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Battle of the hemichannels – connexins and pannexins in ischemic brain injury

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Abstract

Perinatal ischemic brain injury can occur as a result of a global ischemic insult or focal ischemic stroke in the preterm or full-term neonate. One of the most striking features of HI injury is that after initial recovery of cellular oxidative metabolism, there is a delayed, 'secondary' mitochondrial failure that spreads over time from the most severely damaged areas outwards, into previously undamaged regions. This secondary failure is accompanied by transient seizure activity and cytotoxic edema.

The specific mechanisms of this spread are poorly understood, but it is at least partly associated with spreading waves of depression that can trigger cell death in neighboring uninjured tissues. Both connexin and pannexin hemichannels may mediate release of paracrine molecules that in turn propagate cell death messages by releasing intracellular mediators such as ATP, NAD(+), or glutamate or by abnormally prolonged opening to allow cell edema. This review will discuss the controversy around the relative contribution of both connexin and pannexin hemichannels and mechanisms by which they may contribute to the spread of ischemic brain injury.

Keywords. Cerebral ischemia; connexin43; pannexin; hemichannel; neuroprotection; fetal sheep

Introduction

Ischemia is a reduction in tissue blood flow that leads to a reduction in delivery of both oxygen and energy substrates (e.g. glucose) below metabolic requirements. During the perinatal period, this can occur as a result of focal ischemic stroke, where a blood vessel in the brain becomes blocked, leading to localized tissue damage, or global hypoxia-ischemia resulting from birth asphyxia or cardiovascular collapse.[1, 2] Perinatal arterial ischemic stroke occurs in approximately 1/2300 to 1/5000 live infant births, and is more common in the term or near-term infant but can also occur in the preterm infant.[1, 3, 4] Moderate to severe hypoxic-ischemic encephalopathy resulting from a global insult occurs in approximately 1-3/1000 live full-term births.[5] Currently, the only treatment for full term infants suffering from hypoxic-ischemic encephalopathy is therapeutic hypothermia. Although hypothermia significantly reduces death and disability, it is only partially effective.[6] There is no clinical treatment available for infants suffering perinatal arterial ischemic stroke, likely due to difficulties in and timing of diagnosis. HI also contributes to the high burden of neurodevelopmental disability in infants born preterm, who now constitute 7-12% of all live births.[7] There are no clinical treatment options available for preterm infants suffering ischemic brain injury. Therefore novel treatment strategies for preterm and full-term infants exposed to focal or global ischemic brain injury are needed.

Propagation of injury from damaged areas of the brain into previously healthy regions is common to ischemic injury. In ischemic stroke, a characteristic pattern is a focal ischemic core comprised of dead tissue and a surrounding penumbral region that has the potential to either progress to death or be salvaged. The central focal ischemic stroke core extends out into the penumbral regions over the following hours and days. In the case of perinatal HI, brain injury is not uniform and some areas are more prone to early damage. This lesion also

propagates over a number of days to encompass initially uninjured regions.[8] This results in a biphasic pattern of evolving neural injury, with the acute period of HI per se representing the primary phase of injury, followed by a latent phase after tissue reperfusion in which most parameters transiently normalize. From 6 to 15 hours after moderate to severe insults there can be a phase of secondary deterioration that extends the volume of affected tissue, in association with metabolic failure, seizure activity, edema and cell death.[9-11]

Gap junctions have been implicated in the spread of injury in a wide array of central nervous system disease models including ischemic brain injury, spinal cord injury, epilepsy and even trauma.[12-20] However, early studies have relied on the use of non-specific compounds such as octanol and carbenoxolone, which can block multiple membrane channels, including both gap junctions and hemichannels. Long-term blockade of gap junctions can be deleterious after ischemic brain injury, likely due to disruption of the astrocytic syncytium.[21, 22] In our own studies, infusion of a gap junction channel blocking peptidomimetic, Peptide 5, in the near-term fetal sheep at dose levels likely to cause uncoupling of gap junctions as well as blocking hemichannels,[23] was associated with impaired EEG recovery, increased secondary cell swelling and an apparent trend to higher lactate concentrations and mortality after 30 min of global cerebral ischemia.[24]

There is, however, increasing evidence that it is specifically connexin hemichannels that are associated with the spread of brain injury after ischemic stroke and global hypoxic-ischemic brain injury in the fetus/neonate,[25-28] as well as other central nervous system disorders, such as spinal cord injury and retinal stroke.[23, 29, 30] Recent evidence suggests that pannexin hemichannels, membrane channels that are similar in structure to connexin hemichannels, may also contribute to the spread of ischemic brain injury although this has not been studied directly in the developing brain.[31, 32]

The specific roles and the relationship between connexin and pannexin hemichannels in the spread of ischemic brain injury are highly controversial. This review discusses the evidence for roles of the connexin and pannexin hemichannels in ischemic brain injury, with a particular emphasis on how they might contribute to injury in the fetus and neonate.

Introduction to connexin and pannexin hemichannels

Gap junction plaques are clusters of intercellular channels that link adjacent cells directly and are permeable to small molecules, ions and second messengers with a molecular weight less than one kilodalton (kDa).[33, 34] Gap junctions are formed by docking of two connexons together, one contributed from each adjacent cell (Figure 1). A connexon or hemichannel is a hexamer that consists of six sub-units called connexins. There are 21 known connexins in the human genome, 11 of which are expressed in the central nervous system and are named according to their theoretical molecular mass in kDa (as reviewed in [35]). The type of connexin expressed is highly dependent on the brain region, cell type and stage of development. Connexin43 is the predominant connexin found in astrocytes, along with Connexin30.[36] Astrocytes are linked by gap junctions to other astrocytes and oligodendrocytes but not to mature neurons.[36] Connexin43 is also abundant at the astrocytic end-foot processes that surround blood vessels, and contribute to the blood-brain barrier, and on the astrocytic processes in close proximity to chemical synapses.[37] Connexin43 is also expressed in normal capillary endothelium and at low levels in microglia.[38, 39]

It is now becoming clear that connexin hemichannels are active under normal physiological conditions, including contributing to purinergic signaling by regulated release of ATP.[40, 41] Connexin43 hemichannels also mediate nicotinamide adenine dinucleotide (NAD^+) transport, and functionally interacting with the plasma membrane ectoenzyme cyclic adenosine diphosphate (ADP)-ribose hydrolase CD38 that converts NAD^+ to the calcium ion mobilizer,

cyclic ADP-ribose (cADPR).[42, 43] Although the mechanism is not yet clear, there is recent evidence that connexin hemichannels may contribute to spontaneous depolarizations within the human fetal cortex during the second trimester of gestation.[44]

Pannexins share 20% sequence homology with the invertebrate innexin proteins that form invertebrate gap junctions, but have no homology with connexins.[45, 46] However, connexin and pannexin membrane topology is very similar and both channels are blocked by a number of commonly used compounds, such as carbenoxolone and flufenamic acid.[47] It has been suggested that in vertebrates pannexins cannot form gap junctions, as interaction between pannexin hemichannels is prevented by their extensive glycosylation and therefore they exist only in the hemichannel form.[48] Only a single study has shown pannexin hemichannels being involved in cell-cell channel formation following pannexin expression in mouse C2C12 cells.[49]

Three pannexin genes have been identified, Pannexin1, 2 and 3. The Pannexin1 and Pannexin2 are expressed in neurons extensively throughout the brain including in the cortex, striatum, olfactory bulb, hippocampus, thalamus and cerebellum.[50] In contrast, Pannexin1 mRNA has not been detected in glial cells *in vivo.*[51, 52] Pannexin1 has been shown to form hemichannels when expressed in Xenopus oocytes.[50]

Similar to connexin hemichannels, pannexin hemichannels are also thought to be involved in purinergic signaling under physiological conditions. Knockdown of Pannexin1 hemichannels has been shown to significantly reduce ATP release from astrocytes in response to 3-O-(4-benzoyl)benzoyl adenosine triphosphate (BzATP), a P2X7 agonist.[53] However, this concept has recently been challenged by evidence that ATP release was no different in Pannexin1/Pannexin2 deficient astrocytes compared to wild type in response to BzATP stimulation.[54] This release of ATP was blocked by carbenoxolone, an inhibitor of both

pannexin and connexin hemichannels, suggesting that connexin hemichannels were mediating the release of ATP from these astrocytes.

Connexin hemichannels in ischemic brain injury

In addition to their roles in normal physiological functioning of the brain, connexin hemichannels may contribute to the spread of injury under pathological conditions such as ischemia. Unregulated opening of such a large and relatively non-specific channel linking the intra- and extracellular space could compromise ionic gradients required for resting membrane potentials and membrane transport as well as permitting unregulated movement of metabolites and second messenger molecules.

The first convincing evidence of connexin hemichannel opening came from Paul and colleagues,[55] who showed that Xenopus oocytes transfected with Connexin46 mRNA depolarized and lysed within 24 h unless osmolyte was included in the bathing medium. A voltage dependent current was subsequently shown to result from reduced extracellular Ca^{2+} concentrations in the bathing medium of the Connexin46 cDNA injected oocytes.[56] Opening of Connexin43 hemichannels has also been shown in Connexin43 transfected cells that display sensitivity to low extracellular Ca^{2+}.[57] Thus, It is possible that the large drop in extracellular Ca^{2+} concentrations ($[Ca^{2+}]_o$) associated with pathological conditions, such as ischemia, is sufficient to open hemichannels. Hemichannels in rat cortical astrocytes have also been shown to open, as shown by intracellular uptake of large dyes, in response to metabolic inhibition of glycolytic and oxidative metabolism using the chemical inhibitors antimycin A and iodoacetic acid.[58] Similar findings have been demonstrated in isolated ventricular myocytes, in which metabolic inhibition was induced by the glycolytic inhibitor sodium iodoacetate and the mitochondrial uncoupler, carbonyl cyanide m-chlorophenyl-hydrazone.[59]

These data suggest that pathological opening of connexin hemichannels may occur during the ischemic insult itself. More recently, an elegant study from Orellana *et al* provides strong evidence for opening of Connexin43 hemichannels *after* hypoxia in cultured astrocytes.[28] In this study increased dye uptake was reported in Connexin43 containing astrocytes, but not in Connexin43 deficient astrocytes. In addition, known blockers of Connexin43 hemichannels prevented dye uptake and astrocytic demise.[28] Davidson et al have recently provided strong evidence that hemichannels open after cerebral ischemia *in vivo*.[27] An intracerebroventricular infusion of a mimetic peptide at a dose concentration that blocks Connexin43 hemichannels[23] was given after severe ischemia induced by bilateral carotid artery occlusion for 30 minutes in the near-term fetal sheep.[27] The peptide infusions were started 90 minutes after reperfusion, and then continued for either one hour (ischemia-short-infusion) or 25 hours (ischemia-long-infusion). Mimetic peptide infusion was associated with a graded improvement in recovery of EEG power over 7 days recovery, from -13±1.9 decibel (dB) below baseline values after ischemia-vehicle, to -9±1.6 dB after ischemia-short-infusion and -5±1.6 dB after ischemia-long-infusion ($P<0.05$ vs. ischemia-vehicle). Peptide-infusion was also associated with a striking reduction in seizure activity after ischemia, with less frequent status epilepticus, followed by earlier return of sleep state cycling in the fetus. Ischemia-long-infusion (but not ischemia-short-infusion) was associated with improved survival of oligodendrocytes in the intragyral and periventricular white matter, and an intermediate number of surviving neurons in the parasagittal cortex ($2.9 \pm 0.8 \times 10^6$) compared to sham control ($4.3 \pm 0.9 \times 10^6$) or ischemia-vehicle ($1.5 \pm 0.4 \times 10^6$).[27]

Conversely, blockade of connexin 43 hemichannels during ischemia had no effect on EEG recovery or cell death.[26] This suggests that connexin hemichannels play an important role in the downstream propagation of injury after ischemia but do not contribute significantly to the generation of injury during ischemia. This supports connexin hemichannels as a potential

therapeutic target, since treatment after ischemia is more clinically plausible than treatment during ischemia, particularly in the case of neonatal brain injury, where infants must first be born, stabilized and diagnosed before the onset of treatment. Indeed, in the first three controlled trials, therapeutic hypothermia was started a median of 4–4.5 hours after birth, highlighting the considerable difficulties associated with diagnosing and treating these infants.[60-62]

Recently, we have also shown that blockade of connexin 43 hemichannels is neuroprotective after asphyxia in the preterm fetal sheep brain.[25] In this study, an intracerebroventricular infusion of mimetic peptide was given similarly to the ischemia-long infusion protocol described above starting 90 minutes after 25 minutes of complete umbilical cord occlusion. This is a near terminal insult, which results in severe intragyral and periventricular white matter injury and subcortical neuronal loss with cortical sparing. Blockade of connexin hemichannels was associated with earlier recovery of EEG power, reduced neuronal loss in the caudate and putamen and reduced immature/mature oligodendrocyte cell loss in the intragyral and periventricular white matter.

Pannexin hemichannels and ischemic brain injury

The role of pannexin hemichannels during pathological conditions, such as ischemia, remains controversial. Oxygen-glucose deprivation has been shown to open Pannexin1 hemichannels in isolated hippocampal neurons.[63] Opening of Pannexin1 hemichannels during anoxia has been shown to be mediated by Src family kinases, recruited in response to NMDA receptor activation in CA1 pyramidal neurons in acute brain slices from rats and mice.[64, 65] A role for pannexin hemichannels in ischemic brain injury has been supported by an *in vivo* study showing that double knockout Pannexin1/Pannexin2 mice had improved neurological deficits

and reduced infarct size compared to wild type or mice with a single knockout of either Pannexin1 or Pannexin2.[54, 66]

Probenecid is an inhibitor of organic anion transporters that is routinely used as a treatment for gout. It is known to be a powerful inhibitor of Pannexin1 channels, however probenecid also reduces the clearance of prostaglandins, and the endogenous NMDA/glycine receptor antagonist kynurenic acid from the CSF.[67-69] Further, probenecid can inhibit P2X7-induced cationic and anionic dye uptake in stably transfected P2X7 HEK-293 cells in a pannexin independent manner. Treatment with probenecid reduced infarct size, decreased cerebral water content and reduced neuronal death and inflammation in the mouse brain after middle cerebral artery occlusion.[70] Probenecid reduced dye uptake and interleukin-1 beta secretion from human CD14+ monocytes, whereas carbenoxolone and 10Panx1 had no effect. With the use of patch clamping and calcium indicator experiments, the authors concluded that probenecid also blocks the human P2X7 receptor.[71] Therefore, caution should be taken when interpreting data relying on changes associated with probenecid.

In contrast, Madry and colleagues found that pannexin hemichannels did not contribute significantly to the generation of early anoxic depolarization nor the later uptake of dye during ischemia in CA1 pyramidal cells in hippocampal slices.[31] Further, Orellana and colleagues showed that hemichannel activity induced by hypoxia in cortical astrocytes was blocked by Connexin43 mimetic peptide but not by mimetic peptides targeting pannexin hemichannels.[28] Iwabuchi and colleagues have proposed that pannexin hemichannels are part of a complex negative feedback loop, whereby ATP released through Pannexin1 hemichannels early after the onset of ischemia acts via P2X7 receptors to induce closure of Pannexin1 hemichannels. This process has been called ‘ATP-induced suppression of ATP release’.[72]

Hemichannels and seizures

Interestingly, while connexin 43 hemichannel blockade was associated with a significant reduction in seizure burden and status epilepticus after ischemia in the near-term fetal sheep,[27] it was not associated with a significant effect on seizure activity in the preterm fetal brain.[25] This is likely due to differences in the presentation of seizure activity at different maturational ages, with prolonged periods of status epilepticus being common in the near-term fetus after ischemia and more discrete seizures being predominate in the preterm fetus.[73, 74] There is considerable evidence implicating gap junctions and/or connexin hemichannels in the initiation, propagation and particularly in the continuity of seizure activity in astrocytes in vitro and in rat models in vivo.[75-77] Thus, a plausible interpretation is that connexin hemichannel blockade attenuated the propagation of abnormal electrical activity rather than the initial generation of seizures. This would explain why a greater effect was seen on the continuous seizure activity characteristic of status epilepticus in the near-term fetus after ischemia,[27] in contrast with no effect on the discrete seizures seen in the preterm fetus after asphyxia.[25] Further, the severity of injury may alter hemichannel expression and opening given cortical neuronal loss and infarction are seen in the near-term but not preterm fetal brain after hypoxia ischaemia.[25, 27] Little is known about the normal development of the astrocytic syncytium and how this may affect the response to injury and the generation of seizure activity. Current anti-convulsants have poor efficacy and many putative toxic effects,[78] and thus connexin hemichannel modulators may have potential for treatment of status epilepticus.

In contrast, little is known about the contribution of pannexin hemichannels. Intriguingly, the P2X7 receptor-Pannexin1 complex reduced muscarinic acetylcholine receptor-mediated seizure susceptibility in mice compared to knockout littermates.[79] However, increased

expression of Pannexin1, Pannexin2 and Connexin43 has been seen in the hippocampus in response to seizures induced by Co^{2+}.[80]

How connexin and pannexin hemichannels contribute to the spread of ischemic brain injury

It is now well established that cell death after ischemia is not a single, homogenous event, but rather it involves a continuum of apoptotic, necrotic and even autophagic pathways.[81, 82] There is evidence that opening of the mitochondrial permeability transition pore leading to mitochondrial depolarization is important for many of these delayed cell death pathways, although there is evidence that it is regulated differently during development compared to in the adult brain.[83]

In both the adult and the developing brain, pathological opening of connexin and pannexin hemichannels is likely to be a critical 'upstream' event that triggers cellular dysfunction and multiple pathways leading to cell death. Some of the proposed mechanisms by which connexin and/or pannexin hemichannels contribute to abnormal brain activity and cell death after hypoxia ischemia are discussed below.

Altered purinergic signaling

There has been much debate over the relative contribution of connexin versus pannexin hemichannels in ischemic brain injury. However, recent evidence suggests connexin and pannexin hemichannel function may be integrated by purinergic and glutamatergic signaling (figure 2). Orellana and colleagues showed that astrocytes exposed to the inflammatory compound CM-Aβ before a sublethal hypoxia/reoxygenation episode, released glutamate and ATP through Connexin43 hemichannels, which induced permeabilization of cortical neurons via neuronal Pannexin1 hemichannels in astrocyte-neuron co-cultures.[32] Activation of neuronal Pannexin1 hemichannels was dependent on activation of both NMDA and P2

receptors, as inhibition of either receptor alone only partially reduced Pannexin1 activation, while inhibition of both receptors simultaneously completely prevented the neurotoxic response.

Opening of both connexin and pannexin hemichannels has been closely associated with changes in purinergic signaling. Both types of hemichannels have been shown to release ATP.[40, 41] Activation of P2Y receptors by ATP can transiently increase intracellular calcium, further enhancing opening of hemichannels in a process known as 'ATP-induced ATP release.'[40, 84, 85] This allows calcium waves to spread through the astrocytic syncytium, and in spinal cord injuries, ATP release and peri-traumatic areas has been found to be associated with excessive neuronal firing.[86] Supporting this hypothesis, there is evidence that poorly coupled cell lines (C6 glioma, HeLa and U373 glioblastoma cells) show a significant increase in ATP release after connexin expression.[87] In addition, Ca^{2+} wave propagation was reduced by purinergic receptor blockers. Further, dye uptake and ATP release were observed in C6 glioma cells expressing Connexin43 but not in Connexin43 deficient parent cells.[40] The connexin hemichannel activator, quinine, evoked ATP release and Ca^{2+} signaling, which was inhibited by the connexin channel blockers flufenamic acid and gadolinium.[40] However, as above, pannexin hemichannels may also contribute to "ATP-induced suppression of ATP release" early after the onset of ischemic stress in astrocytes [72]. It is not yet clear whether this negative feedback loop that should prevent excessive ATP release actually occurs in neurons and astrocytes in vivo.

In addition to contributing to purinergic signaling via the release of ATP, it has been proposed that Pannexin1 hemichannels can associate with P2X7 receptors, normally a non-selective cation channel, and so contribute to the formation of a large membrane pore that can mediate apoptotic cell death.[88] This hypothesis was supported by a study showing that dye

efflux from N2A cells in response to ATP and BzATP was blocked by inhibitors of both Pannexin1 and P2X7 receptors individually.[89]

The astrocytic syncytium

Connexin43 is the predominant astrocytic connexin in the brain while Pannexin1 is found on neurons throughout the brain, and astrocytes in culture. It is not yet clear whether Pannexin1 is found in astrocytes in vivo.[51, 52, 86] Although traditionally neurons have been the central focus of most studies of brain injury, the importance of astrocytes and other glial cells is becoming better understood. Astrocytes are electrically non-excitable glial cells that communicate by Ca^{2+} signaling, that is facilitated by gap junctions and hemichannels.[90] They play a critical role in maintaining homeostasis of the neural environment. For example, astrocytes critically buffer extracellular glutamate levels and so help prevent excitotoxicity.[91] The astrocyte glutamate transporter-1 (GLT-1) and the glutamate/aspartate transporter (GLAST) take-up glutamate from the extracellular space, where it is then converted into glutamine and released from the astrocytes for re-uptake by neurons. This process may be reversed in injured tissues leading to release of excessive amounts of glutamate into the extracellular fluid.[92]

Astrocyte gap junctions also play at least some part in normal, physiological K^+ buffering, as shown by Wallraff *et al* in mouse brain slices.[93] The first potential mechanism of K^+ buffering is spatial buffering, involving the uptake of K^+, cytosolic diffusion through the functional syncytium created by the inter-astrocytic gap junctions, followed by release of K^+ at a distant site. This prevents ionic imbalances from occurring in metabolically active brain regions. The second proposed method of K^+ buffering involves the net uptake of K^+ by astrocytes.[94] This is associated with activity of the Na^+-K^+ ATPase, Na^+-K^+-$2Cl^-$ co-transporter and separate K^+ and Cl^- channels. Astrocyte gap junctions may also have an

indirect role in the "net uptake model" of K^+ buffering by stabilization of associated intracellular ion concentrations.[95] Extracellular K^+ under normal conditions is approximately 2.7-3.5 mM, but can reach 50-80 mM during ischemia.[96] Sustained exposure to elevated extracellular K^+ levels that exceed the capacity of the physiological buffering systems above, can promote cell depolarization, and increase cell death in fetal rat neuronal cultures.[97]

Normal function of astrocytes is likely to be important for neuronal survival in a number of other ways. First, they contribute to energy supply to neurons under compromised conditions through the 'lactate shuttle', the process by which astrocytes convert glycogen to glucose and glucose to lactate (as reviewed in [98]). Lactate can then be transported to neurons where it is used as a source of energy through mitochondrial metabolism. This has been shown in anesthetized neonatal baboons where lactate is taken up by the brain and presumably used as an energy substrate.[99] Critically, there is evidence that in mouse hippocampal slices connexin 43 and 30 are essential for intracellular trafficking of glucose from the vasculature to hippocampal neurons through astroglial networks, in an activity dependent manner.[100]

Further, there is increasing evidence that astrocytes respond to the synaptic release of glutamate with an increase in intracellular Ca^{2+} and can modulate neuronal excitability by release of neuroactive substances called gliotransmitters.[101-103] These gliotransmitters include glutamate, ATP, D-serine and tumor necrosis factor α.[84, 104-106] Such research has led to the concept of the 'tripartite synapse', in which astrocytes are hypothesized to be involved in synaptic transmission along with the pre- and post-synaptic terminals.[101] For example, ATP release by astrocytes can modulate neuronal excitability via purinergic receptors, while ATP that is degraded to adenosine can inhibit excitatory synapses.[107, 108] Some have suggested that this concept should be extended to include the contribution of microglia, suggesting that effectively there may be a so-called 'quad-partite synapse'.[109]

Finally, astrocytes also secrete a number of soluble factors called neurotrophins that are involved in neuronal survival, maturation, differentiation and development (as reviewed in [110]). These include insulin like growth factor 1 (IGF-1), nerve growth factor, glial cell-line derived neurotrophic factor, the neurokine superfamily and the non-neural growth factor superfamily that act on tyrosine receptor kinase.[110, 111] Given the important contribution of astrocytes to neuronal homoeostasis, disruption to astrocytic function following ischemia may contribute to a toxic environment that is detrimental to neuronal survival. The opening of connexin and potentially pannexin hemichannels will contribute to astrocytic dysfunction after ischemia. The end result is ultimately neuronal cell death, whether directly, or indirectly through impaired astrocyte function, as well as compromise of other brain cells such as oligodendrocytes.

The blood brain barrier and vessel leak

Further, astrocytes play a crucial role in maintenance of the blood brain barrier, which is well-known to become leaky following ischemia or infection. Capillary dysfunction occurs in a number of degenerative central nervous system (CNS) diseases including Alzheimer's and Parkinson's,[112, 113] age related macular degeneration [114, 115] and diabetic retinopathy.[116] Interactions between leukocytes and endothelial cells are early events in both acute and chronic inflammation, and in wound defense.[117] These interactions correlate with Connexin43 expression[118] and trans-endothelial inflammatory cell migration can be reduced by the gap junction blockers octanol and 18α-glycyrrhetinic acid *in vitro.*[119] In mouse lungs inflamed by intratracheal instillations of *Pseudomonas aeruginosa* lipopolysaccharide, Connexin43 is upregulated independently of neutrophils.[120] In heterozygous Connexin43-/+ mice, airway neutrophil counts were reduced by over half, but conversely, neutrophil numbers increased in lipopolysaccharide challenged mice expressing a modified Connexin43

with higher channel conductivity. Specific down-regulation of Connexin43 with antisense oligodeoxynucleotides attenuates recruitment of both neutrophils and macrophages at skin wound sites [121, 122] and following spinal cord injury.[16]

Evidence is mounting that connexin hemichannels may play a direct role in mediating loss of endothelial cells, damaging the vascular wall. de Bock and colleagues have reported that Connexin43 mimetic peptides can prevent trans-endothelial leak *in vitro* and *in vivo* induced by application of the inflammatory peptide, bradykinin.[123] They concluded that, unlike the loss of endothelial barrier function when gap junctions are inhibited,[124] inhibition of hemichannels appeared to preserve barrier function. In the CNS, retinal ischemia-reperfusion induced significant leakage from retinal vessels between 1-24 hours after the insult, peaking just four hours after reperfusion, in parallel with significant up-regulation of Connexin43 at the same times.[29] A single intraperitoneal injection of Connexin43 mimetic peptide at a final concentration expected to block hemichannels, without uncoupling gap junctions, significantly reduced vascular leak (as shown by systemic infusion of Evans Blue dye) to 14% of controls at the time of peak leak, 4 hours after ischemia. The peptide induced reduction in vessel leak was associated with diminished inflammation and, downstream, a reduction in retinal ganglion cell (neuron) loss at 7 and 21 days post-ischemia, from 35% to 9 and 14%, respectively. In parallel *in vitro* studies, the mimetic peptides reduced both human and rat vascular endothelial loss following ischemia from 25–30% to less than 7%. Ultrastructural analysis provided evidence of endothelial membrane degeneration after focal cerebral ischemia.[125] These data indicate that direct endothelial cell loss can occur as a result of hemichannel opening after ischemia, leading to vessel leak and downstream inflammatory events and neural loss.

Translational status of Pannexin and Connexin hemichannel blockers

Ultimately, an important goal of this research is to translate hemichannel inhibitors into clinical application. Non-specific channel blockers (carbenoxolone, octanol, flufenamic acid, glycyrrhetinic acid) are unlikely to have practical application for CNS disorders to any great extent owing to their off-target effects. Although the precise role of pannexin channels after injury remains to be defined, the pannexin-1 mimetic peptide 10Panx1 has been shown to effectively block pannexin hemichannels *in vitro*,[71] but has only been tested preclinically so far. The non-specific pannexin channel blocker, probenecid is FDA-approved and although it also has off target effects some of these may also be neuroprotective.[67-69]

For blockade of connexin channels, Connexin43 specific antisense oligodeoxynucleotides have been shown to significantly improve outcomes after CNS injury in some animal studies,[16, 126] have completed Phase 2 clinical trials for venous and diabetic leg ulcers (see www.codatherapeutics.com) and there is evidence that they may promote healing of human ocular burns.[127] These agents are applied topically, however, in their present form cannot be delivered systemically, and have not been tested after intraventricular administration.

In contrast, three connexin mimetic peptides have shown benefits in multiple animal models. ACT-1 is a 25 amino acid peptide mimicking the cytoplasmic carboxy- terminal of the Cx43 protein. It is said to mediate gap junction channel aggregation and so sequestration of hemichannels from regions surrounding gap junctions.[128, 129] This peptide has completed phase 2 clinical trials for leg ulcers,[130] but is not hemichannel specific and has not yet been used to treat CNS injury. Peptide5 at appropriate concentration is hemichannel specific,[23] acts across more than one connexin isoform, and acts externally to the cell and so is easily delivered. As described in this review, Peptide5 has been shown to markedly improve outcomes after ischemic CNS injury in a number of models but has not been tested in humans.

Other similar extracellular loop mimetic peptides, including Gap26 and Gap27 have shown benefit in animal models but are considered to be gap junction blockers (for review see [131]). Gap19, is a peptide which blocks interactions between the Cx43 carboxy-tail and the cytoplasmic loop. This peptide needs to enter the cell to be active but is unique in preventing hemichannel opening whilst maintaining gap junction channel function.[131, 132] It remains to be determined whether this effect to fix gap junction opening, that will be associated with an inability of the cell to regulate its own cell-cell communication, will be detrimental in any clinical settings. Nevertheless, this peptide has been effective in animal models and so offers significant promise for CNS injury when treatment should normally be transient. Moreover, the preclinical studies reviewed here suggest that after cerebral ischemia would be desirable to block hemichannels but to maintain the integrity of the astrocytic syncytium.[132] This peptide is at a preclinical stage of development but it, and Peptide5 may have potential for clinical translation.

Conclusion

These data highlight the therapeutic potential of blockade of connexin and pannexin hemichannels to improve outcome after ischemic brain injury in adults and newborns. Further *in vivo* studies are required to better understand the relative contributions and the relationship between connexin and pannexin hemichannels in the spread of ischemic brain injury and to dissect key variables that may affect therapeutic success, including dose, timing of treatment after insult and types of insult. At present, the highly promising preclinical data discussed above suggest that connexin and possibly pannexin hemichannel opening may be important therapeutic targets after cerebral ischemia.

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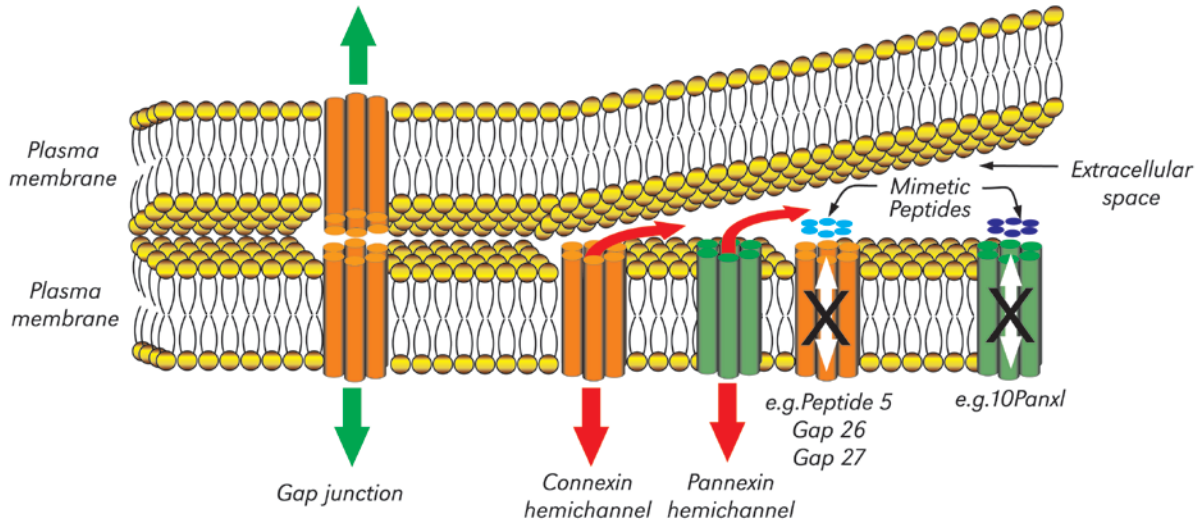
Figure legends

Figure 1. Schematic diagram showing that gap junctions link the cytoplasm of neighboring cells and are formed by two adjacent connexin hemichannels, while pannexin hemichannels do not form gap junctions. Opening of connexin or pannexin hemichannels under physiological or pathological conditions may provide a conduit linking cytoplasm with the extracellular space, allowing release of glutamate and ATP from the cell and uptake of water into the cell. Specific mimetic peptides can be used to block connexin (Peptide5, Gap26 and Gap27) and pannexin (10Panx1) hemichannels.

Figure 2. Flow diagram of the potential role of astrocytic Connexin43 hemichannel and neuronal pannexin hemichannel opening in seizures, secondary energy failure and cell death after ischemia.


Plasma membrane
Plasma membrane
Extracellular space
Mimetic Peptides
e.g.Peptide 5
Gap 26
Gap 27
e.g.10Panxl
Gap junction
Connexin hemichannel
Pannexin hemichannel

Potential role of Cx43 and Px1 hemichannels in seizures, secondary energy failure and cell death following ischemia