Astrocytes and microglia in acute cerebral injury underlying cerebral palsy associated with preterm birth

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Abstract

Cerebral palsy (CP) is one of the most devastating consequences of brain injury around the time of birth, and nearly a third of cases are now associated with premature birth. Compared to term babies, preterm babies have an increased incidence of complications that may increase the risk of disability, such as intraventricular hemorrhage, periventricular leukomalacia, sepsis and necrotizing enterocolitis. The response to injury is highly dependent on brain maturity and although cellular vulnerability is well documented, there is now evidence that premyelinating axons are also particularly sensitive to ischemic injury. In this review we will explore recent evidence highlighting a central role for glia in mediating increased risk of disability in premature infants, including excessive activation of microglia and opening of astrocytic gap junction hemichannels in spreading injury after brain ischemia, in part likely involving release of ATP and over-activation of purinergic receptors, particularly in white matter. We propose the hypothesis that inflammation-induced opening of connexin hemichannels is a key regulating event that initiates a vicious circle of excessive ATP release. This in turn, propagates activation of purinergic receptors on microglia and astrocytes. This suggests that developing effective neuroprotective strategies for preterm infants requires a detailed understanding of glial responses.

Introduction

Cerebral palsy (CP) is one of the most devastating consequences of perinatal brain damage and affects approximately 2/1000 live births. Over a third of all cases are associated with premature birth (1), and a further 15% with acute encephalopathy in term infants (2). CP has one of the very highest indices of burden of disease, that is from loss of potential productive members of society and direct burdens on the individual, family, and social institutions that last the entire life (3).

The *seminal concept* established by recent research is that the injury leading to CP is *not* static, making treatment possible. In term animals acute, reversible hypoxia-ischemia, is associated with a "latent" phase of recovery often lasting 6 – 8 h followed by "secondary" mitochondrial failure associated with seizures and cell swelling and ultimately cell death (4). Although considerable cell death occurs in the secondary phase, this injury can then trigger a chronic or tertiary phase of further progressive cell death, repair and remodeling (5). Confirming that many cells are still viable in the first hours after injury, prolonged, moderate hypothermia started in the latent phase, well after reperfusion, can dramatically reduce ischemic brain damage in near-term fetal sheep. Through the efforts of many researchers and large multi-center controlled trials, it is now well established in term infants that mild to moderate hypothermia induced in the first 6 h after birth with neonatal encephalopathy, and continued for at least 72 h, can significantly improve survival without disability (6). This striking result confirms the effectiveness and relevance of decades of preclinical research.

The challenge is now that hypothermia alone is only partially protective; many infants still die or survive with disability (6). Further, hypothermia may not be applicable to preventing CP in premature infants. There is a strong historical link between mild hypothermia and increased

mortality in preterm newborns (7), and recently a phase 1 trial of cooling for preterm infants was stopped by the U.S. Food and Drug Administration because of potential concerns about intracerebral hemorrhage. Furthermore, unlike term infants, the etiology of injury in preterms appears to be multifactorial, and it is often unclear precisely when brain injury occurred, partly because neurological signs are much more subtle than at term, and partly because the underlying insults are more diffuse (8). Given that hypothermia is only effective in a narrow window after injury (4), the hypoxic-ischemic event would have to be close to birth for treatment to be effective. Thus in order to establish protective protocols for the preterm infant there is a need for better understanding of underlying mechanisms of damage in this population. Our goal in this review is to highlight evidence for novel mechanisms of brain injury that may contribute to disability in premature infants.

Acute perinatal hypoxia-ischemia and infection/inflammation in preterm infants

Modern neuroimaging studies of surviving preterm infants show a consistent pattern of early white matter injury followed by abnormal development of grey matter structures (9). The precise causes of the long-term maldevelopment are still surprisingly controversial, but now there is increasing evidence for a complex mixture of acute neuronal and axonal damage affecting the cerebral white matter, cerebral cortex, basal ganglia, brain stem, and cerebellum, and secondary abnormalities of maturation (8, 10). In preclinical studies the response to injury is highly dependent on brain maturity. The susceptibility of the periventricular white matter does not appear to be related to greater ischemia in that region, at least in the sheep (11). Rather it is closely correlated with relative vulnerability of late oligodendrocyte progenitors in the rabbit,

sheep and human (11, 12). Further, *in vitro*, central premyelinating axons are reported to be much more vulnerable to ischemic injury than adjacent myelinated axons or smaller axons that had not reached the stage of radial expansion (13), likely contributing to the high risk of axonopathy in necrotic periventricular white matter lesions (14).

Early imaging, postmortem and EEG recordings suggest that the acute neural injury occurs broadly around the perinatal period in approximately two thirds of cases, while many cases occur well before the onset of labor, whereas injury after the early neonatal period represents only 10% of cases (15, 16). Both overt asphyxia, including need for resuscitation at birth, and frank/clinical infection at the time of birth ostensibly account for some cases (17, 18). Recent studies, however, have highlighted a role for both earlier and more subtle insults in the majority of cases. For example, the Extremely Low Gestational Age Newborns (ELGAN) Study reported that severe intrauterine growth restriction and evidence of placental vascular thrombosis were associated with impaired neurodevelopmental outcome at 2 years of age (19), consistent with long-standing, prenatal hypoxia. After birth, although low blood pressure is common, the evidence for an association between hypotension and adverse outcomes is limited, as reviewed (20). However, blood pressure is only a surrogate marker of changes in cerebral circulation; robust measurements of cerebral blood flow in gray and white matter that could specifically link cerebral ischemia with long term outcome are now essential.

Similarly, subclinical infection is more common than frank sepsis and also highly associated with adverse outcomes (18, 21). For example, increased cord blood tumor necrosis factor(TNF)-alpha levels has been associated with depression of the EEG in the first few days of life, white matter injury on cranial ultrasound and risk of handicap at 2 years of age (22). In the ELGAN study, necrotizing enterocolitis or bacteremia combined with ventilation on day 14 were strongly

associated with impaired development (23). Strikingly, the combination of intrauterine growth retardation with increased inflammatory markers in the first two weeks of life was associated with greater increase in risk of severe neurodevelopmental impairment compared with either alone (24). These findings raise the possibility that both chronic low-grade injury/substrate limitation and infection/inflammation either independently or together may impair brain development.

Clinical evidence for microglial activation in preterm brain injury

Microglia, which represent 5–15% of brain cells, constitute the pool of immune competent resident cerebral macrophages (25). The normal preterm brain shows transient clusters of amoeboid microglia in the periventricular crossroads of growing axonal pathways in the white matter (26). The periventricular fiber crossroads are rich in axonal guidance and extracellular matrix molecules and it is believed that microglia in these regions may help regulate the development of axonal bundles and elimination of excessive transcallosal projections during brain development (27). The crossroads appear to be particularly vulnerable to injury in the very preterm brain and in post mortem material are highly associated with both focal and diffuse white matter injury (10, 28). Given that microglia release pro-inflammatory cytokines, particularly TNF-alpha and to a lesser extent interleukin(IL)-1beta, at the early stages of white matter damage in newborns, these human postmortem studies implicate inflammation as important contributors to white matter injury (29).

Stages of microglia activation in the immature brain

The initiation of microglia activation is often unknown and diverse experimental insults all activate microglia. In preterm fetal sheep, hypoxia-ischemia (11), systemic asphyxia (30) and exposure to endotoxins are associated with significant activation of microglia and induction of pro-inflammatory cytokines and other inflammatory mediators (31, 32). Direct fetal exposure is not required to trigger inflammation and neural injury. In fetal sheep uteroplacental inflammation induced by endotoxin is associated with significant microglial activation and macrophage infiltration in the fetal brain (33). Similarly, in rabbits, after maternal endotoxin exposure in late gestation motor deficit in the rabbit pups was associated with microglia activation detected by PET imaging (34), although the observed motor deficits were mild to moderate. Thus, neuro-inflammation appears to be a critical series of events involved in white matter damage regardless of the initial trigger of injury.

Experimental studies, using neonatal rodents, have examined the time course of microglia responses induced by excitotoxicity or hypoxia-ischemia. An increased expression of markers for microglia-macrophages is seen within the first 24 hours around lesions after the initial insult. Microglia accumulation peaks around 2-4 days, but may persist for days to weeks in some brain regions (35). At least in the acute phase after neonatal stroke in rats, the inflammatory cells in the brain appear to consist of resident microglia (CD45low/medium/$CD11b^+$), rather than invading monocytes (CD45high/$CD11b^+$) (36). This is followed by several waves of inflammation from 24 h to 7 months after injury, with early expression of $CD11b^+$ and $CD11c^+$ antigen presenting cells and naïve $CD45rb^+$ T-lymphocytes. In contrast, three months after hypoxia-ischemia pronounced activation of $CD45rb^-$ T-lymphocytes expressing CD69 and CD25 was seen in the damaged hemisphere (37). Similarly, in preterm fetal sheep umbilical cord occlusion is associated with widespread activation and proliferation of microglia, and with a significant

influx of neutrophils into the brain 7 days after asphyxia (38). Taken together, these data suggest that acute injury leads to sustained inflammation in the preterm brain, that may in turn increase the risk of future cognitive dysfunction or increase vulnerability to further injury (39).

Nevertheless, it is important to appreciate that chronic inflammation may not be 'all bad'. Microglia can adopt complex phenotypes, allowing them to participate in cytotoxic responses, immune regulation, and injury resolution at different times (40). Thus, while activated microglia can contribute to brain injury through the production of excitatory amino acids, proteases, nitric oxide, reactive oxygen species and other pro-inflammatory mediators (41), they can also have important restorative functions after injury. Exogenous microglia can be neuroprotective (42) and depletion of microglia worsened injury in both adult (43) and neonatal (44) rodents. These data support the importance of time and place for understanding the role of inflammatory processes and are consistent with the variable impact of anti-inflammatory drugs on neuroprotection, discussed next.

Anti-inflammatory treatment for neuroprotection

Many drugs have been proposed to attenuate brain damage by inhibiting inflammation. The broad-spectrum antibiotic minocycline, a derivate of tetracycline, reduced focal brain infarction and had strong anti-inflammatory effects in adult rodents (45) and nearly completely prevented brain damage after hypoxia-ischemia in neonatal rats (46). Also in endotoxin-induced models of brain damage, minocycline given repeatedly, both before and after LPS injection was associated with decreased number of activated microglial cells, decreased elevation of IL-1beta, TNF-alpha and reduced number of iNOS expressing cells (47). However, although minocycline ameliorated

hypoxic-ischemic brain injury in the neonatal rat pups, treatment increased injury in mouse pups (48). Further, minocycline only transiently reduced the volume of injury at 24 h but not 7 days after middle cerebral artery occlusion in 7-day-old rat pups; importantly the transient amelioration of damage was largely independent of microglial activation (49).

Nuclear factor-kappa B (NF-kB), an important modulator of inflammation that also controls transcription of genes promoting apoptosis and other cellular injury response genes, is induced biphasically after hypoxia-ischemia in neonatal rats, maximal at 3 – 6 and 24 hours (50). Ethyl pyruvate, a stable and lipophilic derivative of pyruvate, has been proposed to ameliorate ischemic injury in neonatal rodents by inhibition of NF-kB (51). Subsequent studies, however, found no effect of ethyl pyruvate (52) and there are no studies in other species than rats, which limits the interpretation of the results. Decoy oligonucleotides (IgGkB) that penetrate into the brain and bind and inhibit NF-kB, reduce transcription of mRNA for pro-inflammatory cytokines (53) and treatment before neonatal hypoxia-ischemia reduced brain injury (54). Others have shown that selective NF-kB inhibition has neuroprotective effects that are predominantly mediated via anti-apoptotic mechanisms, including prevention of accumulation of p53, independent of cytokine production (55). The timing of NF-kB inhibition was critical as neuroprotection was only observed when early activation was blocked, whereas prevention of both early and late NF-kB-activity aggravated damage (50). Overall, the studies suggest that totally blocking inflammation is not a reliable strategy for neuroprotection.

Mechanisms of spread of inflammation-induced injury through hemichannels and purinergic receptors in glia cells

One of the most striking aspects of hypoxia-ischemia is the progressive evolution of damage into previously uninjured areas over days to weeks after the insult (56). The mechanisms of this spread are not understood, but gap junctions between glia have been suggested to play an important role through a "gap junction mediated bystander effect" (57). Gap junctions are intercellular channels that link the cytoplasm of adjacent cells, permitting the exchange of small molecules and ions. Under normal physiological conditions gap junctions function in an open state, but undocked hemichannels have either been reported to remain closed or contribute to purinergic signaling (58). Non-specific, global gap junction blockers such as carbenoxolone, glycyrrhizic acid, heptanol and octanol are reported to provide neuroprotection *in vitro* (59), after stroke in adult rats (60) and in rat pups after intrauterine hypoxia-ischemia (61).

More recent evidence now implicates connexin hemichannels in the propagation of injury. Hemichannels, or connexons, are half of a gap junction channel that sits in the unopposed membrane of a cell, prior to the formation of new channels. Opening of connexin hemichannels has been shown as a result of ischemia, as well as oxygen glucose deprivation, metabolic inhibition or low extracellular calcium ion (Ca^{2+}) levels (62-64). This may cause disruption of the resting membrane potential, release of cytotoxic levels of ATP (58) and glutamate, (65) and uptake of water resulting in cell swelling and rupture (66).

The timing of hemichannel opening remains unclear, as much of the literature does not clearly distinguish between even occurring *during* as opposed to *after* ischemia. A study from Thomson et al showed that hemichannels can open within 9.7 min after the onset of oxygen glucose deprivation in acutely isolated hippocampal neurons (67). In contrast, astrocyte cultures exposed to 3 or 6 hours of hypoxia in artificial cerebrospinal fluid, showed increased dye uptake following one hour of reoxygenation, which was abolished by pharmacological treatments

known to inhibit Connexin43 (Cx43), but not pannexin hemichannels, suggesting that hemichannel opening occurs in consequential amounts after the end of hypoxia-ischemia (64). In contrast, metabolic inhibition of cultured astrocytes with antimycin A and iodoacetic acid resulted in increased dye uptake from 40 min after the onset of inhibition, suggesting hemichannel opening during metabolic inhibition (63). Conversely, 48 hours of hypoxia reduced ATP release in endothelial cell cultures, suggesting hemichannel closure during hypoxia (68). In addition, hypoxia resulted in Cx43-serine368 phosphorylation, which causes hemichannels to switch from an open to a closed state (68). Cell type-specific changes may be critical. Protein expression of Cx32, predominantly expressed in oligodendroglia, are normally highest at 60% gestation in fetal sheep, while Cx43 increases with age (69). Studies in Cx43 knockout mice show greater injury after ischemia than in control animals (70), likely indicating adverse effects of loss of gap junctions as opposed to hemichannels.

We have previously reported that prolonged blockade of Cx43 hemichannels with a specific mimetic peptide (50 μmol/kg/h for 1 h, followed by 50 μmol/kg/24h), significantly improved neurological outcomes in the near-term fetal sheep when applied from 90 min after 30 min of cerebral ischemia (71). This included a dramatic reduction in seizures and the incidence of status epilepticus, improved recovery of electroencephalogram (EEG) power and sleep state cycling and improved survival of oligodendrocytes with intermediate neuronal survival between sham controls and vehicle treated animals. The improved recovery of EEG activity with post ischemic hemichannel blockade may be related to better cortical cell survival as suggested by the strong correlation between EEG power and cortical neuronal number (71). Improved cell survival was likely related to a combination of acutely reduced injury spread and subsequently reduced astrogliosis and chronic inflammation as shown recently after spinal injury in adult rats (72).

Although oligodendrocyte numbers were improved in the near-term fetal sheep, it is unclear whether blockade of Cx43 hemichannels would also be effective in the more preterm fetal sheep, at an age where premyelinating white matter is enriched in oligodendrocyte progenitors (11), similar to the preterm human infant.

Post-ischemic opening of hemichannels can lead to greater release of ATP (58, 73). Activation of P2Y receptors by ATP can transiently increase intracellular calcium, which consequently further enhances opening of hemichannels, resulting in "ATP-induced ATP release" (73-75). This release of ATP potentiates the spread of calcium waves through the astrocytic syncytium, and in spinal cord injuries ATP release in peritraumatic areas was associated with excessive neuronal firing (76). Of particular relevance to preterm brain injury, in vitro, P2X7 receptor antagonists, enzymatic ATP degradation and hemichannel blockade all reduced oligodendrocyte mitochondrial depolarization and oxidative stress, reduced ischemic damage to optic nerves (77), and improved functional recovery after ischemia (77). These data are highly consistent with the finding of reduced seizure activity after hemichannel blockade (71).

Activated microglia stimulate hemichannel opening in astrocytes

Recent quantitative human data demonstrate that white matter injury in preterm infants is consistently associated with a diffuse glial reaction involving both astrocytes and microglia (10). Consistent with this finding, there is increasing preclinical evidence from older ages that the microglial and astrocytic responses to inflammation are closely linked. Both release of cytokines such as IL1-beta and TNF-alpha by activated microglia *in vitro*, and Staphylococcus aureus brain infection in mice, are associated with reduced gap junctional communication but paradoxically

increased Cx43 hemichannel activity (78). Intriguingly, hemichannel activity was increased by cytokine exposure in vitro, through a p38 mitogen-activated protein kinase-dependent pathway, even though Cx43 levels were reduced *in vitro* (79), whereas bacterial infection was associated with induction of Cx43 (78). Enhanced hemichannel opening resolved by 7 days after bacterial infection (78), highlighting the importance of timing of exposure. We propose that inflammation-induced opening of connexin hemichannels is a key regulating event that initiates a vicious circle of excessive ATP release. This in turn, promotes activation of purinergic receptors on microglia and astrocytes. Thus, in the early phase after injury, release of ATP links astrocyte impairment to persistent activation of microglia, which can result in injury to neurons, axons (Figure 1) and oligodendrocytes. Beyond these early effects, astrocytes are recognized to play multiple roles in the pathogenesis of chronic brain injury, including release of a broad array of neurotrophic factors as well as factors inhibitory to axonal regeneration and oligodendrocyte maturation (80). Thus, it is not unreasonable to speculate that connexin hemichannels may also play a role in the evolution of the chronic phase of gray or white matter injury.

Conclusions

Astrocytes and microglia, key supporting cells in the brain, are now accepted as important players in the great disaster of perinatal brain injury. However, it is easily forgotten that they may have multiple, time dependent effects, such that under some circumstances they promote injury while at other times or under different conditions may protect neurons and oligodendrocytes and support neurorestoration. The studies reviewed here suggest that astroglia are likely to be important mediators of early injury after hypoxia-ischemia and infection, through

overexpression of connexin hemichannels, leading to ATP release and activation of purinergic receptors and propagation of pro-inflammatory microglia that add to injury in the early post-injury phase. However, in the medium to longer-term after brain insults microglia may contribute to resolution of injury and repair by adopting restorative functions.

There are many remaining gaps in our knowledge particularly with respect to the "time and place" of these responses. What determines the microglial switch from damage to repair after injury? When do astrocytes mediate injury and when do they transition to supporting cell survival after injury? What is the role of the interactions between astrocytes and microglia? When and how does astrogliosis inhibit rather than support neurorestoration? Better understanding of these relationships is essential to design appropriate interventions to help protect the injured preterm brain.

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Figure Legend

Figure 1. Flow diagram outlining the hypothesized relationship between microglia activation and astrocytic connexin 43 hemichannel opening and preterm brain injury. We propose that inflammation-induced opening of connexin hemichannels is a key regulating event that initiates a vicious circle of excessive ATP release. This in turn, propagates activation of purinergic receptors on microglia and astrocytes. Thus, in the early phase after injury, the release of ATP links astrocyte impairment to persistent activation of microglia, which can result in injury to neurons, axons (shown) and oligodendrocytes (not shown for brevity).

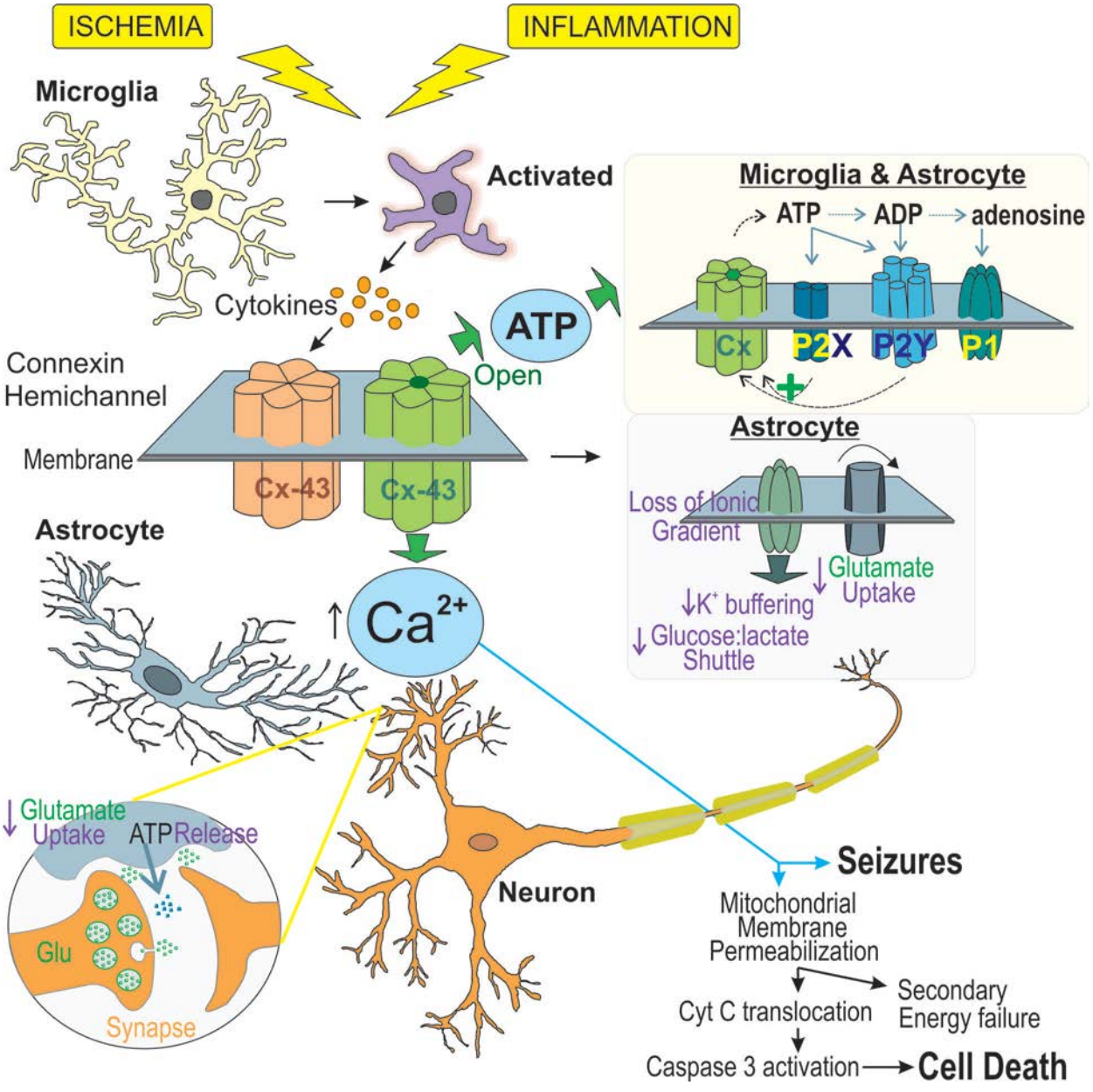
ISCHEMIA
INFLAMMATION
Microglia
Activated
Cytokines
ATP
Open
Connexin
Hemichannel
Membrane
Cx-43
Cx-43
Astrocyte
Ca2+
Microglia & Astrocyte
ATP
ADP
adenosine
Cx
P2X
P2Y
P1
Astrocyte
Loss of Ionic
Gradient
Glutamate
Uptake
K+ buffering
Glucose:lactate
Shuttle
Glutamate
Uptake
ATP Release
Glu
Synapse
Neuron
Seizures
Mitochondrial
Membrane
Permeabilization
Cyt C translocation
Secondary
Energy failure
Caspase 3 activation
Cell Death

Figure 1.