	Permeability
P2X7	BBG, oATP and AZ11645373 [49]	Iodide propidium (+)	–
	A-438079, AZ11645373 and probenecid [50]	Ethidium bromide (+) and Lucifer yellow (−)	–
	A-740003, PPADS and BBG [51]	Yo-Pro-1 (+)	–
Connexin Hemichannel	Heptanol, octanol, CBX, La^{3+}, FFA and AGA [52]	Lucifer yellow (−)	–
	CBX and Gap26 [53]	Ethidium bromide (+)	–
	CBX and mefloquine [54]	Iodide propidium (+)	–
	CBX, FFA, AGA and NFA [55]	Not used	–
Pannexin	^{10}Panx [50, 53]	Ethidium bromide (+)	No change

Nomenclature: –, decreased permeability/no permeability. BBG, brilliant blue G; oATP, periodate-oxidized adenosine triphosphate; CBX, carbenoxolone; AGA, 18alpha-glycyrrhetinic acid; FFA, flufenamic acid; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid; NFA, niflumic acid. The (+) and (−) indicates valency of the dye used.

Table 1.
P2X7, connexins and pannexins antagonists blocked the permeability to anionic and cationic dyes.

burden. In this scenario, ATP and adenosine are released to the extracellular space after injury through vesicles from neurons, besides exocytosis and membrane channels including ABC transporters, pannexin 1, calcium homeostasis modulator 1 (CALHM1) channels and P2X7 pore in glial cells [59]. Estimated extracellular ATP concentrations have been described to reach up to 700 nM after ischemic events leading to tissue necrosis, whereas adenosine levels can reach up to 1000 nM [17], due to ectonucleotidases' activity, which catabolizes AMP and generates adenosine consequently. These extracellular concentrations of adenosine and ATP after ischemic events are high enough to activate P1 and P2 receptors [60].

Even though purinergic receptors in the CNS are believed to play different roles during HIE, while the protective role of adenosine P1A1 receptors during ischemia is well accepted, through the reduction of infarct volume and improvement of neurological functions [61, 62], P2 receptors activation have been associated with inflammation, neurodegeneration and cell death [45, 63]. The evidence supporting an "adenosine neuroprotection" dates from 1997, when Halle and cols reported that pretreatment with 2-amino-3-benzothiophene (PD 81,273)—to increase adenosine binding to P1A1 receptors—conferred neuroprotection following HIE [24]. As expected, the neuroprotection afforded by adenosine is antagonized by theophylline (dimethylxanthine), an inhibitor of phosphodiesterase (PDE) isoenzymes, which break down cyclic nucleotides in the cell and leads to increased concentrations of [cAMP]i and [cyclic 3′,5′ guanosine monophosphate]I [64, 65], antagonizing P1A1 and P1A2 receptors at therapeutic concentrations [23]. Along the same line, Ådén et al. (2003) reported that the absence of P1A2A receptors (P1A2A KO mice) aggravated brain damage after neonatal HI [62].

All mammalian cell types express multiple P2 receptor subtypes, each of which presenting variable affinities for purine and pyrimidine nucleotides [66]. In the neonatal HI CNS, Wang et al. (2009) suggested that P2X7 downregulation in oligodendrocyte precursor cells confers neuroprotection [67], while the use of P2X7 antagonists, such as, A-438079 and JNJ-47965567, can antagonize the occurrence of seizures. In this context, P2X7 could represent a novel and effective therapeutic target in the condition mentioned above [68]. There is accumulating evidence that P2X1, P2X2, P2X3, P2X4 and P2X5 are potentially involved in direct neuronal death [69, 70] and that P2X7 are expressed in glial cells [71]. It remains a matter of debate whether neurons express P2X7.

3. Connexins

Connexins are a family of trans-membrane proteins related to several functions, such as, intercellular communication and tissue differentiation. These proteins are best known for being the assembling subunits of connexons, a hexameric structure composed of six units of connexins [72–74]. The apposition of connexons in adjacent cells forms intercellular conduits named gap junctions (GJs), which allow the transfer of small molecules, ions and second messengers between cells [75]. The connexins family comprises 21 genes in the human genome [76], and their genetic expression varies according to the tissue and extracellular conditions. It is noteworthy that several cell types can express more than one type of connexin simultaneously [73, 77]. Connexins isoforms possess very similar and conserved structures, consisting of two extracellular loops, four transmembrane domains, one cytoplasmic loop, the C-terminus and the N-terminus [78]. When six units of identical connexins assemble, the resulting connexon is termed homomeric, whereas a connexon formed of different types of connexins is named heteromeric. Following the same rule, gap junctions are homotypic if derived from two identical connexons or heterotypic when two different connexons assemble [73] (**Figure 1**).

Among all connexins isoforms, connexin 43 (Cx43) is the more widely expressed, being present in more than 30 different tissues [77]. Predominantly found in astrocyte's membrane, novel studies suggest important participation of Cx43 connexons and GJs in the pathophysiology of several diseases [73, 79–82]. In the field of CNS hypoxic-ischemic insults, including neonatal asphyxia, some groups argue that GJs allows redistribution of nutrients, such as, glucose, and ATP [19], hence providing neuroprotection in this context. On the other hand, a myriad of studies presents Cx43 hemichannels as protagonists in processes of brain blood barrier disruption, calcium disbalance and mitochondrial dysfunction [83–85]. In addition, Cx43 may play an important role in the propagation of SD waves, possibly promoting the expansion of the initial injury to previously undamaged areas [26].

Under normal conditions, GJs may open or close accordingly to cell demands, while undocked connexons remain closed [86, 87]. Nevertheless, in cell death scenarios, hemichannels may open, allowing molecules to flow through the cell membrane, from and into the extracellular space [84, 88–90]. The release of substances, such as, ATP, glutamate and nicotinamide adenine dinucleotide (NAD^+) in the extracellular medium promotes paracrine signaling to the surrounding cells, triggering cell death cascades and, consequently, expanding the primary injury [87].

Purinergic receptors in both neurons and glial cells can be activated from dying cell's ATP release, leading to calcium influx through the receptor channel [83]. An increase of intracellular calcium might induce or worsen mitochondrial dysfunction in the context of brain injury, which leads to both secondary energy deficit and apoptosis [83]. In addition, ATP can also activate pattern recognition receptors (PRR), highlighting NOD-like receptor protein-3 (NLRP3) [84]. Finally, P2X7 and P2X4 receptors are involved in inflammasome activation and the secretion of pro-inflammatory molecules.

One of the most widely characterized approach intending to prevent the inflammatory responses of purinergic receptors activation is Cx43 blockade. However, the effects of Cx43 blockade are still controversial. Several *in vitro* and *in vivo* models in the literature resulted in a reduction of cell death, tissue swelling, and lesion spread [91, 92]. In contrast, some groups described an association between Cx43 blockade, apoptosis and higher infarct volume [93, 94]. This divergence may be explained by the limited pharmacologic agents and approaches described to promote Cx43 blockade. Currently, some of the most commonly used agents in the literature include Cx43 mimetic peptide, carbenoxolone, and octanol [19]. Once agents with greater specificity for Cx43 are established and their mechanisms are characterized, a better understanding of the role of Cx43 in pathological conditions such as HIE will be possible.

4. Pannexins

Pannexins are a class of monomeric proteins capable of forming cell membrane hemichannels, resembling structural and functional homology with the connexins as mentioned earlier. Likewise the formers, these molecules can assemble as oligomeric forms, namely pannexons. There are several different subtypes of pannexons in which pannexins can be assembled, depending on the cell type and physiologic condition of the cell surroundings. Among these, the three human isotypes are expressed virtually in all tissues: Panx1 (426 amino acids, 47.6 kDa), Panx2 (677 amino acids, 74.4 kDa) and Panx3 (392 amino acids, 44.7 kDa) [84, 95, 96].

The characterization of the assembled pannexin polymers upon cell membrane is still controversial since several groups suggest their action to be in the hemichannel fashion—such as connexins—while other findings indicate their function to

resemble full transmembrane channels between cells [84, 95, 96]. Despite the fact pannexins and connexins share several similar pharmacologic properties and expression patterns, unlike connexons, it is now believed that pannexons are not capable of forming functional gap junctions between neighbor cells and hence not being capable of promoting the connection between their cytoplasm [95, 97, 98].

Regarding molecular characterization, human pannexin isotypes share 50–60% of membrane sequence similarity, and their extracellular C-terminal sequences are the most variable among them, defining their subtype characterization based on their pattern of glycosylation [96, 98]. Novel structural studies of pannexin membrane characterization suggest that the physiologic interplay between Panx2 and other subtypes, and functional gap junction formation are very unlikely in experimental scenarios due to glycosylated domain ionic repulsion [97, 98]. Panx2 is believed to be exclusive to CNS cells, even though its molecular properties are not elucidated as well as Panx1 subtypes [95, 96] (**Figure 1**).

In contrast, Panx1 subtypes can be subdivided accordingly to their different expressed conformations, mostly determined by the several possible types of channel activation [97, 99]. Recent studies suggest that in the absence of specific ligands, Panx1 is a Cl-selective channel expressed in astrocytes, oligodendrocytes, and microglia [100]. In contrast, once activated by K^+ ions, Panx1 can isomerize into a highly conductant non-selective channel (500 pS), permeable to molecules such as ATP and other nucleotide polymers. Alternatively, voltage-activation of Panx1 opens a lower conductance conformation (50 Ps) [97, 99]. Nonetheless, it is important to mention that caspase activity, mechanical stretching, osmotic changes, purinergic receptor activation (such as P2X7 bound to adenosine nucleotides), and other cell biomolecular survival pathways are indicated as panx1 activators [19, 43, 101]. Additionally, this suggests that tissue injury and cell death processes can mediate Panx1 activity and might explain one of the early mechanisms of ATP leakage through the cell membrane [97, 99] and intercellular activation through purinergic receptors, and coupling through functional gap junctions. The influence of extracellular ATP in astrocytes downstream signaling is further discussed in this review.

The evidence against the pannexin role as intercellular hemichannels is based on three major aspects of these protein characteristics. Firstly, their well-recognized function as ATP and other macromolecule releasers. In addition, their distribution is described mostly in the apical domains of ubiquitous cells, which makes hemichannel coupling very unlikely [95]. Lastly, the aforementioned glycosylated extracellular domain acts repealing the near plasmatic membrane and does not talk in favor of their gap junction formation [95–98].

Although it seems that hemichannel opening occurs only at the downhill of cell stress, Panx opening is also observed under physiological situations, such as, the glucocorticoid release throughout the circadian cycle and neuronal coordination under sleep pattern changing [102, 103]. Panx1 role was also described in inflammasome formation and the release and cleavage of IL-1β [101, 104], which might suggest a role in the triggering of early inflammatory response and cell death [105].

5. Spreading depression

In 1906, Sir William Gowers described migraine pathophysiology as an intense, yet slow activity of the cortical centers of the brain spreading as "ripples in a pond into which a stone is thrown"—"occupying 20 min or so in passing through center affected" leaving a state of "molecular disturbance of the structures" [106]. This phenomenon was confirmed and described 38 years later in rabbit's cerebral cortexes

by Aristides Leão, who coined the term spreading depression (SD) [107], nowadays associated not only with migraine pathophysiology but also with several other neurological disorders, such as, traumatic brain injury, spinal cord injury, subarachnoid and intracranial hemorrhage, stroke and HIE [28, 108–111] (**Figure 2**).

Characterized as an intense change in ionic homeostasis and extracellular space (ECS) features, SD is an all-direction depression of neural activity that does not respect any tissue cellularity boundaries in the cortex [30, 112]. Although it is described as a negative shift in DC moving a few millimeters per minute, SD also involves fast electrical events, as seen in a burst of activity at the wavefront, followed closely by the numbness of preview neuronal tissue [27, 28, 30, 113, 114]. SD used to be described by Professor Hiss Martins-Ferreira as an "electrical tsunami" spreading among a neuronal mass that encompasses dendritic swelling, gap junction channels assemble and opening, mitochondrial changes, up-regulation, and down-regulation of several other proteins of the CNS [26, 115, 116].

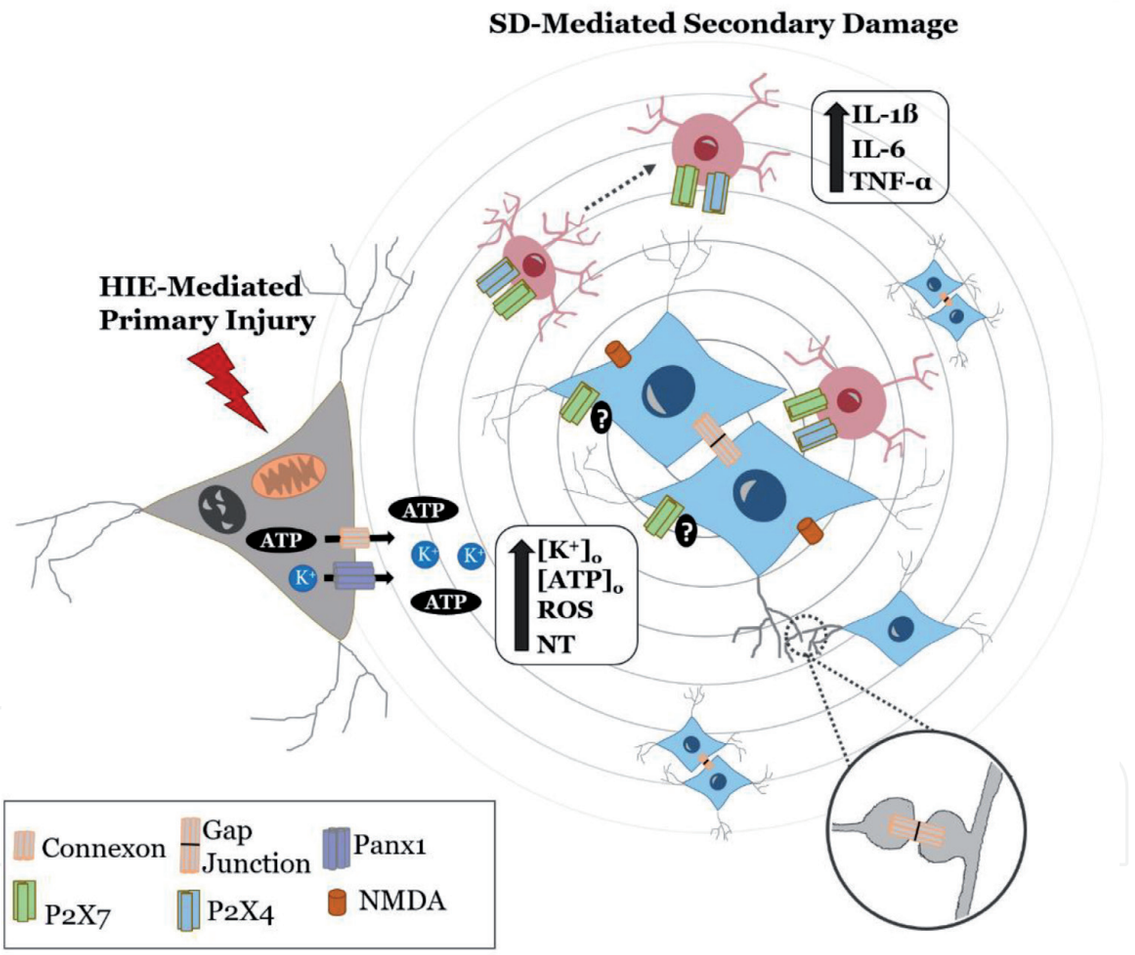

Figure 2.
Waves of Leão spreading depression (SD) and purinergic receptors. Three-step models have been proposed to explain genesis and propagation of SDs, a phenomenon believed to play a major role in the pathophysiology of HIE. Firstly, HIE-mediated primary injury leads to a surge in $[K^+]o$, ATP, reactive oxygen species (ROS) and a number of neurotransmitters (NT) in the extracellular space through Cx hemichannels and Panx1 opening in dying cells (gray cell). The upcoming activation of purinergic receptors at glia [71] and/or at neighboring neuron cell surfaces (blue cells)—a matter of debate (?)—triggering SD phenomenon. Besides, glutamate excitotoxicity is also triggered, leading to Ca^{2+} influx in the adjacent cells through NMDA receptors. Transmembrane ionic shifts during SD leads to a large drop in $[Na^+]o$, $[Ca^{2+}]o$, $[Cl^-]o$, simultaneously with the extracellular DC potential shift. Subsequently (third step), reentrant waves spread throughout the penumbra mediated by gap junctions, promoting secondary damage—via apoptosis—and lesion enlargement [30]. Electrotonic junctions in the neonatal cerebral cortex (see inset—bottom) are mostly dendrodendritic—although these same neurons can also be interconnected by chemical synapses [38]—and are believed to provide an important path for propagation of the waves [42]). Microglia (pink cell) is also activated via P2X4 and P2X7 receptors and releases proinflammatory cytokines.

Among this complex electrochemical phenomenon that revolves in the CNS extracellular space, the intracellular spaces of neuronal-glial populations, and cellular coupling, many factors have been attributed as key players to its genesis and sustenance, such as, Ca^{2+}, K^+, and glutamate and purinergic receptors [30]. Highly increased K^+ concentration was demonstrated in SD scenarios, which lead to several experimental models intending to recapitulate SD waves through artificially increasing this ionic concentration on the cortical surface [29]. It was also observed that the levels of this ion in the ECS reaches typically a plateau, being trespassed once SD is elicited [117] and those higher amounts of K^+ deliverance increase wave frequencies. Moreover, the fast electrical burst at SD wavefront is possibly explained by high extracellular K^+ concentrations inducing high neuronal activity. Imaging studies assessing initial changes in Ca^{2+}, glutamate, and K^+ concentrations at SD wavefront revealed earlier and more significant changes in K^+, suggesting its role as the first responder in SD initiation and propagation [118] (**Figure 2**).

Astrocytic Ca^{2+} waves are also intrinsically related to SD, sharing its "velocity of propagation and stimulation paradigms" initiation stimuli, refractory period, and signaling characteristics, which are postulated not only as involved with the initiation of SD, but also as fundamental for its propagation [30, 119–121]. However, Ca^{2+} waves are not a sine qua non player for SD genesis, as observed in a Ca^{2+} free medium, in ion chelating experimental contexts, in which SD genesis was not inhibited, albeit it caused a lower onset and faster recovery [119] (for further evidence against this Ca^{2+} role, see [122–124]). It is noteworthy that, gap junction blockers, purinergic blockers, ATP hydrolase inducers, and desensitization of the purinergic receptors were reported as potential Ca^{2+} wave inhibitors [30, 125–128].

The myriad of aforementioned cationic changes in SD scenarios implies in several anionics unbalances in the ECS, being Cl^- the most affected inorganic anion [30]. Consequently, Na^+ follows the elicited Cl^- influx, decreasing ECS tonicity leading to cell swelling and morphologic changes in dendritic spines [129]. The dendritic spines recovery depends on mitochondrial membrane potential integrity, an already impaired function in hypoxic-ischemic scenarios [130].

Glutamate concentration is not only found to be higher during and after SD but also its release to be synchronic and excitotoxic processes are triggered by NMDAr activation. Despite these events, the blockade of this neurotransmitter is not able to inhibit SD onset [131–133].

The roles of purinergic signaling in neurodegeneration following perinatal HIE contexts is also well documented. Levels of extracellular ATP in the brain increase during ischemia, activating both P1 and P2 receptors expressed on neural cells (neuronal and glial). Hence, extracellular ATP levels are postulated to be involved in the pathophysiology of post-ischemic inflammation and extent of brain injury [17, 45, 81, 83, 134]. Despite this factor is being considered to be one of the key players in triggering SD through Panx1 and Cx hemichannels in dying cells, the propagation of the waves requires the participation of gap junctional channels within the ischemic penumbra [19, 30, 115, 135]. Therefore, the hypothesis of a functional interplay between P2X7, Panx1, and Cx, and its impact in the injury enlargement is very plausible in the context of the HI newborn cerebral cortex development [19, 104, 130, 136]. In consonance with this conclusion, the blockade of genesis and propagation of SD through purinergic antagonists and GJ blockers may represent a viable therapeutic approach, not only to prevent post-HIE neonatal brain injury expansion, but also in other neurovascular disorders, including migraine, trauma, and ischemic stroke [19] (**Figure 2**).

Mitochondrial activity is fundamental to cell maintenance, carrying in itself an intricate and distinct pattern of protein, ionic and electrical activity [137–139]. Both oxygen and glucose deprivations unbalance the primary source of cellular

ATP production through the disruption of mitochondrial oxidative phosphorylation chain [140–143]. This described powerhouse organelle failure generates a toxic $[Ca^{2+}]i$ increase, caspase activation, and ROS production, which induces necrotic features of the HIE-mediated primary injury [142, 143]. We are lead to believe that this characteristic of cell membrane rupture is the main contributing path for Cx hemichannel opening; acting synergically with the Panx1 heightening of ATP and K^+ in ECS. Henceforth, we postulate this described cascade of hemichannel events to be the first step for SD genesis and propagation in HIE [95, 99, 136, 144]. The opening of these pores induces several other cell-signaling events, such as P2X7 activation and glutamate excitotoxic activity that is leading to significant Ca^{2+} concentration increase in the ECS. Ionic shifts and DC change is followed by gap junction-mediated spiral waves, promoting secondary SD-mediated injury [29] (**Figure 2**).

In conclusion, Cx hemichannels opening may elicit Ca^{2+} waves—responsible for the faster onset of SD-ATP, caspases, NO and other second signaling molecules flow, contributing to the bystander cell killing effect among coupled astroglial cells [145]. This highway-rail for SD waves intensifies its onset, as longs as $[Ca^{2+}]i$ augmentation is followed by Cl^-, Na^+ influx, leading to ECS changes ("Chemical Tsunami") [30]. Depolarizing wavefront will also elicit neuronal bursts, contributing to K^+ and ATP increase in the biophase [146]. The cited secondary injury appears to behave in a positive feedback-like fashion, into which further ionic and ATP release will lead to SD frequency increase and the ECS changes to mechanical panx1 activation.

6. Biomarkers

Inflammation plays a critical role in HIE [147, 148] by the complex interplay between neutrophils, lymphocytes, adhesion molecules, cytokines, and chemokines, causing injury in neurons, glial cells, and white matter. The blood-brain barrier is disrupted leading to egress of brain chemicals, normally only found in the CNS, and circulate peripherally. These chemicals can serve as important biomarkers that may help in disease risk stratification and clinical decision-making.

Hypoxia-ischemia induces activation of microglia and astrocytes [149, 150], resulting in secretion of inflammatory cytokines and chemokines that influence neuronal viability and recovery [149]. Following brain injury, purinergic P2 receptors and extracellular ATP play an important role in the microglial inflammatory response [151]. While P2 receptors are activated during oxygen-glucose deprivation (OGD) leading to microglia activation with cytokine release [70, 152] and subsequent neuronal death, the enhanced expression of P2X4 mediates ATP induced amoeboid microglial cell activation for production of proinflammatory cytokines [153] in postnatal hypoxic rats. In particular, P2X7 expression is increased 24 h after neonatal hypoxia-induced seizures in mouse pups following global hypoxia and injection of P2X7 antagonist reduced the frequency of electrographic seizures and EEG abnormality [68]. Therefore, purinergic biomarkers have been proposed for both cerebral ischemia diagnosis and prognosis [45].

Microglia responds vigorously to hypoxic-ischemic attack and produce excess inflammatory cytokines [148]. Newborns with HIE have higher levels of interleukins IL-1, IL-6, IL-8, IL-10, tumor necrosis factor TNF-α, transforming growth factor TGF-β and monocyte chemoattractant protein MCP-1 that correlate positively with brain injury severity [154–157]. IL-1β plays an important role in brain injury during ischemia [158]. The mechanism of brain damage induced by IL-1β involves the release of free radicals, enhancing the toxicity of excitatory amino acids and

increasing vascular permeability resulting in secondary cerebral edema. Blood and cerebrospinal fluid (CSF) IL-1β, IL-6, and TNF-α concentrations were found higher in HIE neonates compared to control group and a high CSF/serum ratio suggested cytokine production in the brain in addition to the systemic cytokines crossing the blood-brain barrier [154, 159]. Therapeutic hypothermia (TH), a well established treatment for neonates with HIE [8, 10, 157, 159, 160], has been shown to perform a role in the prevention of inflammatory process by maintaining proinflammatory IL-6 at low levels and anti-inflammatory IL-10 at high levels [160] (**Figure 2**).

7. Discussion

HIE remains one of the leading causes of neonatal deaths [5, 6]. Although multiple factors have been implicated in this disorder, the HIE underlying mechanisms are yet to be elucidated. To the best of our knowledge, we provide herein a summary of the described functional interplay among purinergic signaling, pannexins, connexins, and SD waves in HIE pathophysiology. Based on this interaction, the present work intends to review the current information on potential biomarkers on HIE diagnosis and prognosis.

SD waves spread across the cortex at rates of 2–5 mm/min [30, 107, 161], accompanied by a slow negative extracellular voltage and ions movement depolarizing neurons and astrocytes followed by a period of electrical suppression of distinct neuronal populations. The propagation of the SD waves is more significant in fields enriched with dendrodendritic synapses [162], such as, those described in the inner plexiform layer of the retina [30]. At this time, there are no comprehensive reviews of SD, from genesis to sustained propagation for which gap junctions have been reported to be essential [30]. However, the fact that the temporal and spatial characteristics of intercellular Ca^{2+} waves in astrocytes are remarkably similar to those of SDs "contributed, in part, to the mischaracterization of this phenomena" [30, 113]. Even though both events fail when gap junctions are blocked, the latest (i.e., Ca^{2+} waves) is neither antagonized by glutamate receptor blockers nor by purinergic receptor antagonists [30] (**Tables 1** and **2**).

Considering the role of spreading depression waves in HIE etiology, a new panel of biomarkers are being proposed to improve accuracy in diagnosis and prognosis, especially in the disease earlier stages or milder presentations. Unfortunately, ATP and adenosine, with short half-lives, do not offer good performance as biomarkers [174]. Recent studies have supported the use of proton magnetic resonance spectroscopy (MRS), a quantitative, noninvasive method of detecting energy metabolism disturbances in the neonate's brain as a marker in outcome prediction for HIE patients [175]. In these studies, it seems that the ratios including Lactate/N-acetylaspartate and N-acetylaspartate/creatine could be a potential prognostic biomarker to evaluate neurodevelopmental outcomes [176, 177]. However, larger prospective multicenter studies with a standardized protocol for both measurement protocols and analysis methods are required to validate such protocols.

As discussed, cytokines are key players in the inflammatory mechanism, contribute to the progression of ischemic damage and are released by SD-activated glia. Waves of SD cause a considerable perturbation of the ionic environment in the brain, which are readily detected by microglia—although the role of microglial activation in SD-related neurological disorders remains a matter of debate. There is increasing evidence that supports that glia (astrocytes, oligodendrocytes and microglia) may play a key role in triggering SD waves. It is known that CNS microglia become activated due to the increase in extracellular ATP from the depolarization of neurons and glia, by propagated waves of SD and from the release

Channel	Model	Blocker(s)	Effects
P2X7	*In vivo* MCAO (rat)	RB2 (10–100 mg/kg)	RB2 improved neurological score and reduced brain-damaged area [163]
	In vivo 4-VO (rat)	BBG and oATP (1, 5 and 10 μg) and A-438079 (0.03, 0.3 and 3 μg)	P2X7 antagonists increased neuronal survival and improved behavioral deficits. They reduced mortality, glial activation, and cytokine transcription [164].
	In vivo 4-VO (rat)	BBG (50 mg/kg) and A-740003 (0.04 mm/kg)	P2X7 antagonist, BBG, reduced cell death, microglial microvesicle-like components, expression of IL-1β, p-38 phosphorylation and glial activation. BBG and A-740003 improved memory functions [165]
	In vivo carotid arteries occlusion and post-conditioning (mouse)	BBG (20–40 mg/kg)	BBG abolished neuroprotective effects produced by post-conditioning, such as memory, and motor performance improvement [166]
P2Y	Culture of rat primary cortical neurons subjected to OGD	N/A	Ischemic tolerance [167]
	Culture of astrocytes exposed to sublethal OGD and subsequent lethal OGD	N/A	Ischemic tolerance [168]
Connexin	Perinatal ischemia-intrauterine hypoxia-ischemia (rat)	Carbenoxolone (105 mg/kg)	Decreased neuronal death, apoptosis, histopathologic damage and developmental impact. Decreased clustering of dying cells [169]
	Perinatal ischemia-bilateral carotid ligation (sheep)	Cx43 mimetic peptide (50 μmol/kg)	Reduced seizure activity and status epilepticus. Improved neuronal and oligodendrocytes survival [170]
	Neonate 7D hypoxia/ischemia-carotid ligation and hypoxic chamber (rat)	Cx43 mimetic peptide (25–50 μg/kg)	Reduced infarct volume, astrogliosis, glutamate release and improved neurological function [171]
Pannexin	In vivo 4-VO (rat)	Probenecid (2 mg/kg)	Probenecid attenuated neuronal death, cathepsin B translocation in neurons and glial reactivity [172]
	In vivo MCAO (Mouse)	Probenecid (1 mg/ml)	Probenecid reduced infarct size, neurological deficit, brain water content, astrocytic activation and inhibited HMGB1 and AQP4 expression [173]

MCAO, middle cerebral arterial occlusion; 4-VO, four-vessel occlusion; RB2, reactive blue 2; BBG, brilliant blue G; oATP, periodate-oxidized adenosine triphosphate; OGD, oxygen-glucose deprivation; N/A: not applied.

Table 2.
Key findings of P2X7, P2Y, connexins and pannexins blockade in brain ischemia models.

of chemicals through damaged plasma membranes of dying cells [178]. Activated microglia secrete pro-inflammatory mediators such as cytokines and develop phagocytic and major histocompatibility complex (MHC) class II-restricted antigen presenting characteristics. Although microglia express almost all P2X members (P2X1, P2X4, P2X5, and P2X7), these receptors are expressed in different levels and contribute distinctly to neuroinflammation [148]. One of the central cytokines produced by these cells is IL-1β, which plays essential roles in brain injury during ischemia, such as, IL-6 secretion [89, 179]. In bone marrow-derived macrophages, P2X7 activation plays a significant role in the release of IL-1β [180]. In microglia, however, evidence suggests that other receptors, highlighting P2X4, may be protagonists along with P2X7 in IL-1β release [180]. Hence, despite the fact that purinergic biomarkers are not available, cytokines induced by P2X activation are measurable in serum and might be useful as diagnostic and prognostic indicators.

Although TH is a current standard therapy for neonatal HIE [8, 10, 157, 159, 160], no serum biomarker is in current clinical use for this high-risk population. Using a serum panel of biomarkers rather than a single biomarker and combining them with acid-base values, Apgar score, clinical signs of encephalopathy, early EEG and MRI could help in identifying the infants with the highest risk of compromise. None of the proposed biomarkers, so far, has yet been established as clearly better than clinical evaluation of HIE for recruitment of infants at risk of adverse outcomes. Also, even though some of the putative biomarkers show good correlations with outcome, they do so only after the onset of the subsequent deterioration (e.g., 12 or 24 h or even later). In practice, this means that the search continues for a panel of biomarkers with high diagnostic and prognostic accuracy that can be identified early in the disease process to aid the bedside clinician in tailoring treatment to individual HIE newborns.

List of abbreviations

4-VO	four-vessel occlusion
AGA	18alpha-glycyrrhetinic acid
BBG	brilliant blue G
CALHM1	calcium homeostasis modulator 1
cAMP	cyclic adenosine monophosphate
CBX	carbenoxolone
CNS	central nervous system
CSF	cerebrospinal fluid
Cx	connexin
ECS	extracellular space
FFA	flufenamic acid
GJ	gap junction
GPCRs	G coupled protein receptors
HI	hypoxic-ischemia
HIE	hypoxic-ischemic encephalopathy
MCAO	middle cerebral arterial occlusion
MCP-1	monocyte chemoattractant protein-1
MHC	major histocompatibility complex
MRS	proton magnetic resonance spectroscopy
NFA	niflumic acid
oATP	periodate-oxidized adenosine triphosphate
OGD	oxygen-glucose deprivation

Panx	pannexin
PDE	phosphodiesterase
PPADS	pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid
RB2	reactive blue 2
ROS	reactive oxygen species
SD	spreading depression
TBI	traumatic brain injury
TH	therapeutic hypothermia

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