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Gap junctions and hemichannels: communicating cell death in neurodevelopment and disease

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Abstract

Gap junctions are unique membrane channels that play a significant role in intercellular communication in the developing and mature central nervous system (CNS). These channels are composed of connexin proteins that oligomerize into hexamers to form connexons or hemichannels. Many different connexins are expressed in the CNS, with some specificity with regard to the cell types in which distinct connexins are found, as well as the timepoints when they are expressed in the developing and mature CNS. Both the main neuronal Cx36 and glial Cx43 play critical roles in neurodevelopment. These connexins also mediate distinct aspects of the CNS response to pathological conditions. An imbalance in the expression, translation, trafficking and turnover of connexins, as well as mutations of connexins, can impact their function in the context of cell death in neurodevelopment and disease. With the ever-increasing understanding of connexins in the brain, therapeutic strategies could be developed to target these membrane channels in various neurological disorders.

Background

The complexity of the mammalian central nervous system (CNS) is due in large part to the various cell types from which it is composed, as well as the different forms of cellular interactions. These include neuronal interactions via neurotransmission, as well as glial interactions mediated by direct cell-cell contact in addition to paracrine signaling (gliotransmission). Furthermore a number of glial-neuronal interactions exist, and these have been implicated in both normal information processing as well as neuronal protection in the brain. One mechanism mediating such interactions involves gap junctions (GJs), clusters of intercellular membrane channels which provide for direct cytoplasmic continuity between adjacent cells [1].

Gap junctions and connexins in the CNS

GJs allow the passive intercellular diffusion of small molecules, such as glutamate, glutathione, glucose, adenosine triphosphate (ATP), cyclic adenosine monophosphate (cAMP), inositol 1,4,5-trisphosphate (IP$_3$), and ions (Ca^{2+}, Na$^+$, K$^+$) [2]. In addition, cells are able to control the open probability of these channels, providing a mean of including the channels in the general signaling and physiology of the cells and tissues. A single GJ channel consists of two opposing hemichannels, also known as *connexons*, which are made of six proteins called *connexins* [3]. Hemichannels can also function in their own right, having distinct roles in communicating between intracellular and extracellular compartments. Connexins are encoded by a multi-gene family consisting of 20–21 members in mammals [4].

Within the mammalian brain, the various cell types express over ten different connexins, making it a very diverse organ regarding intercellular communication. With regard to the different cell types, neurons express seven different connexins (see below), astrocytes express up to three (Cx43, Cx30, Cx26), as do oligodendrocytes (Cx32,

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Cx29, Cx47), microglia (Cx43, Cx32, Cx36) and endo-thelial cells (Cx37, Cx40, Cx43) [5–7]. These channels have distinct functions within the different cell types and their expression can change dramatically during neuro-development and injury (see below). GJs in oligodendro-cytes have been shown to be essential for proper myelination [8], as well as potassium buffering [9]. Endothelial functions are closely regulated by junctional interactions with astrocytes; specifically important are the connexins expressed in astrocytic endfeet [10, 11]. In this context, astrocytes and endothelial cells do not form gap junctions between them, but rather the connexin in astrocytic endfeet may solely function as hemichannels.

Cx43 is the most highly expressed connexin in the brain because it is involved in extensive GJ coupling (GJC) between astrocytes, the most abundant cell type in the brain. GJ communication is also critical for the proliferation and differentiation of neural stem cells [12]. Although microglia have been reported to express Cx43 and form GJs [13], others have not observed Cx43 im-munoreactivity in microglia [14, 15]; while another group showed that Cx43 does not form GJs in microglia [16], rather it forms hemichannels [17].

Connexins are expressed in both neurons and astro-cytes, and are regulated by numerous factors in healthy and pathological conditions. Neuronal GJs and astrocytic GJs are regulated during development and disease. How-ever, given the nature of the tripartite synapse, neuroglial interactions must also be considered in this context of synaptic malfunction.

Expression and regulation of neuronal connexins in neural development and adulthood

During development, neurons of the rodent CNS ex-press a number of different connexins. These include Cx36 [18–20], Cx30.2 [21], Cx31.1 [22], Cx40 [23], Cx45 [24] and Cx50 and Cx57 in the retina [25]. This presum-ably reflects the diversity of neuronal cell types, express-ing a range of connexins, and/or varying functions of those connexins in the developing CNS. However, knockout of specifically Cx36 results in near complete loss of neuronal GJC in the mature CNS, indicating that it is the primary neuronal connexin [26–29]. Cx36 in various regions of rodent CNS, and Cx35 (the fish orthologue of Cx36) expressed in goldfish Mauthner cells, are often present in mixed chemical and electrical synapses. Cx36 GJs have been observed in close proximity to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) [25, 30] as well as N–methyl–D–as-partate (NMDA) receptors (NMDARs) [30–32]. Multiple reports have demonstrated that Cx36 GJs can be tethered to the cytoskeleton via complex formation with multiple intracellular proteins. Constituents of Cx36-interacting complexes include structural proteins, regulators of

channel activity and gene transcription, as well as factors involved in protein transport, assembly and localization [33–35]. Data suggest that Cx36 may bind these proteins simultaneously at some GJs within the same neuron and the binding requires a four amino acid motif (SAYV) present in the C-terminus of the Cx36 protein [34, 35]. The interaction of Cx36 with these proteins appears to be necessary for addition into electrical synapses, since the SAYV motif is required for incorporation [36].

Cx36 is phosphorylated and its activity is influenced by a number of kinases including cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase, protein kinase C (PKC) and casein kinase II [37–39]. Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) interacts with and phosphorylates Cx36 in mouse infer-ior olive neurons and Cx35 in synapses of teleost Mauthner cells [40, 41]. The binding of Cx36 to CaMKII may not be limited to a substrate-enzyme interaction. Rather, there is some indication that the interaction is associated with changes in expression and/or stability of the kinase. It is noteworthy that neurons of Cx36 knock-out mice have reduced CaMKII levels [42].

Neuronal GJC is finely regulated among individual plaques (i.e., clusters of GJ channels). A given neuron can be coupled to a variable number of neighboring neurons and display different degrees of conductance with each of its coupled partners [32]. Similarly, binding to CaMKII [40] and the phosphorylation status of Cx36 [43] is not uniform within a neuron. Thus, the nature of signaling complexes associated with individual GJs pre-sumably facilitates the fine-tuning of individual synapses and cell-type specific activity [43]. The interactions listed above have also been reported for non-neuronal connex-ins (e.g., Cx43) suggesting that modulation of GJ activity by these interactions may be a general feature [44].

Spatial and temporal variation in GJC during develop-ment of the mammalian CNS has been well documented [45–49]. The expression of Cx36 and associated GJC in-crease during the first two postnatal weeks in most rodent CNS regions (including the cortex and hypothalamus). This initial, relatively robust expression declines during the third and fourth postnatal weeks [18, 50, 51]. This is in contrast to other regions of the CNS, such as the spinal cord, where Cx36 expression and coupling is highest during the late embryonic period followed by a decline in the first postnatal days [18, 52, 53]. The developmental decline in GJC is paired with changes in localization of Cx36 GJs to specific neuronal subtypes in the mature CNS [54, 55]. In the developing CNS in rodents, GJC is observed between disparate neurons; glutamatergic cells (including pyramidal cells) were found to couple with interneurons [20, 56] and neurons may couple with glial cells [57]. However, in the mature CNS, Cx36 GJs are found mostly between GABAergic interneurons

(GABA, γ-aminobutyric acid). It should be noted that connexins other than Cx36 also form GJs in the developing CNS [23, 58].

Despite the fact that chemical synaptic transmission is absent while Cx36 expression initiates in development [59], chemical neurotransmitter receptors regulate the developmental expression of neuronal GJC. In the rat and mouse hypothalamus and cortex, Cx36 expression is up-regulated by chronic activation of group II metabotropic glutamate receptors (mGluRs) and involves cAMP/PKA-dependent pathways. Conversely, $GABA_A$ receptor activation blocks the developmental increase in Cx36 expression [51] and is dependent upon developmental depolarization and Ca^{2+}/PKC-dependent signals. The developmental programs of Cx36 expression are executed using transcriptional (up-regulation) and translational (down-regulation) regulatory mechanisms [51]. These multiple mechanisms contributing to the developmental expression of Cx36 and formation of GJC likely explain the interregional differences in the developmental timing of GJC. The magnitude of the effects of neurotransmitter pathways on Cx36 expression suggests a modulatory rather than a definitive role in developmental regulation of neuronal GJC, primarily during the period when both electrical and chemical synaptic pathways are being laid down.

Regulation of Cx36 and GJC in the mature CNS occurs as well. It allows rapid modification of neuronal connectivity and signaling, including modulation of channel opening probability and alterations in connexin protein homeostasis. Similar to developmental regulation, these regulatory mechanisms are influenced by neurotransmitter signaling. For example, activation of D1 and D2 dopamine receptors, serotonin ($5-HT_2$) receptors, β-adrenoreceptors and elevation of nitric oxide reduces dye coupling between rat cortical neurons within minutes [60]. Similarly, activation of β-adrenoreceptors decreases electrotonic coupling between rat hippocampal interneurons [61] and nitric oxide uncouples striatal neurons [62]. Activation of group II mGluRs in developing mouse cortical neurons induced a rapid increase followed by a decrease in Cx36 protein over a 24-h time period; Cx36 mRNA levels were unchanged during this time, suggesting regulation of protein homeostasis [63]. However, whether Cx36 channel opening probability is regulated in developing neurons is not yet known.

The activity and regulation of Cx36 and GJC in neuronal injury and cell death

Every region of the mature CNS expresses Cx36 and has GJs, though at levels below that observed during development [54]. Transient elevation of Cx36 and GJC occurs following a wide range of neuronal insults, including ischemia [64–66], spinal cord injury and traumatic brain injury (TBI) [67–69], retinal injury [70], epilepsy [71, 72]

and inflammation [73]. The up-regulation of Cx36 and GJs in neurons following injury is very rapid, occurring 1–2 h post-injury with a decline in the subsequent 24–48 h [66, 68, 69, 72, 73]. This is in stark contrast to the developmental expression program outlined earlier, which occurs on the timescale of weeks; this difference in timing suggests that disparate regulatory mechanisms may be operating in development versus injury.

The regulation of GJC expression and activity during neuronal injury is a potential therapeutic target for reducing post-injury neuronal death. Rapid upregulation of neuronal GJC and Cx36 expression was observed following multiple types of neuronal injury in adult mice [66] and coincides with the period of massive glutamate release from injured cells [74, 75]. The post-injury elevation in coupling and Cx36 was prevented by blockade of group II mGluRs and involves post-transcriptional mechanisms since no change in Cx36 mRNA levels were observed [66]. In contrast to what was observed during developmental regulation, $GABA_A$ receptors were found to be only indirectly involved in reducing Cx36 expression after injury, likely via inhibition of electrical activity. Given that neuronal GJC has been shown to be, predominantly, pro-death for neurons following injury (see below), manipulation of pathways that modify expression of Cx36 might be a strategy for neuroprotection.

The morbidity and mortality of stroke is a direct result of neuronal death due primarily to ischemic injury and necrosis [76–79]. Contributing to this is excessive glutamate release from ischemic cells producing NMDAR-mediated excitotoxicity and apoptosis [80–82]. Multiple types of CNS insult beyond stroke, including TBI, epilepsy and inflammation, can produce significant neuronal death, which also involves, in part, NMDAR excitotoxicity [80, 83–86]. While studies have reported a role for GJs in cell death and survival during glutamate-mediated excitotoxicity and neuronal injury, their predominant association has been with cell death.

A few specific studies have reported a "pro-survival" role of Cx36 and GJC. For example, pharmacological blockade of GJs, using non-specific agents, augmented glutamate-induced neuronal death in mouse neuronal cortical cultures [87]. Similarly, secondary neuronal loss in the mouse retina, following infrared laser photocoagulation was most prominent 24–48 h post-injury, was increased by GJ blockade (using non-selective and relatively selective blockers for Cx36) and in Cx36 knockout mice [70]. Both studies support the notion that GJC contributes to cell survival.

The preponderance of reports, however, indicates that Cx36 GJC promotes neuronal death, independent of initiating injury. As noted earlier, sustained activation of group II mGluRs increased neuronal GJC and Cx36 expression during development; this increase amplified

NMDAR-mediated excitotoxicity. Consistent with this, blockade of group II mGluRs prevented increased Cx36 expression and dampened neuronal death from excitotoxicity. These findings support a model where group II mGluRs regulate the developmental program of Cx36 GJC and by doing so, contribute significantly to death decisions in developing neurons [51]. Group II mGluR activation mediates injury-associated increases in neuronal GJC. Not surprisingly, blockade of group II mGluRs reduced injury-mediated neuronal death in multiple injury models [66, 88].

Systemic administration of NMDA induced NMDAR-mediated excitotoxicity in the forebrain of adult wild-type mice, which was prevented by co-administration of the GJC blocker mefloquine [89]. Similarly, blockade of GJC by mefloquine significantly reduced ischemic neuronal death [89] and secondary neuronal death from controlled cortical impact in mice (a model of TBI) [88]. Cx36 knockout provided the same reduction in neuronal death in both models as pharmacological blockade. Mefloquine did not provide additional survival benefit in Cx36 knockouts, suggesting the drug is working primarily through inhibition of Cx36 GJs [88, 89]. Additional studies also reported a pro-death role for neuronal GJC in NMDAR-mediated excitotoxicity and injury models [90–93]. From this and other work, a model of glutamate-mediated excitotoxicity centered on neuronal GJs as the primary determinant of magnitude of the secondary neuronal death following injury has been proposed [94, 95]. Despite the difficulty of making broad generalizations about the role of GJC in cell death, particularly in non-neuronal tissues [96, 97], blockade of GJC as a strategy to limit neuronal death following stroke, TBI or other insult remains very attractive.

Mechanisms by which neuronal Cx36 and GJC contribute to neuronal death and survival

Understanding how neuronal GJs contribute to cell death and survival is critical, if manipulation of GJC is used as an approach to reduce neuronal damage and death in a variety of neurological diseases. Although, some of the studies discussed below were conducted with the use of non-neuronal cells, they provided an important information, which potentially may be applicable to neurons too.

Based upon numerous observations of passage of chemical substances via GJ channels, it has been proposed that the contributions of GJs to cell death and survival are by propagation between the coupled cells of, respectively, "pro-death" and "pro-survival" GJ-permeable signals [98–100]. Though the identity of these signals remains obscure, signaling molecules such as IP$_3$ and reactive oxygen and nitrogen species have been proposed as "pro-death" signals [100–102]. Conversely, molecules such as those involved in energy homeostasis (glucose and ATP), and free

radical scavengers (ascorbic acid and reduced glutathione) may be GJ-permeable "pro-survival" signals [96, 103]. Recently, a study was conducted in cultured neurons obtained from Cx36 knockout mice, in which the neurons were transduced with lentiviral vectors expressing one of three wild-type connexins, including neuronal Cx36 and non-neuronal Cx43 and Cx31 [93]. Ischemia and NMDAR excitotoxicity were used to induce neuronal death in those cultures. The study showed that each of the three wild-type connexins induced functional (channel-permeable) GJs and supported neuronal death. The data suggested that the role of neuronal GJs in cell death is connexin type-independent and presumably relies on channel activities of GJ complexes among neurons [93].

Another model for the role of GJC in cell death postulates that connexins are not involved in cell death mechanisms via their channel activities, but through direct or indirect regulation of transcriptional programs and apoptotic pathways [96, 104]. This model is based upon the following observations (however, mostly obtained for non-neuronal connexins). In osteocytes, Cx43 protein serves as part of a trans-membrane signal transduction pathway that alters the activity of pro-apoptotic Bcl-2 protein, Bad [105]. In non-neuronal cancer cells, Cx26 and Cx43 are co-localized with Bcl-2 proteins (Bak, Bcl-xL and Bax) and participate in cell death pathways via direct interaction with these pro-apoptotic factors [106, 107]. Overexpression of Cx43 in U251 glioblastoma cells does not increase GJC, but is pro-apoptotic [108]. During ischemia in cardiomyocytes, Cx43 serves as part of a multiprotein complex in mitochondrial membranes and controls homeostasis of mitochondria [109, 110]. Interference with expression of various connexins changes the expression of subsets of apoptotic factors (multiple studies reviewed in [96]) that presumably occurs through direct transcriptional control via the "connexin response elements" in pro-apoptotic genes [111] or via direct interaction of connexins with transcriptional regulators (e.g., β-catenin) [112]. In addition, a connexin-dependent induction of apoptosis can be connexin- and cell-specific as apoptosis in umbilical vein endothelial cells is induced by overexpression of Cx37 (but not Cx40 or Cx43), however, overexpression of Cx37 in rat NRK kidney epithelial cells is not pro-apoptotic [113]. A recent study in neuronal cultures utilized a lentiviral transduction of four mutant connexins (including various Cx36 and Cx43 mutants), each of which induced dysfunctional (channel-impermeable) GJs [93]. None of those mutant connexins supported neuronal death caused by ischemia or NMDAR excitotoxicity. This supported the notion that Cx36 unlikely plays a role in neuronal death via channel-independent mechanisms, but likley plays a role via channel-dependent mechanisms. It remains to be explored

whether or not other neuronal connexins (e.g., Cx45) may contribute to neuronal death through the channel-independent mode of action.

Finally, the role of connexin hemichannels in cell death and survival has been proposed. Specifically, for the nervous system, the contribution of glial hemichannels via release of various channel-permeable "pro-death" agents has been discussed [96, 103, 114, 115]. These agents presumably include glutamate, ATP, reactive oxygen and nitrogen species. The existence and role of neuronal hemichannels in neuronal death also has been suggested based on experiments with the use of various neuronal injury models [11, 116]. However, other studies did not support the role of neuronal hemichannels in neuronal death following ischemia and NMDAR-mediated excitotoxicity [91, 93]. Moreover, the role of Cx36 hemichannels in neuroprotection via release of ATP has been suggested [117], adding an additional layer of complexity on the contribution of hemichannels in particular, and connexins in general, to neuronal death and survival.

Expression and regulation of astroglial connexins
In the CNS, astrocytes are highly coupled to each other by GJs and play a significant role in the metabolic and trophic support of neurons [118]. These GJs are composed primarily of the channel protein Cx43, and to a lesser extent Cx30 [119] and Cx26 [120]. GJs form a functional syncytium of coupled astrocytes, contributing to spatial buffering, in dealing with elevated concentrations of extracellular potassium ions (K^+) during increased neuronal activity; GJs assist in dispersal of K^+ accumulated by astrocytes [121].

A major advance in understanding astrocytic GJs and connexins was made through transgenic and knockout mice (reviewed in [122]). The role of astrocytic GJs has been demonstrated in mouse hippocampal slices, with Cx43/Cx30 double knockout mice showing impaired extracellular K^+ buffering compared to wild-type mouse slices [123]. It has also been shown that Cx43 plays a role in transient intracellular K^+ buffering by mitochondria [124]. Pannasch et al. [10] reported key changes in astrocytic and neuronal properties in the absence of Cx43 and Cx30, revealing a major role for astrocytic networks in glutamate clearance, K^+ buffering, and volume regulation of the extracellular space during synaptic activity. Failure to efficiently clear K^+ and glutamate results in prolonged neuronal AMPA and NMDA currents, as well as astroglial membrane depolarization. Further clarification of the role of Cx30 was obtained by examining the hippocampus of single Cx30 knockout mice, demonstrating that Cx30 modulates astrocyte glutamate transport, thereby controlling hippocampal excitatory synaptic transmission [125]. In this case it was shown that Cx30 controls astrocytic processes at the synaptic cleft by modulating their morphology. Glutamate clearance by astrocytes was altered due to these morphological changes.

In addition to forming GJs, Cx43 also forms hemichannels, with single connexons communicating directly with the extracellular space [126]. Hemichannels predominantly exist in a closed state under normal physiological conditions, due to ambient levels of Ca^{2+} [127]. However, various cell stresses, such as hypoxia/reoxygenation and metabolic stress, have been reported to cause opening of hemichannels in cultured astrocytes [128]. Hemichannels enhance neuronal injury under ischemic and proinflammatory conditions [129, 130].

Regulation of Cx43 in neural development
Due to the high level of GJC observed during neurodevelopment, particularly in the cortex [131, 132], it is not surprising that connexins have been shown to be involved. While neurons predominantly express Cx36 postnatally in the rat and mouse (see above), at prenatal stages neural progenitor cells (NPCs), including radial glia, are highly coupled and express Cx43 and Cx26 [47, 133–136]. Different approaches to determine the role of these connexins have been reported, including knockout of Cx43 [133, 137, 138] and knockdown of Cx43 and Cx26 [134]. While there are some variations in the phenotypes obtained, attributed in part to strain differences [138], the role of Cx43 appears to be due to adhesive functions [134], and the C-terminal region is critical for NPC migration [133, 136]. Cx26 knockdown was also shown to impede NPC migration in the developing rat cortex [134]. Cx26 has been demonstrated to be a substrate for focal adhesion kinase (FAK), or to function in stabilizing cell contacts, possibly through interactions with ZO-1 [139]. The authors suggest that FAK could act as scaffold protein, a function also suggested for Cx43. Since Cx30 is not expressed until 15 days after birth in the mouse [119] it is not considered in this context.

The activity and regulation of Cx43 and GJC in neuronal injury and cell death
The level of GJC between astrocytes, as well as hemichannel activity [140], have been shown to be regulated by a number of factors, including neurotransmitters and neuromodulators [141–145], extracellular ion concentrations [146] and various pharmacological agents [147–150]. Astrocytic GJC and Cx43 expression are altered in various brain pathologies, including ischemia [151], stroke [152, 153], brain tumours [154], multiple sclerosis [155], brain abscess [156], Alzheimer's disease [14, 157], and epilepsy [158, 159].

In addition, microglial response to brain injury and disease leads to the release of proinflammatory cytokines, including IL-1β and TNF-α, which impair astrocytic GJC,

but enhance hemichannel activity [160]; this leads to increased neuronal injury. Short application of NMDA induces delayed neuronal injury due to excessive release of glutamate after removal of NMDA [161]. However, neurons in contact with astrocytes are protected against such glutamate toxicity [162, 163]. This neuronal protection was attributed to glutamate uptake by astrocytes [164], and as GJs are permeable to glutamate [165], GJC in astrocytes could improve glutamate uptake contributing to its dissipation, and thus to neuronal protection. In addition, GJC enables the intercellular trafficking of glucose and its metabolites through the astroglial network from blood vessels to distal neurons in an activity-dependent manner [166]. This pathway could sustain neuronal survival in pathological situations that alter energy production, such as hypoglycemia or anoxia/ischemia.

Consideration of pannexins

A three-member family of cell membrane channels, the *pannexin(s)* (Panx 1, 2 and 3), should also be considered in the context of GJ channels and hemichannels in the CNS. Panxs were discovered due to their homology to the invertebrate GJ proteins, *innexins* [167, 168]. Panx1 and Panx2 are present in the CNS [169] and have been linked to different CNS injury models [170–172]. Because of the ubiquitous expression of Panx1 [168], it has been the most widely investigated member of the Panx family [172, 173], however, a recent study reported that Panx2 expression is not only limited to the CNS [174].

Like the connexins, the Panxs traverse the cell membrane, but these large pore\channels allow the passage of large signaling molecules (*e.g.*, ATP; glucose and glutamate) only between intra- and extracellular compartments of neurons and possibly astrocytes [175–177], but not between adjoining cells. The evidence for Panx channels not forming intercellular GJ channels, but rather the equivalent of a connexin hemichannel, has been recently addressed [178] and is attributed to the steric hindrance provided by the extracellularly glycosylated arginine residue, which interferes with the coupling of two opposing Panx channels [179, 180]. Similarly to connexins, the Panx C-terminal domain, particularly from Panx1, has been shown to interact with a host of intracellular factors under specific physiological and pathophysiological conditions [181–183].

Because Panx1 forms channels in the plasma membrane, it likely participates in non-synaptic forms of communication to regulate synaptic function under normal conditions, in addition to astrocytic Ca^{2+} wave propagation and regulation of vascular tone [184–186]. Unlike the protective effects of Cx43, however, Panx1 activation under pathological conditions is detrimental, contributing to ischemia-induced excitotoxicity and ATP-dependent cell death [176, 187–191]. Under ischemic conditions, as seen with oxygen-glucose deprivation in cortical and hippocampal slices, Panx1 channels are irreversibly activated (opened) to promote a progressive and uncontrollable depolarization of neurons, sustained increments in extracellular concentrations of glutamate and aspartate, and subsequent activation of downstream apoptotic and necrotic pathways [192]. Others have shown similar association between Panx1 activity and neuronal death, in different types of neurons [190, 193]. This suggests that Panx1-dependent cell death may be a common mechanism in injured neurons.

In addition to the anoxic depolarization mechanism associated with Panx1, it has also been implicated in contributing to inflammation [188, 194]. For example, cells undergoing apoptosis release chemotactic inflammatory factors to promote phagocytic removal of dead cells. ATP and UTP represent important signaling molecules throughout the inflammatory cascade, also thought of as danger signals that are released from damaged and necrotic cells, at least during the initial stages of ischemia [195], but also more importantly through the Panx and connexin hemichannels. Several studies have reported that Panx1-mediated ATP and UTP release is induced by caspase activity (caspase 3 and 7) in apoptotic cells [176, 187, 189]. Two potential caspase cleavage sites were identified in the C-terminal of Panx1 [189]. The C-terminal cleavage-site-B of Panx1 is evolutionarily conserved among Panx1 homologues, indicating that caspase-dependent cleavage of Panx1 and ATP release may be a conserved mechanism in apoptosis [189]. Caspase-mediated C-terminal cleavage of Panx1 results in irreversible channel opening, inducing higher if not uncontrollable extracellular release of ATP. Findings from several different organ systems collectively suggest that this irreversible activation of Panx1 leads to a cascade of maladaptive immunity, to include sustained cytokine release, improper resolution of inflammation, impaired immune cell chemotaxis, and ultimately cell death [196]. Panx1 activity has also been associated in triggering activation of the inflammasome complex [188], however, this association is not fully elucidated and maybe cell-type specific [176, 188]. Of relevance here, in an *in vivo* experimental model of retinal ischemic injury in male mice, genetic ablation of Panx1 suppresses interleukin production and protects retinal neurons from injury, highlighting the link between Panx1 and inflammation [193].

Interestingly, the reported predominance of caspase activation after ischemic injury in female mice [197] raises the intriguing possibility that the endogenous requirements for Panx1 to regulate neuronal responses to ischemic injury are different between the two sexes. With respect to connexins, whether Panx membrane channels affect connexin activity, under physiological or pathological conditions, is unknown and potentially a fruitful avenue for investigation.

Therapeutic avenues

As discussed above, multiple studies have indicated that blockade of GJs and hemichannels provides neuroprotection in various models of neuronal injury. This suggests a possibility for using the GJ/hemichannel blockade as a novel therapeutic approach. Conceptually, blocking the propagation between the neurons of GJ-permeable toxic signals or blocking the release of toxic agents via hemichannels would create a "firebreak", reducing the extent of cell death. This would prevent excessive neuronal death following ischemic stroke, TBI and epilepsy, significantly reducing morbidity and mortality. Because clinical trials for NMDAR antagonists as neuroprotective agents largely failed [198], development of new neuroprotective agents based on manipulation of neuronal and astroglial GJs and hemichannels should prove to be a valuable alternative approach.

Conclusion

However, as also discussed in the present review, a number of reports suggest that blockade of GJs and hemichannels increases cell death. Clearly, the data on whether GJs are pro-death or pro-survival are conflicting and a convincing, evidence-supported explanation of this phenomenon is absent. This provides the significant barrier for translating the above-described findings to clinical practice. Without resolution of conflicting studies, manipulation of GJC in the clinic, as a novel approach to reduce neuronal death, cannot be advocated. This represents loss of a potentially extraordinary benefit to people suffering a range of brain insults. Identifying the underlying mechanisms and determining conditions for the clinical use of GJ blockers that will not compromise their strong neuroprotective effects should be the major focus of future studies.

Abbreviations

5-HT$_2$: Subfamily of serotonin receptors; AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; ATP: Adenosine triphosphate; CaMKII: Ca^{2+}/calmodulin-dependent protein kinase II; cAMP: Cyclic adenosine monophosphate; CNS: central nervous system; FAK: Focal adhesion kinase; GABA: γ-aminobutyric acid; GJ: Gap junction; GJC: Gap junction coupling; IP$_3$: Inositol 1,4,5-trisphosphate; mGluR: Metabotropic glutamate receptor; NMDAR: N–methyl–D–aspartate receptor; NPC: Neural progenitor cell; Panx: Pannexin; PKA: Protein kinase A; PKC: Protein kinase C; UTP: Uridine triphosphate

Declarations

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Authors' contributions

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There are no requirements for ethics and consent.

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