

The two faces of pannexins: new roles in inflammation and repair

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Abstract: Pannexins belong to a family of ATP-release channels expressed in almost all cell types. An increasing body of literature on pannexins suggests that these channels play dual and sometimes contradictory roles, contributing to normal cell function, as well as to the pathological progression of disease. In this review, we summarize our understanding of pannexin “protective” and “harmful” functions in inflammation, regeneration and mechanical signaling. We also suggest a possible basis for pannexin’s dual roles, related to extracellular ATP and K⁺ levels and the activation of various types of P2 receptors that are associated with pannexin. Finally, we speculate upon therapeutic strategies related to pannexin using eyes, lacrimal glands, and peripheral nerves as examples of interesting therapeutic targets.

Keywords: pannexin, Panx1, ATP, purinergic signaling, inflammation, regeneration

Introduction

Cell–cell and cell–matrix interactions are fundamental properties of multicellular organisms. Gap junctions, formed by connexins and innexins in vertebrate and invertebrate animals, respectively, allow direct passage of ions and small molecules (<2,000 Da) from cell to cell (Figure 1A).¹⁻³ In addition to gap-junction channels, connexins may form hemichannels (HCs), termed “connexons” (Figure 1B),^{4,5} which are hexamers of connexin monomers (Figure 1A), each containing four transmembrane domains, two extracellular loops, and cytoplasmic N and C termini (Figure 1C).⁶ The vertebrate homologues of innexins, called “pannexins”, form mostly HCs, or pannexons (Figure 1B), due to the high level of glycosylation in their extracellular domains (Figure 1D).⁷⁻¹² Similar to connexins (Figure 1C), pannexins have a cytosolic N-terminal domain, four transmembrane domains with two extracellular loops, and a cytosolic C-terminal domain (Figure 1D).¹³ However, pannexins have no homology to the vertebrate connexin gap-junction protein,⁸ and unlike connexins, which have multiple cysteine residues in both extracellular loops, pannexins have only two cysteine residues per loop (Figure 1C and D, black ovals).¹³

The pannexin family consists of three proteins, Panx1, Panx2, and Panx3, all of which have been shown to form a single-membrane channel.^{14,15} Panx1 is ubiquitously expressed in almost all cell types, including those in the nervous and immune systems, eye, muscle, olfactory epithelium, blood vessels, exocrine glands (eg, lacrimal and salivary glands), thyroid, prostate, kidney, and liver (Table 1).¹⁶⁻²⁰ Panx2 transcripts are highly expressed in the central nervous system (CNS).²¹ Lower levels of Panx2 transcripts have been detected in nonneural tissues, including the testis, kidney, retina, and

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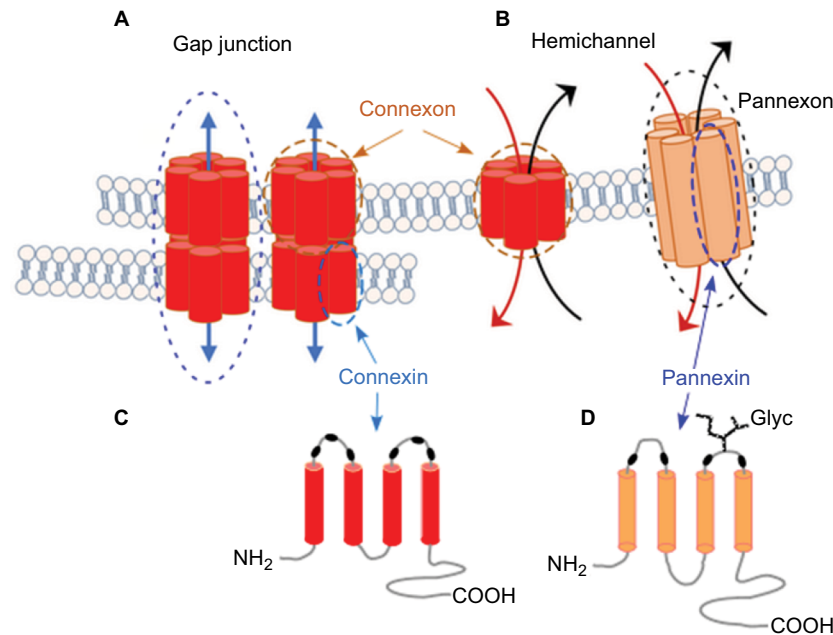


Figure 1 Connexins and pannexins.
Notes: (A, B) Connexin and pannexin share a similar structure, despite the absence of sequence homology. Connexin and pannexin form functional connexon and pannexon hemichannels, respectively. (C, D) Connexins and pannexins are transmembrane proteins with four transmembrane domains, two extracellular loops, one cytoplasmic loop, and cytoplasmic N- and C-terminal domains. Connexin channels can assemble into a gap junction (A) that mediates intercellular communication, while pannexin's extracellular loop has a high level of glycosylation in mammalian cells (D), which prevent the formation of gap junctions.
Abbreviation: Glyc, glycosylation.

Table 1 Expression and localization of pannexin mRNA and protein

	Panx1	Panx2	Panx3
mRNA-expression pattern and levels of expression	Ubiquitously expressed in mammalian ²¹¹ and chicken tissues ²¹²	Cerebral cortex, cerebellum: neurons and immature astrocytes, ²¹ hippocampus (high); ²² kidney tubular cells (low), seminiferous duct cells in the testis (moderate), salivary glands (excretory), and striated ducts (weak) ²¹	In humans, Panx3 mRNA found in the testis, stomach, spleen, salivary gland, lung, heart, duodenum, and adrenal tissue; ²¹³ in mice, Panx3 mRNA found in developing skeletal structures ²¹⁴
Protein expression	Ubiquitously expressed in many mammalian tissues, ^{211,215} with lower levels in the lung, kidney, and heart ventricles ¹³	Protein is expressed in many tissue types, including gastrointestinal tract glandular and epithelial cells, parietal cells, columnar epithelial cells of the human colon ²¹⁶ (strong), and mouse retina, lung, and skin	Extensive expression throughout the developing skeleton during chondrocyte and osteoblast differentiation, ²¹⁴ skeletal muscle, ²⁵ lactating mammary tissue, sebaceous glands, and the small intestine ¹⁵
Localization within the cell	Mostly membrane, ^{13,26,217} but may form permeable channels in the endoplasmic reticulum ²⁷	Membrane, ^{24,26} predominantly cytoplasmic ²¹	Membrane, may form channels in the endoplasmic reticulum and gap junctions after overexpression, ^{218,219} diffuse cytoplasmic in the epidermis ²²⁰
Subunits	6	6–8 ²²¹	6
Key domains (conserved in all isoforms)	Transmembrane domains (×4), extracellular loops (×2), intracellular N-terminus, C-terminus, and intracellular loop		
Glycosylation site	Second extracellular loop	First extracellular loop	First extracellular loop

gastrointestinal tract, while Panx3 mainly localizes in the skin, osteoblasts, and chondrocytes (Table 1).^{15,20–24} Panx3 has also been found in skeletal muscle,²⁵ lactating mammary glands, sebaceous glands, and the small intestine.¹⁵ Interestingly,

Panx2 protein appears to be more ubiquitously expressed than initially predicted by mRNA expression²¹ (Table 1).

Endogenous Panx1 and Panx3 proteins are localized primarily at the plasma membrane,^{13,26} while Panx2 is highly

expressed in the cytoplasmic compartment,²¹ suggesting a unique intracellular function for Panx2. However, several studies have reported the cytoplasmic localization of Panx1 and Panx3 proteins when these proteins were overexpressed in cells.^{21,27} For example, Abee et al²⁷ demonstrated cytoplasmic localization of Panx1 transiently expressed in LNCaP cells, where it formed Ca²⁺-permeable channels in the endoplasmic reticulum (Table 1). It is quite possible that high levels of pannexin protein expression could lead to both membrane and endoplasmic reticulum-channel formation, thus contributing to sustained increases in intracellular Ca²⁺.

Pannexins are ATP-release channels that can be activated by caspase cleavage of their pore-associated C-terminal tail, the autoregulatory region controlling channel permeability. The regulated ATP (nucleotide) release through pannexin HCs is implicated in a number of normal physiological functions and in response to stressors or pathological states in cells and tissue.^{25,28,29} Well-characterized functions of pannexins include regulation of cell differentiation and migration, tissue development and regeneration, inflammation, wound healing and cell death.²⁸ However, mechanistic explanations of how these proteins perform sometimes contradictory roles remain unclear.

In this review, we attempt to clarify existing controversies in the literature on the “protective” and “harmful” roles of pannexin HCs by addressing a question: How do pannexins acquire these different and often opposing roles? We seek to obtain deeper understanding of pannexin signaling, participants in which represent a potential source of novel and promising therapeutic targets in a variety of pathologies. Our focus is entirely on pannexins, with a full understanding that pannexin and connexin HCs have both distinct and complementary but often overlapping functions, particularly in ATP release and inflammation; therefore, we refer the reader to several excellent reviews comparing the roles of these channels.^{30–33}

Pannexins, inflammation, and inflammasome activation

The involvement of pannexins in the induction of inflammation has been reported in multiple publications.^{28,34–36} Inflammation is the major protective function maintained by the evolutionarily conserved innate immune system in response to harmful stimuli, such as pathogens, stress, injury, or cell death. Acute (short-term) inflammation stimulates a regenerative response, while persistent (chronic) inflammation can cause systemic inflammatory diseases.³⁷ Activation of inflammasomes, facilitating the release of interleukin-1 β (IL1 β) and IL18 in response to pathogens and tissue injury, is a key function of the innate

immune system. The inflammasomes, first characterized in monocytes in 2002³⁸ and in neural cells in 2008,³⁹ are multi-protein complexes mediating proteolytic maturation of Casp1, Casp11, IL1 β , and IL18. Proteolytic cleavage of IL1 β and IL18 precursors is executed by active Casp1 (Figure 2);^{40,41} and the release of the mature cytokines occurs via megapores, formed by N-terminal domains of the Casp1/11-processed recently identified pore forming protein gasdermin D.^{42–46} A large body of experimental evidence identifies Panx1 and its associated P2X receptors as essential upstream regulators of inflammasomes and proteolytic activation of Casp1 and Casp11.^{47–51} Panx1 has been reported to activate inflammasomes in many cell types, including macrophages,^{52,53} microglia,⁵⁴ neurons, and astrocytes;⁴⁹ however, the data on particular cell and inflammasome types remain controversial.^{55,56} Currently, the bulk of published data support a pivotal role for Panx1 in CNS/retinal inflammasome regulation.^{55–58} As such, strong suppression of its major components, including Casp1, Casp11, IL1 β , and apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), is observed in both *Panx1*^{−/−} mice and wild-type retinas after Panx1 blockade by probenecid.^{16,54,59}

There are two major regulatory arms for inflammasome activation (Figure 2): signal 1 pathways sense environmental signals via surface TNF, Toll-like, and IL1 receptors and facilitate inflammasome “priming”, ie, transcriptional activation via MyD88–NF κ B-mediated pathways;^{60,61} and signal 2 pathways regulate inflammasome assembly and processing of Casp1/11, IL1 β , and IL18 precursors. This arm is regulated via the Panx1–P2X signalosome to facilitate ATP and K⁺ release, as well as uptake of extracellular Ca²⁺ and danger/pathogen-signaling patterns.^{62,63}

Though a role for Panx1 in the inflammasome regulatory cascade appears to be generally conserved across cell types, Qu et al⁵⁵ suggested that pannexin is “dispensable” for inflammasome formation. In particular, LPS-primed bone marrow-derived macrophages were successfully able to activate Casp1 and secrete its associated inflammatory cytokines (IL1 β and IL18) in response to a number of stimuli in the absence of Panx1. Moreover, the authors also concluded that P2X₇ and Panx1 can function independently and may be involved in distinct signaling pathways.⁵⁵ These controversial views on Panx1 function could be explained by cell-type-specific differences and potential variation in culture conditions, and need to be resolved.

Mechanisms of pannexin-channel activation

Several diverse mechanisms regulating pannexin-channel function have been proposed to date. Pannexin channels

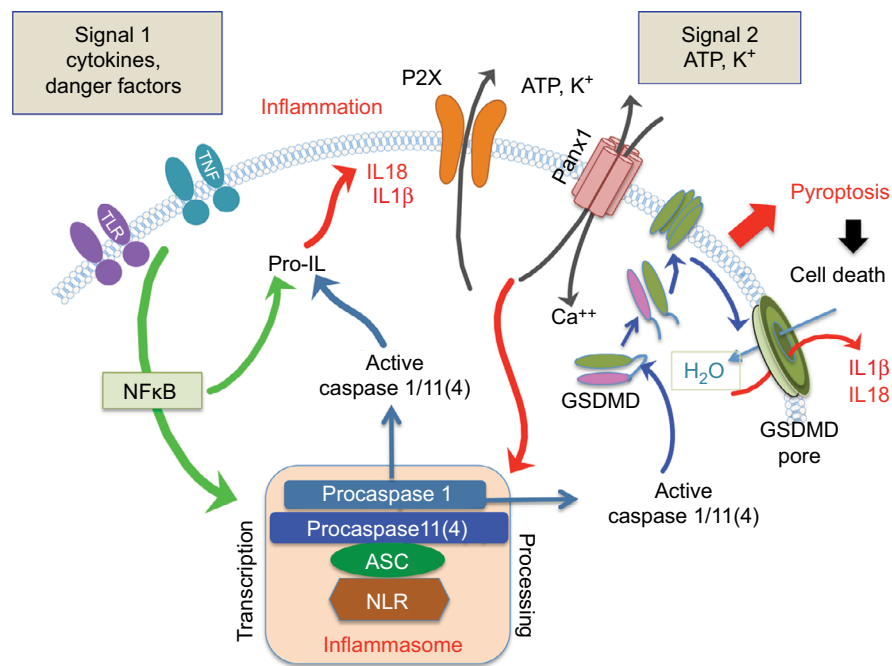


Figure 2 The two signaling arms of the inflammasome-activation cascade.

Notes: Signal 1 pathways sense environmental signals via surface Tumor necrosis factor (TNF), Toll-like (TLR) and IL-1 receptors and facilitates transcriptional priming of inflammasome components via the NFκB pathway and upregulates the expression of precursor proteins of IL1β, caspases 1/11 (also known as caspase 4), and pro-Nod-like receptors (NLR). Signal 2 facilitates activation of the complex via proteolytic processing and assembly. This arm responds to mechanical stress, activation of a ligand-sensing system within the cytosol or extracellular ATP sensing via Panx1–P2X receptor signalosomes. Upon activation, protease activity of caspases regulates the maturation and release of IL1β and IL18. Recent studies showed that Gasdermin D (GSDMD) is a novel membrane pore-forming protein. Cleaved by inflammatory caspases Casp1 or Casp11(4), GSDMD binds to phosphoinositides in the plasma membrane and oligomerizes to generate membrane pores of ~10–14 nm in diameter.²²² This pore size can allow the passage of mature IL1β, IL18, and caspase 1. The formation of the GSDMD pores also disrupts osmotic potential, resulting in an inflammatory form of cell death known as pyroptosis.

Abbreviation: ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain.

have been posited to be activated by caspase-mediated channel cleavage in apoptotic immune cells, G-protein-coupled receptors in vascular smooth muscle,^{64,65} low oxygen tension in erythrocytes and neurons,⁶⁶ high extracellular K^+ in various cell types,^{49,67} and mechanical stretch.^{68,69} Progressive Panx1-channel opening is directly linked to ion- and large-molecule transport, and occurs during both irreversible (caspase-mediated cleavage)⁷⁰ and reversible G-protein-coupled receptor (including α_1 -adrenoceptor-mediated) forms of channel activation.⁷¹ Panx1 activation by caspase-mediated cleavage enables the release of ATP as a “find me” signal that recruits phagocytizing macrophages to apoptotic T lymphocytes.^{65,70} This mechanism is critical for the fast clearance of apoptotic and dead cells during acute inflammation.^{28,55,65,72} Cleavage activation of Panx1 is also involved in pyroptotic cell death (Figure 2).⁷³ A recent study employing electron microscopy and single-channel recordings of full-length and caspase-cleaved pannexin concatemers with defined numbers (0–6) of intact and truncated C termini revealed that Panx1 activation was increased in a sequential manner by stepwise removal of the autoinhibitory C termini. This also resulted in a graded increase in current and ATP/dye permeation.⁷¹ On the other

hand, the reversible G-protein-coupled receptor-mediated mechanism is independent of caspase-mediated pannexin cleavage.⁷⁴ Comparison of α_1 -adrenoceptor-activated with cleavage-activated Panx1 channels indicated that α_1 -adrenoceptor-activated Panx1 channels had a shorter mean open time, but progressively increasing conductance, suggesting that despite differences in gating kinetics, activation of Panx1 channels by both signaling mechanisms involves cumulative changes in open-channel properties.⁷¹

Pannexin signaling via ATP release

Panx1 channels can release ATP under physiological conditions and play critical roles in many pathological processes. ATP is a prominent extracellular signaling molecule in both physiological and pathological conditions. For example, ATP release is important for muscle differentiation and function,^{75–78} and ATP-receptor activation plays a role in regulation of cell proliferation, DNA synthesis, cell differentiation, and cell survival during the course of CNS development.^{79,80} At the same time, ATP may also serve as a major danger signal for cells,⁵⁰ despite it having a very short half-life due to rapid degradation by surface ecto-ATPases.⁸¹

ATP is released from apoptotic, injured, and viable cells that are challenged by assorted cytokines, as well as mechanical or ischemic stress in the presence of elevated K^+ .⁸²

ATP-mediated activation of Panx1, the ATP-release channel, typically ramps up in a vicious cycle only to a certain level, due to a retrograde feedback mechanism regulating activity of Panx1 HCs via a low-affinity ATP binding site.⁸³ Therefore, the permeant (ATP) can inhibit the permeating channel when high extracellular ATP concentration is reached. Importantly, however, this inhibition is abrogated by an increased extracellular concentration of potassium ions (K^+),⁸² suggesting a mechanism of toxicity of extracellular ATP in Panx1-expressing cells. In agreement with this mechanism are the findings that massive activation by Casp3/7 cleavage or expression of constitutively active Panx1 HCs results in cell death.^{65,84} Therefore, the balance between physiological and pathological activities of Panx1 depends on the open-state probability of the channel, which in turn is influenced by the increase in intracellular Ca^{2+} and extracellular ATP and K^+ . An additional level of Panx1-channel regulation is achieved via interactions with purinergic P2 (eg, P2X and P2Y) receptors, which are activated by binding extracellular ATP at the plasma membrane.⁸⁵ Several salient aspects of Panx–P2 interactions, including the mechanisms and significance of such interactions, as well as their sensitivity and specificity, are detailed in the following sections.

Functional interactions of Panx HCs with purinergic P2X and P2Y receptors

There are two major families of purinergic P2 receptors: ionotropic P2X and metabotropic P2Y receptors. Reciprocal interactions, whereby P2 receptors directly activate Panx1 channels,^{86,87} suggest that these proteins can form a signaling complex at the cell surface^{34,88} that mediates both paracrine and autocrine purinergic communication.

The P2X-receptor family contains 7 isoforms ($P2X_{1-7}$), and P2X receptors are classified as ligand-gated channels whose activation regulates cellular membrane potential and intracellular Ca^{2+} levels.^{89,90} More precisely, though, P2X family members are ATP-gated cation channels, selective for Na^+ , K^+ , and Ca^{2+} ions.⁹¹ In the nervous system, P2X receptors are pivotal transducers of ATP-mediated paracrine signals and have been implicated in physiological functions, such as chemotactic cell migration, intercellular calcium-wave propagation, as well as in nervous system dysfunction, leading to neuropathic pain or cell death.⁹² Five isoforms – $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, and $P2X_7$ – have been shown to interact

with Panx1,^{93,94} among which $P2X_4$ and $P2X_7$ are the most common interaction partners in different cell types.^{51,95,96} Both $P2X_4$ and $P2X_7$ isoforms are calcium channels known to dilate into larger pores upon activation.^{97,98} $P2X_7$ -receptor activation results in the appearance of HC-like currents, reflecting the channel permeability for molecules up to 1 kDa and identified as Panx1 HC.⁵² Under pathological conditions, overactivation of the Panx1– $P2X_7$ signalosome complex has been implicated in inflammation, cell death, and neuropathic pain.^{34,96,99,100}

The P2Y-receptor family contains eight isoforms ($P2Y_{1,2,4,6,11-14}$). P2Y receptors are metabotropic G-protein-coupled receptors that couple to G_q , G_s , or G_i in an isoform-specific manner, and their activation modulates intracellular inositol triphosphate, Ca^{2+} , and cAMP levels.^{101,102} Different isoforms of purinergic P2Y receptors are activated by ATP and its degradation products ADP and UTP, and couple to distinct G proteins to induce cAMP production, activation of phospholipase C, or intracellular Ca^{2+} via inositol triphosphate second-messenger systems.^{103,104} Recent publications have implicated P2Y receptors and Panx1 signaling in the regulation of endothelial cell activation in vascular inflammation¹⁰⁵ and cell-volume regulation.¹⁰⁶ ATP release via Panx1 channels activates $P2Y_2$ receptors to amplify signaling in sensing chemotactic gradients in neutrophils.^{107,108} Polarization of surface expression by translocation of Panx1, $P2Y_2$, adenosine A_3 receptors, and ENTPD1 ectonucleotidase to the leading cell edge allows neutrophils to polarize within the gradients.

Sensitivity and specificity of Panx1–P2X signaling during normal tissue function and chronic inflammation

The activity and downstream consequences of several $P2X_4$ - and $P2Y_2$ -receptor isoforms are dependent on ATP binding, with varying sensitivity. $P2X_7$, expressed by microglia, astrocytes, and neurons, is the most studied isoform, and is implicated in cyto- and neurotoxicity via direct interaction with Panx1 channels.^{34,87,88} Due to its relatively low affinity to ATP (EC_{50} 936 μ M), it can only be activated by nonphysiological (≥ 1 mM) increases in extracellular ATP, which is locally achievable only at sites of injury or proximal to activated Panx1/Cx43 HCs. Conversely, the activation at low to medium (<900 μ M) extracellular ATP levels can be mediated via an interaction between the high-affinity $P2X_4$ receptor ($P2X_4$; EC_{50} 2.3 μ M)¹⁰⁹ and Panx1^{49,110} (Figure 3A and B). This synergistic interaction was shown to coactivate $P2X_7$, resulting in massive local efflux of ATP via the Panx1 channel, a forward-feeding autocrine amplification loop (Figure 3C).^{111,112}

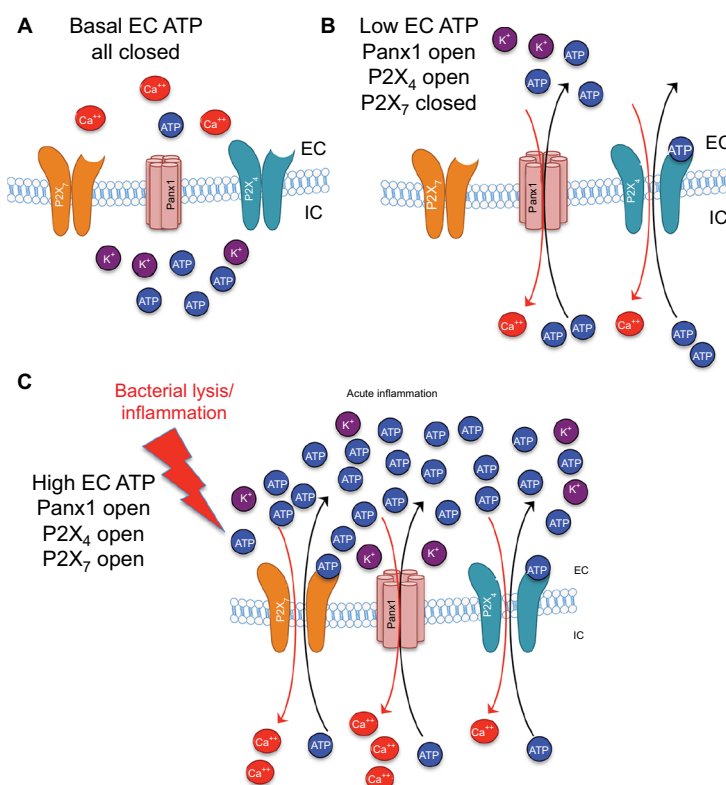


Figure 3 Differential ATP and ion movement depending on Panx1, P2X₄, and P2X₇ activity.

Notes: (A) Basal levels of EC ATP and normal concentration gradients of ATP and ions. (B) Panx1 opening with low levels of EC ATP results in P2X₄ activation, but no P2X₇ activity. (C) Higher levels of EC ATP, such as those resulting from bacterial lysis or chronic inflammation, result in opening of P2X₄ and P2X₇ channels and substantial movement of ATP and ions along their concentration gradients.

Abbreviations: EC, extracellular; IC, intracellular.

Functional synergy between P2X₄ and cytotoxic P2X₇^{110,113–115} is known to be pivotal for Panx1-dependent extracellular ATP-induced cell death,¹¹⁶ which can be suppressed by a blockade of either component in the Panx1–P2X_{4/7} complex or by extracellular ATP removal with apyrase.^{51,96} Similarly, in the retina, genetic ablation or pharmacological inhibition of Panx1,¹¹⁷ P2X₇, or P2X₄^{118–120} has protected retinal ganglion cells (RGCs) in both acute and chronic ocular hypertension (OHT)-injury models. In other studies, a similar blockade was shown to protect neurons and other cell types from death via a rise in ionized Ca²⁺ and the induction of the inflammasome in various injury paradigms.^{34,121,122}

In contrast to P2X₇, interactions between P2X₄ receptor and Panx1 and their link to RGC loss and inflammasome in the retina still require exploration. However, strong evidence of a key cellular role of P2X₄ in response to sublethal levels of ATP has been suggested in experiments on channel blockade with the 5-BDBD antagonist in macrophages,¹¹⁶ as well as on inflammasome activation in various tissue types.^{51,123} In contrast to P2X₇, P2X₄ blockade has been shown selectively

to suppress IL1 β but not IL18 cytokine levels,¹¹⁰ which was reported as potentially neuroprotective.^{124–126}

More recently, an interesting phenomenon in acute-wound healing following the use of intracellular ATP delivery was described. In this study, ATP application was accompanied by a massive increase in macrophage trafficking, in situ proliferation, and direct collagen production within the wound.¹²⁷ Although the signaling mechanism of this phenomenon has not been determined, other research¹²⁸ has demonstrated that the recognition and clearance of dying cells and debris from focal points of inflammation is critical in both the induction and resolution of inflammation.²⁸ Moreover, Panx1-mediated vesicular nucleotide transporters (responsible for ATP accumulation in secretory vesicles)-mediated ATP release have been shown to recruit neutrophils/macrophages to injury sites.^{129–131} It is quite possible that in some types of acute injury, an increase in or acceleration of postinjury inflammation may lead to more rapid resolution of inflammation through ADP or other signaling mechanisms.

Other mediators of Panx signaling and inflammation

In addition to Panx HCs, significant amounts of ATP can be released by bacteria, which trigger Panx1/P2X activity (Figure 3C).¹³² Bacterial ATP may affect different types of cells and lead to the production of proinflammatory cytokines and growth factors. A recent study showed that commensal bacteria-derived ATP activates CD70^{high}CD11c^{low} cells in the intestinal lamina propria, induce IL6 and IL23 production, as well as TGF- β pathway activation. This then led to local differentiation of IL17-producing CD4⁺ T lymphocytes (T-helper T_H-17, cells involved in host defense and several immune disorders).¹³³ Moreover, systemic or rectal administration of ATP into germ-free mice resulted in a marked increase in the number of lamina propria T_H17 cells. The specific effect of ATP on T_H17 differentiation was mediated by P2X and P2Y receptors, and ATP-induced T_H17 differentiation was inhibited by P2X- and P2Y-receptor blockade. Interestingly, this mechanism commonly operates during the differentiation of both “naturally occurring” and “pathogenic” T_H17 cells.^{127,133}

Although ATP-gated unselective cation P2X channels are induced mainly by ATP, some studies report that they also may be activated by other molecules.¹³⁴ β -Toxin produced by *Clostridium perfringens* is a key virulence factor in fatal hemorrhagic enterocolitis and enterotoxemia. This toxin belongs to a family of β -pore-forming toxins. The results of a recent study suggested that Panx1 opening is achieved through the interaction of β -toxin with the P2X₇ receptor. Then, ATP released by Panx1-channel opening promotes oligomer formation of the toxin, leading to cell death.¹³⁴ These studies suggest that Panx1 HC is an important contributor to P2X₇-receptor signaling and provides a mechanistic link among bacterial stimuli, P2X₇-Panx1 signaling, and inflammation.

Pannexins, mechanical signaling, and the cytoskeleton

A mechanosensitive role for connexin HCs in the propagation of intracellular calcium, initiated by the extracellular binding of ATP, was first noted in 1990.¹³⁵ Numerous reports since then have demonstrated the sensitivity of connexin HCs to extracellular Ca²⁺, which are believed to keep connexin HCs in a closed state at physiological Ca²⁺ levels.^{136–138} In contrast, Panx HCs are not gated by external Ca²⁺,¹³⁹ and the mechanical sensitivity of pannexin HCs was not noted until 2004, when single-channel currents were elicited by changes in pressure imposed pneumatically upon membrane patches of *Xenopus* oocytes expressing Panx1.¹⁴⁰ Since then,

mechanosensitive purinergic signaling pathways, including pannexin-mediated ATP release, have been demonstrated in many cell types in response to mechanical stimuli. For example, inhibition of pannexin function suppressed hypertonic stress-induced ATP release and reduced downstream transcriptional activation induced by hypertonicity,^{95,141} and inhibition of Panx1 and several P2X receptors reduced downstream transcriptional activation induced by hypertonicity. Similarly, pannexin- and/or P2-receptor-dependent ATP release has been observed in RGCs,⁶⁹ lens epithelial cells,¹⁴² fibrosarcoma cells,¹⁴³ urothelial cells,¹⁴⁴ and astrocytes¹³⁰ that were subjected to hypoosmotic conditions. In addition to altered tonicity, shear stress has been shown to activate mechanosensitive pannexin channels. Indeed, bone cells and red blood cells have demonstrated robust pannexin-mediated ATP release in response to oscillatory fluid shear stress.^{145,146} Consistent with this function, it was recently suggested that pannexin activity induced by transient fluid shear during media changes and manipulation of tissue-culture containers could confound the interpretation of cell-culture experiments.¹⁴⁷

In contrast to the bulk of the literature, only one study in HEK293 cells subjected to hypotonic media has suggested that pannexin HCs are not directly mechanosensitive.¹⁴⁸ It is likely that this controversial observation may have reflected a unique feature of the examined cell type and/or methodological differences. The authors' choice to use ethidium bromide internalization as an indicator of pannexin HC activity may have led to different outcomes compared with more conventional and commonly used indicators of pannexin activity, such as dye uptake or ATP release.

While mechanical activation of pannexin HCs has been studied primarily in a general context, there is increasing evidence that mechanical signaling can facilitate pathological states, such as edema that stretches the plasma membrane. Mechanical strain was recently reported to trigger a robust inflammatory response, transcriptional priming of NLRP3 inflammasome formation, and IL1 β production via activation of Panx1–P2X₇ signaling.⁵⁴

The eye has emerged as an important model in understanding pathological mechanotransductive roles for Panx1. When the retina is exposed to mechanical stress, ATP is released physiologically by glia and neurons via Panx1 channels.^{29,69,130} In OHT-injured retina, synergistic effects of mechanical stress induced by elevated pressure and massive ATP release facilitate sustained extracellular ATP elevation and prolonged activation of the Panx1–P2X pathway, a combination that is particularly toxic to RGCs, which are

highly enriched in Panx1.^{119,130,149} Experimental data generated in the murine eye indicate that RGC loss and axonal damage strongly correlate with mechanical deformation¹⁵⁰ and repetitive intraocular pressure spikes.^{29,130,151} An increase in extracellular ATP has been reported in eyes exposed to acute or chronic OHT in animal models, as well as in human primary open-angle glaucoma.^{69,111,149,151,152} Conversely, strain-activated, pannexin-regulated release of cytokines may also serve a protective role, as demonstrated by increased IL3 and IL6 expression in RGCs subjected to a 4% chronic strain in vitro or increased intraocular pressure in vivo.¹⁵³ Furthermore, the release of IL18 via inflammasome activation has also been reported to be neuroprotective.^{125,154}

Concurrent with Panx1-mediated ATP release, Cx43 HCs have been demonstrated as another key pathway of ATP release. Although the contribution of Cx43 vs Panx1 to ATP release has been heavily debated recently,^{155–157} the current consensus indicates that Panx1 channels initiate and Cx43 HCs facilitate the bulk of ATP release from macroglia, especially in the presence of TNF α and IL1 β .^{158–160} Consistent with this view, upon their exposure to TNF α and IL1 β cytokines, glial cells become activated and release ATP via Cx43 HCs.¹⁶¹

Given its structural role, the cytoskeleton is a leading candidate to participate in mechanical signaling. Actin dynamics, particularly those mediated by ARP2/3, have been reported to be regulated by pannexin activity,¹⁷ and pannexin interacts physically with actin (through the C terminus of Panx1), but not tubulin/microtubules.^{24,162} In a recent publication, it was reported that the Panx1–P2X₇ autocrine loop induced by ATP increased the migration speed of dendritic cells by promoting reorganization of the actin cytoskeleton.¹⁶³ In addition, pannexin influences a number of cellular changes that require cytoskeletal plasticity, including migration, differentiation, and proliferation.^{162,163} A role for actin is emphasized by evidence that Rho-kinase pathways, which have long been implicated in regulating actin dynamics, are dependent on pannexin-channel activity.¹⁶⁴ The importance of pannexins in regulating cytoskeletal dynamics has also been suggested by the localization of pannexin to the actin-rich filopodia of directionally migrating or path-finding cells.¹⁷ We have shown that loss or inhibition of Panx1 increases neurite extension and branching in sensory neurons ex vivo.¹⁶⁵ This finding suggests that pannexins may play a suppressive role in neuronal growth. Finally, pannexin channels may also somehow play a role in the mechanical sensitivity of other mechanosensitive channels, such as TRPV4 and TRPV1.^{166–168} It is not yet clear how the activity of these channels is coupled to pannexin activity. Such regulation may also be mediated by the cytoskeleton,

the rigidity of which likely influences mechanosensitive-channel response and the stability of which may be regulated by pannexin–P2-mediated pathways, such as those already noted. Therefore, mechanosignaling through Panx HCs may play a role in normal cell migration, growth, and differentiation. However, persistent exposure to mechanical stress may facilitate sustained activation of a Panx1–P2X-signaling loop, contributing to chronic inflammation (Figure 4A and B).

Pannexin and receptor plasticity

The magnitude and persistence of an activating signal have been noted to influence the functional plasticity of pannexin and pannexin-associated purinergic receptors (Figure 4A and B). In addition to the low-affinity binding already mentioned, another negative-feedback mechanism response to increased extracellular ATP involves rapid internalization of Panx1 into endosomes (Figure 4C) in as little as 15 minutes, possibly through signaling initiated by P2X receptors.¹⁶⁹ Positive feedback mechanisms include coregulation of pannexins with purinergic signaling proteins, detected in experiments with hypertonic saline treatment, which triggered both pannexin-channel activity and expression levels of P2X receptors in Jurkat T cells.⁹⁵ Similar coregulation was observed in chronic mechanical strain in astrocytes that resulted in an increased expression of Panx1, -2, and -3 both in vitro and in vivo.¹³⁰

Therapeutic implications of Panx1 inhibition

Pannexin has been implicated in regulating normal and pathological cellular function in a wide range of tissue

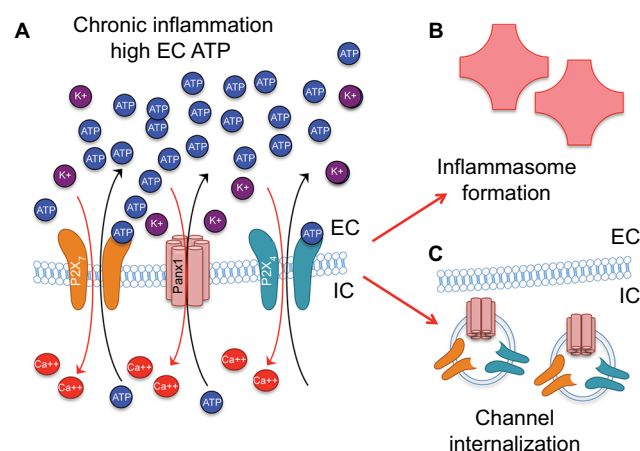


Figure 4 Persistent Panx1 and P2X activation (A) leads to inflammasome formation (B) and channel/receptor plasticity, including channel internalization through endocytosis (C).

Abbreviations: EC, extracellular; IC, intracellular.

types. In normal physiology, pannexin has been shown to modulate vascular tone,¹⁷⁰ brain development,⁹ memory, sleep,^{166,171} skeletal muscle homeostasis,^{172,173} red blood-cell biomechanics,¹⁴⁶ retinal signaling, response to ischemia,^{117,174} and leukocyte emigration.¹⁰⁵ In pathology, pannexin-mediated signaling has been implicated in brain ischemia,^{64,175} ischemic stroke,¹⁷⁶ pain,^{177,178} cardiomyocyte fibrosis,¹⁷⁹ microbial infection,^{59,180} cancer,¹⁸¹ brain inflammation (autoimmune encephalomyelitis/multiple sclerosis),^{182–185} and immunogenic cell-death-inducing antineoplastic agents.^{96,186} On one hand, this potential breadth of functions renders pannexin a powerful and widely applicable therapeutic target. On other hand, this same breadth suggests potentially significant side effects of anti-pannexin therapy, manifested within the same cell type, within the same tissue, or systemically. In addition, as demonstrated by varying results of different commonly used pharmacological inhibitors of pannexin or P2 receptors, including probenecid, Panx1-blocking peptide (¹⁰Panx),¹⁰⁵ carbenoxolone, P2-receptor-inhibiting peptides, and the extracellular ATP scavenger apyrase, the specificity of inhibition can appreciably impact a phenotypic response. In the following sections, we briefly describe possible outcomes related to the authors' expertise, in which the diverse functions of Panx1 must be considered within translational therapeutic strategies.

Panx1 inhibition in the eye

Panx1 forms an ATP-, K⁺-, and Ca²⁺-permeable membrane channel that is highly expressed in the retina, making this easily accessible neural tissue a good model system for delineation of Panx1 function. In the retina, Panx1 has been shown to be activated by mechanical stress,^{29,69,111,130} intracellular Ca²⁺,¹¹⁷ extracellular K⁺,¹⁸⁷ interactions with transient-receptor-potential channels,^{142,188} N-methyl-D-aspartate receptors,¹⁸⁹ activation of C²⁺-dependent caspases 1/11 and NLRP1/3 inflammasomes,^{29,117,190,191} and purinergic receptors upon binding extracellular ATP.⁸⁸ Several of these stressors and agonists are activated in the retina challenged by ischemia, OHT stress, or glaucoma, which can synergize to sustain prolonged Panx1 opening. Consistently with this observation, therapeutic Panx1 blockade protects RGCs and other neurons against mechanical stress and ischemia.^{29,174} However, due to the physiological significance of Panx1, only transient blockade and partial suppression represent therapeutically feasible options, as they are sufficient to block inflammasome and ionized Ca²⁺ influx without affecting global retina functionality.

Panx1 inhibition increases lacrimal-gland repair

Recent studies have proposed distinct roles for both Panx1 and P2X₇ receptors in the control of inflammasome activation, leading to the release of mature IL1 α and IL1 β . These data support the model in which Panx1–P2X₇ signaling is the key regulator of inflammatory response.^{52,192} Probenecid, a well-studied inhibitor of Panx1 and P2X₇ receptors^{193,194} and organic anion transporters, has been traditionally used to treat an inflammatory gout disease.¹⁹⁵ Treatment with probenecid has been found to affect ATP release¹⁹⁵ and suppress neuronal death in ischemic stroke^{176,196} and cerebral edema.¹⁹⁷ This suggests that modulation of Panx1 signaling may prevent inflammatory damage of brain tissue. Another study reports that in vivo administration of the P2X₇R antagonist A438079 in the mouse model of salivary gland exocrinopathy could ameliorate salivary gland inflammation and enhance saliva secretion.¹⁹⁸

Panx1 and P2 receptors are strongly upregulated during acute and chronic inflammation of the lacrimal gland,¹⁸ the primary contributor to the aqueous layer of tear film in humans. Moreover, lacrimal-gland injury due to inflammation leads to aqueous tear-deficiency dry eye. Most current therapies to treat lacrimal-gland disorders suggest topical treatments, including usage of artificial tears and autologous serum eye-drops, but they do not treat the cause of the disease and lead to limited success. Cell-based regenerative therapies may provide better and longer relief to dry-eye patients; however, survival of transplanted cells strictly depends on the degree of inflammation.^{199,200} We recently have shown that the best cell engraftment is observed when Panx1 has been blocked with specific Panx1 inhibitors, including ¹⁰Panx and self-deliverable RNAi (sdRNAi) specific to Panx1.²⁰⁰ Moreover, lacrimal-gland treatment with Panx1 sdRNAi resulted in significant reduction in IL1 β and Nlrp3 expression in *TSPI*^{−/−} mice, a mouse model of aqueous tear-deficiency dry eye.²⁰⁰ These findings have implications for therapeutic strategies targeting Panx1-signaling pathways for suppression of inflammation and/or increasing donor lacrimal-gland progenitor-cell engraftment.

The role of Panx1 in peripheral nerve disease and repair

Pannexins have been implicated in a number of pain-sensitization pathways, in both the peripheral nervous system and the CNS, through activity within and communication between neurons and their supporting cells.^{99,100,201,202} Zhang et al provided initial evidence that cell bodies of sensory neurons release ATP in response to electrical stimulation.

Further, this release stimulated activation of P2X₇ receptors and subsequent release of the inflammatory cytokine TNF α in neighboring glial cells.²⁰³ Pannexin interactions with various isoforms of P2 receptors may be localization-specific, as P2X₄ receptors have also been implicated in purinergic pain pathways: in this case, through activity at sensory endings in the skin.²⁰⁴ Interestingly, P2X₃ receptors have also been noted to play a prominent role in pannexin-mediated signaling in DRG neurons.^{203,205,206} Consistently with a role for pannexin-P2X₃ activity in pain pathways, the neurotoxin BomoTx, from the Brazilian lancehead pit viper, activates ATP release through pannexin HCs and downstream P2X₃-receptor activation, resulting in inflammatory pain, thermal hyperalgesia, and mechanical allodynia. Further, nerve injury results in increased *Panx1* gene and protein expression due to epigenetic mechanisms.¹⁷⁸ In contrast, a recent study revealed that Panx1 inhibition, genetically or through pharmacological reagents, reduced hypersensitivity induced by nerve injury.²⁰⁷

Although studied less comprehensively, pannexins also likely play an important role in peripheral nerve development and regeneration. For example, a role in myelination has been suggested for both P2X₇ receptors and pannexin,²⁰⁸ likely due to communication between stimulated neurons and their flanking Schwann cells.²⁰⁹ In addition, a recent study by our research team indicated that pannexin negatively regulated developmental and regenerative growth of peripheral neurons, as suggested by the increased caliber of axons in the sciatic nerves of *Panx1*^{-/-} mice and increased regenerative outgrowth and branching of cultured DRG explants harvested from *Panx1*^{-/-} mice, as well as in wild-type DRG explants treated with inhibitors of the Panx1-signaling pathway, including apyrase, probenecid, and ¹⁰Panx.²¹⁰ Based on its physiological properties and roles in inflammasome activation, it is feasible to suggest that pannexin activity can modulate the neuroregenerative environment, which is enriched in inflammatory cytokines, as well as immune and activated glial cells that respond to inflammatory cues. As such, pannexin reduction may be an effective strategy to reduce pain and promote regeneration after nerve injury.

Conclusion

The switch between normal (minor) and pathological (massive) ATP release from Panx HCs and downstream P2 receptors and other channels can determine whether an outcome will be “good” or “bad”. Low levels of extracellular ATP and K⁺ produced by physiological pannexin activity is required for homeostatic cell function (Figure 5A). Conversely, high levels of extracellular ATP and K⁺ and overload of the intracellular compartment with Ca²⁺ synergistically

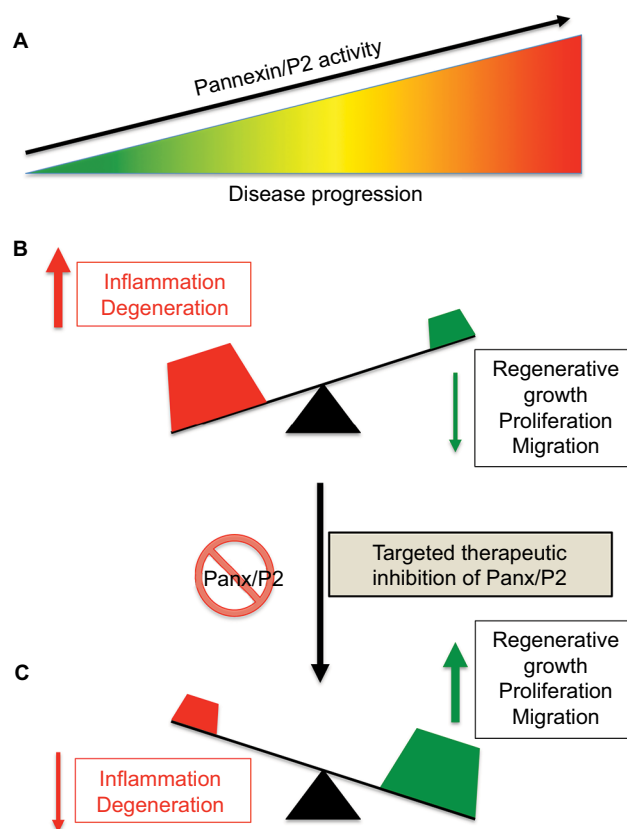


Figure 5 Effect of therapeutic suppression of pannexin function.

Notes: (A) Disease progression often correlates with increased Panx/P2-receptor activity and chronic inflammation (B). We hypothesize that therapeutic suppression of pannexin function may tilt the balance of a progressing disease from that of inflammation and degeneration to one of enhanced cellular growth, proliferation, and/or migration (C).

lead to sustained activation of Panx1–P2X₇ signaling and inflammasome pathways, inducing Casp1/11-dependent pyroptotic cell death (Figure 5B). Therefore, therapeutic modulation of Panx1 channels represents a feasible new strategy to reduce inflammation and promote regeneration (Figure 5C).

Acknowledgments

This work was supported by National Institutes of Health National Eye Institute grants 1R01EY026202 (to HPM) and R01EY021517 (to VIS), Russian Science Foundation grant N17-15-01433 (to VIS), and the Department of Veterans Affairs (Merit Award 1 I01 RX001471 to SBS).

Disclosure

The authors report no conflicts of interest in this work.

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