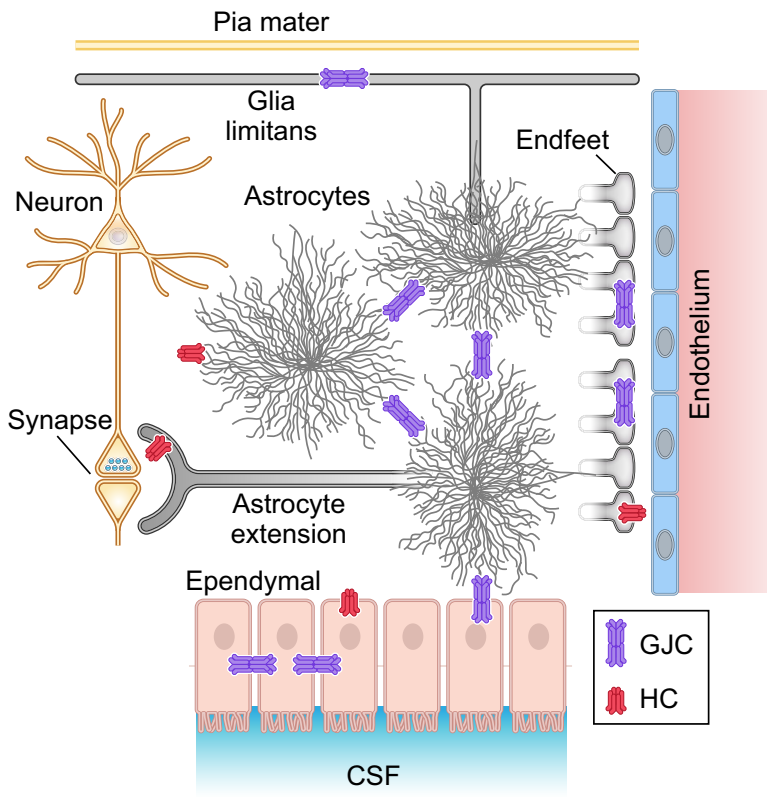
Physiological Reviews

Review Article

GLIAL CONNEXINS AND PANNEXINS IN THE HEALTHY AND DISEASED BRAIN

GRAPHICAL ABSTRACT



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KEYWORDS

blood-brain barrier; brain metabolism; calcium waves; glial syncytium; gliotransmission

CLINICAL HIGHLIGHTS

- Brain glial cells strongly express connexins, especially in astrocytes and oligodendrocytes where these channel-forming proteins have specialized functions.
- Connexins form gap junction channels that directly connect astrocytes among each other as well as with myelin-forming oligodendrocytes and brain ventricle-lining ependymal cells forming a "panglial syncytium."
- Astrocytes project extensions to synapses as well as blood vessels, thereby influencing synaptic activity, communicating to blood vessels and the blood-brain barrier, and distributing energy molecules like glucose and lactate, all involving crucial connexin contributions.
- Potassium and glutamate are spatially buffered over the junctionally coupled network to help control the composition of the interstitium. In epilepsy, the astrocytic spatial buffer protects against seizure activity, but astrocytic coupling may also inappropriately facilitate synchronization and enhance seizure activity.
- Connexins also form hemichannels that are normally closed but open under ischemic, inflammatory, and neurodegenerative conditions. Uncontrolled hemichannel opening facilitates transmembrane ion fluxes and the escape or entry of low-molecular-weight molecules (<1.5 kDa), which may disturb cell function and enhance cell death. Hemichannels release ATP that may exert a variety of effects through purinergic paracrine signaling. Pannexins also form glial channels that resemble connexin-based hemichannels and may contribute to brain disease.
- Therapeutically targeting gap junctions is difficult as their inhibition may compromise physiological processes, while enhancing their function may facilitate spreading of cell death. Recent work with specific hemichannel blockers demonstrates protective potential in animal models of epilepsy and stroke.

Giaume et al., 2021, *Physiol Rev* 101: 93–145
April 23, 2020;
https://doi.org/10.1152/physrev.00043.2018

american physiological society

Physiol Rev 101: 93–145, 2021
First published April 23, 2020; doi:10.1152/physrev.00043.2018

GLIAL CONNEXINS AND PANNEXINS IN THE HEALTHY AND DISEASED BRAIN

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Giaume C, Naus CC, Sáez JC, Leybaert L. Glial Connexins and Pannexins in the Healthy and Diseased Brain. *Physiol Rev* 101: 93–145, 2021. First published April 23, 2020; doi:10.1152/physrev.00043.2018.—Over the past several decades a large amount of data have established that glial cells, the main cell population in the brain, dynamically interact with neurons and thus impact their activity and survival. One typical feature of glia is their marked expression of several connexins, the membrane proteins forming intercellular gap junction channels and hemichannels. Pannexins, which have a tetraspan membrane topology as connexins, are also detected in glial cells. Here, we review the evidence that connexin and pannexin channels are actively involved in dynamic and metabolic neuroglial interactions in physiological as well as in pathological situations. These features of neuroglial interactions open the way to identify novel non-neuronal aspects that allow for a better understanding of behavior and information processing performed by neurons. This will also complement the "neurocentric" view by facilitating the development of glia-targeted therapeutic strategies in brain disease.

blood-brain barrier; brain metabolism; calcium waves; glial syncytium; gliotransmission

"*Cell junctions require careful analysis because they reflect not only the biology of individual cells, but also their 'sociology'; that is, the cooperativity with other cells and the relations to the environment.*" [Mugnaini (336)]

I. INTRODUCTION

The initial experimental description of an electrical spread between cardiac cells (558), as well as between neurons (161), initiated the concept of direct cell-to-cell communication between the cytoplasm of adjacent cells (120, 428, 578). This mode of cell interaction via unique channels called gap junctions (GJs) was then extended to small signaling molecules (294, 389, 502) and led to the notion of direct intercellular communication supporting electrical and biochemical/metabolic coupling, referred to as gap junctional intercellular communication (GJIC). Since then, ultrastructural and molecular approaches have revealed that such GJIC occurred at a specialized apposition of membranes of two neighboring cells, the GJ, where a specific protein family, the connexins (Cxs), form intercellular channels with specific assembly and turnover properties **(FIGURE 1*A*)** (see Ref. 269). More recently, functional data have indicated that in addition to gap junction channels (GJCs) formed by the docking of two hexameric Cx channels, hemichannels (HCs) themselves may be functional in certain conditions (28, 191) by supporting transmembrane fluxes as well as autocrine/paracrine communication. Finally, the existence of non-channel functions of Cxs (495, 538) has enlarged the physiological roles played by these proteins. In this review, we focus on glia in the central nervous system (CNS) and will also consider another family of membrane proteins, the pannexins (Panxs) that share several features and functional properties with Cxs. **FIGURE 2** illustrates a timeline marking major papers related to glial Cxs and Panxs.

Given the complexity of neural circuits, involving neuronal as well as glial contributions, our main scope is primarily

- Brain glial cells strongly express connexins, especially in astrocytes and oligodendrocytes where these channel-forming proteins have specialized functions.
- Connexins form gap junction channels that directly connect astrocytes among each other as well as with myelin-forming oligodendrocytes and brain ventricle-lining ependymal cells forming a "panglial syncytium."
- Astrocytes project extensions to synapses as well as blood vessels, thereby influencing synaptic activity, communicating to blood vessels and the blood-brain barrier, and distributing energy molecules like glucose and lactate, all involving crucial connexin contributions.
- Potassium and glutamate are spatially buffered over the junctionally coupled network to help control the composition of the interstitium. In epilepsy, the astrocytic spatial buffer protects against seizure activity, but astrocytic coupling may also inappropriately facilitate synchronization and enhance seizure activity.
- Connexins also form hemichannels that are normally closed but open under ischemic, inflammatory, and neurodegenerative conditions. Uncontrolled hemichannel opening facilitates transmembrane ion fluxes and the escape or entry of low-molecular-weight molecules (<1.5 kDa), which may disturb cell function and enhance cell death. Hemichannels release ATP that may exert a variety of effects through purinergic paracrine signaling. Pannexins also form glial channels that resemble connexin-based hemichannels and may contribute to brain disease.
- Therapeutically targeting gap junctions is difficult as their inhibition may compromise physiological processes, while enhancing their function may facilitate spreading of cell death. Recent work with specific hemichannel blockers demonstrates protective potential in animal models of epilepsy and stroke.

directed to review results from works that have the highest level of relevance such as for instance in vivo studies when available. We also include data, where indicated, obtained from acute brain slices and in vitro cell culture preparations, experimental approaches that can reveal further mechanistic insights. However, some of the earlier investigations from cell culture studies and general properties of Cx/Panx proteins will not be considered in detail, since these have been thoroughly reviewed elsewhere (103, 114, 182, 484, 512). Last but not least, we limited our scope to the CNS focusing specifically on the brain, while not discussing results from retina, cochlea, peripheral ganglia, or other related areas.

A. Connexins and Pannexins

In vertebrates, GJCs are formed by a multigenic family of Cxs (480), with 21 and 20 different Cxs identified in the human and mouse genomes, respectively (344, 563). Considering brain glial cell populations, with the exception of NG2 cells (see below), two conclusions can be made; they all express more than one Cx isotype, and each cell type expresses a specific set of Cxs. This implies that various molecular combinations of GJCs may occur **(FIGURE 1*B*)** with various differences in biophysical properties, regulation, and function.

While for some time it has been taken as dogma that Cxs can only function as GJCs, there is conclusive evidence now that HCs can be functional in brain cells (28, 180) (see **FIGURE 1*B***). Indeed, before their aggregation at the junctional plaque and subsequent formation of GJCs, hexameric rings of Cxs were initially assumed to remain always closed. An obvious reason for this was that, as GJCs are "poorly" selective for ions and permeable to low-molecular-weight molecules, open plasma membrane HCs would result in a loss of cell integrity or at least would require substantial energy to maintain an energetically unfavorable condition. Moreover, HCs may also be formed by another membrane protein family called Panxs. Panxs generally do not form GJs (exceptions, see Ref. 451) and have therefore been recommended to be called channels rather than HCs (488). Three members have been identified and have the same membrane topology and share some pharmacological properties, but exhibit no significant sequence homology with Cxs (56, 379). With the genomic characterization of Cxs (563) and Panxs (20), it has been determined that there are at least 11 different Cxs and 2 different Panxs expressed in the rodent CNS.

B. Glial Cell Populations in the CNS

Macroglial cells are composed of several subpopulations that are distinctive in their morphology, location, properties, and functions. Astrocytes are identified based on their stellate morphology, and many of them are positive for specific markers such as the glial fibrillary acidic protein (GFAP). They have an important role in the CNS since they are in close relationship with neurons, contributing to the "tripartite synapse" (14), and also are part of the glio-vascular interface (244). The second group is formed by the oligodendrocytes, the myelinating glial cells in the CNS. However, there are also nonmyelinating oligodendrocyte lineage cells that include perineuronal or satellite oligodendrocytes and NG2 cells. The latter are now considered as a separate glial cell population that has an antigenic phenotype of adult oligodendrocyte precursor cells (OPCs). Finally, microglia and mast cells are members of the innate immune system present in the CNS. Microglia are the CNS tissue macrophages and show different phenotypes, ranging from resting to activated states (252). Mast cells are located on the brain side of the blood-brain barrier (BBB) and in leptomeninges in close contact with microvasculature (518).

II. CONNEXIN AND PANNEXIN EXPRESSION IN GLIAL CELLS

A. Connexin and Pannexin Expression in Glial Cell Subpopulations

A number of Cxs, as well as Panx1, have been detected in glial cells **(FIGURE 3)**, and in most cases their presence has

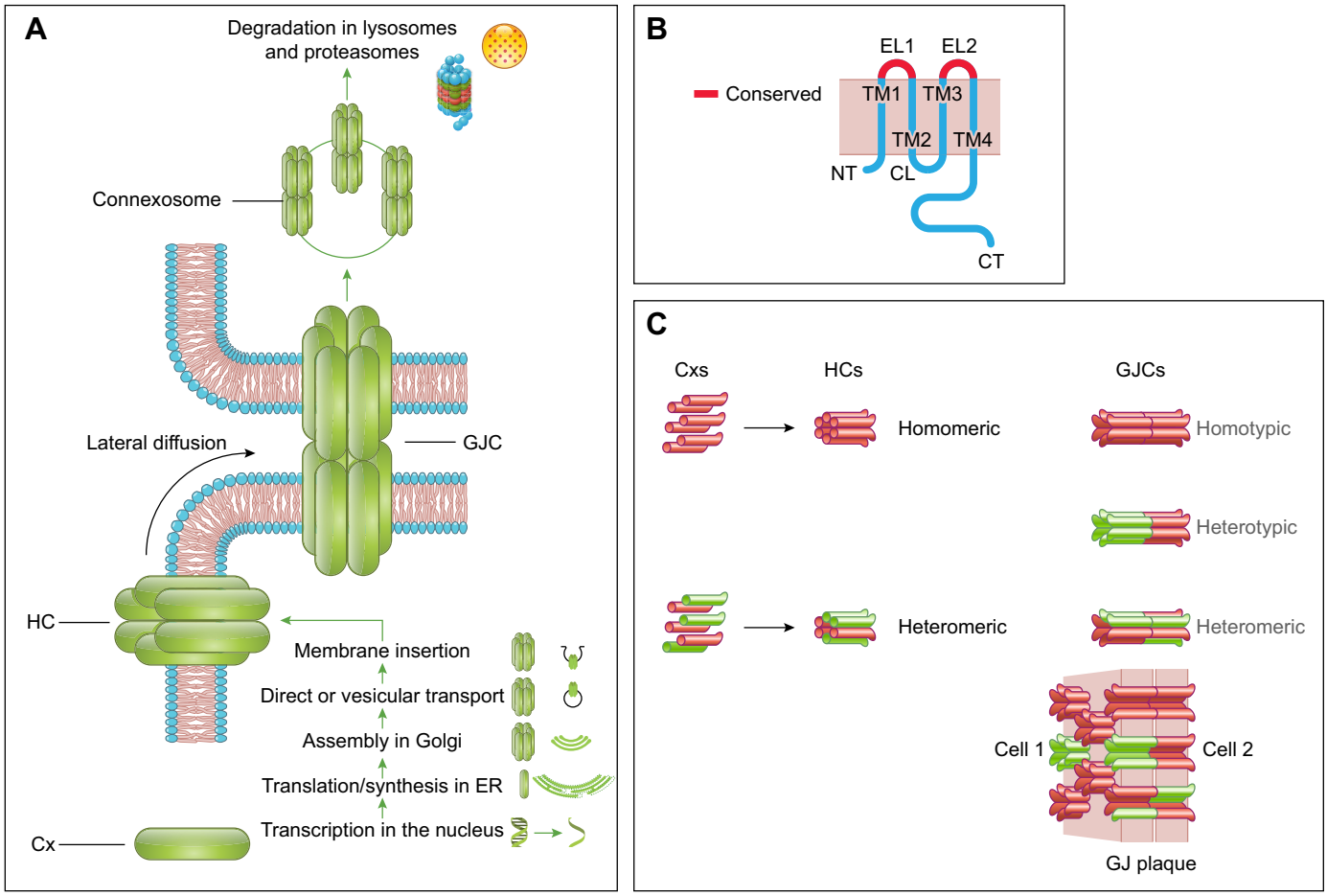

FIGURE 1. The connexin life cycle and channels. *A*: the making and breaking of connexin (Cx) channels. Cx biosynthesis in the endoplasmic reticulum (ER) is followed by forward trafficking to the plasma membrane, involving the secretory pathway. In this process, Cxs oligomerize in the Golgi apparatus and *trans*-Golgi network and assemble as hexameric hemichannels (HCs) that are transported to the plasma membrane via microtubules and actin filaments (280). The head-to-head docking of HCs results in the formation of a gap junction (GJ) channel (GJC). Subsequently, GJCs move by lateral diffusion and further organize as plaques (*C, bottom right*) composed of multiple densely packed GJCs; the actual plaque size may vary. Cxs have a very short lifetime on the order of hours and therefore have a very high turnover rate, necessitating highly organized internalization and degradation mechanisms. Currently, the best characterized pathway consists of the uptake of an entire GJ plaque as a connexosome, also called annular junction because of its ring-shaped form. Degradation of the connexosome then further proceeds through lysosomal and proteasomal pathways. Details of the life cycle may differ between different Cx isotypes. *B*: membrane topology of Cxs, which are composed of 4 transmembrane domains (TM1 to TM4), 2 extracellular loops (EL1, EL2), an intracellular cytoplasmic loop (CL), and NH_2- and COOH-terminal tails also inside the cell (NT, CT). ELs and TMs contain well-conserved sequences, while the CL, NT, and CT have sequences that strongly differ between different Cx isotypes. The membrane topology of Panxs is similar to the Cx topology. *C*: Cxs can assemble as homomeric or heteromeric HCs. The docking of two homomeric HCs composed of the same Cx isotype results in a homotypic GJC; docking of two HCs composed of distinct isotypes yields a heterotypic GJC. Docking of heteromeric HCs results in a heteromeric GJC.

been confirmed by functional tests for their channel functions.

1. Astrocytes

This major glial cell population expresses mainly Cx43 as well as Cx30. The respective contribution of these two Cxs to GJIC has been validated by functional tests in acute brain slices, using *hGFAP-cre*:*Cx43*$^{fl/fl}$ and global Cx30 knockout (KO) mice (110, 511), as well as Cx30$^{-/-}$/hGFAP-Cre:Cx43$^{fl/fl}$ double KO mice (382, 440, 441, 542) (further called astrocytic Cx43/Cx30 dKO mice). These two Cxs are expressed in astrocytes with variable proportions in a number of brain structures (see Refs. 344, 481). To a lesser extent, Cx26 is also detected in some astrocytes (9, 298, 347), although a reporter allele revealing Cx26 gene expression demonstrated that it is not expressed in glial cells in the mouse (150). Although the expression of Panx1 and Panx2 in neurons is well established (540), their presence in astrocytes remains a matter of debate depending on the physio-

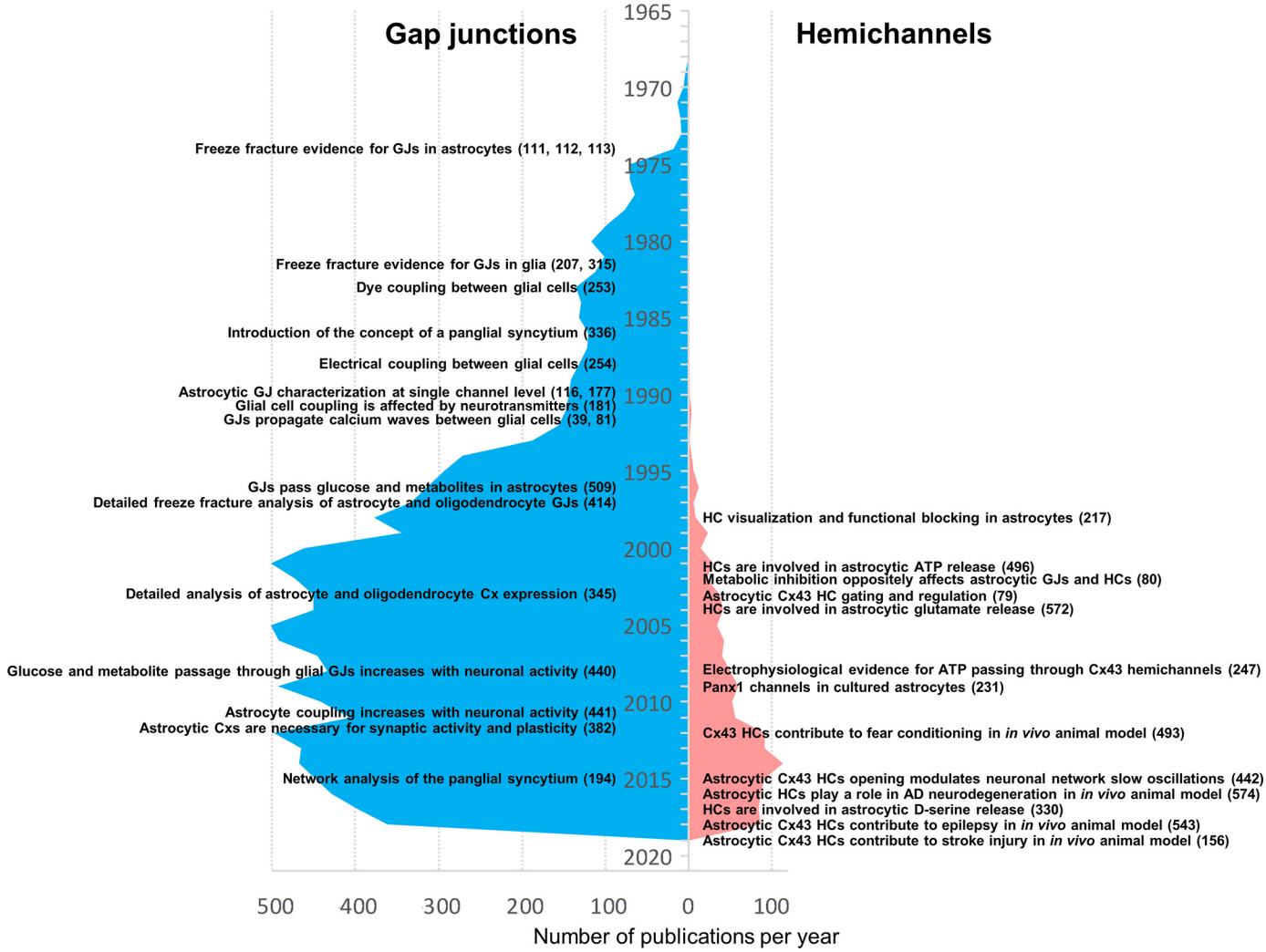

FIGURE 2. Time line marking major papers regarding gap junctions (GJs) and hemichannels (HCs) in glial cells, based on a PubMed search for the key words "gap junctions" and "hemichannels" over the period 1965–2019. Cx, connexin. Numbers in parentheses refer to reference list.

logical condition (it is currently unknown whether Panx2 forms functional channels). Nevertheless, in pathological models, there is a consensus for Panx1 expression and its contribution to HC-like function in reactive astrocytes (248, 318, 372, 454, 474, 574). Discrepancies regarding Panx1 or Panx2 expression in astrocytes could come from differences in the investigated cell models, especially in culture, since for instance the presence of microglial cells, whose proportion varies according to protocols, influences HC activity in astrocytes (325, 423). Also, the stress status of the preparations could result in the expression of functional Panx1 channels at the membrane (372).

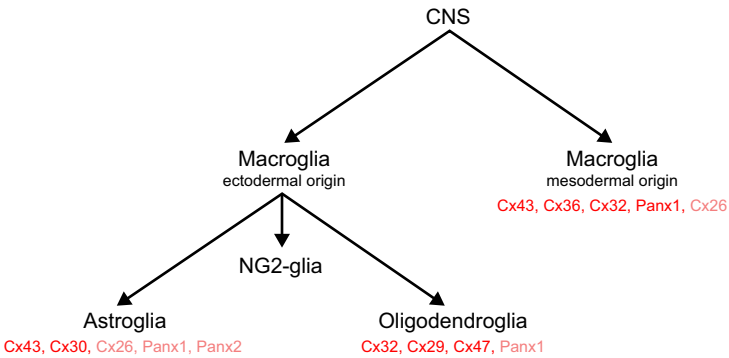

FIGURE 3. Connexin (Cx) and pannexin (Panx) expression in different classes of glial cells. Cxs and Panxs that have been unequivocally identified are shown in red, while those in faint red are candidates that remain to be confirmed. CNS, central nervous system.

2. Oligodendrocytes

While their progenitors do not express Cxs, differentiated oligodendrocytes express Cx29, Cx32, Cx45, and Cx47 (194, 306, 318, 344, 349, 512). Interestingly, depending on their compatibility profile, heterotypic GJs have been observed with astroglial Cx43 and Cx30 based on freeze-fracture replica immunogold labeling (344, 415) and functional dye coupling (194, 306, 307, 554). In the oligodendrocyte lineage, Panx1 activation has been observed after oxygen and glucose deprivation (127) and in a mouse model of multiple sclerosis (199). Also, the expression of Panx1 has been detected in mouse hippocampal oligodendrocytes and is upregulated by stress (318).

3. NG2 cells

Cx or Panx expression have not been found in adult OPCs and NG2 cells with the exception of the detection of Cx32 in a subset of $NG2^+$ and platelet-derived growth factor-receptor positive ($PDGFR^+$) cells in early oligodendrocyte progenitors in the dentate gyrus of adult mice (324). Moreover, double whole cell patch-clamp recordings did not provide evidence for junctional currents in pairs of $NG2^+$ cells in acute hippocampal slices (568).

4. Microglial cells

These cells express Cxs and Panxs in amounts depending on their state of activation. In basal conditions, nontreated cultured microglia express low amounts of Cx43 (142, 174), Cx36 and Cx32 (124, 304), and Cx45 (124), as well as Panx1 (371). Cx43 and Panx1 have been shown to support HC activity, and when treated with pro-inflammatory cytokines, Cx43-mediated GJ coupling is observed in vitro (142, 449). Heterotypic coupling between microglial cells and neurons has also been found in cocultures, based on dye coupling and double patch-clamp recording of junctional currents (124).

B. Developmental Pattern of Glial Connexin and Pannexin Expression

1. Connexins

Early reports highlighted glial Cx expression (117), as well as the extensive nature of GJCs between cells in the developing CNS, particularly in the neocortex (391, 577). It has been established that GJCs are prevalent in neural precursor cells including neural stem cells, as well as neuronal and glial progenitors (35). These Cxs have been shown to be involved in a number of developmental processes in the CNS.

Since many Cxs are expressed in the developing brain and their presence varies with different regions, we will focus mainly on the neocortex as an example of temporal and spatial expression of glial Cxs in mice. At embryonic days E14–18, a time of dynamic cortical development, an analysis of Cxs demonstrated the expression of Cx26, Cx36, Cx37, Cx43, and Cx45, while Cx30 and Cx32 are absent, and Cx40 is expressed at a very low amount (73). While expression of both Cx26 and Cx43 occurs in $MAP2^+$ and $nestin^+$ cells, and Cx36 is expressed in neurons (77), the cell types for the other Cxs are for the most part not conclusively determined. By 3 wk after birth Cx30 is expressed in astrocytes (267), while expression of Cx32 occurs in oligodendrocytes coincident with development of myelin sheaths (117). The most highly expressed Cx during neurodevelopment is Cx43 (391, 577). At prenatal stages in mouse, Cx43 and Cx26 are expressed in neural progenitor cells, including radial glia (73, 74, 137). This underlies the adhesive function of Cxs that has in large part been implicated in neuronal migration and development of neocortical circuits (136, 391).

The expression of Cx45 in embryonic mouse neocortex might be due to developing oligodendrocytes, which are known to express high levels of Cx45 (115). Double immunofluorescent staining experiments showed Cx45 immunoreactivity in some $MAP2^+$ neurons as well as non-neuronal cells (73, 78). Using a Cx45 LacZ-reporter mouse and immunoblot analysis experiments, Maxeiner et al. (319) found high amounts of Cx45 protein in the cerebral cortex of a variety of brain regions at E18.5 to postnatal day 8 (P8), with expression decreasing to become restricted to neurons in the adult within cerebral cortical, hippocampal, and thalamic neurons and in basket and stellate cells of cerebellum.

2. Pannexins

During neurodevelopment in the rat brain, Panx1 and Panx2 mRNAs are widely expressed and exhibit distinct distributions (540). Panx1 is abundantly expressed in the embryonic and postnatal brain and declines with maturation in the adult. In contrast, Panx2 expression is low in the prenatal brain with increasing expression during postnatal development. Additional studies have clarified that Panx1 is expressed in postnatal neural stem and progenitor cells ($nestin/GFAP^+$) in the ventricular zone, where it has been proposed that these channels influence proliferation through the release of ATP (560).

C. Regional Distribution of Glial Connexins and Pannexins

Astroglial Cx43 is widely expressed in the brain (221), while the presence of Cx30 is detected in the grey matter but lacking in the white matter (348). In line with this, coupling between fibrous astrocytes in white matter is less extensive compared with coupling between protoplasmic astrocytes

in grey matter (197). In addition, the balance between the expression of these Cxs differs according to brain regions: in the olfactory glomeruli, the thalamus and the anterior hypothalamus Cx30 prevails, while in the hippocampus Cx43 is dominant, and they are rather equally distributed in the cortex (194, 348). Such diversity in their cellular expression is even more pronounced since in the thalamus Cx43 was not found in all astrocytes (194). In the somatosensory cortex, astrocytes located between two whisker barrels express low amount of these two Cxs compared with astrocytes within barrels (225); a similar feature is also observed in the glomerular layer of the olfactory bulb (441).

In oligodendrocytes, Cx32 is expressed mostly in the white matter and localized in myelin sheaths of large diameter fibers, where it typically forms reflexive GJCs between stacked myelin layers at paranodes (245). In contrast, Cx47 is expressed in both white and grey matter oligodendrocytes. Cx29 appears to be restricted to oligodendrocytes that myelinate small caliber fibers (9, 245, 259, 285). Moreover, Cx29 reporter mice reveal widespread expression with high amounts observed in all white matter tracts and grey matter regions (9). Based on the mRNA distribution, the amount of Cx32 varies greatly between CNS regions being particularly abundant in the midbrain, pons, and medulla, while cerebellum and forebrain regions are less enriched for this Cx (352); Cx32 is also found in the grey matter by immunohistochemistry (328). Interestingly, Cx47 colocalizes with Cx32 in oligodendrocyte cell bodies and processes while Cx29 rarely colocalizes with these two Cxs (9). With regard to Cx29, there is currently no evidence that this specific isotype does form functional channels. A HC function has been proposed based on electron micrograph evidence demonstrating organized alignment and apposition of myelin adaxonal membrane Cx29 with axonal Kv1.2 channels in the internode area (413).

Concerning the regional pattern of Cx expression in microglia, no specific data are available, mainly because to our knowledge this question has not been directly addressed.

Finally, the expression of Panx1 is also heterogeneously distributed in the CNS, mainly in the cortex, striatum, olfactory bulb, hippocampus, thalamus, and cerebellum (56). However, this has been mainly attributed to neuronal rather than glial expression. Similarly, while Panx2 has been shown to be present in various CNS regions, glial localization has only been definitively demonstrated in astrocytes following ischemia (580).

D. Heterotypic Gap Junctions in Glia

Having several glial subpopulations associated with high expression of multiple Cxs gives rise to a so-called "panglial syncytium," whereby GJCs are formed between astrocytes and other glial or non-glial cells (414). In particular, astrocytic GJCs connect to oligodendrocytes and to ependymal cells lining the ventricles, with astrocyte-oligodendrocyte GJs occurring twice as frequent compared with those between astrocytes and ependymal cells (413, 414) **(FIGURE 4)**. Because astrocytes and oligodendrocytes express nonoverlapping sets of Cxs, the intercellular channels formed between them must be asymmetric with regard to their Cx content, i.e., these junctions are heterotypic **(FIGURE 1*C*)**. Work in coculture systems with electrophysiological and dye coupling approaches indeed indicated that these cells were functionally coupled (412, 527). Dye coupling studies in acute brain slices of mice lacking Cx32 and/or Cx47 further confirmed that oligodendrocyte Cx47 can form heterotypic channels with astrocyte Cx43 or Cx30 but not Cx26, whereas oligodendrocyte Cx32 can functionally interact with astroglial Cx30 or Cx26 but not Cx43 (554). More recently, analysis of mice with cell type-specific deletion of Cxs showed that deletion of Cx30 and Cx47 led to complete loss of panglial coupling, which can be restored when one allele of either Cx is present (194). Of note, coupling between oligodendrocytes was, based on ultrastructural evidence, initially suggested to only occur via heterotypic GJs to an intermediately positioned astrocytic extension (315). However, morphological and functional evidence now indicates that direct coupling is also possible (306, 554).

III. CONNEXIN AND PANNEXIN CHANNEL PROPERTIES IN GLIA: GAP JUNCTION CHANNELS AND HEMICHANNELS

A. Gating and Permeability Properties

In general, it is accepted that different Cx-based channels show distinct permeability to ions and second messengers as well as metabolites and may fulfill in this way their specific role in different physiological events. The narrowest diameter of the Cx channel pore is much wider than the diameter of hydrated atomic ions like K^+, Na^+, and Cl^- (~15 Å vs. 2.4–3.6 Å range) (555). Despite this, connexin channels display charge selectivity, even for atomic ions (526). For larger charged molecules, which are more likely to interact at the level of the narrowest pore site, charge selectivity is more pronounced and sometimes very different between different connexins. Panx1 channels have been reported to display anion selectivity. The low conductance (~60 pS range) state indeed shows an anion preference in the rank order of $NO_3^- > I^- > Br^- > Cl^- > F^- >>$ aspartate$^-$ ≈ glutamate$^-$ ≈ gluconate$^-$ (300), while the high conductance (~500 pS range) state has been demonstrated to pass ATP^{2-} (546) but also the positively charged dye molecule $DAPI^{2+}$ (203).

So far, in situ studies on the biophysical features of GJCs and HCs are very sparse. However, exogenous expression systems have provided relevant information on gating

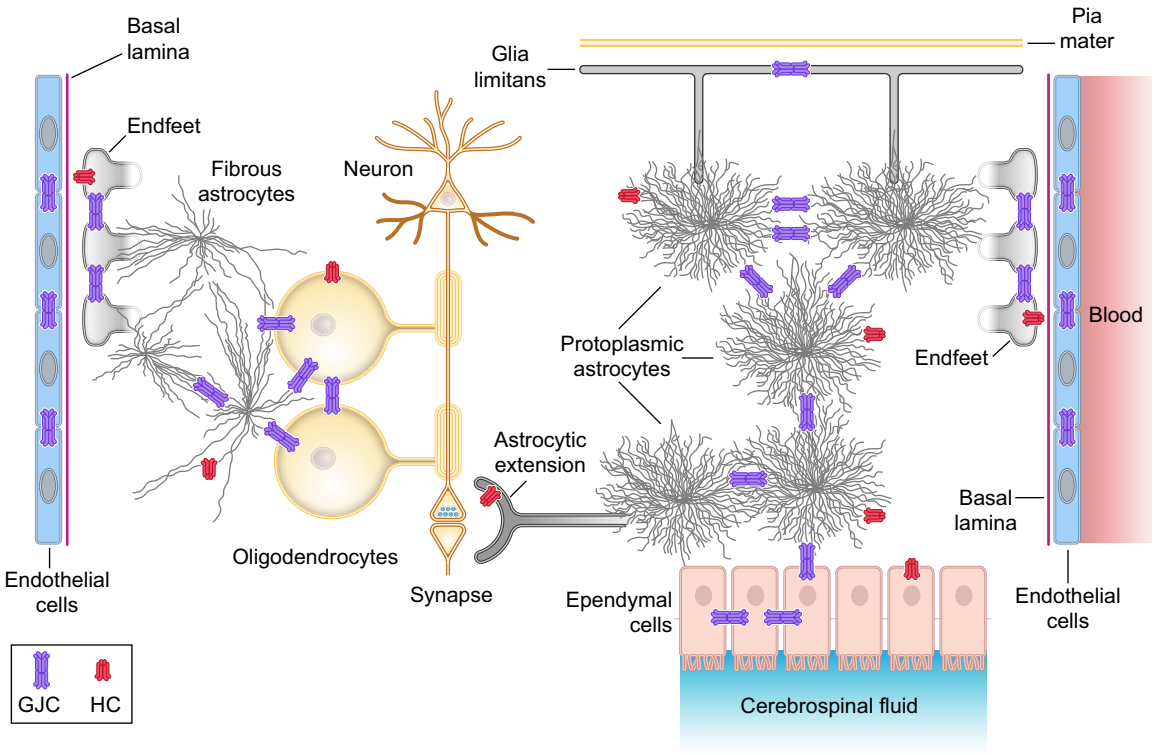

FIGURE 4. The glial network and syncytium. Glial cells form a large network in the central nervous system that is in contact with/juxtaposed to multiple cell types including neurons, ependymal cells that line the brain ventricles, endothelial cells of brain blood vessels, and the thin fibrous tissue layer of the pia mater, the innermost layer of the meninges. At some of these contacts, gap junctions (GJs) (in purple) can be found, e.g., between astrocytes and ependymal cells (414) that, like tanycytes, are glial cells (419, 564). At other sites, there is no evidence for GJs, e.g., at astrocyte extensions in contact with synapses or at the astrocyte-vascular interface, where astrocytic endfeet juxtaposed to capillary endothelial cells are physically separated by an intermediate basal lamina (475). Of all glial cells, astrocytes display the highest degree of GJ coupling, thereby forming a "functional syncytium." Not all astrocytes are equally well coupled: protoplasmic astrocytes in grey matter display stronger coupling than fibrous astrocytes in white matter (197, 387). Astrocytes are also coupled to oligodendrocytes (414) and to olfactory ensheathing glial cells (26), thereby forming what has been called the "panglial syncytium" (reviewed in Refs. 413, 512). Importantly, the syncytium is not uniform but rather consists of local networks of coupled cells with defined spatial organization, e.g., as observed in the barrel cortex (225), in barreloid structures in the thalamus (76), or in olfactory bulb glomeruli (441). In these networks, oligodendrocytes can be directly coupled with each other (306, 554) or via an intermediate astrocyte (413 and references therein). In addition to GJs, hemichannels (HCs) (colored red) may also contribute to signaling surrounding brain cells, e.g., as a mechanism contributing to gliotransmitter release at synapses (330, 543). The glial GJ syncytium plays an important role in spatial K^+ buffering (see **FIGURE 8**), in glutamate removal (382), and in the distribution of glucose and its metabolites in the brain (440).

mechanisms as well as permeability properties (62, 131, 135, 373, 523, 525). Interestingly, the selective permeability of both Cx HCs and GJCs is regulated by protein phosphorylation (19, 133) and redox potential (423); regulation of Cx channels by phosphorylation and redox potential has been extensively reviewed in References 134, 310, 402, 482–484 and 169, 421, 425, respectively. In general, the unitary conductance of Cx HCs in glial cells is about twice that of GJCs formed by the same Cx type (448). In addition, the charge selectivity concluded from permeation experiments with cationic and anionic dyes ranged from highly cation selective (526) to slightly anion selective (503) (data from GJCs composed of Cx26, Cx32, and Cx43), suggesting that the pores of specific Cx GJCs have distinct characteristics and divergent conductance and permeability properties.

Since Cx43 is the most abundant Cx expressed by glial cells, we will first focus on the most relevant biophysical features of GJCs and HCs formed by this protein. With the use of double patch-clamp analysis of hippocampal astrocytes in acute brain slices, it was found that junctional currents are independent of the transjunctional voltage (V_j) over a range from −100 to +110 mV (327). Electrical coupling between astrocytes also elicited weak low-pass filtering properties as compared with the more strongly pronounced low-pass coupling observed in neurons (327). However, astrocytes display strong electrical coupling (multiples of nS conduc-

tance) while neurons are weakly coupled (54, 249, 507). As a result, the unexpected V_j independence of astrocytic junctional currents (junctional Cx43 currents are normally reduced by V_j above 30 mV) as well as the weak low-pass filtering properties may have resulted from poor space clamp conditions.

In oligodendrocytes, Cx47 KO (Cx47-deficient mice) reduces the number of coupled cells by ~80%, while coupling completely disappears in Cx32/Cx47-double-deficient mice. Cx47 ablation furthermore abolishes coupling of oligodendrocytes to astrocytes. Based on these data, the oligodendrocyte contribution to the functional syncytium is thought to entirely depend on Cx47 and Cx32 (306). In the context of oligodendrocyte-astrocyte coupling, Cx47/Cx43 heterotypic channels show current rectification and form a directional reporter-dye diffusion barrier between oligodendrocytes expressing Cx47 to astrocytes expressing Cx43 (145). The Cx47P90S mutation associated with leukodystrophy eliminates the restrictive permeability of Cx47 channels and the diffusion barrier of Cx47/Cx43 channels, suggesting a relevant function for the asymmetric channel behavior observed in the wild type (145) (for a further discussion on theoretical issues of preferential directionality of Cx channels, see Refs. 57, 151, 323).

In mouse astrocytes, high extracellular K^+ concentration (474, 501) as well as membrane stretch have been shown to activate Panx1 channels, through mechanisms that involve interaction of the COOH terminus with F-actin (31). Despite the frequent suggestion that Panx1 channels might be activated by stretch in different cell types, this mechanism has also been challenged since Panx1 channels were not activated by cell swelling (429). On the other hand, Panx1 channels are closed by high extracellular ATP concentration (408), which should be distinguished from the Panx1 clustering and endocytosis that also occur after 15-min exposure to high extracellular ATP (50). Chiu et al. (69) provide a recent overview of various Panx1 channel-activating conditions.

Panx1 channels display smaller unitary currents at positive than at negative potentials, mainly because subconductance states are more prevalent at the positive side (18, 514). The unitary conductance of Panx1 channels also depends on the activation condition, and values range from ~60 to ~500 pS; this issue has been recently reviewed in detail (547).

Both Panx and Cx channels are, in addition to voltage, also affected by the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$). Elevation of $[Ca^{2+}]_i$ results in the closing of GJCs (297, 395), but the concentrations vary widely (from hundreds of nanomolar to micromolar concentrations; Refs. 85, 275, 297, 435, 490), most probably because of the involvement of microdomain Ca^{2+} signaling that is poorly reflected in global cytoplasmic $[Ca^{2+}]_i$ measurements. In contrast, moderate $[Ca^{2+}]_i$ elevation up to ~500 nM stimulates the opening of Cx43 HCs (reviewed in Ref. 280); HC opening activity starts to decrease when $[Ca^{2+}]_i$ rises above 500 nM and HCs are fully closed again at 1 μM (102, 403, 404). Panx1 channels have a more graded increase in opening activity with increasing $[Ca^{2+}]_i$, which is reportedly linear up to high concentrations outside the physiological range (100 μM), at least as observed in a *Xenopus* oocyte expression system (293). Besides intracellular, extracellular Ca^{2+} also affects Cx HCs, which are since long known to open upon lowering of the extracellular Ca^{2+} concentration (242, 282). Ca^{2+} interactions with conserved charged residues forming electrostatic networks near the extracellular HC pore mouth normally keep the HCs closed, but upon withdrawal of extracellular Ca^{2+}, the lost interactions provoke gating rearrangements that result in HC opening (296). Low extracellular Ca^{2+} has also been reported to enhance the opening of Panx1 channels by an indirect mechanism involving low Ca^{2+}-triggered ATP release, subsequent $P2X_7$ receptor activation, and increased $P2X_7$-Panx1 association (406).

B. Regulation and Modulation of Gap Junction Channels

GJIC in astrocytes is influenced by a large array of factors and conditions **(FIGURE 5)**. This includes neurotransmitters, neuromodulators, vascular signals, ubiquitous signaling molecules, ionic changes like extracellular K^+ elevation and intracellular elevation of the H^+ and Ca^{2+} concentration, various growth factors, and pro-inflammatory cytokines (reviewed in Refs. 179, 411, 438). Extracellular accumulation of glutamate and K^+ is a hallmark of increased neuronal activity and impacts the degree of astrocytic GJIC that has been proposed to be activity dependent, i.e., dependent on the frequency of neuronal firing (179). This was supported by experimental evidence obtained in olfactory glomeruli (441) and in the thalamus (76).

1. Extracellular potassium ions

Activity-dependent effects on glial cell coupling were first obtained from in vitro studies performed by Enkvist and McCarthy (141) and later on frog optic nerve by Marrero and Orkand (311). Electrical stimulation of the optic nerve triggered an increase of Lucifer yellow (LY) dye coupling in glial cells, which was associated with a depolarizing shift in membrane potential. Importantly, K^+ channel block with Ba^{2+} suggested that the glial depolarizing response played a role in the increased dye coupling, and work by others indicated that the K^+-induced increase in gap junctional coupling is mediated by Ca^{2+} activation of calmodulin-dependent kinases (99). In olfactory glomeruli, neuronal activity increases astrocytic coupling, which can be prevented by tetrodotoxin (TTX) or sensory deprivation and restored by

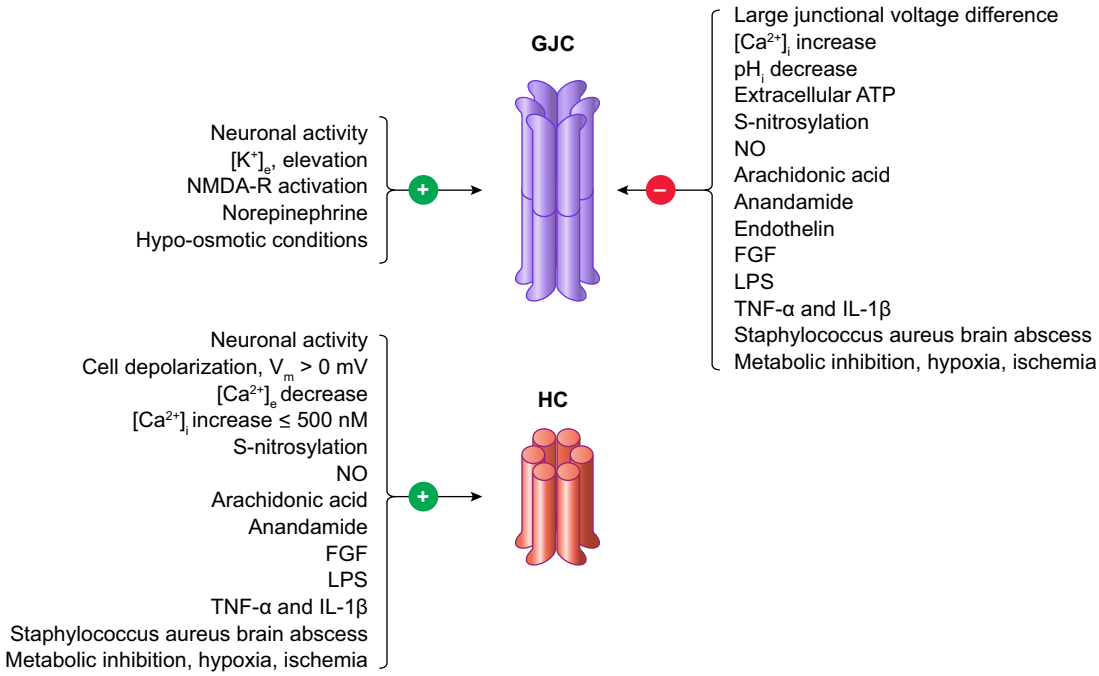

FIGURE 5. Modulatory effects on gap junctions (GJs) and hemichannels (HCs) in the brain. Increased neuronal activity enhances astrocytic GJ coupling via several mechanisms, including elevation of extracellular K^+ ($[K^+]_e$), increased glutamate release acting on neuronal α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and *N*-methyl-D-aspartate (NMDA) receptors, or increased concentrations of the modulatory neurotransmitter norepinephrine acting on astrocytes. Coupling is also increased under hypo-osmotic conditions. GJ coupling is decreased by large junctional voltage differences (differences in the membrane potential V_m of GJ-coupled cells), by an elevation of intracellular Ca^{2+} ($[Ca^{2+}]_i$), decrease of intracellular pH (pH_i), elevated extracellular ATP, *S*-nitrosylation redox signaling, e.g., in response to nitric oxide (NO), by arachidonic acid and its cannabinoid metabolite anandamide, and by the vasoconstrictor endothelin. GJ coupling is furthermore decreased by members of the fibroblast growth factor (FGF) family, by the Gram-negative bacterial wall component lipopolysaccharide (LPS), the pro-inflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-1β, by the Gram-positive bacterium *Staphylococcus aureus*, and by metabolic inhibition and hypoxia/ischemia. Most notably, many of these conditions have opposite effects on HCs that switch from a normally closed baseline state to a state with increased opening activity. The majority of the effects shown apply for connexin (Cx)43-based channels, and effects may differ for other Cxs. Effects of pH_i on HCs and of modulation by phosphorylation are not shown. Phosphorylation effects vary with the kinase and phosphorylation site involved (402) and are reviewed in Refs. 134, 310, 402, 482–484, 402, 134. Cx channel modulation is further discussed in section III and illustrated in **TABLE 1**.

increasing extracellular K^+. Gap junctional modulation by neuronal activity was lost upon Cx30 KO while not affected by Cx43 KO, pointing to a major role of Cx30. K_{ir} channels were suggested to be involved based on channel inhibition experiments with Ba^{2+} (441). In the ventral posterior nucleus of the thalamus, astrocytes and oligodendrocytes also form coupled networks that are limited by the barreloid borders (76). Experiments with biocytin or the fluorescent nonmetabolizable glucose probe 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxyglucose (2-NBDG) demonstrated that coupling was decreased by suppression of neuronal activity with TTX.

2. Glutamate

Work by Rouach et al. (440) in mouse hippocampal brain slices has demonstrated that glutamatergic synaptic activity mediated by AMPA receptors strongly stimulates the intercellular trafficking of 2-NBDG via astrocytic GJCs. Surprisingly, modulatory effects on dye coupling were absent when biocytin or sulforhodamine B were used as markers, indicating that increased 2-NBDG spread was not caused by increased GJIC but was rather the result of increased glucose demand at a distance from the location where the patched astrocytes were dye-injected. The absence of modulatory effect on biocytin diffusion stands in contrast to results obtained by Serrano et al. (468) on the same preparation who demonstrated that NMDA application to the slices enhanced the intercellular spread of biocytin, which was inhibited by TTX. This response was only observed in cells with linear electrical membrane properties corresponding to cells highly coupled by GJs, while it was not present in outwardly rectifying astrocytes that are not coupled and presumably

correspond to NG2 glial cells. Possibly, the distinct outcomes of these two studies regarding modulation of intercellular biocytin diffusion may relate to differences in glutamatergic signaling, involving NMDA receptors in the Serrano et al. study (468) and predominantly AMPA receptors in the Rouach et al. study (440).

Mouse cerebellar slices have been used to demonstrate that kainate activation of AMPA receptors is associated with decreased GJIC between Bergmann glial cells, shown with electrophysiological approaches and LY dye spread (337). The effect disappeared with removal of extracellular Ca^{2+}, suggesting that Ca^{2+} entry in Bergmann glial cells mediated the decreased GJIC.

3. Norepinephrine

Norepinephrine is an important neurotransmitter, released by locus ceruleus neurons that widely connect in the brain, thereby influencing neurons as well as glial and vascular cells. Norepinephrine modulates astrocytic GJIC; norepinephrine and pharmacological agonists and antagonists mediate α_1-adrenergic stimulation inhibiting LY dye coupling in striatal astrocyte cultures (181). This effect involved both phospholipase C (PLC)-Ca^{2+}-protein kinase C (PKC) and phospholipase A_2 (PLA_2)-arachidonic acid signaling. β-Adrenergic stimulation with isoproterenol slightly increased coupling, which was promoted by addition of a phosphodiesterase inhibitor pointing to involvement of cAMP signaling. Thus adrenergic signaling can either inhibit or enhance astrocyte GJIC, involving Ca^{2+}-PKC or cAMP signaling, respectively. Interestingly, α_1-adrenergic stimulation also reduced intercellular Ca^{2+} wave propagation in cultured hippocampal astrocytes (340), a process that is dependent on the amount or degree of GJIC and HC activity (184, 460) (glial Ca^{2+} waves are discussed in sect. VI*D*).

4. Cannabinoids

Cannabinoids are another class of neuromodulators with effects on various neurotransmitter systems. Anandamide, an arachidonic acid metabolite and endogenous ligand of CNS CB_1 receptors, was demonstrated to inhibit GJIC in cultured striatal astrocytes (527). The effect was not mediated by CB_1 receptors and resulted in decreased intercellular Ca^{2+} wave propagation. Additionally, arachidonic acid itself, as a substrate of several phospholipase pathways, inhibits astrocytic GJIC, as do various other *cis*-unsaturated fatty acids, and inhibition has been linked to activation of PKC (274), Ca^{2+} entry, or arachidonic acid byproducts (313). Further details on cannabinoid modulation of Cx channels are given in section III*C*.

5. Endothelins

These peptides are considered the strongest endogenous vasoconstrictive agents currently known, and thought to be mainly involved in pathological responses. Two endothelin isoforms, endothelin-1 and endothelin-3, were reported to strongly inhibit LY dye coupling in an extracellular Ca^{2+}-dependent manner in confluent astrocyte cultures (176). This observation was then validated in acute hippocampal slices (37).

6. Extracellular ATP

The ubiquitous extracellular messenger ATP also inhibits LY dye coupling and Ca^{2+} wave propagation in rat astrocyte cultures, and the effect depends on P2Y receptors and activation of the PLC-Ca^{2+}-PKC signaling cascade (140). Inhibition was also documented using dual whole cell recording on astrocyte pairs in hippocampal slices and was shown to be potentiated by pretreating the cells with the cytokine interleukin (IL)-1β (326). ATP inhibition of GJIC may appear contradictory given its crucial role as an extracellular messenger of intercellular Ca^{2+} wave propagation (281) (see sect. VI*D*). It is however known that GJIC is not inhibited by the passage of a Ca^{2+} wave while it is inhibited by a sustained (minutes) $[Ca^{2+}]_i$ elevation induced by ionomycin (72). Thus, given the ATP-P2Y-Ca^{2+} linkage, minute-long ATP presence may inhibit GJs, while short-lived dynamic ATP changes occurring during Ca^{2+} wave propagation may exert less pronounced GJ effects.

7. Nitric oxide

Nitric oxide (NO) is another universal messenger that not only affects but also permeates GJCs (149). Evidence for its effects on astrocyte GJIC comes from experiments with lipopolysaccharide (LPS), a bacterial endotoxin and immune activator. Challenging cultured rat astrocytes with LPS upregulates inducible NO synthase, with increased NO production that inhibits astrocytic GJIC (11, 41, 65, 109, 288, 423). The effect on GJIC appears to be mediated by peroxynitrite formed by NO interaction with superoxide anions (41).

8. Lipopolysaccharide

LPS was demonstrated to inhibit LY dye coupling in cultured astrocytes via Toll-like receptor 4 (TLR4) signaling and subsequent downregulation of caveolin-3 (288), upregulation of phosphorylated forms of stress-activated protein kinase/c-Jun NH_2-terminal kinase and increased ubiquitin-proteasome proteolytic degradation of Cx43 (287). LPS lowering of astroglial Cx43 was confirmed in an in vivo mouse model with intraperitoneal injection of LPS (368). Interestingly, intraperitoneal LPS injection also leads to loss of Cx47 GJs in oligodendrocytes (368). Reduced astroglial Cx43 has been suggested to dampen the inflammatory reaction (582). However, LPS lowering of Cx43 seems to depend on the presence of microglial cells: astrocyte-microglial cocultures showed no effect of LPS on the amount of

Cx43 in cocultures with 5% microglial cells, while Cx43 was decreased by LPS in cocultures with 30% microglial cells (146, 198, 213; see also Ref. 423). It should be noted that the presence of microglial cells by itself already decreases the amount of astrocyte Cx43 when kept in coculture (439). Treating microglial cells with LPS before coculturing with astrocytes further decreased the amount of Cx43 and LY coupling between astrocytes (213, 325), indicating a role of microglial factors described further below. However, LPS did not decrease dye coupling in hippocampal brain slices, and this was not due to compensatory upregulation of Cx30 (5).

9. Pro-inflammatory cytokines

The LPS effect on astrocyte-microglial cocultures was mimicked by adding conditioned media from LPS-activated microglial cultures to astrocyte monolayers, resulting in decreased Cx43 expression and the degree of GJIC (213, 325). In follow-up work, tumor necrosis factor (TNF)-α and IL-1β were identified as two major microglial pro-inflammatory cytokines responsible for this effect (423), which is in line with the earlier finding of John et al. (241) that IL-1β inhibits GJs in primary human fetal astrocytes.

10. Fibroblast growth factor

Several members of the fibroblast growth factor (FGF) family act as inhibitors of astrocyte GJIC. FGF-2 reduced dye coupling in astrocyte cultures derived from cortex and striatum but had no effect on mesencephalic astrocytes (426). In contrast, FGF-5 reduced coupling in mesencephalic astrocytes but not in cortical or striatal astrocytes (427). FGF-9 reduced coupling in all three types of astrocytes. Moreover, it has been shown that FGF-1 reduced dye coupling in spinal cord astrocytes (175). Growth factors from the transforming growth factor-β (TGF-β) family also affect dye coupling: TGF-β1 increased astrocyte coupling but decreased coupling of the poorly coupled C6 glioma cells; this was associated with increased and decreased Cx43 phosphorylation, respectively (434). Moreover, TGF-β3 decreased coupling of cortical and striatal, but not mesencephalic astrocytes (426).

11. Hypo-osmotic conditions

Finally, exposure of astrocytes to hypo-osmotic solutions has been reported to promote GJIC, possibly in response to $[Ca^{2+}]_i$ changes (461).

C. Regulation and Modulation of Hemichannels

In many cases, HCs are distinctly regulated compared with GJCs, and often the responses to modulatory influences are oppositely directed **(TABLE 1)**. Here, we discuss other conditions/triggers leading to HC opening based on evidence obtained in brain slices, neural tissues, or in vivo.

1. Effect of pro-inflammatory cytokines

Cell culture work has demonstrated that LPS activation of microglia or direct application of TNF-α/IL-1β to astro-

Table 1. *Agents/conditions provoking opposite responses of Cx43 hemichannels and gap junction channels*

Conditions/Agents	Reference Nos.
Conditions/agents that enhance HC opening while closing GJCs	
Quinine	GJCs (309); HCs (496)
G138R, G60S, and R76W Cx43 mutants	125, 126, 264, 517, 569
Intracellular loop-tail interaction in the Cx43 protein	GJCs reviewed in Ref. 107; HCs (235, 403, 552)
$[Ca^{2+}]_i$ elevation up to ~500 nM	GJCs (275, 297); HCs (330)
Arachidonic acid and metabolites	GJCs (181); HCs (80, 101)
Anandamide	GJCs (528); HCs (524)
Metabolic inhibition	GJCs (80); HCs (80, 452)
TNF-α/IL-1β	GJCs (325); HCs (423)
LPS	GJCs (146, 198, 287, 288); HCs: direct effects of LPS (101), indirect effects via microglial activation (15, 158)
Staphylococcus aureus	GJCs/HCs (248) (also Cxs other than Cx43)
FGF-1/FGF-2 and others	GJCs (426, 427); HCs (101, 175) (FGF-2 effects depend on cell type)
Aß peptides	GJCs (325); HCs (164, 373, 571)
Agents that close HCs and enhance GJ coupling	
L2 peptide, RRNY peptide	GJCs reviewed in Refs. 107, 534; HCs (163, 403)
Paroxetine	237

$[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; Cx, connexin; FGF, fibroblast growth factor; GJCs, gap junction channels; HCs, hemichannels; IL, interleukin; LPS, lipopolysaccharide; TNF, tumor necrosis factor.

cytes increases HC open probability (423). Importantly, these experiments were performed at a normal extracellular Ca^{2+} concentration of ~1.8 mM, i.e., under realistic conditions that do not promote HC opening by themselves (low extracellular Ca^{2+} conditions have been applied to enhance responses induced by other trigger conditions; e.g., Ref. 496). HC opening in response to TNF-α/IL-1β were absent in astrocytes isolated from Cx43 KO mice. TNF-α/IL-1β treatment furthermore decreased the cell surface levels of Cx43, suggesting that enhanced HC opening was the result of increased HC open probability rather than an increased pool of HCs in the plasma membrane (423). Exposure times to LPS-conditioned microglial media or to TNF-α/IL-1β in this study were 24 h; however, recent data indicate that TNF-α enhancement of HC opening is already observed within a time frame of a minute (J. C. Sáez and L. Leybaert, unpublished data). Interestingly, the effects on both GJCs and HCs involved a p38 mitogen-activated protein kinase (MAPK)-dependent pathway. In contrast, the sulfhydryl reducing agent dithiothreitol rapidly reversed the effect on HCs but not on GJCs (423), indicating that only HC modulation is redox-dependent. Modulatory cannabinoid agonists prevent the LPS-triggered microglial release of TNF-α/IL-1β (158), thereby silencing the inflammation-related HC opening. Finally, inflammatory brain abscesses following inoculation of the Gram-positive bacterium *Staphylococcus aureus* in mice striatum have confirmed that HC function is indeed increased and GJIC is decreased in the vicinity of the abscess (248).

2. Effects of FGF

Members from the FGF family have also been reported to oppositely modulate HC versus GJC functional state. Work performed on cultured spinal cord astrocytes demonstrated that FGF-1 promotes Cx43 HC opening while reducing GJIC (175). However, studies on astrocytes isolated from Cx43 KO mice and siRNA-based interference with astrocytic Panx1 expression indicated that Cx43 HC opening was preceded by Panx1 channel opening. This cascade of channel openings involved FGF-1-induced ATP release via vesicular (botulinum toxin-sensitive) mechanisms that sparked these events via $P2X_7$ receptor activation (175). For FGF-2, work in C6 glioma cells stably transfected with Cx43 has demonstrated that this growth factor triggers Cx43 HC opening (101). In contrast, in astrocyte cultures and cultured hippocampal slices, FGF-2 and epidermal growth factor were found to inhibit HCs via the MAPK cascade, an effect that was counteracted by IL-1β (335).

3. Effects of metabolic inhibition

Exposure of cultured astrocytes to metabolic inhibition induced by antimycin A, an inhibitor of oxidative phosphorylation (complex III inhibitor), and iodoacetic acid, a glycolysis inhibitor (targeting glyceraldehyde-3-phosphate dehydrogenase), triggered ethidium bromide (EtBr; mol wt 394) dye uptake after 30 min exposure, which plateaued near 90 min. Moreover, astrocytes isolated from hGFAP-Cre:$Cx43^{fl/fl}$ mice did not show LY (~457 mol wt) dye uptake under the same conditions, pointing to the involvement of Cx43 HCs (80). Interestingly, GJIC was reduced by metabolic inhibition, indicating opposite regulation compared with HCs. In terms of the signals leading to HC opening, the authors proposed involvement of Cx43 dephosphorylation. Indeed, metabolic inhibition increased the number of HCs on the cell membrane assayed by biotinylation of surface proteins and Western blot analysis, and this response was followed by increased dephosphorylation and *S*-nitrosylation of Cx43 (422). The effects of metabolic inhibition on astrocytic Cx43 channel gating currently remain uncharacterized; however, work in cardiomyocytes has demonstrated that metabolic inhibition with iodoacetic acid and carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP) increases opening activity (552).

Follow-up work in cultured astrocytes demonstrated HC dye uptake not only in response to antimycin A/iodoacetic acid but also to oxygen and glucose deprivation (OGD). The HC response involved Ca^{2+} signaling and signaling via the serine/threonine protein kinase Akt, which consequently increased the plasmalemmal Cx43 pool and thereby enhanced dye uptake (452). Making use of a similar in vitro model of OGD-exposed astrocytes, Li et al. (286) reported that Gap26 peptide (see **FIGURE 6)**, which has been demonstrated to rapidly block HCs and with some delay also GJs (reviewed in Ref. 280), decreased the post-OGD presence of Cx43 in the cytoplasm and increased Cx43 presence in the plasma membrane. The decreased presence of Cx43 in the cytoplasm was explained by Cx43 ubiquitination and degradation, while increased plasma membrane presence was linked to increased Akt phosphorylation of Cx43, promoting forward transport. These results suggest that Gap26 may have effects beyond its channel-inhibiting effects, which needs to be further scrutinized.

OGD has also been demonstrated to enhance opening of Panx1 channels in cultured astrocytes, based on ATP release measurements (233). Here, Panx1 channel opening was explained by a suppression of $P2X_7$ receptor activity. However, this needs to be contrasted with other studies reporting $P2X_7$ receptors rather enhance Panx1 channel function (175, 392, 450). In any case, the interactions between $P2X_7$ and Panx1 are complex and characterized by bidirectional effects at the level of molecular interactions, ATP release and others (52).

4. Hemichannel opening depends on neuronal activity

Work on acute hippocampal slices from mice has demonstrated spontaneous uptake of EtBr in stratum radiatum astrocytes under basal nonstimulated conditions (68). EtBr uptake was inhibited by the nonselective Cx HC inhibitor

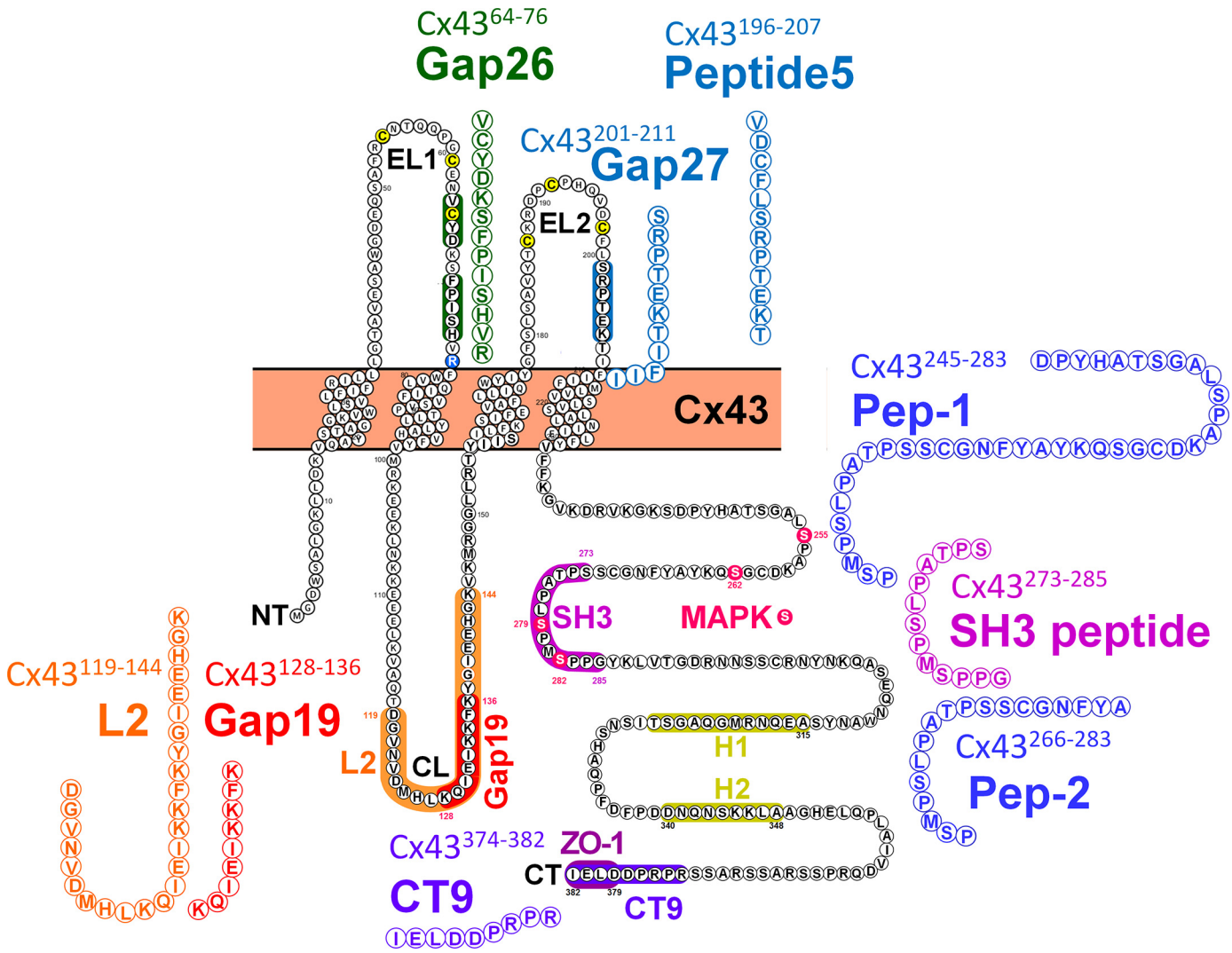

FIGURE 6. Peptides affecting connexin (Cx) channel function. The figure illustrates the Cx43 topology with indication of various mimetic peptide sequences affecting Cx43 channel function. L2 and Gap19 inhibit Cx43 hemichannels (HCs) without inhibiting gap junctions (GJs) (403, 552); L2 additionally prevents GJ closure (reviewed in Ref. 107). RRNY acts like L2/Gap19 (163, 534). Gap26 and Gap27 rapidly inhibit HCs (minutes), more slowly inhibit GJs (hours), and affect Cx43 but also other Cxs (reviewed in Ref. 280). Peptide5 inhibits HCs at low concentration and GJs at higher concentration (365); it acts on Cx43 and presumably also on other Cxs. Pep-1 rescues lost Cx43 HC function in astrocytes from mice where the mitogen-activated protein kinase (MAPK) phosphorylation site is inactivated by substituting Ser by Ala residues (156). Pep-2 acts as a cSrc inhibitor that inhibits Cx43 HCs in astrocytes (167). CT9 and SH3 peptides enhance Cx43 HC opening (40, 235, 403). The MAPK phosphorylation sites are indicated on red background circles. The yellow Cys residues in EL1/EL2 are conserved residues involved in HC docking and GJ formation. Several important domains are illustrated including the VCYD/FPISH motifs on EL1, SRPTEK on EL2 and the L2, Gap19 sequences on the CL. The CT-located SH3- and ZO-1-binding domains are interaction sites for the L2 and Gap19 peptides. The two α-helical domains H1 and H2 are organized domains within an otherwise intrinsically disordered CT.

carbenoxolone (Cbx; 200 μM) and Gap26 (100 μM) and reduced in slices from astrocytic hGFAP-Cre:Cx43$^{fl/fl}$ mice. In contrast, EtBr uptake was not affected by the Panx1 channel blocker ^{10}Panx1. Gap26 furthermore inhibited basal ATP release from brain slices. Apparently, the released ATP promoted basal neuronal excitatory synaptic activity as the excitatory postsynaptic current (EPSC) amplitudes were reduced by PPADS and RB2, two broad-spectrum P2 receptor antagonists. In the presence of P2 receptor blockade, Gap26 had no effect, suggesting that HC-linked ATP release impacts neuronal activity and sets the tone of excitatory synaptic signaling.

Evidence for activity-dependent enhancement of astrocytic HC opening also came from work performed in mouse olfactory bulb slices (442). In this preparation, mitral cells display spontaneous slow (~1 Hz) oscillations between "up" and "down" states that are correlated with glomerular local field potentials and network activity. Interestingly, the slices showed EtBr uptake in glomerular astrocytes that

was decreased when the spontaneous slow oscillatory activity was depressed by TTX. Dye uptake was also decreased in brain slices from astrocytic Cx43/Cx30 dKO mice, supporting the possibility that HC activity occurred in astrocytes. In addition, work with Gap26 applied for a short time (<15 min, to prevent effects on GJCs; see sect. IV*B*) showed dye uptake inhibition in brain slices from $Cx30^{-/-}$ mice, indicating involvement of Cx43-based HCs. Further studies on the effect of dKO on the slow oscillations between up and down states revealed that the upstate amplitude (a depolarized perithreshold state amplitude) was decreased in dKO animals, consequently resulting in a lower firing rate. In line with the dye uptake HC studies, $Cx30^{-/-}$ had no effect on the upstate amplitude, while astrocyte-specific Cx43 KO (hGFAP-Cre:$Cx43^{fl/fl}$) reduced it, confirming Cx43 as the major Cx involved.

Neuronal activity has significant effects on the extracellular concentration of K^+ and Ca^{2+}, with extracellular K^+ more than doubling and extracellular Ca^{2+} decreasing to half the normal concentration (202). Torres et al. (517) investigated this further by using photoactivation of the Ca^{2+} buffer diazo-2 to locally reduce extracellular Ca^{2+} in acute mouse hippocampal slices. This induced local ATP release and astrocyte $[Ca^{2+}]_i$ dynamics including slowly propagating Ca^{2+} waves (~4 μm/s) and oscillations following the wave. Stimulation of synaptic activity by local photoactivation of caged glutamate also provoked extracellular Ca^{2+} lowering and induced ATP release that subsequently triggered slow intercellular Ca^{2+} waves. These responses were inhibited by 100 μM Cbx and were strongly reduced in brain slices from Cx30/Cx43 dKO mice while deletion of Cx30 alone had no effect. Finally, they demonstrated that high-frequency electrical stimulation produced a similar cascade of events (extracellular Ca^{2+} lowering, ATP release, intercellular Ca^{2+} waves), but the Ca^{2+} waves were found to propagate at two different speeds: one propagating fast and associated with neuronal activity, and the other propagating more slowly and associated with a glial activity **(FIGURE 9)**. These results elegantly demonstrate that Cx43 HCs can be activated under physiological condition by neuronal synaptic activity as a result of a lowering of extracellular Ca^{2+}. Functional consequences of HC opening and associated Ca^{2+} waves are summarized in **TABLE 2**.

Evidence for basal and stimulated HC activity in astrocytes is also available from in vivo dye uptake studies (524). The authors applied EtBr to the exposed cortex of anesthetized mice and followed dye uptake into deeper lying cells making use of two-photon microscopy. They found basal uptake in astrocytes but not in microglial cells, which was inhibited by Gap26 and the Cx channel inhibitor flufenamic acid while ^{10}Panx1 peptide had no effect. Dye uptake was increased in cells in the vicinity of a laser ablation lesion (~15 μm diameter). The local laser-induced brain parenchymal injury triggered fast migration of microglial cells towards the ablation site, encapsulating the injury within 40 min. Migration of microglial cells was driven by astroglial ATP release through HCs, based on experiments with apyrase and flufenamic acid. Also arachidonic acid metabolites and the cannabinoid neurotransmitter anandamide increased astrocytic HC opening and enhanced the microglial response to the acute laser-induced injury, based on genetic inactivation of the anandamide-degrading enzyme fatty acid amide hydrolase (FAAH); the effects were dependent on cannabinoid CB1 receptors. Thus, while anandamide inhibits GJCs as described above (528), it enhanced HC activity, similar to the effects of pro-inflammatory conditions, FGF, and metabolic inhibition **(TABLE 1)**.

IV. PHARMACOLOGY: A GENERAL UPDATE

There is a need for selective modulators of Cx- and/or Panx-based channels due to two main reasons: *1*) to enhance or reduce their functional state to elucidate their role in different cell types and tissues, and *2*) to demonstrate the importance of gain or loss of function of these channels in different genetic or acquired pathological conditions. Several excellent in depth reviews on blockers of Cx- and Panx-based channels provide a background for what is discussed below (38, 88, 280, 535, 562).

Table 2. *Functional effects of glial Cx-based intercellular Ca^{2+} waves*

Brain Region (species)	In Vitro/In Vivo, Anesthesia	Wave Trigger	Evidence for Cx Involvement	Functional Effect	Reference Nos.
Embryonic cortical plate (rat)	In vitro slices, partial brain preparation	Spontaneous	Cbx, octanol, low pH	ATP-dependent synchronization of radial glia cell cycle	559
Hippocampus (mouse)	In vitro slices	Low $[Ca^{2+}]_e$, glutamate	Cbx, Cx30/Cx43 dKO	ATP-dependent activation of inhibitory interneurons	517
Brain cortex (mouse)	In vivo, ketamine	Laser ablation	Flufenamic acid and Cbx	ATP-dependent microglial migration	91
Cerebellum (mouse)	In vivo, urethane	Spontaneous	Cbx	↓ local field potentials; ↓ blood flow	266

$[Ca^{2+}]_e$, extracellular Ca^{2+} concentration; Cbx, carbenoxolone; Cx, connexin; dKO, double knockout.

A. Cx Channel Inhibitors

Connexin channels are inhibited by a large family of molecules with diverse chemical structure that invariably affect both GJCs and HCs. Traditional GJC/HC blockers are long carbon chain alcohols or derivatives of glycyrrhetinic acid, oleamide, anandamide, and fatty acid derivatives. They are liposoluble molecules with EC_{50} in the micromolar range, implicating they are low-affinity nonselective blockers of GJCs. For instance, in primary cultures of microglia exposed to pro-inflammatory conditions, GJCs and HCs have been blocked with traditional GJC blockers including 100 μM 18α-glycyrrhetinic acid and 0.5–1 mM octanol (142, 174, 312, 449). In striatal astrocytes GJCs are inhibited by anandamide (528) and in rat glial cells by 100 μM oleamide (196). Cbx, a glycyrrhetinic acid derivative, has been extensively used as a Cx channel blocker because of its good water solubility. However, Cbx needs tens of micromolars to inhibit GJCs, which also inhibits Panx1 channels (further discussed below) (55, 491). Since the aforementioned nonselective GJC blockers are lipophilic, it is reasonable to think that their site of action might be located within lipophilic pockets of the Cx protein facing the membrane lipids.

In addition, Cx channels are also inhibited by anesthetics and anti-inflammatory compounds. General anesthetics including propofol, etomidate, halothane, enflurane, isoflurane but not diazepam, morphine, ketamine, thiopental and clonidine inhibit GJCs in cultured astrocytes (308). In acute cortical slices, propofol has a strong inhibitory effect on GJCs and HCs, while ketamine and dexmedetomidine are weaker inhibitors (290). With regard to anti-inflammatory compounds, fenamates like flufenamic acid, niflumic acid, and meclofenamic acid are nonsteroidal cyclooxygenase inhibitors that inhibit GJCs with an IC_{50} in the order of 25–40 μM (204, 492) and also inhibit HCs (50 μM flufenamic acid; Ref. 189). Flufenamic and niflumic acid additionally inhibit Panx1 channels at millimolar concentrations (301). Mefloquine is, like quinine, an antimalarial drug that has some specificity for Cx36, a microglial but also neuronal Cx, which is inhibited with an IC_{50} of ~300 nM, making it one of the most potent GJC inhibitors currently known (86). At tens of micromolar concentrations, it also inhibits GJCs composed of Cx43, Cx32, and Cx26, as well as vascular Cx40 (570) and also Cx26-based HCs (IC_{50} of ~16 μM; Ref. 278). The flip side is that the substance has several side effects as discussed in Ref. 535, so experimental conditions should be well controlled to avoid these interfering effects, as is the case with most small molecule inhibitor substances.

While the substances discussed so far inhibit both GJCs and HCs, some substances have slightly different and in some cases more selective effects on the two channel types. For example, some glycyrrhetinic acid derivatives have been claimed to specifically inhibit HCs (510); Cbx furthermore inhibits HCs at lower (~10 μM) concentrations than GJCs (~50 μM) (491, 572). Cx antibodies directed against extracellular loop (EL) domains rapidly inhibit HC activity (217, 370, 371, 472) but also display delayed inhibition of GJCs, presumably because the bulky molecule interferes with HC docking (reviewed in Ref. 432). Synthetic peptides identical to or mimicking defined domains in various locations of the tetraspan Cx protein have more clear distinctive effects on HCs and GJCs. Gap26 and Gap27 peptides that are identical to a stretch of amino acids on EL1 and EL2, respectively **(FIGURE 6)**, inhibit HCs within minutes but, like antibodies, result in delayed inhibition of GJCs (reviewed in Ref. 280). Peptide5 that partly overlaps with Gap27 inhibits HCs at micromolar concentrations while GJs need hundreds of micromolars for inhibition (256, 365). L2 peptide that is identical to an amino acid stretch on the cytoplasmic loop of Cx43 prevents GJC closure upon intracellular acidification (107, 466), while inhibiting Cx43 HCs (403). Gap19, a 9 amino acid sequence within the L2 region, inhibits Cx43 HCs while not inhibiting GJCs (4, 156, 330, 552, 557). Interestingly, antidepressant drugs applied at clinically relevant concentrations also appear to distinctly modulate dye coupling versus dye uptake in primary cortical astrocyte cultures, with fluoxetine (10 μM) inhibiting GJCs and HCs, paroxetine (5 μM) enhancing GJC coupling while inhibiting HCs, and duloxetine (5 μM) not affecting GJ coupling but inhibiting HCs (237).

B. Cx Hemichannel Inhibitors

As introduced above, there are several substances that, under certain conditions, preferentially inhibit HCs relative to GJCs. Lanthanum ions (La^{3+}) inhibit HCs while not inhibiting GJCs (11, 79). However, this trivalent ion has a large array of other effects including inhibition of Ca^{2+} channels (334, 576), limiting its use to in vitro recording conditions that allow control or avoidance of side effects. With respect to a more rationalistic design of Cx channel inhibitors with improved selectivity, synthetic peptides currently offer the best opportunities. As a result, peptide HC inhibitors have opened opportunities for use in translational animal disease models (108).

1. Gap26 and Gap27 peptides

Gap26 and Gap27 were the first mimetic peptides identical to defined extracellular loop sequences of the Cx protein that were demonstrated to inhibit GJCs (553). Eight years later, it was realized these peptides inhibit HCs largely before they start inhibiting GJCs (51, 143; reviewed in Ref. 280). Indeed, Cx43 HCs are inhibited by Gap26 or Gap27 with a time constant of ~150 s and ~225 s, respectively, while the latency for effects on GJCs is variable and ranges between tens of minutes up to >6 h (95, 104, 118, 375). The IC_{50} for Cx43 HC inhibition is ~80 μM for Gap26 and ~160 μM for Gap27 (551), and typical concentrations of 200–300 μM are normally used. These concentrations are

higher than the IC_{50} of 20–30 μM reported to inhibit rhythmic vasomotor activity in isolated endothelium-denuded arterial rings, which is obviously not a direct measure of GJC coupling (64). The high concentrations necessary for HC inhibition have been criticized to deliver nonspecific inhibition by steric block of the channel pore as judged from experiments on $Cx32E1^{43}$ (chimeric Cx32 with EL1 replaced by the corresponding Cx43 sequence) in the *Xenopus* oocyte expression system (549). Wang et al. (551) scrutinized this by testing inactive scrambled Gap26/Gap27 sequences and found them to nonspecifically inhibit Cx43 HCs starting from 1 mM concentrations on; lower concentrations of these inactive peptide versions had no inhibitory effects, indicating that 200–300 μM of active Gap26/Gap27 sequences do not inhibit HCs by nonspecific effects. The Gap27 inhibition of fear memory consolidation by microinfusion in the rat basolateral amygdala (BLA) of 1 mM of this peptide (493) may possibly include unspecific steric pore block effects at these high concentrations.

The sequences mimicked by Gap26 and Gap27 peptides include the VCYD and FPISH motifs on EL1 (Gap26) and SRPTEK on EL2 (Gap27) **(FIGURE 6)**. The presence of a Cys residue in Gap26 makes this peptide chemically less stable compared with Gap27. Outside these conserved motifs, the Gap26/Gap27 sequences may slightly differ between species and also between Cx isotypes. Gap26 and Gap27 sequences mostly used are those from Cx43 (for a comparison of human Gap26 and Gap27 sequences for astrocytic Cx43, Cx30, and Cx26, see Table 1 in Ref. 566). The Cx43-based sequence of Gap27 is the same as for Cx37, and Gap27 will thus also inhibit vascular Cx37 HCs/GJCs when used in vivo. Although the Cx43 Gap26 sequence is different from the corresponding one of Cx37, Cx43-based Gap26 inhibits Cx37 HC opening (375), again pointing to possible vascular side effects when used in vivo. It is currently unknown whether the Cx43 version of Gap26/Gap27 inhibits astrocytic Cx30 channels; it is likely they do not affect Cx26 channels, as demonstrated for Gap27 (566). Of note, Gap27 (200 μM) has been reported to inhibit Panx1 channels in the *Xenopus* oocyte expression system (549), but others failed to find any effect of Gap27 on Panx1 currents in a HEK293 cell readout (392). As mentioned in section IIIC*3*, Gap26 peptide has been demonstrated to increase the presence of Cx43 in the plasma membrane, suggesting it may have non-channel effects as well (286).

Gap26/Gap27 have been widely applied in in vitro experiments on glial cells and acute brain slices, but there are only a few studies where these peptides were applied in an in vivo brain disease context. One of these studies involved proinflammatory intravenous stimulation of rats with bradykinin, which induces BBB leakage assessed 30 min later; intravenous injection of Gap27 (25 mg/kg) together with bradykinin suppressed barrier leakage (95). A second study concerns a rat brain hypoxia/ischemia model, in which pretreatment with Gap26 or Gap27 significantly reduced cerebral infarct volume, while post-treatment with Gap26 24 h post-ischemia improved several functional recovery readouts (286). In this study, Gap26/Gap27 was intraperitoneally administered, with Gap26 showing most strong effects in a concentration range of 10–50 μg/kg while 5 μg/kg or below was ineffective.

2. Peptide5

Peptide5 is, like Gap27, composed of the Cx43-based SRPTEK sequence on EL2 preceded by the VDCFL sequence at its NH_2-terminal side and an extra Thr residue at its COOH-terminal side **(FIGURE 6)**. Interestingly, some evidence based on competition with other EL2-based peptides points to interaction of Peptide5 with EL2 (256); in contrast, the nature and location of the interaction sites for Gap26 and Gap27 are currently still unknown. Peptide5 inhibits HCs at ~5 μM, while GJs need 500 μM for inhibition (365). Given the conserved nature of its SRPTEK backbone, Peptide5 is expected to also affect HCs other than those composed of Cx43. However, the peptide's HC effects appeared to be very sensitive to sequence alterations in the preceding VDCFL sequence, with HC inhibition disappearing upon removal of this nonconserved sequence; this suggests that some Cx isotype specificity may be possible, which needs further verification. In contrast to this, Peptide5 inhibition of GJCs is not affected by removal of the VDCFL sequence (256).

Peptide5 has been tested in several animal disease models, which demonstrated a neuroprotective potential; below we highlight routes of peptide administration and doses used in brain ischemia and other ischemia models. In a sheep model of fetal brain ischemia induced by 30 min of reversible bilateral carotid artery occlusion, intracerebroventricular infusion of Peptide5 (50 μmol/kg in 1 ml) 90 min after ischemia reduced post-ischemic seizures/status epilepticus and reduced cell death of oligodendrocytes and neurons 1 wk later (94). These effects were more pronounced when post-ischemic infusion was prolonged to 25 h instead of being applied for only 1 h; application of the peptide during ischemia gave no protection (93). Long (25 h) post-ischemic treatment with Peptide5 also protected GABAergic neurons (166). Work in an in vivo retinal ischemia model demonstrated that Peptide5 also limits vascular leakage (90), which may mechanistically contribute to the observed protection against post-ischemic brain damage. A further account on the use of Cx channel blockers in the context of ischemia/stroke is given in section VIIC and in **FIGURE 10**.

3. L2 peptide

L2 is a 26-amino acid long peptide identical to a sequence on the cytoplasmic loop of Cx43 **(FIGURE 6)**, which prevents Cx43 GJC closure under acidifying conditions. This

effect was proposed to be mediated by binding of L2 to the COOH-terminal tail, thereby preventing tail-loop interaction that acts to close GJCs upon intracellular acidification according to a particle-receptor model (466; reviewed in Refs. 107, 280). Unexpectedly and in contrast to its preventive effect on acidotic Cx43 GJC closure, L2 peptide was found to inhibit Cx43-based HCs (403; reviewed in Ref. 234). The H126K/I130N mutant version of L2 (amino acid numbers corresponding to their location on the Cx43 protein) is an inactive control peptide that does not inhibit HCs (403). Because the L2 target (COOH-terminal tail of Cx43) is inside the cells, the peptide needs to be fused to a membrane translocation peptide such as the TAT peptide. TAT-linked L2 peptide (TAT-L2) inhibited Cx43 HC responses with an IC_{50} of ~10 μM (403). TAT-L2 was demonstrated to inhibit fear memory consolidation when in vivo microinfused into rat BLA at concentrations starting from 10 nM on with 1–10 μM giving complete inhibition of fear memory consolidation, while inactive TAT-$L2^{H126K/I130N}$ peptide had no effect (493). TAT-L2 inhibition of fear memory consolidation was counteracted by microinfusing a mix of putative gliotransmitter substances consisting of D-serine, glutamate, glutamine, glycine, ATP, and lactate (493). A further discussion of work performed with TAT-L2 can be found in section VI*B*.

4. Gap19

Gap19 is another Cx43 HC-inhibiting peptide consisting of a 9-amino acid stretch within the cytoplasmic loop-located L2 sequence (552). The peptide contains 6 amino acids (residues 130–136 of the Cx43 protein; see **FIGURE 6**) that play key roles in COOH-terminal tail interaction with the cytoplasmic loop of Cx43-based channels (48, 129, 215). Modification of residue 130 to a Thr (Gap19-I130T mutant) completely removes its HC-inhibiting effect (552). As already referred to in the context of L2 peptide, tail-loop interactions act to close Cx43 GJCs while they are necessary for HCs to become available for opening with electrical or chemical triggers (403, 552; reviewed in Ref. 280). Based on surface plasmon resonance experiments, the loop L2 domain interacts with at least two different regions of the Cx43 COOH-terminal tail, one located in the last 9 COOH-terminal amino acids (552) and a second that comprises a well-known SH3-binding domain (amino acids 273–285) (235) **(FIGURE 6)**. These two tail-located sites are the interaction sites for Gap19 and L2 peptides. Interestingly, co-addition of CT9 peptide, composed of the last 9 COOH-terminal amino acids, together with Gap19, dose-dependently counteracts Gap19 HC inhibition (552). When added alone, CT9 acts as an enhancer of Cx43 HC opening (40, 97, 235). Tail-loop interaction is disrupted when $[Ca^{2+}]_i$ rises above ~500 nM, which sets a brake on HC function (404). However, exogenous CT9 peptide can substitute for endogenous tail-located CT9 thereby counteracting the high $[Ca^{2+}]_i$ brake, keeping HCs available for opening, thus resulting in enhanced function; SH3 peptide (amino acids 273–285, **FIGURE 6**) has a similar enhancing effect on HC function (235). HC enhancing CT9 peptide together with inactive Gap19-I130T mutant peptide are good control tools for Gap19 experiments.

In terms of its GJC effects, Gap19 was previously identified as a control peptide for Gap26/Gap27 that had no effects on GJCs (personal communication with Dr. W. H. Evans, Cardiff University); Wang et al. (552) looked more carefully into this and found long Gap19 exposures to significantly enhance GJC coupling (13% increase after 24 h). Unlike L2 peptide that prevents acidification-induced GJC closure, Gap19 does not seem to prevent GJC closure induced by $[Ca^{2+}]_i$ elevation with ionomycin (557). Importantly, Gap19 does not inhibit Panx1 channels (552).

Gap19 has some intrinsic membrane permeability that is improved by adding a membrane translocation sequence such as TAT peptide. For comparison, the IC_{50} for Gap19 inhibition of Cx43 HCs is ~47 μM, while it is lowered to ~7 μM for TAT-Gap19 (552). TAT-Gap19 has been applied in several in vivo animal brain studies to investigate its effects on memory, convulsions, neurodegeneration in the context of Parkinson's disease, and stroke. In mice, intracerebroventricular microinfusion of TAT-Gap19 at a concentration of 1 nmol/μl (2.7 μg/μl) for 2 min at 0.5 μl/min significantly impaired spatial short-term memory in a delayed spontaneous alternation Y-maze task, without affecting locomotor activity or spatial working memory (544).

Below we discuss routes of administration and doses that have been used in various animal disease models (seizure/epilepsy, Parkinson's disease, stroke). TAT-Gap19 microdialyzed at 2.7 μg/μl (1 μM) in the hippocampus of freely-moving mice for 60 min (pretreatment) significantly decreased seizure duration induced by subsequent microdialysis of pilocarpine, a focal limbic seizure model (543). Pilocarpine seizure induction also increased microdialysate D-serine levels, which were suppressed by TAT-Gap19 pretreatment. The anticonvulsant action of TAT-Gap19 was furthermore reversed by D-serine microdialysis, suggesting that TAT-Gap19 protection against seizures is mediated by lowering extracellular D-serine levels. The anticonvulsive properties of TAT-Gap19 were further confirmed in electrical seizure mouse models, i.e., an acute 6 Hz model of refractory seizures (2.7 μg/μl TAT-Gap19 intracerebroventricular or 25 mg/kg ip administration) and a chronic 6 Hz corneal kindling model (TAT-Gap19 50 mg/kg ip) (543). In an MPTP neurotoxin Parkinson's disease mice model, TAT-Gap19 (4 × 23 mg/kg ip during the day of MPTP intoxication and once a day for another 2 days) protected against dopaminergic neuron degeneration (303). Neuroprotection was stronger in animals with astrocytic Cx30-driven glucocorticoid receptor KO ($GR^{Cx30CreERT2}$ mutant mice), in which case TAT-Gap19 also reverted microglial activation. In a permanent middle

cerebral artery occlusion (pMCAO) mice stroke model, 7.5 μmol/kg ip TAT-Gap19 administered 2 h post-stroke induction reduced the infarct volume to one-fifth of vehicle-treated controls 4 days after stroke; 0.75 μmol/kg ip still halved the infarct volume (156). In a transient MCAO mice stroke model, Gap19 was intracerebroventricularly injected at 300 μg/kg (10 μl), resulting in reduced infarct size (66).

As a final note, it is important to take into consideration that, when using peptides like TAT-Gap19 (or others like Gap26/27), these compounds have short half-lives in the blood, in the order of below 20 min (based on calculations described in Ref. 317). As a result, intraperitoneal administration is preferred over intravenous administration when delayed outcome assessment is done in the order of several hours post-administration. It has been shown that intraperitoneal injected TAT-Gap19 is present in GFAP positive astrocytes 80 min after injection, where it was associated with Cx43 (156).

A further account on Cx channel inhibition in the context of these disease models can be found in section VII, *A*, *C*, and *E2*.

5. Boldine

While the above discussion demonstrates some potential of Cx HC inhibiting peptides in animal disease models, neurodegenerative diseases like Alzheimer's disease, Parkinson's disease, and epilepsy are chronic in nature, therefore necessitating approaches that inhibit HCs over several months or even years. This requires further research to develop stable nontoxic HC inhibiting compounds, including small molecule compounds. With respect to this, boldine, an anti-inflammatory compound from the Chilean endemic Boldo tree, has been demonstrated to inhibit Cx43 HCs and Panx1 channels when applied at 100–500 μM in cultured astrocytes, without affecting GJCs; 1–5 mM are necessary to inhibit EtBr uptake in acute brain slices (573). Three months long in vivo oral boldine treatment (30 mg/kg added to the drinking water) of Alzheimer's disease APPswe/PS1dE9 mice prevented increased glial HC activity and diminished hippocampal neuritic dystrophies in the neighborhood of Aβ plaques (573) (for a more detailed account, see section VII*E3*). Given the known anti-inflammatory effects of this compound, the inhibitory effect of boldine on Ca^2-permeable HCs has been proposed to occur upstream from activation of Ca^2-dependent intracellular inflammatory pathways (573).

C. Panx Channel Inhibitors

The arsenal of Panx channel inhibitors involves small molecules like Cbx, probenecid, trovafloxacin, mefloquine, Brillant Blue FCF, spironolactone, and the ^{10}Panx1 peptide. Cbx is a nonspecific inhibitor that blocks Panx1 channels, including heteromeric Panx1/Panx2 channels, with an IC_{50} in the range of 2–5 μM (55, 301, 392), a concentration that does not inhibit HCs, which need 20–30 μM for half-maximal inhibition [data for Cx26 and Cx38 (430, 431)]. Panx1 inhibition by Cbx involves interactions with the Panx1 EL1 (333). However, others have reported that Cbx inhibits Cx HCs at an IC_{50} of ~5 μM (572) and inhibits $P2X_7$ receptors with an IC_{50} of 175 nM (498), i.e., at concentrations more than 10 times lower.

Probenecid is an organic anion transport inhibitor that has been used in the past to prevent tubular penicillin secretion in the kidney to achieve higher plasma concentrations and is nowadays a well-known gout drug that inhibits urate reabsorption in the kidney and therefore facilitates its excretion. It inhibits Panx1 channels with an IC_{50} of 150 μM while not inhibiting Cx32E1^{43} and Cx46-based HCs in a *Xenopus* oocyte expression system (473). Concentrations of 500 μM have been used to inhibit Panx1 channels in cultured microglia or astrocytes exposed to β-amyloid peptides or stress are inhibited by 500 μM probenecid (372, 373); 2 mM has been used to inhibit Panx1-linked hypotonic swelling-induced ATP release in cultured rat optic nerve astrocytes (24). Probenecid inhibition of Panx1 is, like Cbx, mediated by interactions with the EL1 of Panx1 (333). Like Cbx, it also inhibits $P2X_7$ receptors with an IC_{50} of ~200 μM (32). Probenecid traverses the blood-brain barrier but has a restricted distribution in the brain because of efficient removal mechanisms; intravenous infusion of 75 $mg.kg^{-1}.h^{-1}$ has been reported to result in ~100 μM probenecid in the hippocampus (105), which is below the IC_{50} mentioned above.

Trovafloxacin is a quinolone antibiotic that inhibits Panx1 channels with an IC_{50} of ~4 μM (405). In vivo intraperitoneal delivered trovafloxacin (60 mg/kg) has been demonstrated to mitigate neuroinflammation and improve outcome after traumatic brain injury in mice (173).

The anti-malaria compound mefloquine inhibits, in addition to its high-affinity inhibition of Cx channels, also Panx1 channels, but the effects seem to depend on the enantiomeric composition. This compound is available as a racemic mixture, and the (±)erythro-mefloquine form from selected providers has been reported to inhibit Panx1 channels with an IC_{50} of ~50 nM (232). However, mefloquine additionally inhibits $P2X_7$ receptors with an even higher affinity as reflected from the IC_{50} of ~2.5 nM (enantiomeric form not specified; Ref. 498). Given the mutual influences and interactions of Panx1 channels with $P2X_7$ receptor channels, combined with the strongly different IC_{50} of different enantiomeric forms, makes the interpretation of mefloquine effects on Panx1 a complicated task. The food dye Brilliant Blue G is another substance that affects both $P2X_7$ receptors and Panx1 channels; in contrast to this, Brillant

Blue FCF, another food dye, inhibits Panx1 channels with an IC_{50} of 270 nM without affecting $P2X_7$ receptors up to 100 μM concentration (548). These food dyes do not affect Cx43 channels. Spironolactone, a diuretic acting at the mineralocorticoid receptors, also has Panx1 inhibiting effects (190).

^{10}Panx1 is a 10-amino acid long peptide identical to a sequence on the EL1 of Panx1 (Trp74–Tyr83 of human Panx1). It inhibits Panx1 currents with an IC_{50} in the 30–50 μM range, achieving steady-state inhibition within 4–15 min (392). At 200 μM, the peptide has been shown to also inhibit Cx46 HC currents in the *Xenopus* oocyte expression system (549). For work with a scrambled inactive version of ^{10}Panx1, see Reference 513.

In conclusion, current approaches for inhibiting Panx1 channels in acute brain slices or in vivo should ideally be based on the use of several inhibitors, e.g., probenecid, ^{10}Panx1 peptide, and Brillant Blue FCF. For general Cx channel inhibition, Cbx remains the standard tool for the purpose of determining whether Cx channels are involved in a given response of interest. Microglial Cx36 channels can be inhibited by mefloquine, but this Cx is also present in neurons. Although Gap26/27 peptides have previously been claimed to inhibit GJCs, this effect invariably follows their more rapid action on HCs. For HCs, Peptide5 can be used, and for HCs composed of Cx43, TAT-coupled Gap19 or L2 peptides are the best choice currently available. ^{10}Panx1 and Peptide5 are not useful for in vivo use as they do not pass the BBB when systemically administered; in contrast, TAT-Gap19 passes the barrier and associates with astrocytic Cx43. A further account on distinguishing Panx from Cx channels can be found in Reference 388.

V. NON-CHANNEL FUNCTIONS OF GLIAL CONNEXINS

While for decades Cxs were only considered as channel constituents, an emerging concept relates to their involvement in other cellular functions. Indeed, the Cx43 COOH terminus contains multiple domains involved in protein interactions that permit crosstalk between Cx43 and cytoskeletal as well as regulatory proteins, with these domains endowing Cx43 with the capacity to affect cell growth and differentiation (277). More generally, non-channel functions have been shown in the context of cell growth, adhesion, migration, apoptosis, and signaling (538, 583). Here, we will limit our interest to channel-independent functions of Cxs that have been found in glia.

A. Brain Development and Neuronal Migration

Brain development is a highly dynamic and complex process involving cell proliferation, migration and differentiation, as well as extensive cell death (471). Glial cells are instrumental in orchestrating the migration of neurons to their definitive location within the developing CNS, and interactions in the context of migration are critical for proper morphogenesis. During this process, Cx43 and Cx26 are expressed in the radial glia contacting neurons migrating toward the cortical plate (35, 342) and in migratory neural crest cells (227). Given the highly regulated expression of Cxs during neurodevelopment, they are likely involved in several coordinated processes. Cx43 has long been associated with multiple aspects of cell proliferation (1, 537). This is consistent with a role for Cx43 to impact neurogenesis through regulating the cell cycle. One of the most dynamic processes underlying CNS development is cellular migration. One possible avenue for involvement of Cx43 in neuronal migration might be via the spread of intercellular Ca^{2+} waves (ICWs) (460) (See sect. VI*D*). This process has been demonstrated in cortical radial glia, astrocytes, and neuronal precursors (459, 559).

The most abundantly expressed Cx during CNS development is Cx43 and was the first Cx to be targeted in a KO strategy (418). This provided a unique opportunity to directly examine the role of Cx43 in CNS development, particularly in migration. Using BrdU labeling at different prenatal times, Fushiki et al. (162) demonstrated a delay in neuronal migration in the Cx43 KO mouse cortex. This was later confirmed by other KO studies (74, 561) as well as through the use of RNAi to knockdown Cx43 and Cx26 (137). A non-channel mechanism for Cx43 has been proposed through an adhesive role between neuronal progenitors and radial glial to facilitate migration (137). This is based on the extracellular domains of Cx43 interacting and docking with similar extracellular Cx domains exposed on adjoining cells to provide this adhesive force. However, the intracellular COOH-terminal region is also critical for neuronal precursor migration as indicated from experiments in haplodeficient mice expressing K258stop CT-truncated Cx43, in which neuronal progenitors have normal GJC function but exhibit disturbance in their migration to the cortical plate (74). This suggest that channel-independent intracellular signaling and cytoskeletal mechanisms are also involved in this migratory process.

B. Cell Signaling

In cultured astrocytes, it was found that deletion of Cx43 causes a rightward shift in the concentration-response curve to $P2Y_1R$ agonists, thus affecting glial Ca^{2+} signal transmission (459, 499). To discriminate whether these changes were due to a decrease in GJIC or to protein-protein interactions, $P2Y_1R$ function and expression levels were evaluated in Cx43 KO astrocytes transfected with full-length Cx43 as well as selected Cx43 domains. Results indicate that restoration of $P2Y_1R$ function is independent of GJIC and that the Cx43 COOH terminus spanning the SH3 bind-

ing domain (amino acids 260–280) participates in the rescue of $P2Y_1R$ pharmacological behavior without affecting its expression levels (458). These findings suggest that the Cx43 SH3-binding domain on the COOH-terminal tail (CT) provides an interaction/binding site for an intracellular molecule, most likely a member of the c-Src tyrosine kinase family, which affects $P2Y_1R$-induced Ca^{2+} mobilization. It was proposed that a channel-independent function of Cx43 is to serve as a decoy for these kinases. Such modulation of $P2Y_1R$ is expected to influence several neural cell junctions, especially under inflammatory and neurodegenerative conditions where the expression amount of Cx43 is decreased (458). The CT-located SH3-binding domain of Cx43 (see **FIGURE 6**) is part of an intrinsically disordered CT region that functions as a signaling hub where several proteins interact, including the protooncogene c-Src (186, 486, 487). This Cx43-Src interaction inhibits the oncogenic activity of Src and promotes a conformational change in the structure of Cx43 that allosterically modifies the binding to other important signaling proteins (508).

C. Control of Process Morphology in Astrocytes

The expression of Cx30 was reported to be involved in behavioral and basic cognitive processes, being upregulated in mice raised in enriched environments known to promote structural changes in the brain and to enhance learning and memory performance (410). Also, its deletion was shown to be associated with increased emotionality and decreased rearing activity in the open-field along with neurochemical changes (110). However, at that time, there were no transcriptomic studies dealing with gross systems level changes in gene expression that could account for these behavioral alterations. More recently, Cx30 KO work has demonstrated that astroglial Cx30 controls hippocampal excitatory synaptic transmission and plasticity through the modulation of astroglial glutamate transporters, thereby impacting synaptic concentration of glutamate levels (381). In particular, Cx30 KO mice showed decreased excitatory synaptic transmission mediated by AMPA receptors and impaired synaptic plasticity through modulation of synaptic glutamate levels. Interestingly, this effect is mediated by morphological changes controlling the insertion of astroglial processes into the synaptic cleft. Indeed, serial section electron microscopy has established the presence of Cx30 as a key molecular determinant of astroglial synapse coverage. Electrophysiological and behavioral tests performed with Cx30 KO and wild-type (WT) mice demonstrated its involvement in synaptic strength and memory. It was concluded that by setting excitatory synaptic strength, Cx30 plays a role in long-term synaptic plasticity and in hippocampus-based contextual memory. As a whole, these results identified Cx30 as a regulator of synaptic strength by controlling the synaptic location of astroglial processes and glutamate transporters and setting synaptic concentration of glutamate (381).

In addition to the influence of Cx30 on process formation, evidence is also available for Cx43. Primary cultures of mouse astrocytes, which express only Cx43 (261, 267), have been studied by transcriptomic and proteomic analysis (366). Transcriptomic studies of Cx43 null astrocytes reveal changes in cytoskeletal and other genes (229, 230). To analyze the influence of Cx43 on the astroglial proteome, RNA interference was used to downregulate the expression of this Cx in cultured mouse astrocytes. Gel electrophoresis analysis was used to compare silenced astrocytes with control astrocytes, and six regulated proteins were found to belong to a group of cytoskeletal proteins involved in cortical formation. These observations were confirmed by quantitative immunocytochemistry and immunoblotting that revealed an upregulation of actin, tubulin, tropomyosin, EB1, transgelin, and GFAP and a downregulation of Ser-3-phosphorylated cofilin. Finally, Cx43 silencing led to changes in cell morphology, migratory activity, and cell adhesion. In conclusion, this study links the expression of Cx43 to differentially expressed components of the cytoskeleton, which are involved in process formation and migration, thereby offering the chance to gain further insights into the mechanisms by which astrocytes can achieve changes of their structural phenotype.

VI. ROLES OF GLIAL CONNEXINS AND PANNEXINS IN BRAIN FUNCTIONS

A. Gliotransmission

The concept of gliotransmission was initially introduced to account for the active information transfer of neuroactive molecules from glia to neurons (30). This gliotransmission is favored by the so-called "tripartite synapse" where astroglial perisynaptic processes are closely associated to the pre- and postsynaptic elements **(FIGURE 7*A*)** (14). Recent insights indicate that the tripartite synapse should be rather considered as a multipartite entity that has been dubbed "the synaptic cradle," which additionally involves microglial cells facilitating synaptic plasticity (e.g., dendritic pruning) and extracellular matrix in the synaptic cleft and also present extrasynaptically (357, 530). Although gliotransmitters can in principle be released from several classes of glial cells, most data available show that they are primarily released from astrocytes **(TABLE 3)**. Evidence for gliotransmission was first revealed when increases in $[Ca^{2+}]_i$ in cultured astrocytes were shown to induce glutamate release followed by neuronal activation (384). Several gliotransmitter release pathways have been identified, including via vesicular (SNARE mediated, lysosomes, etc.) mechanisms, by channel- and transporter-mediated mechanisms, as well as by decreased transporter mediated neurotransmitter uptake in astrocytes. However, it should be kept in mind that recent

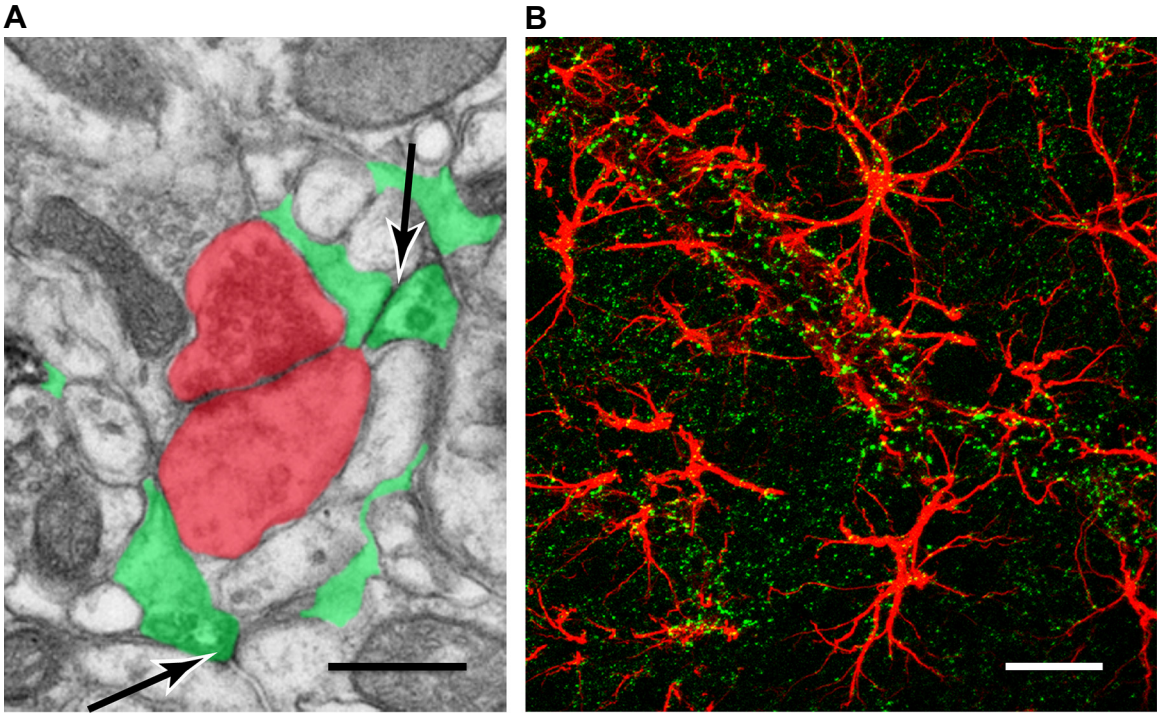

FIGURE 7. *A*: electron micrograph showing the closeness of two modes of intercellular communication: a synapse for neuronal communication and gap junctions (GJs) between astrocytes. Note that pre- and postsynaptic elements (in red) are surrounded by astrocytic processes (green). Arrows indicate GJ plaques between two astrocytic processes. *B*: astrocyte glial fibrillary acidic protein (GFAP)-positive (in red) and Cx30 staining (green) to reveal their expression between and within astrocytic domains at endfeet that wrap blood vessels. Size bar in *A* is 0.5 μm and in *B* is 25 μm. [Modified from Giaume et al. (179).]

observations have questioned the role of $[Ca^{2+}]_i$ in gliotransmitter release and thus require a revision of the relevance of Ca^{2+}-dependent gliotransmission in neuronal signaling (13, 148, 456). So far, at least three gliotransmitters have been demonstrated to contribute to astrocyte-neuron signaling, including glutamate, ATP, and D-serine that can be released by a Ca^{2+}-dependent vesicular mechanism in astrocytes (587). Of note, the astrocytic origin of D-serine has been challenged as serine racemase, a key enzyme in its synthesis, was recently reported to be mainly present in neurons and far less so in astrocytes; cell-targeted suppression of this racemase also seems to point to neurons as the main synthesis site (565).

Under normal conditions, gliotransmitter release could operate directly due to the rather high permeability of Cx and Panx channels as indicated by the uptake of fluorescent molecules with a molecular weight ranging from 350 to 650 Da (LY, EtBr, and others) that are traditionally used to study HC activity (183). In fact, so far only ATP has been extensively documented to permeate through Cx43 HCs in a C6 glioma cell expression system, based on combined single-channel recording and bioluminescence ATP measurements and ion substitution experiments (247).

While there are reports that link Cx43 or Panx1 to neuroactive molecules, there is no definitive demonstration that their release occurs directly through HCs. Indeed, since the pioneering work of Ransom's group, who observed glutamate release by astrocytes exposed to a divalent cation-free solution (572), several other studies have reported that Cx or Panx1 channels are involved in gliotransmitter release. In these studies, the main evidence was based on the use of HC/GJC blockers or KO mice affecting either Cx43 or Panx1. Alternatively, Cx/Panx HCs could be involved indirectly by being an upstream element of the release process. Cx43-based HCs are permeable to Ca^{2+} (464), and both Cx43 HCs and Panx1 channels facilitate ATP release that indirectly increases $[Ca^{2+}]_i$ via P2 receptors; as such, these $[Ca^{2+}]_i$ changes may activate downstream Ca^{2+}-dependent release events. Also, at least for Cxs, the homeostatic function of GJIC could contribute to gliotransmission either via the networking of coupled astrocytes or through reflexive GJCs that connect the fine arborized astrocytic extensions (the "gliapil") forming the majority of the astrocyte's volume (33). In sum, Cx43 HCs and Panx1 channels may be involved in gliotransmitter release, but the exact nature of their contribution needs to be further established.

B. Impact of Glial Connexins and Pannexins in Synaptic Activity and Plasticity

An exciting step forward over the last decade in the field of dynamic neuroglial interactions concerns the impact of gliotransmission on neuronal activity, in particular on syn-

Table 3. *Hemichannel involvement associated with gliotransmitter release*

Gliotransmitter	Stimulation	Cx/Panx Involvement	Contribution of $[Ca^{2+}]_i$	Pharmacology	Experimental Models	Reference Nos.
Glutamate	Divalent cation-free solution	Cx43	Independent	Carbenoxolone, flufenamic acid, α-GA	Cultured astrocytes	572
Glutamate	LPS	Cx43	Not tested	Carbenoxolone, Gap26, Cx43 KO	Hippocampal slices	5
Glutamate/ATP	Ultrafine particles	Cx43, Panx1	Not tested	Carbenoxolone, Gap27, ^{10}Panx1	Cultured astrocytes	556
ATP	Basal condition	Cx43	Not tested	Gap26, Cx43 KO	Hippocampal slices	68
ATP	Basal condition	Cx43	Not tested	Carbenoxolone, gadolinium, NPPB	Cultured astrocytes, hippocampal slices	247
ATP	Basal condition	Cx43	Not tested	Gap26, Cx43 KO	Olfactory bulb slices	442
ATP	ATP-induced ATP release	Panx-1	Not tested	Panx1 KO	Cultured astrocytes	231
D-Serine	ATP, divalent cation-free solution	$P2X_7R$/Panx1	Independent	Carbenoxolone, probenicid, ^{10}Panx1	Cultured astrocytes	378
D-Serine	Basal condition	Cx43	Ca^{2+} dependent	Gap26, carbenoxolone	Prefrontal acute slices	330
D-Serine	Pilocarpine-induced seizures	Cx43	Not tested	TAT-Gap19	In vivo microdialysis	543

$[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; Cx, connexin; KO, knockout; LPS, lipopolysaccharide; Panx, pannexin.

aptic transmission and plasticity (13). Recognition of this astrocyte-synapse interaction has not only contributed insight into the development and remodeling of synapses (8), but has consolidated the concept of "tri-partite synapse" which functionally associates perisynaptic astroglial processes to the pre- and postsynaptic elements (13, 14, 209). Initial evidence that Cx channels play a role in such dynamic neuroglial interactions was based on the observation of GJC-mediated astrocyte-to-neuron interaction using coculture models and acute brain slices. It was observed that $[Ca^{2+}]_i$ increases generated in astrocytes triggered a Ca^{2+} response in cocultured neurons (355). This intercellular signaling was abolished by Cx channel blockers, suggesting that astrocytic-neuronal signaling is mediated by GJCs rather than synaptic mechanisms as concomitantly proposed by the Haydon group (384). Although it is highly likely that the observed astrocytic-neuronal coupling was an artefact of the non-natural condition of the in vitro coculture system used, the work demonstrated that, under certain circumstances, direct GJ coupling was possible (at the time of these experiments, HCs were not yet taken into consideration). Work performed a few years later in acute slices of the locus ceruleus confirmed the existence of glial-neuronal GJ coupling (10). These authors reported that spontaneous oscillations in the membrane potential of a subset of glial cells were synchronous to the firing of neurons, were insensitive to various receptor antagonists and disrupted by Cbx, and displayed neuron-to-glia dye coupling and electrical coupling (10). Gap junctional coupling between glial cells and neurons have also been documented in rat cerebellar slices, showing electrical coupling between Bergmann glial cells and Purkinje neurons that was inhibited by octanol and halothane (377) and between freshly isolated and short-term cultured satellite glial cells and neurons from the trigeminal ganglion (489); the latter study demonstrated that glial alterations in electrical potential influenced excitability of the connected neuron. **TABLE 4** gives an overview of several studies reporting heterotypic coupling between glial cells and neurons.

Later studies that made use of the astrocytic Cx43/Cx30 dKO mice have provided evidence for the involvement of astroglial Cxs in synaptic plasticity (382). The removal of extracellular glutamate and potassium ions, accumulating in the extracellular space upon neuronal activity, occurs through the modulation of clearance rate and extracellular space volume controlled by astrocytes, a process that is facilitated by astrocytic GJCs (179). As a result, removal of astrocytic GJCs/HCs in Cx43/Cx30 dKO affected synaptic plasticity in hippocampal acute slices with decreased long-term potentiation and enhanced long-term depression in the CA1 region due to effects at the level of neuronal excitability, neurotransmitter release probability, and insertion of postsynaptic AMPA receptors (382).

More recent studies have focused on the role of astrocytic HC function in the control of neuronal activity and synaptic plasticity in basal conditions. The available evidence is based on acute lowering of extracellular Ca^{2+}, a well-known HC opening stimulus, on the use of Gap26 that rapidly blocks HCs and with some delay also GJCs, and on Cx30/Cx43 dKO. Torres et al. (517) demonstrated in hippocampal acute slices that localized lowering of extracellular Ca^{2+} by photoactivation of the Ca^{2+} buffer diazo-2

Table 4. *Heterotypic coupling between glial cells and neurons*

Preparation	Experimental Approach	Cell Identification	Observations	Conclusion	Reference Nos.
A-N cocultures	Ca^{2+} imaging		Ca^{2+} wave in A induces Ca^{2+} responses in N; blocked by octanol	GJ-mediated A-N communication	355
A-N cocultures from rat forebrain (E18–E20 or P2)	Dye coupling (LY), DWCR	N: β-tubulin III; A: GFAP	A-N dye coupling; bidirectional electrical coupling, blocked by octanol	A-N coupling is present embryonically but disappears postnatally	157
Locus ceruleus slices from 4- to 10-day-old rat pups	Dye coupling (biocytin), patch-clamp recording, immunohistochemistry	N: tyrosine hydroxylase; A: GFAP, S100β	Synchronization between spontaneously firing N and A responses, blocked by Cbx; A-N dye coupling; involvement of Cx26 and Cx32 (A-A, N-A)	Depolarization of an A increases neuronal excitability	10
Human fetal hippocampal cultures (week 20–23)	Dye coupling (LY), DWCR	N: NF-66; A: GFAP	Bidirectional electrical coupling; Cx identification at transcriptional level	Bidirectional A-N coupling in cultures isolated from human second trimester hippocampal cells	445
Cortical slices from P7 and P14 rats	Dye coupling (neurobiotin), immunohistochemistry	N: NeuN, GABA	Heterotypic coupling; involvement of Cx26, Cx36, and Cx43	A-N coupling 2 wk postnatally involving pyramidal and nonpyramidal neurons	34
Cerebellar slices of P15–P30 rats	Dye coupling (Alexa Fluor 488), DWCR	Location and morphology based	Symmetrical electrical coupling between Purkinje N and Bergmann glia blocked by octanol and halothane	Glio-neuronal coupling	377

A, astrocyte; Cx, connexin; DWCR, double whole cell recording; E, embryonic day; GFAP, glial fibrillary acidic protein; GJ, gap junction; LY, Lucifer yellow; N, neuron; P, postnatal day.

triggered astrocytic HC opening involving $[Ca^{2+}]_i$ dynamics and ATP release, which were absent in Cx30/Cx43 dKO and inhibited by Cbx (see also sect. IV*C4*). These responses initiated spike generation in interneurons thereby providing enhanced inhibition of glutamatergic synapses (517). Chever et al. (68) showed that short application of Gap26 suppressed basal neurotransmission activity of excitatory synapses of CA1 pyramidal cells. In this study, ATP release, supposedly through astroglial Cx43 HCs, was proposed to directly activate P2 purinergic receptors on CA1 pyramidal cells, which are known to potentiate glutamatergic synaptic transmission (192, 380). In olfactory bulb slices, short application of Gap26 was found to decrease the upstate amplitude and firing rate of mitral cells, involving HC ATP release and A1 adenosine receptors (442). Finally, a recent study in acute slices from mouse prefrontal cortex demonstrated that the pharmacological and genetic inhibition of Cx43 HC activity reduced the amplitude of NMDA-induced EPSCs in pyramidal neurons without affecting AMPA EPSCs (330). This reduction was rescued by addition of D-serine in the extracellular medium. Moreover, long-term potentiation of NMDA EPSCs after high-frequency stimulation was reduced by prior inhibition (Gap26 or in Cx43 KO) of Cx43 HC activity in astrocytes and was rescued by adding extracellular D-serine. Dye uptake and patch-clamp experiments on isolated astrocytes in primary culture furthermore demonstrated that Cx43 HC opening was Ca^{2+}-dependent; accordingly, inhibiting astrocytic $[Ca^{2+}]_i$ dynamics with EGTA (2 mM) removed the NMDA EPSCs potentiation effect in the slices. Altogether these observations demonstrate that Cx43 HC activity is associated with D-serine release in astrocytes (330).

Finally, in vivo evidence for a HC contribution to cognitive behavior and memory emerged from experiments in which HCs were pharmacologically inhibited. In a pioneering study performed by Stehberg et al. (493), the rat basolateral amygdala was microinfused with TAT-L2 peptide that inhibits Cx43 HCs without inhibiting GJCs. Such in vivo blockade of Cx43 HCs during memory consolidation induced amnesia for auditory fear conditioning, as assessed 24 h after training, without affecting short-term memory, locomotion, or shock reactivity. Moreover, the amnesic effect was transient (it disappeared after a retraining phase without new TAT-L2 administration), and addition of the peptide after the critical 4 h consolidation period following training had no effect, indicating specific targeting of memory consolidation. Fear memory was not affected when TAT-L2 was associated with a mixture of putative gliotransmitters, including glutamine, glutamate, D-serine, glycine, and ATP. These observations suggest that gliotransmission involving Cx43 HCs participates in fear memory consolidation at the BLA. Finally, in another study, the in vivo blockade of Cx43 HCs with TAT-Gap19 microinfused into mouse brain ventricles was shown to significantly impair the spatial short-term memory in a delayed spontaneous alternation Y maze task (544). The TAT-Gap19 concentration in the cerebroventricular fluid was calculated to be on the order of ~29 μM, which is four times larger than the IC_{50}

for Cx43 HC inhibition (~7 μM) (552), indicating that TAT-Gap19 attained concentrations compatible with HC inhibition.

C. Ionic Homeostasis

Although a certain degree of selectivity is well established for GJCs (205), as far as atomic ions are concerned, there is a consensus that they are permeable to most of them, especially those that contribute to the physiological roles of glial cells (K^+, Na^+, Cl^-, Ca^{2+}). Among these, the most studied has been the intercellular movement of K^+ in astrocytes. Indeed, neuronal activity leads to rapid fluctuations of the extracellular K^+ concentration ($[K^+]_e$) due to the restricted volume of the extracellular space. If increases in $[K^+]_e$ are not rapidly corrected, neuronal resting potential would depolarize and affect activation of transmembrane ion channels and the function of transporter proteins and receptors. Accordingly, the control of interstitial $[K^+]_e$ is a crucial canonical function of astrocytes. Such a critical role for glia in maintaining extracellular K^+ homeostasis in the brain was initially proposed by Hertz (211) and further elaborated by Orkand et al. (374). In astrocytes, K^+ channels and transporters allow K^+ fluxes (reviewed in Refs. 60, 212, 260, 367) and spatial K^+ buffering mediated by GJCs helps to dissipate K^+ over a large area (529, 531, 545) and to release it at endfeet apposed to brain capillaries **(FIGURE 8)**. Work in mice hippocampi has demonstrated that Cx30/Cx43 dKO decreases spatial K^+ clearance in the stratum lacunosum moleculare, while it has no effect in the stratum radiatum (542). This difference was explained by the fact that astrocytes in the stratum lacunosum moleculare have no preferential orientation and less extended processes, thereby necessitating GJCs for efficient spatial clearance. In contrast, those in the stratum radiatum are more elongated in a direction perpendicular to the neuronal cell layer and are thereby less dependent on GJCs for spatially buffering K^+ along their axis.

Neuronal activity not only triggers extracellular K^+ elevation but is also associated with transient intracellular Na^+ changes in perisynaptic astrocytes, thereby impacting metabolic homeostasis of, e.g., lactate and glutamate, neurotransmitter uptake as well as K^+ buffering (257). GJCs facilitate equalizing

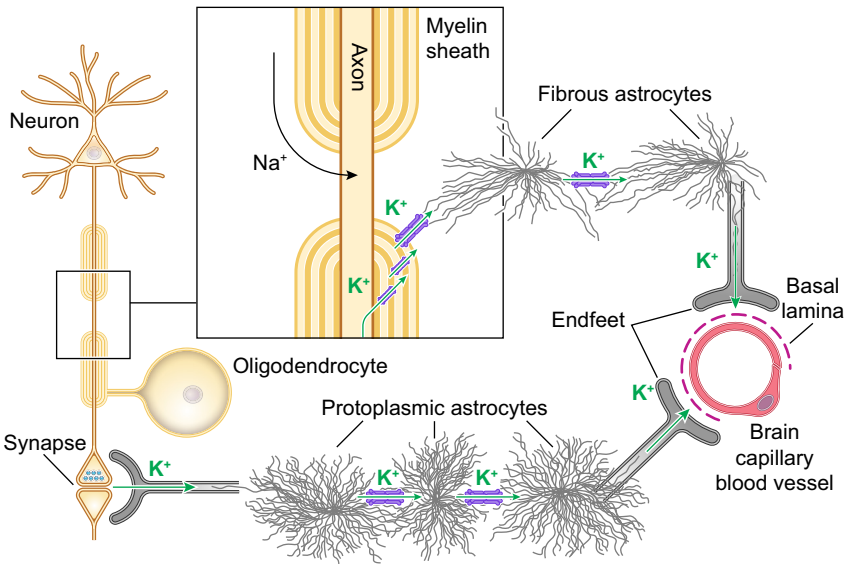

FIGURE 8. Gap junctions and spatial K^+ buffering. Synapses and axons are major sites of extracellular K^+ release during neuronal activity. Na^+ enters the axon at nodes of Ranvier while K^+ is released beneath the myelin sheath in the juxtaparanodal area (413), is taken up via still poorly defined mechanisms [possible involving Kv1 channels and connexin (Cx)29 hemichannels (413)], and is transported by reflexive Cx32-based gap junctions (GJs) between stacked layers of oligodendrocyte membranes. K^+ is then transferred to astrocytes by heterotypic GJs composed of oligodendrocytic Cx47 with astrocytic Cx43/Cx30 or oligodendrocytic Cx32 with astrocytic Cx30 or sparse Cx26. Astrocytic contacts at tripartite synapses (synaptic cradle) take up K^+ via various mechanisms including Na^+-K^+-ATPase, Na^+-K^+-$2Cl^-$ cotransporters, and $K_{ir}4.1$ channels (27). $K_{ir}4.1$ channels can in principle only contribute to K^+ uptake when the astrocytic membrane potential (V_m) is more negative than the K^+ equilibrium potential (E_K); it has been suggested that such condition occurs as a result of isopotentiality within the astrocytic GJ-coupled syncytium, which acts to stabilize V_m below the E_K that is shifted in positive direction as a result of extracellular K^+ accumulation (258, 260, 299). Astrocytic K^+ subsequently diffuses in the astrocytic GJ-coupled network thereby spatially distributing and buffering K^+. Astrocytically buffered K^+ may leave the astrocyte network via endfeet-located $K_{ir}4.1$ channels, ending up in the perivascular fluid of the glymphatic system or eliminated via endothelial K^+ efflux mechanisms towards the circulation. K^+ entry via capillary endothelial $K_{ir}2.1$ channels and consequent upstream arteriolar dilation (295) may facilitate such circulatory clearance.

intracellular sodium ion concentrations between cells and serve to coordinate physiological responses that depend on the intracellular concentration of this ion (29, 436, 437). In acute hippocampal slices, the selective stimulation of a single astrocyte induces an increase in intracellular Na^+ concentration, which then spreads to neighboring cells. This process is disturbed by pharmacological inhibition (Cbx) of GJCs and virtually omitted in Cx30/Cx43 dKO mice (271). Also the intercellular spread of Na^+ between astrocytes in hippocampal slices is primarily independent of Ca^{2+} signaling and gliotransmission, but mainly based on diffusion through GJCs. Thus the spatial spread of Na^+ along coupled astrocytes may add in rapidly correcting intracellular Na^+ concentration to maintain the normal steep inwardly directed sodium gradient.

D. Intercellular Calcium Signaling and Waves

Glial cells are endowed with chemical excitability at the level of Ca^{2+} signals, whereby small $[Ca^{2+}]_i$ increases can be amplified by Ca^{2+}-induced Ca^{2+} release at inositol trisphosphate (IP_3) receptors (385, 532). The large repertoire of astrocytic plasma membrane receptors thereby transduces extracellular messages from neurons and other brain cells into intracellular Ca^{2+} dynamics. GJCs, together with paracrine signaling, can communicate these Ca^{2+} concentration changes to neighboring cells, thereby producing so-called ICWs. Such glial spatiotemporal Ca^{2+} dynamics have been previously discussed in several excellent reviews to which the reader is referred for further background (7, 147, 281, 460). Here, we briefly review the basic mechanisms of ICW propagation via Cx channels followed by an overview of predominantly in vivo evidence of Cx-based glial ICWs.

Cxs contribute in two ways to glial ICWs: by carrying the Ca^{2+} messenger IP_3 via GJCs directly to the cytoplasm of a neighboring cell, and/or by contributing to paracrine signaling whereby HCs function as a release mechanism of ATP **(FIGURE 9)**. As a result, ICWs are inhibited by general Cx channel blockers as well as by selective HC blockers (235, 403). The ATP released via HCs subsequently interacts with G protein-coupled purinergic receptors, which are predominantly of the ATP/ADP-sensitive $P2Y_1$ type in astrocytes (201, 433, 469) and of the ADP-preferring $P2Y_{12}$ type in microglia (210, 332). Of note, ATP can also be released via other channels, including Panx1 channels (87, 231, 501), volume-regulated anion channels (159), $P2X_7$ receptors (498, 500), or vesicular release (49). With regard to the contribution of Panx1 channels to ICWs, work in cultured astrocytes has demonstrated that Panx1 KO increases the wave spread, indicating Panx1 may act as a brake; apparently, this brake effect disappeared under low extracellular Ca^{2+}/Mg^{2+} conditions (501).

GJCs not only communicate Ca^{2+} signals to neighbors by the passage of IP_3 but also by the direct movement of Ca^{2+} itself via these channels; however, clear examples pointing to Ca^{2+} as the major intercellular messenger flowing through GJCs are rare, and no evidence is available for glial cells. Since the recent finding that IP_3 diffuses not faster but equally slow as Ca^{2+} in the cytoplasm, it is possible that Ca^{2+} passage through GJCs is more important than previously thought (122, 279). Clearly, intercellular Ca^{2+} wave propagation is a complex process whereby intra- and extracellular signaling pathways mutually influence each other in often synergistic ways; e.g., IP_3 may flow in from an adjacent glial cell through GJCs but will also be produced by subsequent Ca^{2+} activation of PLC and by extracellular ATP activation of $P2Y_1$ receptors. Moreover, the relative importance of extracellular ATP and intracellular Ca^{2+}/IP_3 signaling may differ between different brain regions (197). Work in cultured astrocytes has furthermore demonstrated that the intra- and extracellular pathways of wave propagation are each involved at distinct phases, with GJCs being more important at the wave initiation site while extracellular ATP being more important at distance (159); modeling work furthermore indicates that too strong a coupling may actually impair wave propagation (270). Ca^{2+} waves triggered in in vitro cultured astrocytes (81) or occurring spontaneously in cultured brain slices (206) often appear as well-organized global events whereby a wavefront clearly sweeps across multiple rows of cells. Prominent spontaneous Ca^{2+} waves also occur in radial glial cells during formation of the cortical plate as observed in acute brain slices and partial brain preparation from E16–E17 rats (559). These waves involve GJs, HCs, and purinergic signaling whereby ATP acts as a crucial mitogenic signal to synchronize the cell cycle of radial glial cells. Spontaneous Ca^{2+} waves also occur in acute brain slices from the ventrobasal thalamus of postnatal rats (P5–P17), but the wave aspect is less clear and cross-correlation analysis is necessary to understand the spatiotemporal relation of the observed $[Ca^{2+}]_i$ changes (386). Interestingly, these astrocytic waves trigger inward current responses in thalamo-cortical neurons resulting from NMDA receptor activation. In contrast to this, in vivo recorded Ca^{2+} waves in the brain rarely appear with a clear wave aspect under normal conditions (33), which only occurs in response to nonphysiological triggers of mechanical or chemical nature (e.g., steplike increases of intracellular Ca^{2+} or IP_3, or of extracellular ATP and glutamate from caged inactive precursors). However, certain pathological conditions like cortical spreading depression or Alzheimer's disease animal models display clearly observable ICWs. Another question most relevant for the field is whether glial ICWs occur in vivo during normal brain activity under nonanesthetized conditions in adult animals. With respect to this, it should be pointed out that anesthesia can have a major impact on wave propagation, as anesthetics are well known to block GJCs and HCs (290, 308) as well as astrocytic Ca^{2+} signaling (515). Below, we discuss in vivo evidence for ICWs.

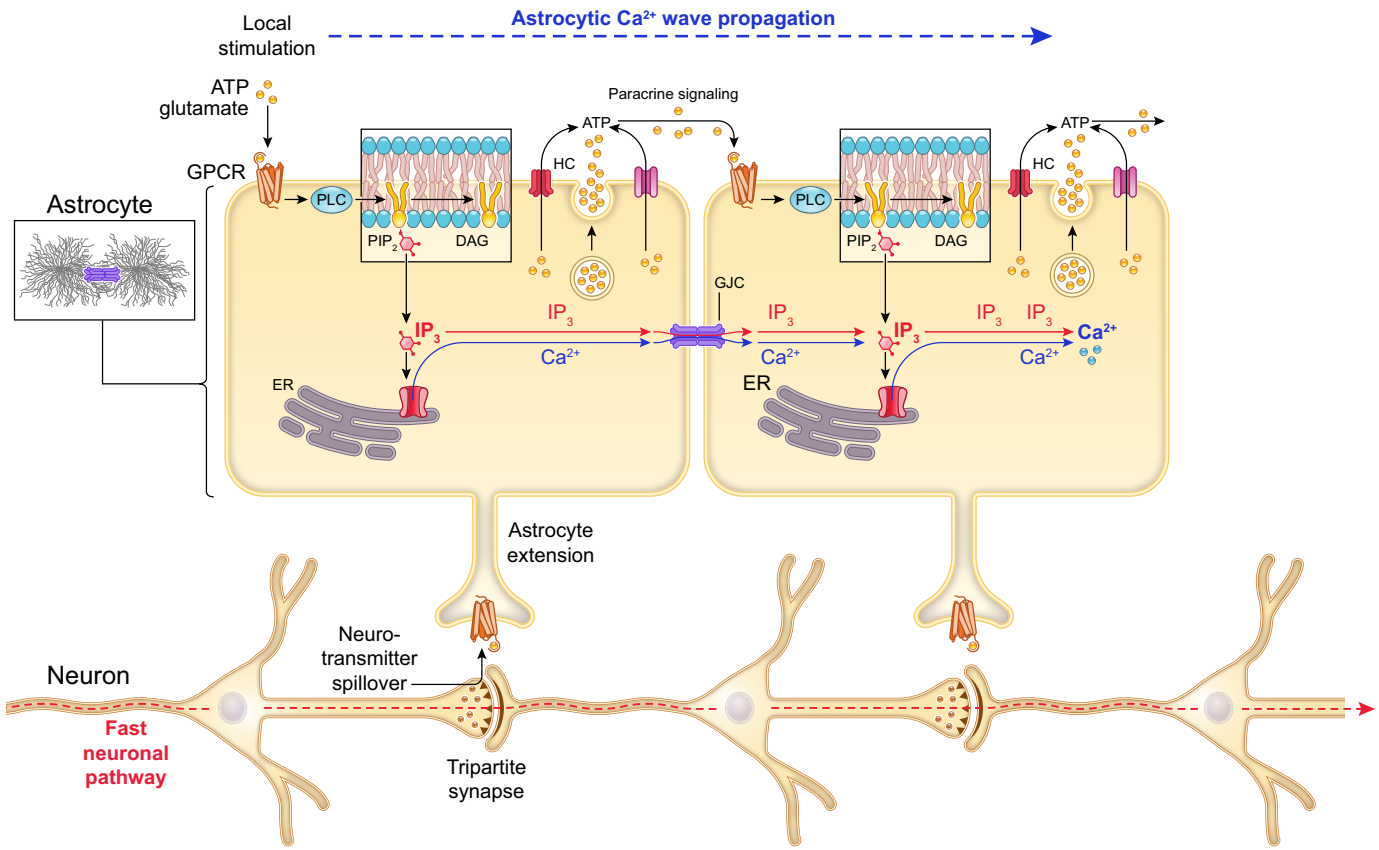

FIGURE 9. Intercellular calcium wave propagation mechanisms in astrocytes. Calcium waves can be initiated by extracellular signals (ATP, glutamate) acting on G protein-coupled receptors (GPCR), thereby activating phospholipase C (PLC) and generating inositol trisphosphate (IP_3). IP_3 diffuses through the cytoplasm towards gap junctions (GJs) that connect adjacent astrocytes. IP_3 also activates endoplasmic reticulum (ER) Ca^{2+} release, which also diffuses to the GJs. IP_3 has been considered to diffuse faster than Ca^{2+}, arriving before Ca^{2+} at the GJs. Recent evidence indicates that IP_3 and Ca^{2+} diffuse at comparable speeds, making them arrive simultaneously at the GJs. As a result, both IP_3 and Ca^{2+} may pass through the GJs, although the case for IP_3 is currently best documented. In parallel to the intracellular pathway, Ca^{2+} waves also propagate via paracrine messengers such as ATP released through vesicular mechanisms or opening of ATP-permeable channels such as connexin (Cx) hemichannels (HCs). In line with this, intercellular astrocytic Ca^{2+} waves are blocked by nonspecific Cx channel inhibitors but also by more specific inhibitors that block HCs only. In brain, the paracrine purinergic pathway is prominently present, especially in white matter, but both extra- and intracellular pathways strongly interact in a synergistic way. Astrocytic Ca^{2+} waves propagate at a speed of maximally ~25 μm/s, but in acute brain slices and in vivo, their propagation speed is often faster, which is caused by a parallel fast neuronal signaling pathway whereby synaptic neurotransmitter spillover may trigger IP_3/Ca^{2+} signals in astrocytic extensions in contact with synapses. In this case, suppression of neuronal activity by tetrodotoxin or other interventions will slow down wave propagation to the slower astrocytic mode. PIP_2, phosphatidylinositol 4,5-bisphosphate.

1. Glial calcium waves in the brain

One of the earliest in vivo brain recordings was reported by Hirase et al. (214) who used two-photon imaging combined with ester-loaded fluo-4 Ca^{2+} indicator to record astrocytic Ca^{2+} signals from the motor cortex of urethane anesthetized postnatal rats. The astrocytic origin of the signal was verified by post hoc immunostaining for S100β, a Ca^{2+} binding protein typically present in astrocytes. These in vivo recordings did not show anything that looks like a truly moving Ca^{2+} wavefront. Instead, cross-correlation analysis of Ca^{2+} dynamics in spatially separated cells was necessary to demonstrate the existence of a temporal relation between spatially distinct astrocytic Ca^{2+} events.

Spontaneous ICW activity was also recorded in the hippocampus of urethane-anesthetized postnatal mice (P9–P25; fluo-4 ester-loaded Ca^{2+} indicator) (266). Ca^{2+} dynamics were recorded in sulforhodamine 101 positive cells, a maker for protoplasmic astrocytes (362). Repetitive ICWs occurred every ~3 min propagating at a rather fast velocity of ~61 μm/s, which were called "glissandi" (266). ICW activity increased with neuronal activity and was reduced by TTX, indicating a neuronal component in wave propagation; it was also inhibited by

Cbx and the non-specific P2 receptor antagonist suramin, indicating involvement of astrocytic Cx channels and purinergic signaling **(FIGURE 9)**.

2. Glial calcium waves in the cerebellum

Extensive in vivo evidence for glial ICWs comes from the cerebellum, specifically from Bergmann glial cells, which are radial glial cells in the molecular layer. Nimmerjahn et al. (363) described spontaneous Bergmann glial ICWs in awake, nonanesthetized mice. In addition to these spontaneous "burst" responses, the authors also described smaller, more localized "sparkle" responses and larger "flare" responses; the latter were triggered by neural activity during locomotion and propagated over Bergmann glial fibers along a distance of hundreds of microns away from the initiation point. Importantly, application of isoflurane anesthesia silenced the flare responses, pointing to the importance of recording in awake rather than anesthetized animals (as observed in Ref. (219). The Cx involvement was not tested, but the authors rightly pointed to the fact that the disappearance of flares with isoflurane may well be linked to inhibition of Cx channels, as documented for other general anesthetics (290, 308).

Brazhe et al. (53) used wavelet-based image analysis to interpret their in vivo two-photon glial Ca^{2+} recordings of spontaneous Bergmann glial ICWs in the medial vermis of the cerebellum in ketamine/xylazine-anesthetized mice. The observed ellipsoid waves were similar to those reported by Hoogland et al. (220) and Nimmerjahn et al. (363), but their analysis showed that secondary waves could bud off from the primary wave, and sometimes circled around the primary wave area without invading it, indicating the primary wave zone experienced a refractory period. The refractoriness was interpreted to result from either increased ATP degradation, desensitization of P2Y purinergic receptors, or Ca^{2+}-linked GJC closure. Interestingly, secondary waves also appeared to continue their propagation along blood vessels when these were located in the neighborhood of the primary wave. Such ICW propagation along small blood vessels resembles earlier observations in rodent acute brain slices whereby Ca^{2+} uncaging in a perivascular astrocyte triggered Ca^{2+} waves that propagated between several juxtaposed astrocytic endfeet (338). Indeed, endfeet are well coupled by GJCs between each other, thereby producing a preferential ICW propagation pathway along small brain blood vessels (475). **TABLE 2** summarizes in vitro and in vivo functional consequences of intercellular Ca^{2+} waves.

Interestingly, the frequency of spontaneous Ca^{2+} waves in cerebellar Bergmann glial cells appears to increase with age, increasing 20-fold between 10- and 80-wk-old mice (316), an observation that has also been reported in the retina (268). The wave frequency elevation with age has been linked to a reduction of oxygen tension with aging (316).

3. Glial calcium waves and microglial activation

Evidence from acute brain slices has demonstrated that astrocytic Ca^{2+} waves induced by electrical or local ATP stimulation trigger membrane currents in microglial cells that involve purinergic signaling (465). Davalos et al. (91) later showed that in vivo laser ablation in the brain (~15 μm diameter ablation site) induced directional convergence of microglial processes and migration towards the injury site. These microglial responses were ATP-dependent and involved Cxs based on their strong inhibition by Cbx and flufenamic acid. As resting state microglial cells do not express Cxs, it was concluded that the target of Cx channel inhibition was astrocytic rather than microglial in nature. Making use of a similar brain laser injury model in zebrafish, Sieger et al. (470) demonstrated that ablation induced an ICW and that the associated microglial branching and migration depended on ATP signaling and $P2Y_{12}$ receptors, as suggested by others (210). However, the ablation-associated Ca^{2+} waves were neuronal rather than glia-based in the zebrafish model.

4. Glial calcium waves and cortical spreading depression

The role of glial Cxs and Panxs in cortical spreading depression (CSD) is discussed in detail in section VII*B*; here we discuss the glial Ca^{2+} waves associated with CSD. The propagation speed of the cortical CSD traveling wave is on the order of 1–5 mm/min, corresponding to 17–83 μm/s, which slightly overlaps with the 10–25 μm/s velocity of glial ICWs. In contrast, purely neuronal Ca^{2+} waves associated with cortical or thalamic neural activity propagate at a rate of ~37 mm/s in vivo (497), much faster than glial Ca^{2+} waves or CSD. The fact that CSD waves propagate slightly faster than glial Ca^{2+} waves but much slower than neuronal Ca^{2+} waves has sparked interest in the role of glial cells (276). Initial experiments with the nonspecific Cx channel blocker heptanol indeed suggested a role for GJCs in the propagation of spreading depression (272). Subsequent work in mouse acute cortical brain slices demonstrated that a high K^+-induced CSD wave, recorded from the intrinsic optical signal, was accompanied by a glial Ca^{2+} wave (399). Both waves propagated at ~40 μm/s, but the Ca^{2+} wave extended beyond the reach of the CSD wave, continuing at a slower speed (~18 μm/s) that is typical for a glial Ca^{2+} wave. NMDA receptor blockade with MK-801 inhibited the CSD wave but not the glial wave, while Cx channel inhibition with Cbx reduced the glial Ca^{2+} wave but not the CSD wave, indicating involvement of distinct signaling processes of the two waves. In vivo rat work (chloral hydrate anesthetized) performed by Chuquet et al. (71) further demonstrated that the glial Ca^{2+} wave was secondary to the primary neuronal event. Two Ca^{2+} waves were distinguished: a fast neuronal Ca^{2+} wave, also recorded in astrocytes (~35 μm/s), and a slower astrocytic Ca^{2+} wave (~6 μm/s) that occurred only in one-third of the cases. The

fast neuronal wave preceded the fast astrocytic wave by ~300 ms, indicating that astrocytic events are consequence rather than origin of the neuronal wave. Interestingly, the fast astrocytic Ca^{2+} wave was linked to a vasoconstrictive response in cortical penetrating arteries and intracortical arterioles. Evidence for a neuronal and glial contribution to the Ca^{2+} waves (see **FIGURE 9**) also comes from CSD induction with the K^+ channel blocker 4-aminopyridine, a seizure-inducing compound, in in vivo isoflurane-anesthetized rats (17). Here, fast Ca^{2+} waves (~1.1 mm/s, spreading over ~15 mm^2) were inhibited by TTX, demonstrating a neuronal component, but also by Cbx indicating GJ/HC involvement in a presumably glial component. Here, the neuronal wave induced a hemodynamic vasodilatory response, which stands in contrast to the Chuquet et al. (71) study where the glial Ca^{2+} wave was linked to a vasoconstrictive response.

All evidence taken together, astrocytic Cxs and ICWs have been hypothesized to be involved in CSD, but there is currently no evidence that would support a leading role for ICWs in this phenomenon. One should be cautious with the use of Cbx to decide on a glial or neuronal contribution to Ca^{2+} wave propagation in CSD as neurons may express Cx36, which are also involved in CSD wave propagation (23). Furthermore, neuronal networks strongly influence astrocytic Ca^{2+} wave propagation as a result of neurotransmitter spillover acting on astrocytes, as illustrated in **FIGURE 9**.

5. Glial calcium waves and Alzheimer's disease

The role of glial Cxs in Alzheimer's disease is discussed in section VII*E3*; below we discuss details of glial Ca^{2+} waves in the disease.

Kuchibotla et al. (265) reported ICWs in transgenic amyloid precursor protein/presenilin 1 (APP/PS1) mice. On the basis of in vivo two-photon imaging of bulk-loaded Oregon Green BAPTA Ca^{2+} indicator combined with SR101 loading of astrocytes, the authors found cross-correlated spontaneous astrocytic Ca^{2+} changes in cells separated by larger distances (~200 μm) in mice with plaques as compared with WT animals (maximally 50 μm separation). Patent intercellular Ca^{2+} waves were observed in six of eight APP/PS1 mice, while none was observed in WT mice. The waves were initiated in astrocytes in the vicinity (~25 μm) of plaques, propagated at a speed of ~23 μm/s over distances in the order of ~200 μm, and were not affected by TTX excluding a neuronal component and pointing to plaque-intrinsic Aβ-mediated toxic effects directed to astrocytes. No Cx-targeting interventions were done to uncover a GJ/HC contribution, but the observed elevation of astrocytic Cx43 expression in the vicinity of amyloid plaques in the human brain (346) may in principle support ICW activity. In vitro evidence furthermore shows that Aβ1–42 peptide enhances intercellular Ca^{2+} waves in a GJ- and ATP-dependent manner in cultured rat cortical astrocytes (208), while Aβ1–40 peptide triggers wave activation by itself (70).

The existence and role of ICWs is certainly one of the most exciting and important questions concerning the physiology and pathophysiology of astrocytes and the contribution of these cells in neuroglial interactions. In this respect, ICWs may be hypothesized as an organized glial response to neuronal activity that in its turn may impact neuronal function through gliotransmitter release. While the above in situ studies provide arguments for their in vivo occurrence, we need more extensive experiments that include characterization of the mechanisms of wave propagation and their possible function. The improvement of in vivo imaging techniques should provide more definitive answers in the future, in particular on the role of glial ICWs in disease models and on the contribution of Cxs/Panxs in their propagation.

E. Brain Metabolism and Energy Substrate Trafficking

Astrocytes are ideally placed to contribute to brain metabolism. Indeed, astrocytes contact blood vessels via their endfeet at the glio-vascular interface where Cxs are expressed **(FIGURE 4*B*)**, while their perisynaptic processes are associated with neurons. As glucose is the main source of energy and synapses are the most energy demanding part of neurons, astrocytes constitute a crucial cellular intermediary element to feed neurons that parallels the extracellular interstitial route of glucose diffusion. Also, in addition to the possibility of enhancing aerobic glycolysis using extracellular glucose as substrate, cultured astrocytes were shown to contain an energy reserve under the form of glycogen (123). Since the pioneering work of Pellerin and Magistretti (393, 394) who established the concept of an astrocyte-neuron lactate shuttle, it is accepted that in vitro astrocytes take up glucose, followed by a glutamate-induced glycolytic degradation producing lactate that is transferred to neurons via monocarboxylate transporters. In 1995, the GJC-mediated glial networking was proposed by Magistretti et al. (305) to be part of the so-called astrocyte-neuron lactate shuttle by hypothesizing a metabolic trafficking between astrocytes **(FIGURE 7*B*)**. This suggestion was confirmed later in cultured astrocytes by using radiolabeled energy metabolites and demonstrating that these substrates were able to diffuse through GJCs in a monolayer of astrocytes (509). This finding was subsequently confirmed making use of the fluorescent deoxyglucose analog 2-NBDG (36). A further step forward was achieved using hippocampal slices and showing that the intercellular diffusion of 2-NBDG in astrocytes was inhibited by Cbx and in astrocyte specific Cx43 KO mice (*hGFAP-cre:Cx43$^{fl/fl}$*) or Cx30$^{-/-}$ animals, and absent in the dKO (440). As already introduced in section III*B2*, intercellular diffusion of a fluorescent deoxyglucose an-

alogue via astrocytic GJCs was found to be activity-dependent and enhanced by focal activation of neurons (440). Recently, such metabolic networking was investigated in the mouse thalamus where barreloid structures seem to represent functional units for somatotopic organization of vibrissae representation (76). In this study, GJC-coupled networks were shaped by barreloid borders and neuronal activity. In this brain structure, the metabolic coupling involved astrocytes and oligodendrocytes, the latter being considered as the cellular elements limiting the communicating panglial network to a single barreloid as uncoupled oligodendrocytes are located at their border. It was suggested that such astrocyte-oligodendrocyte coupling may be important for metabolic support of myelinated axons, and coupling might serve as a back-up pathway to fuel the respective metabolic pathways, for example, lipid synthesis in oligodendrocytes and myelination. This was recently confirmed by showing that in the corpus callosum, the oligodendrocyte GJC-mediated networking provides energy to sustain axonal function predominantly by glucose delivery (331). Thus the panglial networking may play a critical role in energy substrates delivery to neurons, to prevent axon damage and neuronal loss. However, a recent study carried out in vivo showed that neuronal metabolic responses to stimulation do not depend on astrocytic stimulation by glutamate release but rather reflect increased direct glucose consumption by neurons (121). This finding indicates that in addition to metabolic glial networking direct neuronal glycolysis can also play a role in brain energy metabolism.

Glial Cxs also contribute to metabolic pathways is also involved during glial development, as for example in the oligodendroglial lineage where precursor cells (OPCs) undergo a series of energy consuming developmental events. Recently, using coculture models, 2-NBDG was shown to be transferred between astrocytes and differentiated oligodendrocytes through Cx-based GJCs but not between astrocytes and OPCs. Instead, Cx HC-mediated 2-NBDG uptake was demonstrated to support OPC proliferation. Evidence for HC involvement was concluded from the enhancement of 2-NBDG and EtBr uptake by extracellular Ca^{2+}-free conditions and uptake inhibition by Cbx and La^{3+}. A contribution of the Glut-1 glucose uniporter was excluded based on the absence of effects of the inhibitors cytochalasin B and STF31. Interestingly, deletion of Cx43 in astrocytes inhibited OPC proliferation by decreasing matrix glucose levels without impacting on OPC HC properties, a process that was also observed in acute brain slices from corpus callosum (364). This work demonstrates that Cx-based channels have a dual metabolic involvement, with GJCs facilitating glucose exchange between astrocytes and differentiated oligodendrocytes, and HCs functioning as a pathway of OPC glucose uptake.

F. Blood-Brain Barrier Development and Integrity

The BBB resides at the level of vascular endothelial cells of brain capillaries, which form a very thin layer that separates the blood from the brain parenchyma by a basal lamina. This endothelial barrier interacts with multiple neighboring cell partners including astrocytes, neurons, and blood cells (e.g., leukocytes) to form a highly interactive signaling and transport unit (89, 358, 581). Indeed, while its name only refers to its barrier function, it is also a hub for transport systems that shuttle nutrients, metabolites, ions, and water in and out of the brain tissue (2, 21). To coordinate and fine-tune these functions, signaling is crucial, involving multiple pathways that include signaling via Ca^{2+} and Cxs (3, 96, 98). Astrocytes and pericytes play major roles in this complex gliovascular signaling web and thereby strongly influence development, maturation, and maintenance of BBB functions (67, 504). At the time of birth, the blood-brain endothelial interface is still developing its barrier functions (455). At P5, blood vessels are incompletely covered by endfeet, while at P10, coverage is complete based on aquaporin 4 (AQP4) immunostainings largely expressed by endfeet. Cx43 starts to accumulate in perivascular endfeet at P2, and perivascular Cx43 punctae become large and stable at P15 (144). Astrocytic Cx30 also starts to appear at about P10 (mice), becomes concentrated around the blood vessels at P12, and further increases until P20. Thus, at P20, astrocytic Cx30 and Cx43 have their normal distribution in perivascular endfeet, at a time point where the BBB is considered mature.

Interestingly, dKO of Cx30 and Cx43 in mice does not appear to affect the gross organization of the endothelial-astrocyte interface or endothelial tight junction proteins (106, 144). The only notable morphological alteration is the fact that endfeet appear swollen, which may be linked to combined alterations of Cxs, AQP4, and β-dystroglycan (44, 289). Under normal conditions, HCs and GJCs, in addition to Kir4.1 and other ion channels present in endfeet, may provide pathways for ionic fluxes, while AQP4 provides the conduit for associated water fluxes; interestingly, these proteins are encoded and expressed by the local endfeetome (46). As a result of Cx43/30 dKO, the balance between local osmotic and water fluxes is altered, resulting in endfeet swelling. Despite the swelling, BBB permeability as determined from brief in situ brain perfusion with [^{14}C]sucrose was not altered. However, increasing the mean arterial blood pressure significantly increased [^{14}C]sucrose passage across the barrier, indicating decreased barrier function and leakage of this permeability marker (144). Importantly, leakage was only observed when albumin was added to the extra-corporeal brain perfusion administered via carotid artery catheters, indicating that increased blood pressure needs to be associated with increased endothelial shear stress provoked by the presence of circulating albumin. In the latter case, barrier

function was disturbed for both low- ([^{14}C]sucrose) and high-molecular-weight (horseradish peroxidase) substances. Follow-up work demonstrated that BBB leakage did not occur in Cx30 KO animals, indicating that astroglial Cx43 is the main player in terms of its impact on barrier function (45). Interestingly, astrocytic Cx43 KO also impacts immune cell passage through the BBB, with abnormal infiltration of B and T cells, plasmocytes, neutrophils, and macrophages in the brain of hGFAP-Cre:Cx43$^{fl/fl}$ mice (47). The immune cell infiltration resulted from activated barrier endothelial cells that strongly expressed adhesion molecules (P- and E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1) and generated pro-inflammatory cytokines (TNF-α) as well as chemokines (CXCL10, CXCL12, and CCL5), which attracted leukocytes to enter the brain parenchyma. This mounted a slowly appearing modest autoimmune response against an astrocytic protein called von Willebrand factor A-5a. As observed in dKO mice, barrier permeability in astrocyte-specific Cx43 KO mice was only increased upon challenging with elevated vascular pressure and shear stress. Overall, the evidence indicates that the presence of Cx43 in astrocytes helps keep the endothelial barrier closed for immune cell infiltration (44).

G. Myelination

As indicated above (see sect. II*A*), oligodendrocytes and astrocytes express nonoverlapping Cxs, which emerge at specific time points and locations leading to time- and space-specific formation of GJCs between these two cells; Cx KO mouse models have furthermore provided some clues to their role in myelination (82, 283, 328). The levels of Cx47 and Cx32 are much lower in proteolipid-protein mutant rats, which exhibit failure of oligodendrocyte maturation and myelin formation as a result of absence of this major myelin protein (195). Together, these data support the idea that oligodendroglial Cxs are part of the program correlated with myelin gene expression. To address this issue, the function of GJCs formed between astroglial and oligodendroglial lineage cells in vivo needs to be determined. So far, there are only in vitro studies showing a time specific developmental pattern of homotypic GJCs between oligodendrocytes and heterotypic GJCs between oligodendrocytes and astrocytes (16, 254, 527). In this study, OPCs were characterized by the lack of ionic and dye coupling, whereas immature oligodendrocytes exhibited both types of intercellular communication. This heterotypic dye coupling first appears after 3 wk in culture and increases to 25% after 6 wk (527), suggesting that glial GJCs are highly correlated with myelination. Moreover, once myelination becomes intact, GJIC among glial cells is a prerequisite for myelin maintenance and axonal function (519, 520).

Recent in vivo work in a lysolecithin-induced demyelination mouse model demonstrated that astrocyte-specific Cx43 KO (hGFAP-Cre:Cx43$^{fl/fl}$) accelerates the remyelination process, increases the number of mature oligodendrocytes, and reduces glial activation and deposition of myelin debris (284). In WT animals, treatment with boldine also enhanced remyelination, presumably by its anti-inflammatory effect that may in part be explained by its HC-inhibiting properties (see sect. IV*B*). Overall, these data point to astrocytic Cx43 involvement in disturbed myelination, while the role of HCs needs to be further scrutinized.

VII. CONTRIBUTIONS OF GLIAL CONNEXINS AND PANNEXINS TO BRAIN PATHOLOGIES

Given the central role of glial cells in both the developing and mature brain, it is not surprising that many neurological disorders result from, or exhibit, perturbed glial functions (178, 533). In this context, neuroinflammation is considered as a progressive phenomenon characterized by loss of structure and/or function of neurons with outcome of neuronal sufferance or death. Prior to cell death, several cellular and molecular events also occur in glia including the "reactive gliosis" characterized by activated microglia and reactive astrocytes. Such phenotypic changes of glial cells are found in most of brain pathologies, as well as injuries, and are associated with changes in Cxs/Panxs expression and functions. In the following paragraphs, we focus on studies that have been performed in animal models of brain pathologies triggered either experimentally or by genetic manipulation; when available, data from human patients will be mentioned.

A. Epilepsy

Epilepsy is one of the most frequent neurologic diseases that affects humans. In general, epileptic activity can result from blocking or activating synaptic and voltage-gated inhibitory conductances. Hence, it is believed that an imbalance of excitation and inhibition is the leading mechanism of the transition from normal brain condition to seizure. Derived from this notion, the currently used antiepileptic compounds are only symptomatic since they reduce or suppress seizures that reappear if patients stop their treatment. Notably all current treatments focus on neuronal molecular targets; however, several reports have demonstrated the presence of reactive astrogliosis (119).

In epilepsy, gap junctional coupling between astrocytes has been described to completely disappear in human mesial temporal lobe epilepsy as a result of sclerosis (25). This condition strongly reduces the spatial buffering capacity of coupled astrocytes leaving neurons more susceptible to microenvironmental changes, resulting in spontaneous epileptiform activity and reduced threshold for evoking seizure

EPILEPSY	INTERVENTION	STROKE
	Genetic models	
	Global heterozygous KO ($Cx43^{+/-}$)	↑ infarct, ↓ astrogliosis (350,478)
	Astrocyte-specific Cx43 KO	↑ infarct, ↓ astrogliosis (351)
↑ epileptiform activity (slices) (542)	Cx30/Cx43 dKO in astrocytes	
	CT-truncated Cx43 (K258 stop)	↑ infarct, ↓ astrogliosis (263)
	$Cx43^{+/G60S}$ (↓GJ function, ↑HC function)	↑ infarct (264)
	MAPK $Cx43^{S255/262/279/282A}$ (MK4 mutant; loss of HC function)	↓ infarct (156)
	General Cx channel inhibitors	
↓ seizure activity (43,165,171,172,188, 224,321,360,407,457,506)	Cbx	↓ capsase-3 (99)
	Octanol	↓ infarct (409,416)
	18α-glycyrrhetinic acid	↓ cell death (397)
↓ seizure activity (240,360,396,541)	Meclofenamic acid	
	GJ coupling enhancers	
↑ seizure activity (165,322,407,457)	Trimethylamine	
↑ seizure activity (187)	Gap134 (Danegaptide, ZP1609)	↓ infarct (153)
	Peptide inhibitors	
	Gap26	↓ infarct (286)
↓ epileptiform activity (slices) (453)	Gap27	↓ infarct (286)
↓ neuronal damage (slices) (575)	Peptide5 (HC inhibitor)	↓ infarct (93,94)
↓ seizure activity, ↓ D-serine (543)	TAT-Gap19 (Cx43 HC inhibitor)	↓ infarct (156)
	Gap19 (Cx43 HC inhibitor)	↓ infarct, ↓ apoptosis (66)

FIGURE 10. Effect of diverse Cx-targeting interventions on the outcome of epilepsy/seizures and stroke in in vivo animal models. Red indicates worse outcome, while green corresponds to improved outcome. Reference numbers are in parentheses. In vitro work is indicated by "slices." Cbx, carbenoxolone; Cx, connexin; dKO, double knockout; GJ, gap junction; HC, hemichannel; MAPK, mitogen-activated protein kinase.

activity (382, 542). In post-surgical hippocampus of patients with temporal lobe epilepsy and hippocampal sclerosis, a lack of coupled astrocytes has been found while coupled astrocytes were frequently found in specimens of non-sclerotic tissue (25). Similar astrocyte uncoupling has been described in hyperthermia-induced febrile convulsions, supporting the notion that astrocyte-astrocyte uncoupling is a relevant mechanism possibly contributing to epileptogenesis (255). The inhibition of astrocyte coupling during the early phase of epileptogenesis is not caused by reduced amounts of Cxs, but rather associated with changes in the phosphorylation status of Cx43. Thus the development of anti-epileptic drugs could include drug discovery of agents that increase GJIC between astrocytes. However, this conclusion needs to be balanced with the fact that the GJ coupling enhancers trimethylamine (a weak base, inducing intracellular alkalosis that increases coupling; Ref. 398) and Gap134 (a dipeptide coupling enhancer; Ref. 59) worsen seizure outcome while Cx channel inhibition with Cbx, fenamates, or peptide inhibitors act oppositely and improve seizure outcome (reviewed in Refs. 63, 341; illustrated in **FIGURE 10**). These effects may, in addition to modulation of astrocytic coupling and HC function, also be related to actions at the level of neuronal GJCs/HCs. Furthermore, Cbx and fenamates also inhibit Panx1, making firm conclusions rather problematic. Additionally, astrocytic GJs have a Janus-face action in seizures, as they may facilitate spatial K^+ buffering and thereby act anticonvulsively, but they may also convey pro-convulsive synchronous activity over the astroglial network (187).

In an acute model of kainic acid-induced status epilepticus, it has been found that interference with Panx1 ameliorates the outcome and shortens the duration of the status (454). In the temporal lobe cortex of patients with chronic temporal lobe epilepsy, the amount of Panx1 was found to be significantly higher than in the controls (238). However, Panx1 may also have diverse effects on seizures (12, 463), and its role may relate to its neuronal rather than its glial origin.

Astroglial Cx43 HCs open in response to the chemoconvulsant pilocarpine, an effect that is inhibited by TAT-Gap19. From the mechanistic point of view, it was found in vivo that pilocarpine-induced seizures are accompanied by increase in the amount of D-serine, and this was suppressed by TAT-Gap19. Concordantly, the anticonvulsant action of TAT-Gap19 was reversed by exogenous D-serine administration, suggesting that Cx43 HC inhibition protects against seizures by lowering extracellular D-serine. The anticonvulsive outcome of Cx43 HC inhibition was also observed in electrical seizure mouse models, an acute model of refractory seizures and a chronic kindling model (543). The above findings strongly suggest that Cx43 HCs, Panx1 channels, and Cx

GJCs play a critical role in epilepsy and normalization of their functional state could improve the current anti-epileptic treatment. **FIGURE 10** summarizes the effect of various Cx-targeting interventions mostly based on evidence obtained from in vivo animal seizure models. The effects are compared with results obtained in in vivo stroke models and illustrate rather good correlation of the outcome effect for the various interventions shown (except for the GJ coupling enhancing substance Gap134). Further considerations related to this table are discussed in the context of stroke in section VII*C*.

B. Spreading Depression and Migraine

Patients with acute brain injuries or migraine may develop CSD waves (see sect. VI*D4*) that can be monitored by electroencephalography and may disturb brain homeostasis. The electrical depression propagates as a wave that spreads at 2–5 mm/min leading to cell depolarization, breakdown of ionic gradients across the cell membrane, loss of electrical activity, and neuronal swelling that encompasses the distortion of neuronal dendritic spines. Hypoxia or high extracellular K^+ concentration are potent experimental stimuli used to induce CSD in animal models (401, 485). CSD has been considered the origin of the migraine aura phase when it crosses the visual area. The aura phase consists of focal neurologic symptoms preceding or coinciding with migraine headache (reviewed in Ref. 443). Here, we focus on the possible involvement of Cx- and Panx1-based channels in CSD.

The GJC and Cx HC/Panx1 channel blocker Cbx does not prevent high K^+ induced CSD in brain slices (399, 536). Moreover, astrocytic Cx43 KO results in an increase in CSD propagation studied in hippocampal slices (511). Nonetheless, octanol and 18-glycyrrhetinic acid prevented CSD induced by electric stimulation in chicken retinas (314, 356). As such, the contribution of Cx and Panx channels in CSD remains controversial, and further work is needed to investigate the effect of specifically targeting HCs and Panx1 channels. It has indeed been shown in experimental brain trauma models that $[K^+]_e$ may significantly increase up to 60 mM (361), which is a concentration that induces opening of Cx43 HCs (444) and Panx1 channels, the latter leading to inflammasome activation through the caspase-1 pathway in neurons and astrocytes (474). Furthermore, a lowered extracellular Ca^{2+} and increased $[Ca^{2+}]_i$ also affect Cx- and Panx1-based channels.

While increased $[Ca^{2+}]_i$ is associated with a reduction in GJC coupling, it activates Cx HCs and Panx1 channels (100, 102, 293) (see sect. III*A*). To wrap up, Cx- and Panx1-based channels may contribute in some way to CSD, but astrocytes are not the primary drivers of CSD wave propagation (584). Astrocytic ICWs occur at the end of the signaling cascade, after the $[K^+]_e$ and glutamate elevations that primarily drive CSD wave propagation (138) (see sect. VI*D4* for a more extensive discussion of glial ICWs in CSD).

C. Stroke and Ischemia

Stroke results in a neurological deficit due to an acute vascular injury of the CNS, including cerebral infarction, intracerebral hemorrhage, and subarachnoid hemorrhage (447). Ischemic stroke is characterized by cell death usually resulting from a transient or permanent occlusion of a cerebral artery. For the purpose of this review, we focus on ischemic stroke, which accounts for ~80% of all strokes (516). Cerebral infarction initiates a complex series of events affecting all brain cells and extracellular matrix components. Blood flow interruption in the ischemic territory results in low ATP levels, ionic disruption, and metabolic failure, which leads within minutes to cell death upon reperfusion (292). The peri-infarct region surrounding the core is subjected to milder ischemic conditions because of perfusion from collateral blood vessels. Within a limited amount of time (~5–6 h; Ref. 585), this area remains salvageable and is thus a major focus for therapeutic intervention with neuroprotective strategies.

1. Connexins in stroke

A rapid response to brain ischemia involves the activation of "reactive" astrocytes in the peri-infarct region, characterized by increased expression of Cx43 (155, 223). Astroglial Cx43 has been implicated to provide a neuroprotective role in experimental stroke conditions (478, 350, 351). Part of this effect is attributed to spatial buffering mediated through GJCs between astrocytes (462). The strongest evidence for a role of astroglial Cx43 comes from experiments using KO mice. The role of astrocytic Cx43 in stroke has been clarified using the GFAP-Cre: $Cx43^{fl/fl}$ mouse model (351). When subjected to middle cerebral artery occlusion (MCAO), these mice with astrocytes lacking Cx43 exhibited a significant increase in stroke infarct volume and enhanced apoptosis compared with WT controls. It needs to be added that interpretation of Cx43 KO work may be complicated as a result of alterations in other genes as concluded from transcriptomics approaches (229, 230).

Clarification of the role of Cx43 in stroke resulted from investigating a mouse model of oculodentodigital dysplasia (ODDD) (264), a syndrome caused by Cx43 gene mutations, resulting in ocular, nasal, dental, and digital abnormalities, and can include neurological symptoms (383, 390). Several Cx43 mutations can result in reduced GJC formation and gain in HC activity (126, 228, 251, 320). A mouse model of this disorder was identified in a screen following mutagenesis, where a G60S missense point mutation resulted in an ODDD phenotype (152). Heterozygote $Cx43^{+/G60S}$ mice exhibit a similar ODDD-like phenotype as

observed in humans and show a significant increase in infarct volume 4 days after MCAO (264). Such increased neuronal death was shown to correlate with decreased astrocytic GJIC, but also with an increase in HC activity in astrocytes.

The function of Cx43 channels is primarily regulated by phosphorylation. The COOH terminus contains phosphorylation sites for different kinases that directly regulate Cx43 activity. When mice expressing a truncated mutant form of Cx43 lacking the COOH terminus (Cx43ΔCT) were subjected to MCAO, they suffered increased cerebral damage and inflammation compared with controls (302). Further insight was obtained using mice with mutations of specific Cx43 phosphorylation sites. CK1, PKC, and MAPK have all been shown to phosphorylate Cx43 (482) and are also active in ischemic conditions (109, 239, 246). To test if disrupting these phosphorylation sites of the Cx43 COOH-terminal tail impacts stroke outcome, WT and Cx43 phosphorylation null mutants for CK1 ($Cx43^{S325A/328Y/330A}$), PKC ($Cx43^{S368A}$), and MAPK ($Cx43^{S255/262/279/282A}$) (243) mice were subjected to MCAO (155). Of these, only the MAPK mutant mice exhibited a significant reduction in infarct volume, decreased apoptosis and inflammation, as well as an increase in astrocyte reactivity compared with WT controls. Importantly, the MAPK mutation also resulted in a reduction in Cx43 HC activity. The impact of reduced activity of Cx43 HCs on neuroprotection was further confirmed by administration of the Cx43 HC blocker TAT-Gap19 (see sect. IV*B*) in WT mice subjected to MCAO (156). Therefore, accumulating data support the therapeutic efforts towards novel stroke therapies targeting Cx43 HCs (92, 369). **FIGURE 10** illustrates the outcome effect of various Cx-targeting approaches including genetic modifications, general Cx channel modulators (GJ inhibitors and enhancers), and peptide inhibitors of Cx channels, including HC inhibitors, obtained from in vivo animal stroke models. Overall, comparison of these various Cx-targeting approaches shows consistent effects in both stroke and epilepsy/seizure models. Genetic modifications invariably result in worse outcomes except for the MAPK $Cx43^{S255/262/279/282A}$ mutant that is characterized by loss of Cx43 HC function. GJ inhibition with general Cx channel blockers, which inhibit both GJs and HCs, improve the outcome; for GJ enhancers, the result is less clear with Gap134 decreasing stroke infarct size while increasing seizure activity. However, it needs to be taken into account that general Cx channel modulators will also affect neuronal Cxs and may influence Panx1 channels as well. Gap26 and Gap27 peptides that first inhibit Cx HCs and with some delay also GJs also improve outcome, and more specific HC inhibition with peptide5 or Cx43-targeting TAT-Gap19/Gap19 equally result in improved outcomes in both stroke and seizures **(FIGURE 10)**.

2. Pannexins in stroke

Since Panx1 activity has been shown to participate in inflammation (474), apoptosis (65) and necrosis (514), it has been investigated in pathological processes including cerebral ischemia. Most studies have focused on Panx1, which is primarily expressed in neurons (417, 540, 579, 586) but also reported in astrocytes (231). Panx1 KO did not significantly reduce cerebral injury in mice following MCAO (22). However, in Panx1/Panx2 dKO mice, a significant decrease in infarct volume was reported (22). Further investigation on the role of Panx1 in cerebral ischemia, using both Panx1 KO mice and treatment with the Panx1 blocker probenecid, demonstrated that only female Panx1 KO mice exhibited a neuroprotective effect following MCAO (154). However, in male mice subjected to transient focal ischemia, probenecid significantly reduced inflammation, cerebral edema, and neuronal death (567). These disparate results could be attributed to differences in the stroke models used, specifically with regard to transient versus permanent cerebral vessel occlusion. The role of astrocyte Panx1 in the context of ischemic stroke remains to be defined.

D. Dysregulation of Sleep/Wake Cycle

Sleep is a behavioral state that alternates with waking. Interestingly, P. Haydon and collaborators (200) have provided evidence for the involvement of neuroglial interactions in sleep/wake regulation, contributing to move from a "neurocentric" view of this process to an active contribution of astrocytes in which Cxs participate. Indeed, using a conditional Cre-Lox system KO of Cx43 in astrocytes, they observed an excessive sleepiness and a fragmented wakefulness during the nocturnal active phase. Such astrocyte-specific genetic manipulation silenced the wake-promoting orexin neurons located in the lateral hypothalamic area by impairing glucose and lactate trafficking through astrocytic networks (75). Moreover, in the glomerular layer of the olfactory bulb, slow alternations between two distinct membrane potentials in mitral cell neurons generated bistability of membrane potential associated with "up" and "down" states. With the use of a combination of genetic and pharmacological tools, the activity of astroglial Cx43 HCs was shown to be activity-dependent and resulted in the increase in upstate amplitude that impacts mitral cells firing rate (442) (see sect. III*C4*). As these oscillations are reminiscent of neocortical neuron oscillations typically observed during slow-wave sleep or under anesthesia (170, 494) as well as during quiet wakefulness (84), this study suggests that the HC function of Cx43 may play a role in sleep/wake cycle. Finally, transcriptional changes induced by modafinil, a wake-promoting drug that enhances neuronal electrotonic coupling (522), were accompanied by Cx30 expression level enhancement and increase in astrocyte GJIC on cortical slices in an activity-dependent manner (291). Remarkably, application of γ-hydroxybutyrate, a

sleep-promoting drug, has an opposite effect on astrocyte GJIC on cortical slices (291) while the sleep-inducing lipid oleamide blocked the GJ-mediated communications between rat glial cells (196). These pharmacological data suggest that astrocyte GJIC increases during wakefulness and decreases during sleep (400). Altogether they also highlight that the two main Cxs in astrocytes and their two channel functions (GJIC and HC) contribute to sleep/wake regulation.

E. Neurodegenerative Diseases

1. Huntington's disease

Huntington's disease is an autosomal dominantly transmitted neurodegenerative disorder characterized by progressive motor dysfunctions, cognitive impairment, and personality changes. In the caudate nucleus of human patients, immunohistochemical techniques indicated that Cx43 density was increased with Huntington's disease and became located in patches while this Cx was homogeneously distributed in the neuropil of normal brains. Also, GFAP staining of astrocytes was highly increased in the caudate nucleus, indicating a reactive astrocytosis around degenerating neurons associated with an increased expression of astroglial Cx43 (539).

2. Parkinson's disease

Parkinson's disease (PD) is a common neurological disorder characterized by progressive degeneration of dopaminergic neurons. The resulting disruption of dopaminergic neurotransmission in the basal ganglia produces progressive extrapyramidal motor symptoms. A commonly used animal model of PD is the MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-lesioned striatum which triggers the degeneration of dopaminergic neurons. With the use of this model it was initially reported that transient increase in Cx43 mRNA and Cx43-immunoreactive punctae following MPTP lessoning, without any induction of functional coupling between astrocytes and other glial cells as revealed by dye coupling of patched cells in acute striatal slices (446). However, it should be noted that in this study dye injections in striatal acute slices did not reveal functional coupling between astrocytes, not even in controls, which is in contradiction with more recent work showing large GJIC in the striatum in basal condition (6, 249). Furthermore, a rotenone (a mitochondrial complex I inhibitor) model of PD demonstrated increased Cx43 protein levels in vitro as well as in vivo, which were associated with an increase of in vitro in dye coupling (250). In this study an increase of Cx43 was also reported in the striatum of rotenone-treated rats compared with control brains. Finally, recent studies have addressed interesting clues concerning the contribution of astroglial Cxs in PD. First, $GR^{Cx30CreERT2}$ mice, which are Cx30-based conditionally inactivated for glucocorticoid receptor in astrocytes, showed significantly augmented dopaminergic neuronal loss in substantia nigra of MPTP-treated mice compared with controls. This was paralleled by increased Cx43 HC activity and elevated $[Ca^{2+}]_i$ in astrocytes examined in acute midbrain slices from control and mutant mice treated with MPPT. Moreover, $GR^{Cx30CreERT2}$ mutant mice treated with TAT-Gap19 peptide reverted dopaminergic neuronal loss and microglial activation (303). Thus astrocytic glucocorticoid receptors regulate Cx43 HC activity during MPTP-induced Parkinsonism, which in turn affects dopaminergic neurodegeneration. Second, with the use of Cx30 KO mice, it was observed that the loss of dopaminergic neurons was increased compared with wild-type MPTP-treated mice. Also, astrogliosis in the striatum of Cx30 KO mice was attenuated after MPTP treatment, whereas microglial activation was unaffected (160). Altogether these results indicate that, besides Cx43 HCs that have a deleterious role, Cx30 may play a neuroprotective role in the MPTP model of PD.

3. Alzheimer's disease

Alzheimer's disease (AD), discovered more than a century ago, is the most common neurodegenerative disease of the elderly. This pathology is characterized by an abnormal accumulation of the amyloid β peptide (Aβ) generated by sequential proteolytic cleavage of the amyloid precursor protein (APP) by β- and γ-secretase (467). Aβ oligomers aggregate in the brain parenchyma to form extracellular deposits called amyloid plaques, which are a typical histopathological lesion of AD mainly in the hippocampus and cortex. A consistent feature of Aβ accumulation is the strong reactive gliosis that includes activated microglia and reactive astrocytes (343). Nagy et al. (346) reported an increase in astroglial Cx43 expression in brain tissue from human AD patients, an observation that was further confirmed for Cx43 and Cx30 expression in astrocytes associated to Aβ plaques in AD patients (262). Likewise, the acute application of Aβ peptide in cultured astrocytes and hippocampal slices was shown to result in the activation of HCs, first in mast cells (Panx1 and Cx) followed by microglia (Panx1 and Cx43) and then in astrocytes (Cx43). This cellular cascade resulting in glutamate and ATP release from glia induces neuronal cell death (371). Interestingly, with the use of similar models, endogenous and synthetic cannabinoids were recently shown to prevent this deleterious cascade leading to neuronal death (164). Furthermore, studies have focused on astroglial Cx43 in a genetic murine model of AD named the APP/PS1 mouse that develops Aβ plaques in the hippocampus and the cortex leading to neuronal injury based on immunostainings for neuritic dystrophies. In this mouse, Cx43 and Cx30 expression are also increased in astrocytes associated with plaques, and functional studies in hippocampal slices indicate that GJIC is not impacted, while HC activity is highly increased in these astrocytes. This HC contribution is absent when the APP/PS1 mouse was cross-bred with astrocyte-targeted Cx43

KO animals (574), and this was associated with a reduction in neuronal damage in the hippocampus. More recently, the same genetic approach allowed demonstrating that the lack of astroglial Cx43 in the APP/PS1 mouse ameliorates cognitive functions (420). Finally, efforts were performed to identify new pharmacological approaches that target astroglial Cxs and more specifically their HC function. Along this line, boldine, which inhibits Cx HCs and Panx1 channels but not GJs (see sect. IV*B*), was administrated during 3 mo to the AAP/PS1 and resulted in a similar reduction in neuronal injury as observed in APP/PS1/Cx43 KO mice (573). Altogether these works highlight the active contribution of reactive astrocytes in AD and identify Cx43 HCs as a potential target to slow down neurodegenerative processes in this pathology.

F. Gliomas

Malignant glioma is the most common, aggressive, and lethal primary CNS tumor (521) and is believed to develop from glial cells that rapidly proliferate into tumors characterized by intra- and intertumoral heterogeneity, resistance to conventional treatments, and poor prognosis (42). With regard to Cx43 in malignant glioma, there are seemingly conflicting reports describing Cx43 as both a tumor suppressor and a promoter of tumor progression (reviewed in Ref. 477). A tumor suppressive function of Cx43 was first identified in a rat C6 glioma model where overexpression of Cx43 significantly suppressed brain tumor formation in rats (353). Analysis of Cx43 protein in human glioma samples showed an inverse correlation between Cx43 levels and tumor grade. However, later studies found that some high grade gliomas express varying levels of Cx43 (reviewed in Ref. 325). The heterogeneous nature of gliomas and the existence of both channel-dependent and -independent mechanisms in the context of cancer cell growth, migration, and apoptosis may provide insight into these conflicting reports regarding Cx expression in gliomas, but also highlight the complexities associated with developing new therapeutic approaches.

Cxs have been extensively studied in the context of cancer (reviewed in Refs. 1, 329, 354). With regard to brain tumors, many arise from astrocytes or glial progenitors (reviewed in Ref. 521). Cx43 has been considered as a driver of glioma invasion (476), a marker of glioma progression (83), and an inducer of temozolamide (TMZ) resistance in glioma cells (185, 339, 550), and Cx46 plays a role in the context of glioma stem cell phenotype (216). Further consideration must be given to the emerging role for Cxs in brain cells contributing to glioma progression, which provide the microenvironment for glioma progression. In this regard, astrocytes play a critical role since they constitute most of the microenvironment of malignant glioma and account for the increased peritumoral expression of Cx43 (476). In this microenvironment, Cx43-mediated GJIC is expected to occur between tumor cells, between astrocytes, and heterocellularly between tumor cells and astrocytes. This interaction between astrocytes and tumor cells may play a role in chemotherapeutic resistance of gliomas (273). Cx43 also mediates cellular signaling as a HC that permits paracrine communication between the cytosol and the extracellular environment (191). Hemichannel function may modulate exosome trafficking, as it has been suggested that Cx43 HC in an exosome could dock with a HC in a cell, thus providing targeted delivery from exosome to cell (61). This could hypothetically facilitate the process of organ-directed metastasis, which is determined by the integrin expression pattern of the exosome (222). The finding that Cx43 is detected in exosomes lends further support to a role for Cx43 in the complex tumor microenvironment (479). It has been recently shown that GJIC between glioma cells and astrocytes allows the exchange of microRNAs, small, noncoding RNA molecules that regulate several protein targets and are thus able to reprogram normal astrocytes to facilitate glioma invasion (218). Glioma cells have also been shown to extend long membrane protrusions, referred to as tunneling nanotubes, which have been demonstrated to facilitate invasion in the brain and protect from radiation-induced cell death (376). Part of this process has been suggested to involve intercellular communication through microtube-associated GJCs.

Expression of Cx43 is significantly enhanced in tumor-associated astrocytes, especially at the periphery of the tumor core (476). With the use of a mouse model consisting of syngeneic intracranial implantation of GL261 malignant glioma cells into Nestin-Cre:Cx43$^{fl/fl}$ mice, in which Cx43 is selectively eliminated in astrocytes, it was demonstrated that reduction of astrocytic Cx43 decreases the invasion of tumor cells from the tumor core in the presence of an intact immune system (476). The importance of reactive astrocytes in the regulation of glioma resistance and invasion suggests therapeutic value in targeting Cx43 in the glioma microenvironment. Several recent studies have focused on Cx43 mimetic peptides to modulate GJC and HC functions. In the context of gliomas, treatment with αCT1, which has been shown to inhibit HCs, restored TMZ sensitivity in TMZ-resistant glioma cells and inhibited the growth of gliomas in mice treated with TMZ (339). Other studies have shown that cell-penetrating COOH-terminal mimetic peptides targeting the non-receptor tyrosine kinase c-Src binding domain of Cx43 (TAT-Pep-2; **FIGURE 6**) indicate potential in reversing glioma cell phenotype via downregulation of the inhibitor of differentiation (ID1), and the transcription factor Sox-2 which regulates stem cell self-renewal (168). While a peptide targeting a tyrosine kinase binding domain would be expected to have many effects on a cell and tissue, these results show promising therapeutic potential.

VIII. PERSPECTIVES AND CONTROVERSIES IN THE FIELD; QUESTIONS THAT SHOULD BE ADDRESSED IN THE FUTURE

Cxs and Panxs in glia have now found their place among the numerous direct and indirect mechanisms involved in neuroglial interaction (179). Their contribution to this cross-talk ensures that within a multicellular organ like the brain, glial cells sense the activity of neurons, and in turn regulate their activity and survival. This coordination is essential to provide the integrated functioning of the CNS as a whole and has also to be considered in pathological situations. This statement forces us to address or revisit questions that may have been raised long ago, but still remain unanswered. It further generates the following questions that have not yet been comprehensively explored, and which may provide a stimulus for future research efforts.

A. Discrimination Between Connexins and Pannexins

As described above, the distinction between these two families of membrane proteins, that both support channel activity, is still suffering from the limited number of pharmacological tools. Consequently, new efforts need to be undertaken in this direction to be able to specifically inhibit glial Cx and Panx HCs. Also, while several compounds (peptides, drugs, natural molecules) are available to block HC activity in glia without impacting GJIC, there is no way yet to block GJIC without affecting HCs. Also, while genetic approaches have so far provided targeted KO, there is now a need for conditional KOs to better understand the role of Cxs and Panxs in healthy and disease brain.

B. Do Functional Hemichannels Exist?

There has been some critical appraisal of the available evidence related to HC functioning (359, 491). Studies on HC function are based on uptake/release assays of fluorescent molecular markers called "dye uptake/release" assays and on electrophysiological characterization by patch-clamp studies. Both measurements assess different aspects of channel function, i.e., permeability of the channel for molecular markers (molecular mass of several hundreds of Daltons) and the electrical current associated with the flow of atomic ion charge carriers (Na^+, K^+, Ca^{2+}, and others), respectively. The permeation properties of charged molecular markers and atomic ions are poorly interrelated and may differ depending on the Cx isotype (130, 132). If experiments prove difficult to be repeated in different laboratories, the problem is often related to differences in the assays or cellular readout systems. Single-channel patch-clamp studies report both channel behavior and unitary channel conductance, the latter being specific for the channel and Cx isotype being studied. Such information is absent in dye uptake/release studies. The Cx expression system is crucial as the cellular background conditions, e.g., the activity of various kinases, may be quite different (101). With respect to this, the *Xenopus* oocyte expression system is poorly suited for the electrophysiological characterization of HCs composed of Cx43: functional GJs can be formed by the docking of two HCs from apposed *Xenopus* oocytes (505), but free HCs not being part of GJs are electrically nonfunctional unless Cx43 COOH-terminal peptide is added (403) or the extracellular Ca^{2+} is reduced. Thus single-channel patch-clamp evidence is a requirement to conclude on HC involvement, and the evidence should derive from cells in which the investigated properties are relevant for the condition studied, be it physiological or pathophysiological. Related to this, there is currently convincing information available that demonstrates that Cx43 HCs can open in primary cultured astrocytes under realistic conditions and recorded at single-channel resolution. By realistic conditions, we mean cells held at normal negative resting potential and challenged by a modest (200–500 nM) increase of intracellular Ca^{2+} applied through the patch-pipette. Under those conditions, spiking HC opening activity with a single-channel conductance of ~220 pS typical for Cx43 HCs has been observed (156, 330). In our opinion, such HC opening is a demonstration of their functionality, i.e., they can be opened by realistic conditions as recorded by the gold-standard of channel function methods. If possible, the combination of electrophysiology with simultaneous dye uptake/release would be the best of both worlds, as this allows to correlate channel behavior for passing atomic ions versus molecular markers (58). Finally, for Cx43, there is now a range of excellent peptide tools (Gap19, L2, and CT9; **FIGURE 6**) that allow to distinguish HCs from GJCs (see sect. IV*B*).

C. Direct Contribution of Hemichannels to Gliotransmission

This question is critical in a physiological context as well as in pathological situation. Indeed, although we can speculate what permeates HCs, based on what we know about the permeability of GJCs for ions and signaling molecules, there is no final demonstration of their direct involvement as a channel in the release process. Do HCs contribute directly as a diffusive pathway for gliotransmitter release or do they rather contribute to HC Ca^{2+} entry with ensuing $[Ca^{2+}]_i$ changes that activate more complex release mechanisms? While there are strong arguments to consider that Cx HCs and Panx channels act as a pathway for Ca^{2+} entry and ATP release, this question remains unanswered in terms of the HC passage of other molecular actors of neuroglial interaction.

D. What About Cx/Panx Expression and Role in Microglia and Oligodendrocytes?

Based on the works cited and discussed in this review it is clear that we know much less in this domain, and it seems essential that efforts that have been focused on astrocytes now also need to be concentrated on the other types of glia. Ultimately, this should also include a better understanding of panglial networking between astrocytes and oligodendrocytes (76, 194).

E. Development of New Techniques

While in the 1990s most of the investigations on glial Cxs/Panxs were carried out in cell culture models, the following decade has seen the development of approaches in acute brain slices. Now it is clear that there is a need for in vivo investigations in unanesthetized animals, with either imaging or electrophysiological approaches allowing online monitoring of HC and GJC functions. Also, these approaches should be extended to include investigations at the behavioral and cognitive level to make a link between molecular events and their behavioral impact, this being particularly critical for animal models of brain pathologies. These in vivo investigations should be accompanied by concerted in vitro efforts to improve our understanding of distinct gating of free HCs and those incorporated in GJs. Such understanding is necessary to foster novel ideas and targets for developing new drugs distinctly targeting HCs and GJs.

F. Therapeutic Potential

Interestingly, given the role of GJCs and HCs in diverse neurological disorders, the case can be made for considering Cxs and Panxs as alternative therapeutic targets. The approach of targeting the astroglial networking has in fact been pursued by Theranexus, currently conducting clinical trials with modulation of Cxs in combination with neuropsychiatric drugs in narcolepsy (128) and neuropathic pain (236). Zealand Pharma has carried out a phase 2 clinical trial with the GJC modulating peptide danegaptide in acute myocardial infarction (139). Although the trial showed no improvement in salvaging myocardial tissue, preclinical studies have shown benefit in a mouse ischemia-reperfusion model (552). FirstString Research has followed up on the demonstrated preclinical efficacy of Cx43-directed therapeutics for glioma (193, 339). Finally, Ocunexus has focused on mimetic peptide therapeutics targeting retinal injury (256). As a whole, these pioneering studies will hopefully open the door to further opportunities to consider the neuroglial network in new treatments for neurological disorders.

IX. CONCLUDING REMARKS

Glial cells are the supportive cells of the CNS, but they also crucially participate in active brain functions and dysfunctions. By expressing Cxs and Panxs throughout their cellular lifetime, which differs from neurons that mainly express Cxs during brain development, these membrane channels are major players in neuroglial interactions. Although glial HCs are functional in physiological condition in certain brain regions, they are much less active than in pathological situations where they seem to play a deleterious role in several diseases and injuries. In contrast, GJCs ensure a homeostatic role through astroglial and panglial networks, for example, by contributing to metabolic trafficking and potassium or glutamate dissipation. These features should be considered as beneficial roles fostering continued investigations towards the properties of glial Cx and Panx channels to expand the simple and reduced role that was initially attributed to glia: to be "supportive" cells of the CNS.

ACKNOWLEDGMENTS

We are very grateful to Dr. Annette Koulakoff (Collège de France, Paris) who provided the picture of FIGURE 7*B*. We also acknowledge Philippe Michou-Saucet (Collège de France, Paris) for helping us in getting the references in good order. We apologize for any references we might have overlooked.

C. Giaume sadly passed away during preparation of this manuscript; this paper is in honor of his significant contribution to the glial connexin and pannexin field. An in memoriam paper can be found in Tabernero et al. *Glia* 68: 1321–1328, 2020 (DOI: 10.1002/glia.23836).

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GRANTS

This work was partially funded by CRPCEN (Caisse de Retraite et de Prévoyance des Clercs de Notaire) (to C. Giaume); Canadian Institutes of Health Research and the Natural Sciences, Engineering Research Council of Canada, and the Canada Research Chairs program (to C. C. Naus); Iniciativa Científica Milenio-ANID, Project P09-022-F, CINV (to J. C. Sáez); Fondo Nacional de Desarrollo Científico y Tecnológico Grant 1191329 (to J. C. Sáez); Fund for Scientific Research Flanders, Belgium, Grants G.0A82.13N and G.0527.18N (to L. Leybaert); Ghent University Special Research Fund (to L. Leybaert); and the Geneeskundige Stichting Koningin Elisabeth Grant STI.DI2.2017.0004.01 (to L. Leybaert). C. C. Naus and C. Giaume have been jointly supported by the Peter Wall Institute for Advanced Studies at University of British Columbia.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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