2017-04-22

Connexin Hemichannels in Astrocytes: An Assessment of Controversies Regarding Their Functional Characteristics

Nielsen, BS

10.1007/s11064-017-2243-7
Neurochemical Research

This is a the final author's draft of a paper accepted for publication in the journal Neurochemical Research 2017. DOI: https://doi.org/10.1007/s11064-017-2243-7

Connexin hemichannels in astrocytes – a critical assessment of controversies regarding their functional characteristics

[1]Brian Skriver Nielsen, [2]Daniel Bloch Hansen, [3]Bruce R. Ransom, [4]Morten Schak Nielsen and [1]Nanna MacAulay*

[1]Department of Neuroscience and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark, [2] Plymouth University Peninsula Schools of Medicine and Dentistry, Plymouth, United Kingdom, [3]Department of Neurology, University of Washington, Seattle, United States of America, [4]Department of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark

*Correspondence to Nanna MacAulay, University of Copenhagen, Blegdamsvej 3, bldg. 22.3, 2200 Copenhagen N, Denmark. Tel.: (+45)35327566; Email: macaulay@sund.ku.dk

Abstract

Astrocytes in the mammalian central nervous system are interconnected by gap junctions made from connexins of the subtypes Cx30 and Cx43. These proteins may exist as hemichannels in the plasma membrane in the absence of a 'docked' counterpart on the neighboring cell. A variety of stimuli are reported to open the hemichannels and thereby create a permeation pathway through the plasma membrane. Cx43 has, in its hemichannel configuration, been proposed to act as an ion channel and a membrane pathway for different molecules, such as fluorescent dyes, ATP, prostaglandins, and glutamate. Published studies about astrocyte hemichannel behavior, however, have been highly variable and/or contradictory. The field of connexin hemichannel research has been plagued by great variability in the experimental preparations employed, a lack of highly specific pharmacological inhibitors and by confounding changes associated with genetically modified animal models. This review attempts to critically assess the gating, inhibition and permeability of the astrocytic connexin hemichannels and proposes that connexins in their hemichannel configuration act as strictly-gated isoform-specific selective pores when open.

Keywords

Hemichannels, permeability, current, isoforms, gating, connexin, Cx30, Cx43

Acknowledgements

This project was supported by the Danish Medical Research Council (NM).

This is a the final author's draft of a paper accepted for publication in the journal Neurochemical Research 2017. DOI: https://doi.org/10.1007/s11064-017-2243-7

Many mammalian cells are coupled to their neighboring cells by gap junctions. These large-pore intercellular permeation pathways confer connectivity between the cytoplasm of adjacent cells and allow exchange of a variety of molecules, such as atomic ions, second messengers, nutrients, and metabolites (1, 2). Gap junctions are comprised of connexins, six of which assemble to create each half gap junction, called a connexon or a hemichannel. A functional gap junction forms when a hemichannel in one cell docks with a hemichannel in a neighboring cell (3). Connexins exist in 21 different isoforms in the human genome, each named according to their molecular weight in kilo Daltons (4). The single-channel conductance and permeability profile of gap junctions strictly depend on their isoform composition: Different connexin isoforms show large variations in parameters like conductance, cation versus anion selectivity, and permeability to both dyes and biological molecules (1, 2). These variations correlate poorly between parameters, e.g., Cx45 has a small single-channel conductance and high cation to anion selectivity, but still remains more permeable to the negative dye Alexa 350 than channels with larger single-channel conductance and higher preference to anions (2). The different permeants such as ions and fluorescent dyes may, in addition, be differentially regulated (5, 6), underscoring that the permeation pathway through gap junctions display isoform-specific characteristic and permeant-specific gating.

It has become evident that half gap junctions, i.e., hemichannels, may exist in the cell membrane in the absence of a docked counterpart, at least in cultured astrocytes (7). This is not unexpected as connexin proteins have a high turnover rate and are continuously manufactured and inserted into astrocyte membrane to form hemichannels; for at least a brief time, each hemichannel will be unpaired. A number of interrelated questions arise: 1) to what extent do hemichannels open to the extracellular environment, 2) to the extent that hemichannels do open, what molecular cues would prompt them to do so, 3) which solutes would permeate hemichannels in their open conformation, 4) does hemichannel activity depend on connexin isoform composition, and 5) what physiological or pathophysiological role(s) would hemichannels serve in such cases. Connexin investigators are rather divided on these questions with some assigning important physiological roles to connexin hemichannels and others questioning their functional existence. Researchers enjoy much greater concordance on the topic of astrocyte gap junctions: There is general agreement that astrocytes exhibit strong intercellular coupling mediated by gap junctions comprised of connexin isoforms Cx30 and Cx43 (8-10). In this fashion, groups of astrocytes are said to form a 'syncytium' that allows

them to act in a coordinated manner to physiological events, such as spatial buffering-mediated K^+ clearance following neuronal activity (10, 11). This review, however, focuses on the unsettled state of astrocyte hemichannel behavior, especially regarding gating, permeability, inhibition, and functional roles.

Connexin hemichannel activity determined with fluorescent dye uptake

Astrocyte connexin hemichannels were originally demonstrated on cytoplasmic processes and filopodia of subconfluent primary cultured rodent astrocytes using antibodies to the extracellular loop epitopes of Cx43 (7). Connexin hemichannel activity was not demonstrated on these primary astrocytes (although dye uptake was mentioned in passing but not assigned to Cx43) but removal of divalent cations (chelation by 5 mM EGTA) caused Lucifer yellow uptake in ~ 15% of the cells of an immortalized astrocytes (RGCN cell line cultures) with the remaining cells restricting dye uptake under these conditions (7). The Lucifer yellow permeation pathway was assigned to Cx43 because the Cx43 extracellular loop antibody reduced uptake in two out of three experiments (7). Divalent cation-free solution-induced Cx43-mediated uptake of fluorescent dyes into primary cultures of astrocytes has since been demonstrated in another study (12) while others have failed to observe such transport activity assigned to Cx43 upon removal of divalent cations (13-15). As in gap junction research, transfer of fluorescent molecules has been widely employed to monitor hemichannel activity (12, 13, 16, 17). When this transfer is observed, it has often been presumed to mean that biological molecules of smaller size than the fluorescent probe must also permeate the hemichannel. The Cx43-mediated fluorescent dye uptake in astrocytes (in culture/in brain slices) is, in addition, reported to be enhanced by a diverse range of other molecules including metabolic inhibitors, agents that cause oxidative stress, and exposure to growth factors, cytokines, and β-amyloid (13, 15, 18-22). In the case of β-amyloid hemichannel stimulation, prolonged treatment (72h) with the neurotoxic β-amyloid peptide fragment $A\beta_{25-35}$ was required to increase the dye uptake in cultured astrocytes (19, 21) while astrocytic β-amyloid-induced dye uptake occurred in acute hippocampal slices after only 3h exposure (21). While it is still of unknown significance, increased astrocytic Cx43 immuno-reactivity has been observed in the vicinity of β-amyloid plaques (23, 24). In unpublished experiments using a similar experimental design to (21), however, exposure to $A\beta_{25-35}$ failed to induce elevated dye uptake in cultured astrocytes (DB Hansen, MS Nielsen, N MacAulay and BR Ransom), Fig. 1.

At present, the explanation for these apparently contradictory results remains obscure; one obvious possibility is subtle differences in how these two laboratories performed the experiments (e.g., differences in cell preparation or culture conditions/confluence, animal strain, dye application, bath conditions, etc.). Also worth noting is that the 10 μM $A\beta_{25-35}$ necessary to induce hemichannel activity in cultured astrocytes, although commonly used experimentally, is at least 100-fold greater than the concentration which appears to be relevant for human disease (25, 26), raising the possibility that the effect may be a pharmacological curiosity more than a biological principle.

Connexin hemichannel inhibitors

Few pharmacological agents have exclusive actions. The commonly used drugs to block or inhibit connexin hemichannels are prime examples of this principle, and their poor specificity for connexin targets has been a source of confusion and frustration. This shortcoming in our scientific armamentarium complicates exact determination of the functional roles of hemichannels in complex cells such as astrocytes. Flawed as they are, a range of pharmacological inhibitors and mimetic peptides have been proposed suitable for blockade of the diverse actions attributed to hemichannel opening (Table 1). Gap junction inhibitors may be expected to also block unopposed hemichannels, while only a few compounds are reported to specifically inhibit hemichannels (27-29). Many chemical classes appear on the gap junction inhibitor list, including glycyrrhizic acid (GA) and its derivatives 18-α/β glycyrrhetinic acid and carbenoxolone (CBX), long chain alcohols like octanol and heptanol, fenamates such as flufenamic acid (FFA), the long chain fatty acids arachidonic acid and oleic acid, the long chain amides anandamide and oleamide, and volatile anesthetics like halothane (30-38). The majority of these compounds also inhibit the activity of other astrocytic and neuronal membrane channels including other connexins and pannexins, purinergic P2X receptors, and volume-regulated anion channels (VRACs) (29, 39-41). Since the consequences of these agents cannot be reliably attributed to actions on a particular channel, their utility is substantially compromised (see Table 1). CBX is occasionally employed as a connexin hemichannel blocker which illustrates the problem: In cultured astrocytes and Cx43-expressing C6 cells, CBX appears to inhibit the hemichannel activity (12, 42). However, CBX failed to affect Cx43 hemichannel activity in planar bilayers (43) and displayed a rather low potency in blocking Cx43 hemichannels expressed in *Xenopus* oocytes, where Cx30 hemichannels were significantly more sensitive towards this inhibitor (14). In addition, CBX is an efficient

inhibitor of VRACs (41, 44) and of Panx1-mediated conductance (45) although with only a modest reduction of fluorescent dye uptake through Panx1 (14, 46, 47). The ion channel modulator FFA is another extensively used inhibitor towards Cx43 (12, 48, 49) but affects an array of other channels, including chloride channels, TRP channels, gap junctions, P2X receptors, and pannexins, reviewed in (50). The indiscriminate behavior of FFA and CBX makes it challenging to unequivocally interpret results obtained with either agent when applied on complex cell systems expressing many potential binding partners. A potential way to distinguish between gap junctions and connexin hemichannel activity is to use the trivalent heavy metal lanthanides, lanthanum (La^{3+}) and gadolinium (Gd^{3+}), which target connexin hemichannels without blocking gap junctions (13, 49, 51). Gd^{3+} inhibits Cx43 in cultured astrocytes, mammalian cell lines, as well as in Cx43-expressing *Xenopus* oocytes (48, 49, 52), whereas Gd^{3+}-sensitivity of Cx30 has only been described in *Xenopus* oocytes to date (14). La^{3+} efficiently blocks both Cx30 and Cx43 hemichannels in primary cultured astrocytes and cochlear cells (12, 21, 53). Unfortunately, the list of other trivalent metal ion-sensitive channels is also quite extensive and includes other connexin hemichannels, such as Cx46 and Cx50, as well as calcium channels, the maxi anion channels, TRP channels, and stretch-activated ion channels (51, 54-57).

In recent years, sequence-homology peptides have been developed in the hope that they would be connexin-specific hemichannel inhibitors. The peptides mimic short sequences of the connexin protein (hence the name 'mimetic peptides') and were originally designed to interfere with hemichannel docking and thereby block formation of functional gap junctions (58, 59). Two such mimetic peptides, Gap26 and Gap27, are identical to a region in the first and second extracellular loops, respectively. Besides interfering with the gap junctional communication (60, 61), these peptides were subsequently reported to partially inhibit uncoupled hemichannels, such as astrocytic Cx43 (48, 62-64). However, it later became apparent that Gap27 also targets Panx1 and therefore is not specific to connexin hemichannels (65). Moreover, high doses of these peptides in a scrambled form (to eliminate their specificity) may in themselves affect connexin hemichannel activity (66). The more recently developed mimetic peptide Gap19, identical to a part of the intracellular loop of Cx43, appears to affect Cx43 hemichannels without altering gap junctional coupling or Panx1 activity (67, 68). Antibody-mediated inhibition is another approach used to block hemichannel docking and activity. Several antibodies (e.g. aEL2-186, Mabcx43E2, and aEL-46) targeting the extracellular loops

of Cx43 have been demonstrated to block hemichannel activity and, after prolonged exposure, also dye coupling and gap junctional plaque size (7, 66, 69). However, aEL2-186 also inhibits Cx32, raising the question of how selectively such antibodies can target unique connexin proteins. Antibody cross-reactivity with several connexins might even be anticipated because the amino acid sequence of the extracellular loops is highly conserved within the different isoforms (66). A novel antibody, Cx43(E2), is reported to exclusively target the second extracellular loop of Cx43 hemichannels (i.e., without cross reacting with other connexins such as Cx26, Cx32, Cx46, and Cx50) (70), thereby blocking conductance and permeation of small molecules, such as fluorescent dyes, prostaglandin E2, and ATP (21, 70-72). These recent developments show promise in the quest for highly selective connexin hemichannel blocking agents but further validation is needed of their specificity for unique connexin hemichannels and the lack of confounding effects on other connexin isoforms or astrocyte transport mechanisms whose actions might be confused with hemichannel functions. In the absence of such validation, using these blockers as diagnostic tools for connexin hemichannel function may be fraught and results must be interpreted cautiously.

Genetic manipulation of connexins

Alteration of select gene loci (and their protein products) has proven extremely powerful in understanding channel functions. Given the lack of specific inhibitors for the astrocytic connexin hemichannels, the knock-out/knock-down strategy has been employed for both Cx30 and Cx43. Together with pharmacological experiments, genetic manipulation has yielded insights about the diverse roles of these connexin hemichannels, both in normal and pathological situations (e.g., vasoconstriction, synaptic transmission, modulation of excitability of sensory neurons, Alzheimer's disease, neuropathic pain, cell damage in brain trauma, ischemic stroke, HIV-mediated brain dysfunction, and brain alterations following prenatal nicotine exposure, (17, 21, 97-104)). The downside of the gene alteration strategy is the frequent discovery of inadvertent effects on other factors in these mice: The widespread transcriptome alterations observed in mice with knock-out or knock-down of Cx43 (105, 106) can, in some cases, lead to changes in membrane transport mechanisms unrelated to Cx43, but which may then be mistakenly construed to be Cx43 effects. A striking example of an indirect effect of Cx43 alteration was demonstrated in inducible Cx43 knock-out mice, where voltage-gated Na^+ channel (Nav5.1) expression and currents were significantly reduced (107). Along these lines, a mouse strain with selective removal of the last five amino acids from Cx43

exhibited functional gap junctions but mislocalized voltage-gated Na^+ channels (Nav5.1); the misdirected Na^+ channels were the direct cause of the observed lethal arrhythmia (108). Significantly reduced basal ATP concentration has also been reported in astrocytes obtained from $Cx43^{-/-}$ mice (109). Reduced ATP content could lead to a decline in ATP release on the basis of a diminished transmembrane gradient, completely independent of any changes in ATP-permeable Cx43 hemichannels at the cell membrane (109). These examples illustrate that care must be exercised in drawing conclusions about connexin hemichannel function in astrocytes based on genetic modification of the expression of connexin protein in these cells. Unexpected associated changes in other cellular functions may be misinterpreted as due to the targeted change in connexin expression. In summary, the preceding discussion highlights some of the methodological challenges to isolating, with precision, the physiological and pathophysiological actions of specific hemichannels. Given current limitations, it is thus difficult to distinguish the action of a given connexin hemichannel from that of other large-pore channels expressed by astrocytes. In our opinion, these technical factors, and others yet undiscovered, are important contributors to the diversity of results that have been reported regarding the functional roles and biophysical characteristics of different hemichannel isoforms.

Fluorescent dye uptake in heterologously expressed astrocyte connexins

An alternative approach to characterize the activity of connexin hemichannels in isolation is heterologous expression of these in various mammalian cell lines or *Xenopus laevis* oocytes. Cx43-mediated dye uptake was demonstrated in HeLa cells overexpressing this channel upon removal of divalent cations (16, 92, 110), whereas identical rates of dye uptake were observed in parallel experiments on native C6 cells and C6 cells expressing Cx43, suggesting a minor contribution of Cx43 to the collective cellular dye uptake in divalent cation free solution (14). *Xenopus* oocytes are renowned for their gross overexpression of proteins upon microinjection of the cRNA encoding these, with little or no native expression of the multitude of large-pore channels expressed in complex cell types. *Xenopus* oocytes do express an endogenous connexin isoform, Cx38 (111), which may or may not participate in membrane current activity (see later) but does not itself contribute to divalent cation/Gd^{3+}-sensitive fluorescent dye uptake and does not interfere with fluorescent uptake data obtained with connexin-overexpressing oocytes (52, 112). The *Xenopus* oocytes therefore appear to be a useful expression system for biophysical characterization of individual connexin hemichannels in isolation, as they have proven highly

useful for this endeavor in the gap junction research field, e.g., (113-115). Oocytes expressing Cx30 or Cx43 displayed ethidium accumulation similar to that obtained in the uninjected oocytes until divalent cations were removed from the test solution, at which point the connexin-expressing oocytes increased their uptake of this fluorescent dye by 3-4 fold (14). The connexin-mediated ethidium uptake was completely abolished by Gd^{3+}, underscoring the connexins as the molecular origin of this transport activity. Membrane depolarization and dephosphorylation of Cx43 have previously been listed amongst the potential activators of Cx43 hemichannel activity (112, 116, 117) but these molecular cues failed to induce Cx43-mediated dye uptake in subsequent experiments (13, 52, 118). In contrast, Cx30, when activated by removal of divalent cations, closed upon PKC activation, emphasizing isoform-specific regulation of these connexins in their hemichannel configuration (118).

Connexin hemichannels as ion channels

Although no doubt remains regarding the ability of gap junctional channels to conduct currents, one particular point of contention is the ability of certain connexins to conduct atomic ions in their hemichannel configuration. There is general agreement that some connexin isoforms, when open, form hemichannels that conduct current. For example, Cx30 forms current-conducting, Gd^{3+}-sensitive hemichannels that open at physiological membrane potentials upon removal of divalent cations, when expressed in *Xenopus* oocytes or mammalian cell lines (14, 52, 94, 119). The first demonstration that Cx43 hemichannels carried current was in HeLa cells overexpressing this connexin: The cells were whole cell voltage clamped and exposed to a ramp protocol from -100 mV to +100 mV. At membrane potentials more positive than +70 mV, single-channel activity was observed and assigned to Cx43 (Fig. 2, adapted from (86)). Practically speaking, open channel events were exceptionally rare and occurred under extreme conditions: Given the whole cell voltage clamp configuration and the high copy number of Cx43 hemichannels expressed in the HeLa cell plasma membrane, only *one or two* channels opened simultaneously during the voltage ramps employed. Removal of divalent cations caused channel openings at slightly less positive membrane potentials (around +40 mV) in the illustrated representative example, although the effect was not systematically quantified (Fig. 2, adapted from (86)). These data suggest that Cx43 hemichannels are impermeable to atomic ions at physiologically relevant membrane potentials in the negative range (or the low positive range up to +30 mV experienced by the cardiomyocyte Cx43 during the cardiac action potential); ion permeation events, albeit with very low open probability, only occur at highly

positive potentials (86). Similar results in Cx43-expressing HeLa cells or cardiomyocytes were later obtained by other research groups (64, 120) confirming the negligible Cx43-mediated current activity at physiologically relevant membrane potentials. A single study in cultured astrocytes reported current activity upon removal of divalent cations in three out of the five tested cells, with the two remaining cells displaying no divalent cation-sensitive current activity (49). In the cells that exhibited these currents in divalent cation-free solution, the current pattern differed from that seen in Cx43-expressing HeLa cells and cardiomyocytes, since it occurred over the entire tested voltage range. In contrast, we detected no divalent cation/Gd^{3+}-sensitive membrane currents in cultured rat astrocytes at the tested membrane potentials (up to +60 mV) (14). The preponderance of evidence supports the conclusion that Cx43, expressed in native cell types (astrocytes and cardiomyocytes) or in mammalian cell lines, does not exhibit open ion channel events at physiological membrane potentials but may be induced to allow atomic ion permeation at highly positive membrane potentials. Some investigators consider demonstration of current a prerequisite for proving hemichannel involvement (121), despite the fact that Cx43 readily mediates dye uptake and ATP release at physiological potentials (12, 16, 42, 110) but generally fails to conduct current until exposed to highly positive membrane potentials (64, 86). Given the complexity of intact nervous tissue there is always the possibility that 'conducive conditions' present *in vivo* for expression of a particular biological event are simply absent in the reductive *in vitro* environment. While this can be a convenient refuge for proponents of a particular position when expected results are not supported by experimental evidence, it is also a true phenomenon that must be taken into consideration. Thus, it is interesting that large-conductance single-channel current activity in cultured astrocytes has been noted following incubation with conditioned medium from activated microglia (95) or with the neurotoxic β-amyloid fragment ($A\beta_{25-35}$) (19, 21), which correlated with increased surface expression of Cx43 on the plasma membrane in the two latter. The currents were visible at negative membrane potentials in cultured astrocytes (in the presence of K^+ channel inhibitors) in 4/4 tested astrocytes exposed to the conditioned medium from activated microglia but also in 2/10 astrocytes kept in control medium, as also observed in control astrocytes not exposed to $A\beta_{25-35}$ in (21). Unitary current transitions were detected at resting potentials in most untreated cells in a later study (19), in which stimulation with $A\beta_{25-35}$ increased the frequency of unitary events at both positive and negative membrane potentials. This current activity was assigned to Cx43 based on its putative single-channel conductance of 220 pS (19, 95), previously reported for Cx43 hemichannels by some researchers (86), and by its absence

in Cx43 KO cells and blockade by the antibody Cx43E2 (21). Of importance to note, other research groups have obtained Cx43-mediated conductances of 165 pS (42), 120 pS (87) and a substate conductance of 75 pS (86).

Parallel expression of Cx30 and Cx43 in *Xenopus* oocytes revealed large Gd^{3+}-sensitive currents in Cx30-expressing oocytes upon removal of divalent cations while none were apparent in Cx43-expressing oocytes at the tested membrane potentials ≤ +60 mV (despite robust Gd^{3+}-sensitive ethidium uptake in day-matched oocytes, see Fig. 3, (14, 52)). Removal of divalent cations induce membrane depolarization of Cx43-expressing oocytes (52, 122), which was interpreted as an increase in Cx43-mediated membrane conductance (122). However, membrane depolarization following removal of divalent cations occurs to a similar extent in uninjected oocytes, see Fig. 4, suggesting that Cx43 is not the molecular origin of this membrane depolarization. The native oocyte Cx38 has in some early studies been shown to confer significant gap junctional current activity in the uninjected control oocyte (111) whereas Cx38 in its hemichannel configuration did not contribute to the membrane potential or the slope conductance of uninjected oocytes at negative membrane potentials (123), nor to the membrane currents recorded in the presence/absence of divalent cations (52). Only at prolonged (>10 sec) positive voltage steps did Cx38 antisense RNA reduce the current and abolish the tail currents following return back to the holding potential, Fig. 5, left panels and (123). These data indicate that during short-to-medium long voltage pulses (100 ms – 10 s), Cx38 does not contribute significantly to the membrane current in native oocytes, in agreement with (52), whereas it may be involved in the delayed (small) current response at positive potentials and the subsequent tail currents (which are, however, not detectable in our oocyte batches, Fig. 5, right panel, unpublished data). The remainder of the study (123) is based on overexpression of the Cx38 and therefore does not represent the contribution of endogenous Cx38 in native *Xenopus* oocytes. Taken together, it appears that connexin hemichannels, when activated by removal of divalent cations, may display isoform-specific permeability profiles (Cx30 permeable to ethidium and atomic ions and Cx43 permeable to ethidium but not atomic ions) and thus retain the ability to exclude one permeant while allowing another at given membrane potentials. This paradoxical feature is also recognized for Pannexin 1 (dye uptake and ATP release at negative potentials but conductance only at positive potentials whether expressed in mammalian cells or oocytes (45, 109, 124)) and in the otherwise unrelated research field of aquaporin permeability, where several isoforms of this family share the ability to allow permeation of

larger molecules such as glycerol, urea, ammonia etc, while at the same time excluding the much smaller atomic ions (125, 126). It will be of future interest to identify the molecular determinants of the distinctive permeant selectivity of Cx30 and Cx43.

Permeation of connexin hemichannels by biological molecules

As for fluorescent dyes and membrane currents, it has been challenging to determine the permeability profile of connexin hemichannels to biological molecules and their isoform-specific characteristics. Problems arise due to the host of other membrane transport mechanisms transporting the same permeants but also due to the lack of specific inhibitors and the apparent secondary effects of knocking out connexins. Structural studies of the connexins, however scarce, have revealed a sizeable central pore in the Cx26 and Cx43 structures up to 15Å (127, 128) which spurred the notion of these channels allowing permeation towards a host of solutes. However, the Cx43 crystal structure is of a limited resolution and the authors thus point out that with a higher resolution, in which the amino acid side chains would be visible, the Cx43 central pore may be as narrow as 5Å (128), which would put severe limitations to the range of permeable solutes. There is evidence in favor of Cx30 and Cx43 acting as an ATP permeation pathway both in cell experiments (42, 53, 110, 116, 129) and in (patho)physiological contexts (99, 130-132). This feature has, however, been questioned and assigned to other transport proteins and/or reduced cytosolic ATP concentration in the $Cx43^{-/-}$ astrocytes (109). *Xenopus* oocytes expressing Cx30 and Cx43 displayed a small but significant ATP release upon removal of divalent cations, supporting the notion that these connexins may work as low-capacity ATP conduits in their hemichannel configuration (52). Notably, ATP with its negative charges produce a current when traversing a membrane (42). It therefore remains puzzling with the multitude of studies reporting Cx43-mediated ATP release at physiological membrane potentials (42, 53, 110, 116, 129), at which no Cx43-mediated conductance occurs (52, 64, 86, 120). Several other molecules have been suggested to permeate connexins in their hemichannel configuration, i.e. glutathione, glutamate, prostaglandins (12, 41, 72, 133-135), although glutamate release has, in addition, been assigned to other channel types in astrocytes (136, 137) and microglia (138). The permeation profiles of Cx30 and Cx43 were determined upon expression in *Xenopus* oocytes after verification of channel opening as Gd^{3+}-sensitive dye uptake in the absence of divalent cations (52): Under these experimental conditions, radio-actively labelled urea (60 kDa), glycerol (92 kDa), and mannitol (182 kDa), all smaller than ethidium (314 kDa) and routinely used to probe the pore size of various

aquaporin isoforms (125, 126, 139), failed to permeate Cx43-expressing oocytes. Glutathione and lactate did not permeate Cx30 or Cx43, whereas only Cx30 displayed permeability towards glucose and glutamate (52). Neither of these connexin hemichannels acted as pathways for osmotically-driven water transport in a manner analogous to aquaporin-mediated water flux (52). It thus appears that the different astrocytic connexin isoforms form functional hemichannels although with distinct selectively towards a range of different biological permeants. Connexin hemichannels, when activated by removal of divalent cations, therefore do not constitute a non-selective pore but rather a selective permeation pathway which may or may not significantly contribute to a given transport parameter in a complex cell expressing a multitude of different channels and transport proteins.

Future challenges and directions

Although there is ample evidence in favor of (patho)physiological roles for the astrocyte connexins in their hemichannel configuration, uncertainty remains regarding the biophysical characteristics of each connexin isoform and their quantitative contribution to membrane transport under (patho)physiological conditions. With the above-mentioned cross-reactivity of a range of different inhibitors (or the lack of testing thereof) and putative secondary effects of knock-down/knock-out of individual connexins, a successful search for selective isoform-specific inhibitors will be instrumental to unequivocal determination of the physiological role of these membrane proteins. Some laboratories report divalent cation-sensitive Cx43-mediated fluorescent dye uptake in mammalian cell lines (7, 16), primary astrocytic cultures (12), and *Xenopus* oocytes (52) while others report lack of dye uptake assigned to Cx43 with this activation paradigm in primary culture of astrocytes or Cx43-expressing C6 cells (13-15). The diversity in the experimental observations concerning the ability of Cx43 hemichannels to facilitate dye uptake, conduct current and/or release signaling molecules raises the question whether complete opening of Cx43 hemichannels requires an undefined cellular or experimental factor. Factors of interest that may differ between laboratory protocols and cellular systems could be 1) presence or absence of certain interaction partners, 2) presence of a free C-terminal fragment of Cx43 in some cell lines (140), 3) different composition of experimental solutions and/or usage of ultra-pure water in experimental solutions (141, 142), 4) composition of cell culture medium (15, 95), 5) pre-exposure of cells to positive clamp potentials prior to initiation of the experimental clamp protocol (Feliksas Bukauskas, personal communication), 6) non-physiological experimental pH (5.8/8.0) and temperatures (i.e., 4ºC)

(43, 92), and 7) associated mechanical strain upon solution change (49). The baseline uptake of fluorescent dyes is rather significant in many cell types which calls for strict experimental design including *parallel* experiments of equal number of treated versus non-treated cells (or WT versus KO) obtained from a number of separate astrocyte/cell preparations. Connexin-mediated dye uptake is routinely quantified in two distinct manners; either as increased *rate* of dye uptake or increased dye uptake in an entire cell population e.g., (12-14, 16) or as an increased *number* of cells displaying dye uptake, e.g., (7, 87, 92). While the former approach is standard in membrane transport physiology, the latter quantification method appears slightly unorthodox as it raises the question as to why only a certain percentage of the connexin-expressing cells display dye uptake: Is it possible that connexin hemichannel-mediated dye uptake occurs upon reaching a given threshold (and detected as a percentage of cells responding)? Alternatively, the astrocytic responses attributed to Cx43 hemichannels may be mediated by other large-pore channels present in the native systems, possibly Cx30, Panx1, $P2X_7$, volume-regulated anion channels, or maxi ion channels, etc., with functional properties comparable to those expected of Cx43 and with overlapping inhibitor profiles (41, 55, 76, 109, 119), reviewed in (29, 124). It is easier to point out possible explanations for the contradictory results that have been reported in the astrocyte hemichannel field, than it is to provide a clear roadmap for eliminating them. We are optimistic, nevertheless, that these uncertainties will yield to the use of more discriminating methods, coupled with greater sensitivity to the sources of biological variability. In this way, it should be possible to define the physiological role(s), if such exist, of each unique connexin hemichannel expressed by astrocytes, providing important insights into the function of these endlessly fascinating cells.

This is a the final author's draft of a paper accepted for publication in the journal Neurochemical Research 2017. DOI: https://doi.org/10.1007/s11064-017-2243-7

Figure legends

Fig. 1 Lack of Amyloid-β induced dye uptake in astrocytes. Astrocyte cultures were prepared from cortex of neonatal C57BL/6 mice and prepared as detailed in (143). After four weeks in culture, the cells were treated for 72h with 10 µM Amyloid-β peptide ($A\beta_{25-35}$) as described in (21) and dye uptake experiments performed as described in (14). Data are illustrated as dye uptake as a function of time (upper panel, dye introduced as marked) and summarized in the lower panel. The data represent five individual experiments from two different astrocyte preparations, ns; not significant.

Fig. 2 Hemichannel-mediated currents at positive potentials in Cx43-expressing HeLa cells. a) Cx43-expressing HeLa cells were whole-cell voltage clamped and exposed to a voltage ramp from -100 mV to +100 mV. At highly positive potentials, La^{3+}-sensitive currents were observed in Cx43-expressing cells. b) Single-channel activity in a Cx43-expressing HeLa cell was observed at +90 mV, indicating a maximum of 1-2 channels open at a given time in the entire cell. c) Removal of divalent cations shifted the channel activation to less positive potentials in the illustrated representative cell. d) no large-conductance single-channel activity was observed in parental HeLa cells. Figures adapted from (86).

Fig. 3 Dye uptake and membrane conductance are not necessarily linked connexon hemichannel features. a) Representative current traces from an uninjected oocyte and oocytes expressing Cx30 or Cx43 in control solution and after exposure to divalent cation-free solution (DCFS). Inserts illustrate enlarged traces of uninjected and Cx43-expressing oocytes in DCFS. b) Summarized I/V curves obtained as in (a) from uninjected, Cx30- and Cx43-expressing oocytes (n= 17-21 of each from four different batches of oocytes) illustrate large Gd^{3+}-sensitive currents only in Cx30-expressing oocytes. c) Ethidium uptake in uninjected, Cx30- and Cx43-expressing oocytes obtained from the same batch as those from panel A-B in control solution, in DCFS, and in DCFS+Gd^{3+}. Data were obtained from four independent experiments each conducted with five oocytes for each condition, ns; not significant, *; $P<0.05$, **; $P<0.01$. Figure adapted from (52).

Fig. 4 Removal of divalent cations depolarizes Cx43-expressing oocytes to the same extent as uninjected oocytes. Unclamped oocytes (either uninjected (n=11) or Cx43-expressing (n=11) oocytes) were exposed to divalent cation-free solution (essentially as described in (52)) and the membrane potential recorded before (control) and after 4 min exposure to the divalent cation-free solution (DCFS). Data represent 11 oocytes of each kind (uninjected or Cx43-expressing) obtained from four different batches of oocytes, statistical significance was tested between membrane potentials in control solution and membrane potentials in DCFS, ns; not significant.

Fig. 5 Some batches of uninjected oocytes display Cx38-mediated current activity at prolonged positive voltage pulses and others do not. a-b) Uninjected oocytes in control conditions (a) and following injection of siRNA encoding the endogenous Cx38 (b) exposed to prolonged voltage jump from -40 mV to +30 mV in test solution containing divalent cations, adapted from (123). c) These proposed Cx38-mediated currents were not visible in other oocytes batches exposed to a similar experimental protocol (n = 4, unpublished results).

References

1. Harris, A.L. 2007. Connexin channel permeability to cytoplasmic molecules. Prog. Biophys. Mol. Biol. 94:120-143.

2. Harris, A.L., and Locke, D. 2009. Permeability of Connexin Channels. Pages 165-206, in Harris, A.L., and Locke, D. (eds.), Connexins A Guide, Humana Press.

3. Nielsen, M.S., Nygaard, A.L., Sorgen, P.L., Verma, V., Delmar, M., and Holstein-Rathlou, N.H. 2012. Gap junctions. Compr. Physiol 2:1981-2035.

4. Sohl, G., and Willecke, K. 2003. An update on connexin genes and their nomenclature in mouse and man. Cell Commun. Adhes. 10:173-180.

5. Ek-Vitorin, J.F., King, T.J., Heyman, N.S., Lampe, P.D., and Burt, J.M. 2006. Selectivity of connexin 43 channels is regulated through protein kinase C-dependent phosphorylation. Circ. Res. 98:1498-1505.

6. Kwak, B.R., van Veen, T.A., Analbers, L.J., and Jongsma, H.J. 1995. TPA increases conductance but decreases permeability in neonatal rat cardiomyocyte gap junction channels. Exp. Cell Res. 220:456-463.

7. Hofer, A., and Dermietzel, R. 1998. Visualization and functional blocking of gap junction hemichannels (connexons) with antibodies against external loop domains in astrocytes. Glia 24:141-154.

8. Dermietzel, R., Hertberg, E.L., Kessler, J.A., and Spray, D.C. 1991. Gap junctions between cultured astrocytes: immunocytochemical, molecular, and electrophysiological analysis. J. Neurosci. 11:1421-1432.

9. Gosejacob, D., Dublin, P., Bedner, P., Huttmann, K., Zhang, J., Tress, O., Willecke, K., Pfrieger, F., Steinhauser, C., and Theis, M. 2011. Role of astroglial connexin30 in hippocampal gap junction coupling. Glia 59:511-519.

10. Wallraff, A., Kohling, R., Heinemann, U., Theis, M., Willecke, K., and Steinhauser, C. 2006. The impact of astrocytic gap junctional coupling on potassium buffering in the hippocampus. J. Neurosci. 26:5438-5447.

11. Orkand, R.K., Nicholls, J.G., and Kuffler, S.W. 1966. Effect of nerve impulses on the membrane potential of glial cells in the central nervous system of amphibia. J. Neurophysiol. 29:788-806.

12. Ye, Z.C., Wyeth, M.S., Baltan-Tekkok, S., and Ransom, B.R. 2003. Functional hemichannels in astrocytes: a novel mechanism of glutamate release. J. Neurosci. 23:3588-3596.

13. Contreras, J.E., Sanchez, H.A., Eugenin, E.A., Speidel, D., Theis, M., Willecke, K., Bukauskas, F.F., Bennett, M.V., and Saez, J.C. 2002. Metabolic inhibition induces opening of unapposed connexin 43 gap junction hemichannels and reduces gap junctional communication in cortical astrocytes in culture. Proc. Natl. Acad. Sci. U. S. A 99:495-500.

14. Hansen, D.B., Ye, Z.C., Calloe, K., Braunstein, T.H., Hofgaard, J.P., Ransom, B.R., Nielsen, M.S., and MacAulay, N. 2014. Activation, permeability, and inhibition of astrocytic and neuronal large pore (hemi)channels. J. Biol. Chem. 289:26058-26073.

15. Morita, M., Saruta, C., Kozuka, N., Okubo, Y., Itakura, M., Takahashi, M., and Kudo, Y. 2007. Dual regulation of astrocyte gap junction hemichannels by growth factors and a pro-inflammatory cytokine via the mitogen-activated protein kinase cascade. Glia 55:508-515.

16. Orellana, J.A., Diaz, E., Schalper, K.A., Vargas, A.A., Bennett, M.V., and Saez, J.C. 2011. Cation permeation through connexin 43 hemichannels is cooperative, competitive and saturable with parameters depending on the permeant species. Biochem. Biophys. Res. Commun. 409:603-609.

17. Yi, C., Mei, X., Ezan, P., Mato, S., Matias, I., Giaume, C., and Koulakoff, A. 2016. Astroglial connexin43 contributes to neuronal suffering in a mouse model of Alzheimer's disease. Cell Death. Differ. 23:1691-1701.

18. Abudara, V., Roux, L., Dallerac, G., Matias, I., Dulong, J., Mothet, J.P., Rouach, N., and Giaume, C. 2015. Activated microglia impairs neuroglial interaction by opening Cx43 hemichannels in hippocampal astrocytes. Glia 63:795-811.

19. Gajardo-Gomez, R., Labra, V.C., Maturana, C.J., Shoji, K.F., Santibanez, C.A., Saez, J.C., Giaume, C., and Orellana, J.A. 2017. Cannabinoids prevent the amyloid beta-induced activation of astroglial hemichannels: A neuroprotective mechanism. Glia 65:122-137.

20. Garre, J.M., Retamal, M.A., Cassina, P., Barbeito, L., Bukauskas, F.F., Saez, J.C., Bennett, M.V., and Abudara, V. 2010. FGF-1 induces ATP release from spinal astrocytes in culture and opens pannexin and connexin hemichannels. Proc. Natl. Acad. Sci. U. S. A 107:22659-22664.

21. Orellana, J.A., Shoji, K.F., Abudara, V., Ezan, P., Amigou, E., Saez, P.J., Jiang, J.X., Naus, C.C., Saez, J.C., and Giaume, C. 2011. Amyloid beta-induced death in neurons involves glial and neuronal hemichannels. J. Neurosci. 31:4962-4977.

22. Retamal, M.A., Cortes, C.J., Reuss, L., Bennett, M.V., and Saez, J.C. 2006. S-nitrosylation and permeation through connexin 43 hemichannels in astrocytes: induction by oxidant stress and reversal by reducing agents. Proc. Natl. Acad. Sci. U. S. A 103:4475-4480.

23. Mei, X., Ezan, P., Giaume, C., and Koulakoff, A. 2010. Astroglial connexin immunoreactivity is specifically altered at beta-amyloid plaques in beta-amyloid precursor protein/presenilin1 mice. Neuroscience 171:92-105.

24. Nagy, J.I., Li, W., Hertzberg, E.L., and Marotta, C.A. 1996. Elevated connexin43 immunoreactivity at sites of amyloid plaques in Alzheimer's disease. Brain Res. 717:173-178.

25. Bukanova, J.V., Sharonova, I.N., and Skrebitsky, V.G. 2014. Amyloid beta peptide (25-35) in picomolar concentrations modulates the function of glycine receptors in rat hippocampal pyramidal neurons through interaction with extracellular site(s). Brain Res. 1558:1-10.

26. Cirrito, J.R., May, P.C., O'Dell, M.A., Taylor, J.W., Parsadanian, M., Cramer, J.W., Audia, J.E., Nissen, J.S., Bales, K.R., Paul, S.M., DeMattos, R.B., and Holtzman, D.M. 2003. In vivo assessment of brain interstitial fluid with microdialysis reveals plaque-associated changes in amyloid-beta metabolism and half-life. J. Neurosci. 23:8844-8853.

27. Giaume, C., and Theis, M. 2010. Pharmacological and genetic approaches to study connexin-mediated channels in glial cells of the central nervous system. Brain Res. Rev. 63:160-176.

28. Giaume, C., Leybaert, L., Naus, C.C., and Saez, J.C. 2013. Connexin and pannexin hemichannels in brain glial cells: properties, pharmacology, and roles. Front Pharmacol. 4:88.

29. Spray, D.C., Ye, Z.C., and Ransom, B.R. 2006. Functional connexin "hemichannels": a critical appraisal. Glia 54:758-773.

30. Aylsworth, C.F., Trosko, J.E., and Welsch, C.W. 1986. Influence of lipids on gap-junction-mediated intercellular communication between Chinese hamster cells in vitro. Cancer Res. 46:4527-4533.

31. Burt, J.M., and Spray, D.C. 1989. Volatile anesthetics block intercellular communication between neonatal rat myocardial cells. Circ. Res. 65:829-837.

32. Burt, J.M. 1989. Uncoupling of cardiac cells by doxyl stearic acids specificity and mechanism of action. Am. J. Physiol 256:C913-C924.

33. Davidson, J.S., Baumgarten, I.M., and Harley, E.H. 1986. Reversible inhibition of intercellular junctional communication by glycyrrhetinic acid. Biochem. Biophys. Res. Commun. 134:29-36.

34. Davidson, J.S., and Baumgarten, I.M. 1988. Glycyrrhetinic acid derivatives: a novel class of inhibitors of gap-junctional intercellular communication. Structure-activity relationships. J. Pharmacol. Exp. Ther. 246:1104-1107.

35. Dhein, S. 1998. Gap junction channels in the cardiovascular system: pharmacological and physiological modulation. Trends Pharmacol. Sci. 19:229-241.

36. Guan, X., Cravatt, B.F., Ehring, G.R., Hall, J.E., Boger, D.L., Lerner, R.A., and Gilula, N.B. 1997. The sleep-inducing lipid oleamide deconvolutes gap junction communication and calcium wave transmission in glial cells. J. Cell Biol. 139:1785-1792.

37. Harks, E.G., de Roos, A.D., Peters, P.H., de Haan, L.H., Brouwer, A., Ypey, D.L., van Zoelen, E.J., and Theuvenet, A.P. 2001. Fenamates: a novel class of reversible gap junction blockers. J. Pharmacol. Exp. Ther. 298:1033-1041.

38. Srinivas, M., and Spray, D.C. 2003. Closure of gap junction channels by arylaminobenzoates. Mol. Pharmacol. 63:1389-1397.

39. Juszczak, G.R., and Swiergiel, A.H. 2009. Properties of gap junction blockers and their behavioural, cognitive and electrophysiological effects: animal and human studies. Prog. Neuropsychopharmacol. Biol. Psychiatry 33:181-198.

40. Patel, D., Zhang, X., and Veenstra, R.D. 2014. Connexin hemichannel and pannexin channel electrophysiology: How do they differ? FEBS Lett. 588:1372-1378.

41. Ye, Z.C., Oberheim, N., Kettenmann, H., and Ransom, B.R. 2009. Pharmacological "cross-inhibition" of connexin hemichannels and swelling activated anion channels. Glia 57:258-269.

42. Kang, J., Kang, N., Lovatt, D., Torres, A., Zhao, Z., Lin, J., and Nedergaard, M. 2008. Connexin 43 hemichannels are permeable to ATP. J. Neurosci. 28:4702-4711.

43. Brokamp, C., Todd, J., Montemagno, C., and Wendell, D. 2012. Electrophysiology of single and aggregate Cx43 hemichannels. PLoS. One. 7:e47775.

44. Benfenati, V., Caprini, M., Nicchia, G.P., Rossi, A., Dovizio, M., Cervetto, C., Nobile, M., and Ferroni, S. 2009. Carbenoxolone inhibits volume-regulated anion conductance in cultured rat cortical astroglia. Channels (Austin) 3:323-336.

45. Bruzzone, R., Barbe, M.T., Jakob, N.J., and Monyer, H. 2005. Pharmacological properties of homomeric and heteromeric pannexin hemichannels expressed in Xenopus oocytes. J. Neurochem. 92:1033-1043.

46. Huang, Y.J., Maruyama, Y., Dvoryanchikov, G., Pereira, E., Chaudhari, N., and Roper, S.D. 2007. The role of pannexin 1 hemichannels in ATP release and cell-cell communication in mouse taste buds. Proc. Natl. Acad. Sci. U. S. A 104:6436-6441.

47. Qiu, F., and Dahl, G. 2009. A permeant regulating its permeation pore: inhibition of pannexin 1 channels by ATP. Am. J. Physiol Cell Physiol 296:C250-C255.

48. Braet, K., Vandamme, W., Martin, P.E., Evans, W.H., and Leybaert, L. 2003. Photoliberating inositol-1,4,5-trisphosphate triggers ATP release that is blocked by the connexin mimetic peptide gap 26. Cell Calcium 33:37-48.

49. Stout, C.E., Costantin, J.L., Naus, C.C., and Charles, A.C. 2002. Intercellular calcium signaling in astrocytes via ATP release through connexin hemichannels. J. Biol. Chem. 277:10482-10488.

50. Guinamard, R., Simard, C., and Del, N.C. 2013. Flufenamic acid as an ion channel modulator. Pharmacol. Ther. 138:272-284.

51. Eskandari, S., Zampighi, G.A., Leung, D.W., Wright, E.M., and Loo, D.D. 2002. Inhibition of gap junction hemichannels by chloride channel blockers. J. Membr. Biol. 185:93-102.

52. Hansen, D.B., Braunstein, T.H., Nielsen, M.S., and Macaulay, N. 2014. Distinct permeation profiles of the connexin 30 and 43 hemichannels. FEBS Lett. 588:1446-1457.

53. Anselmi, F., Hernandez, V.H., Crispino, G., Seydel, A., Ortolano, S., Roper, S.D., Kessaris, N., Richardson, W., Rickheit, G., Filippov, M.A., Monyer, H., and Mammano, F. 2008. ATP release through connexin hemichannels and gap junction transfer of second messengers propagate Ca2+ signals across the inner ear. Proc. Natl. Acad. Sci. U. S. A 105:18770-18775.

54. Bouron, A., Kiselyov, K., and Oberwinkler, J. 2015. Permeation, regulation and control of expression of TRP channels by trace metal ions. Pflugers Arch. 467:1143-1164.

55. Liu, H.T., Toychiev, A.H., Takahashi, N., Sabirov, R.Z., and Okada, Y. 2008. Maxi-anion channel as a candidate pathway for osmosensitive ATP release from mouse astrocytes in primary culture. Cell Res. 18:558-565.

56. Mlinar, B., and Enyeart, J.J. 1993. Block of current through T-type calcium channels by trivalent metal cations and nickel in neural rat and human cells. J. Physiol 469:639-652.

57. Yang, X.C., and Sachs, F. 1989. Block of stretch-activated ion channels in Xenopus oocytes by gadolinium and calcium ions. Science 243:1068-1071.

58. Dahl, G., Nonner, W., and Werner, R. 1994. Attempts to define functional domains of gap junction proteins with synthetic peptides. Biophys. J. 67:1816-1822.

59. Warner, A., Clements, D.K., Parikh, S., Evans, W.H., and DeHaan, R.L. 1995. Specific motifs in the external loops of connexin proteins can determine gap junction formation between chick heart myocytes. J. Physiol 488 (Pt 3):721-728.

60. Boitano, S., and Evans, W.H. 2000. Connexin mimetic peptides reversibly inhibit Ca(2+) signaling through gap junctions in airway cells. Am. J. Physiol Lung Cell Mol. Physiol 279:L623-L630.

61. Isakson, B.E., Evans, W.H., and Boitano, S. 2001. Intercellular Ca2+ signaling in alveolar epithelial cells through gap junctions and by extracellular ATP. Am. J. Physiol Lung Cell Mol. Physiol 280:L221-L228.

62. Desplantez, T., Verma, V., Leybaert, L., Evans, W.H., and Weingart, R. 2012. Gap26, a connexin mimetic peptide, inhibits currents carried by connexin43 hemichannels and gap junction channels. Pharmacol. Res. 65:546-552.

63. Evans, W.H., Bultynck, G., and Leybaert, L. 2012. Manipulating connexin communication channels: use of peptidomimetics and the translational outputs. J. Membr. Biol. 245:437-449.

64. Wang, N., De, B.M., Antoons, G., Gadicherla, A.K., Bol, M., Decrock, E., Evans, W.H., Sipido, K.R., Bukauskas, F.F., and Leybaert, L. 2012. Connexin mimetic peptides inhibit Cx43 hemichannel opening triggered by voltage and intracellular Ca2+ elevation. Basic Res. Cardiol. 107:304.

65. Wang, J., Ma, M., Locovei, S., Keane, R.W., and Dahl, G. 2007. Modulation of membrane channel currents by gap junction protein mimetic peptides: size matters. Am. J. Physiol Cell Physiol 293:C1112-C1119.

66. Riquelme, M.A., Kar, R., Gu, S., and Jiang, J.X. 2013. Antibodies targeting extracellular domain of connexins for studies of hemichannels. Neuropharmacology 75:525-532.

67. Abudara, V., Bechberger, J., Freitas-Andrade, M., De, B.M., Wang, N., Bultynck, G., Naus, C.C., Leybaert, L., and Giaume, C. 2014. The connexin43 mimetic peptide Gap19 inhibits hemichannels without altering gap junctional communication in astrocytes. Front Cell Neurosci. 8:306.

68. Wang, N., De, V.E., Ponsaerts, R., Boengler, K., Palacios-Prado, N., Wauman, J., Lai, C.P., De, B.M., Decrock, E., Bol, M., Vinken, M., Rogiers, V., Tavernier, J., Evans, W.H., Naus, C.C., Bukauskas, F.F., Sipido, K.R., Heusch, G., Schulz, R., Bultynck, G., and Leybaert, L. 2013. Selective inhibition of Cx43 hemichannels by Gap19 and its impact on myocardial ischemia/reperfusion injury. Basic Res. Cardiol. 108:309.

69. Clair, C., Combettes, L., Pierre, F., Sansonetti, P., and Tran Van, N.G. 2008. Extracellular-loop peptide antibodies reveal a predominant hemichannel organization of connexins in polarized intestinal cells. Exp. Cell Res. 314:1250-1265.

70. Siller-Jackson, A.J., Burra, S., Gu, S., Xia, X., Bonewald, L.F., Sprague, E., and Jiang, J.X. 2008. Adaptation of connexin 43-hemichannel prostaglandin release to mechanical loading. J. Biol. Chem. 283:26374-26382.

71. Batra, N., Burra, S., Siller-Jackson, A.J., Gu, S., Xia, X., Weber, G.F., DeSimone, D., Bonewald, L.F., Lafer, E.M., Sprague, E., Schwartz, M.A., and Jiang, J.X. 2012. Mechanical stress-activated integrin alpha5beta1 induces opening of connexin 43 hemichannels. Proc. Natl. Acad. Sci. U. S. A 109:3359-3364.

72. Orellana, J.A., Froger, N., Ezan, P., Jiang, J.X., Bennett, M.V., Naus, C.C., Giaume, C., and Saez, J.C. 2011. ATP and glutamate released via astroglial connexin 43

hemichannels mediate neuronal death through activation of pannexin 1 hemichannels. J. Neurochem. 118:826-840.

73. Iglesias, R., Locovei, S., Roque, A., Alberto, A.P., Dahl, G., Spray, D.C., and Scemes, E. 2008. P2X7 receptor-Pannexin1 complex: pharmacology and signaling. Am. J. Physiol Cell Physiol 295:C752-C760.

74. Beckel, J.M., Daugherty, S.L., Tyagi, P., Wolf-Johnston, A.S., Birder, L.A., Mitchell, C.H., and de Groat, W.C. 2015. Pannexin 1 channels mediate the release of ATP into the lumen of the rat urinary bladder. J. Physiol 593:1857-1871.

75. Sridharan, M., Adderley, S.P., Bowles, E.A., Egan, T.M., Stephenson, A.H., Ellsworth, M.L., and Sprague, R.S. 2010. Pannexin 1 is the conduit for low oxygen tension-induced ATP release from human erythrocytes. Am. J. Physiol Heart Circ. Physiol 299:H1146-H1152.

76. Suadicani, S.O., Brosnan, C.F., and Scemes, E. 2006. P2X7 receptors mediate ATP release and amplification of astrocytic intercellular Ca2+ signaling. J. Neurosci. 26:1378-1385.

77. Tovar, K.R., Maher, B.J., and Westbrook, G.L. 2009. Direct actions of carbenoxolone on synaptic transmission and neuronal membrane properties. J. Neurophysiol. 102:974-978.

78. Vessey, J.P., Lalonde, M.R., Mizan, H.A., Welch, N.C., Kelly, M.E., and Barnes, S. 2004. Carbenoxolone inhibition of voltage-gated Ca channels and synaptic transmission in the retina. J. Neurophysiol. 92:1252-1256.

79. Braet, K., Aspeslagh, S., Vandamme, W., Willecke, K., Martin, P.E., Evans, W.H., and Leybaert, L. 2003. Pharmacological sensitivity of ATP release triggered by photoliberation of inositol-1,4,5-trisphosphate and zero extracellular calcium in brain endothelial cells. J. Cell Physiol 197:205-213.

80. Seminario-Vidal, L., Okada, S.F., Sesma, J.I., Kreda, S.M., van Heusden, C.A., Zhu, Y., Jones, L.C., O'Neal, W.K., Penuela, S., Laird, D.W., Boucher, R.C., and Lazarowski, E.R. 2011. Rho signaling regulates pannexin 1-mediated ATP release from airway epithelia. J. Biol. Chem. 286:26277-26286.

81. Locovei, S., Scemes, E., Qiu, F., Spray, D.C., and Dahl, G. 2007. Pannexin1 is part of the pore forming unit of the P2X(7) receptor death complex. FEBS Lett. 581:483-488.

82. Rae, M.G., Hilton, J., and Sharkey, J. 2012. Putative TRP channel antagonists, SKF 96365, flufenamic acid and 2-APB, are non-competitive antagonists at recombinant human alpha1beta2gamma2 GABA(A) receptors. Neurochem. Int. 60:543-554.

83. Woodward, R.M., Polenzani, L., and Miledi, R. 1994. Effects of fenamates and other nonsteroidal anti-inflammatory drugs on rat brain GABAA receptors expressed in Xenopus oocytes. J. Pharmacol. Exp. Ther. 268:806-817.

84. Hu, H., Tian, J., Zhu, Y., Wang, C., Xiao, R., Herz, J.M., Wood, J.D., and Zhu, M.X. 2010. Activation of TRPA1 channels by fenamate nonsteroidal anti-inflammatory drugs. Pflugers Arch. 459:579-592.

85. McCarty, N.A., McDonough, S., Cohen, B.N., Riordan, J.R., Davidson, N., and Lester, H.A. 1993. Voltage-dependent block of the cystic fibrosis transmembrane conductance regulator Cl- channel by two closely related arylaminobenzoates. J. Gen. Physiol 102:1-23.

86. Contreras, J.E., Saez, J.C., Bukauskas, F.F., and Bennett, M.V. 2003. Gating and regulation of connexin 43 (Cx43) hemichannels. Proc. Natl. Acad. Sci. U. S. A 100:11388-11393.

87. John, S.A., Kondo, R., Wang, S.Y., Goldhaber, J.I., and Weiss, J.N. 1999. Connexin-43 hemichannels opened by metabolic inhibition. J. Biol. Chem. 274:236-240.

88. Ma, W., Hui, H., Pelegrin, P., and Surprenant, A. 2009. Pharmacological characterization of pannexin-1 currents expressed in mammalian cells. J. Pharmacol. Exp. Ther. 328:409-418.

89. Pelegrin, P., and Surprenant, A. 2006. Pannexin-1 mediates large pore formation and interleukin-1beta release by the ATP-gated P2X7 receptor. EMBO J. 25:5071-5082.

90. Nakazawa, K., Liu, M., Inoue, K., and Ohno, Y. 1997. Potent inhibition by trivalent cations of ATP-gated channels. Eur. J. Pharmacol. 325:237-243.

91. Nathan, R.D., Kanai, K., Clark, R.B., and Giles, W. 1988. Selective block of calcium current by lanthanum in single bullfrog atrial cells. J. Gen. Physiol 91:549-572.

92. Li, H., Liu, T.F., Lazrak, A., Peracchia, C., Goldberg, G.S., Lampe, P.D., and Johnson, R.G. 1996. Properties and regulation of gap junctional hemichannels in the plasma membranes of cultured cells. J. Cell Biol. 134:1019-1030.

93. Li, K., Chi, Y., Gao, K., Yan, Q., Matsue, H., Takeda, M., Kitamura, M., and Yao, J. 2013. Connexin43 hemichannel-mediated regulation of connexin43. PLoS. One. 8:e58057.

94. Valiunas, V., and Weingart, R. 2000. Electrical properties of gap junction hemichannels identified in transfected HeLa cells. Pflugers Arch. 440:366-379.

95. Retamal, M.A., Froger, N., Palacios-Prado, N., Ezan, P., Saez, P.J., Saez, J.C., and Giaume, C. 2007. Cx43 hemichannels and gap junction channels in astrocytes are regulated oppositely by proinflammatory cytokines released from activated microglia. J. Neurosci. 27:13781-13792.

96. Wei, H., Deng, F., Chen, Y., Qin, Y., Hao, Y., and Guo, X. 2014. Ultrafine carbon black induces glutamate and ATP release by activating connexin and pannexin hemichannels in cultured astrocytes. Toxicology 323:32-41.

97. Bol, M., Wang, N., De, B.M., Wacquier, B., Decrock, E., Gadicherla, A., Decaluwe, K., Vanheel, B., van Rijen, H.V., Krysko, D.V., Bultynck, G., Dupont, G., Van, d., V, and Leybaert, L. 2016. At the cross-point of connexins, calcium and ATP: blocking hemichannels inhibits vasoconstriction of rat small mesenteric arteries. Cardiovasc. Res.

98. Chen, G., Park, C.K., Xie, R.G., Berta, T., Nedergaard, M., and Ji, R.R. 2014. Connexin-43 induces chemokine release from spinal cord astrocytes to maintain late-phase neuropathic pain in mice. Brain 137:2193-2209.

99. Chever, O., Lee, C.Y., and Rouach, N. 2014. Astroglial connexin43 hemichannels tune basal excitatory synaptic transmission. J. Neurosci. 34:11228-11232.

100. Orellana, J.A., Saez, J.C., Bennett, M.V., Berman, J.W., Morgello, S., and Eugenin, E.A. 2014. HIV increases the release of dickkopf-1 protein from human astrocytes by a Cx43 hemichannel-dependent mechanism. J. Neurochem. 128:752-763.

101. Orellana, J.A., Busso, D., Ramirez, G., Campos, M., Rigotti, A., Eugenin, J., and von, B.R. 2014. Prenatal nicotine exposure enhances Cx43 and Panx1 unopposed channel activity in brain cells of adult offspring mice fed a high-fat/cholesterol diet. Front Cell Neurosci. 8:403.

102. Orellana, J.A., Avendano, B.C., and Montero, T.D. 2014. Role of connexins and pannexins in ischemic stroke. Curr. Med. Chem. 21:2165-2182.

103. Retamal, M.A., Alcayaga, J., Verdugo, C.A., Bultynck, G., Leybaert, L., Saez, P.J., Fernandez, R., Leon, L.E., and Saez, J.C. 2014. Opening of pannexin- and connexin-based channels increases the excitability of nodose ganglion sensory neurons. Front Cell Neurosci. 8:158.

104. Rovegno, M., Soto, P.A., Saez, P.J., Naus, C.C., Saez, J.C., and von, B.R. 2015. Connexin43 hemichannels mediate secondary cellular damage spread from the trauma zone to distal zones in astrocyte monolayers. Glia 63:1185-1199.

105. Iacobas, D.A., Iacobas, S., Urban-Maldonado, M., and Spray, D.C. 2005. Sensitivity of the brain transcriptome to connexin ablation. Biochim. Biophys. Acta 1711:183-196.

106. Iacobas, D.A., Iacobas, S., Urban-Maldonado, M., Scemes, E., and Spray, D.C. 2008. Similar transcriptomic alterations in Cx43 knockdown and knockout astrocytes. Cell Commun. Adhes. 15:195-206.

107. Jansen, J.A., Noorman, M., Musa, H., Stein, M., de, J.S., van der Nagel, R., Hund, T.J., Mohler, P.J., Vos, M.A., van Veen, T.A., de Bakker, J.M., Delmar, M., and van Rijen, H.V. 2012. Reduced heterogeneous expression of Cx43 results in decreased Nav1.5 expression and reduced sodium current that accounts for arrhythmia vulnerability in conditional Cx43 knockout mice. Heart Rhythm. 9:600-607.

108. Agullo-Pascual, E., Lin, X., Leo-Macias, A., Zhang, M., Liang, F.X., Li, Z., Pfenniger, A., Lubkemeier, I., Keegan, S., Fenyo, D., Willecke, K., Rothenberg, E., and Delmar, M. 2014. Super-resolution imaging reveals that loss of the C-terminus of connexin43 limits microtubule plus-end capture and NaV1.5 localization at the intercalated disc. Cardiovasc. Res. 104:371-381.

109. Iglesias, R., Dahl, G., Qiu, F., Spray, D.C., and Scemes, E. 2009. Pannexin 1: the molecular substrate of astrocyte "hemichannels". J. Neurosci. 29:7092-7097.

110. Ponsaerts, R., De, V.E., Retamal, M., D'hondt, C., Vermeire, D., Wang, N., De, S.H., Zimmermann, P., Himpens, B., Vereecke, J., Leybaert, L., and Bultynck, G. 2010. Intramolecular loop/tail interactions are essential for connexin 43-hemichannel activity. FASEB J. 24:4378-4395.

111. Barrio, L.C., Suchyna, T., Bargiello, T., Xu, L.X., Roginski, R.S., Bennett, M.V., and Nicholson, B.J. 1991. Gap junctions formed by connexins 26 and 32 alone and in combination are differently affected by applied voltage. Proc. Natl. Acad. Sci. U. S. A 88:8410-8414.

112. Bao, X., Altenberg, G.A., and Reuss, L. 2004. Mechanism of regulation of the gap junction protein connexin 43 by protein kinase C-mediated phosphorylation. Am. J. Physiol Cell Physiol 286:C647-C654.

113. Cao, F., Eckert, R., Elfgang, C., Nitsche, J.M., Snyder, S.A., ulser, D.F., Willecke, K., and Nicholson, B.J. 1998. A quantitative analysis of connexin-specific permeability differences of gap junctions expressed in HeLa transfectants and Xenopus oocytes. J. Cell Sci. 111 (Pt 1):31-43.

114. Dahl, G., Miller, T., Paul, D., Voellmy, R., and Werner, R. 1987. Expression of functional cell-cell channels from cloned rat liver gap junction complementary DNA. Science 236:1290-1293.

115. Ek-Vitorin, J.F., Calero, G., Morley, G.E., Coombs, W., Taffet, S.M., and Delmar, M. 1996. PH regulation of connexin43: molecular analysis of the gating particle. Biophys. J. 71:1273-1284.

116. DeVuyst E., Wang, N., Decrock, E., De, B.M., Vinken, M., Van, M.M., Lai, C., Culot, M., Rogiers, V., Cecchelli, R., Naus, C.C., Evans, W.H., and Leybaert, L. 2009. Ca(2+) regulation of connexin 43 hemichannels in C6 glioma and glial cells. Cell Calcium 46:176-187.

117. Ramachandran, S., Xie, L.H., John, S.A., Subramaniam, S., and Lal, R. 2007. A novel role for connexin hemichannel in oxidative stress and smoking-induced cell injury. PLoS. One. 2:e712.

118. Alstrom, J.S., Hansen, D.B., Nielsen, M.S., and Macaulay, N. 2015. Isoform-specific phosphorylation-dependent regulation of connexin hemichannels. J. Neurophysiol. 114:3014-3022.

119. Essenfelder, G.M., Bruzzone, R., Lamartine, J., Charollais, A., Blanchet-Bardon, C., Barbe, M.T., Meda, P., and Waksman, G. 2004. Connexin30 mutations responsible for hidrotic ectodermal dysplasia cause abnormal hemichannel activity. Hum. Mol. Genet. 13:1703-1714.

120. Valiunas, V. 2013. Cyclic nucleotide permeability through unopposed connexin hemichannels. Front Pharmacol. 4:75.

121. Saez, J.C., and Leybaert, L. 2014. Hunting for connexin hemichannels. FEBS Lett. 588:1205-1211.

122. Fasciani, I., Temperan, A., Perez-Atencio, L.F., Escudero, A., Martinez-Montero, P., Molano, J., Gomez-Hernandez, J.M., Paino, C.L., Gonzalez-Nieto, D., and Barrio, L.C. 2013. Regulation of connexin hemichannel activity by membrane potential and the extracellular calcium in health and disease. Neuropharmacology 75:479-490.

123. Ebihara, L. 1996. Xenopus connexin38 forms hemi-gap-junctional channels in the nonjunctional plasma membrane of Xenopus oocytes. Biophys. J. 71:742-748.

124. Scemes, E., Spray, D.C., and Meda, P. 2009. Connexins, pannexins, innexins: novel roles of "hemi-channels". Pflugers Arch. 457:1207-1226.

125. Stroud, R.M., Savage, D., Miercke, L.J., Lee, J.K., Khademi, S., and Harries, W. 2003. Selectivity and conductance among the glycerol and water conducting aquaporin family of channels. FEBS Lett. 555:79-84.

126. Tsukaguchi, H., Shayakul, C., Berger, U.V., Mackenzie, B., Devidas, S., Guggino, W.B., van Hoek, A.N., and Hediger, M.A. 1998. Molecular characterization of a broad selectivity neutral solute channel. J. Biol. Chem. 273:24737-24743.

127. Maeda, S., Nakagawa, S., Suga, M., Yamashita, E., Oshima, A., Fujiyoshi, Y., and Tsukihara, T. 2009. Structure of the connexin 26 gap junction channel at 3.5 A resolution. Nature 458:597-602.

128. Unger, V.M., Kumar, N.M., Gilula, N.B., and Yeager, M. 1999. Three-dimensional structure of a recombinant gap junction membrane channel. Science 283:1176-1180.

129. Cotrina, M.L., Lin, J.H., Lopez-Garcia, J.C., Naus, C.C., and Nedergaard, M. 2000. ATP-mediated glia signaling. J. Neurosci. 20:2835-2844.

130. Chen, M.J., Kress, B., Han, X., Moll, K., Peng, W., Ji, R.R., and Nedergaard, M. 2012. Astrocytic CX43 hemichannels and gap junctions play a crucial role in development of chronic neuropathic pain following spinal cord injury. Glia 60:1660-1670.

131. Huang, C., Han, X., Li, X., Lam, E., Peng, W., Lou, N., Torres, A., Yang, M., Garre, J.M., Tian, G.F., Bennett, M.V., Nedergaard, M., and Takano, T. 2012. Critical

role of connexin 43 in secondary expansion of traumatic spinal cord injury. J. Neurosci. 32:3333-3338.

132. Langevin, H.M., Fujita, T., Bouffard, N.A., Takano, T., Koptiuch, C., Badger, G.J., and Nedergaard, M. 2013. Fibroblast cytoskeletal remodeling induced by tissue stretch involves ATP signaling. J. Cell Physiol 228:1922-1926.

133. Stridh, M.H., Tranberg, M., Weber, S.G., Blomstrand, F., and Sandberg, M. 2008. Stimulated efflux of amino acids and glutathione from cultured hippocampal slices by omission of extracellular calcium: likely involvement of connexin hemichannels. J. Biol. Chem. 283:10347-10356.

134. Ye, B., Shen, H., Zhang, J., Zhu, Y.G., Ransom, B.R., Chen, X.C., and Ye, Z.C. 2015. Dual pathways mediate beta-amyloid stimulated glutathione release from astrocytes. Glia 63:2208-2219.

135. Zhang, J., Zhang, H.Y., Zhang, M., Qiu, Z.Y., Wu, Y.P., Callaway, D.A., Jiang, J.X., Lu, L., Jing, L., Yang, T., and Wang, M.Q. 2014. Connexin43 hemichannels mediate small molecule exchange between chondrocytes and matrix in biomechanically-stimulated temporomandibular joint cartilage. Osteoarthritis. Cartilage. 22:822-830.

136. Fellin, T., Pozzan, T., and Carmignoto, G. 2006. Purinergic receptors mediate two distinct glutamate release pathways in hippocampal astrocytes. J. Biol. Chem. 281:4274-4284.

137. Liu, H.T., Tashmukhamedov, B.A., Inoue, H., Okada, Y., and Sabirov, R.Z. 2006. Roles of two types of anion channels in glutamate release from mouse astrocytes under ischemic or osmotic stress. Glia 54:343-357.

138. Takeuchi, H., Jin, S., Wang, J., Zhang, G., Kawanokuchi, J., Kuno, R., Sonobe, Y., Mizuno, T., and Suzumura, A. 2006. Tumor necrosis factor-alpha induces neurotoxicity via glutamate release from hemichannels of activated microglia in an autocrine manner. J. Biol. Chem. 281:21362-21368.

139. Fenton, R.A., Moeller, H.B., Zelenina, M., Snaebjornsson, M.T., Holen, T., and Macaulay, N. 2010. Differential water permeability and regulation of three aquaporin 4 isoforms. Cell Mol. Life Sci. 67:829-840.

140. Joshi-Mukherjee, R., Coombs, W., Burrer, C., de Mora, I.A., Delmar, M., and Taffet, S.M. 2007. Evidence for the presence of a free C-terminal fragment of cx43 in cultured cells. Cell Commun. Adhes. 14:75-84.

141. Giaume, C., Orellana, J.A., Abudara, V., and Saez, J.C. 2012. Connexin-Based Channels in Astrocytes: How to Study Their Properties. Pages 283-303, in Milner, R. (ed.), Astrocytes Methods in Molecular Biology, Springer Protocols.

142. Schalper, K.A., Palacios-Prado, N., Orellana, J.A., and Saez, J.C. 2008. Currently used methods for identification and characterization of hemichannels. Cell Commun. Adhes. 15:207-218.

143. Ye, Z.C., Ransom, B.R., and Sontheimer, H. 2001. (1R,3S)-1-Aminocyclopentane-1,3-dicarboxylic acid (RS-ACPD) reduces intracellular glutamate levels in astrocytes. J. Neurochem. 79:756-766.