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REVIEW

Connexin: a potential novel target for protecting the central nervous system?

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Abstract

Connexin subunits are proteins that form gap junction channels, and play an important role in communication between adjacent cells. This review article discusses the function of connexins/hemichannels/gap junctions under physiological conditions, and summarizes the findings regarding the role of connexins/hemichannels/gap junctions in the physiological and pathological mechanisms underlying central nervous system diseases such as brain ischemia, traumatic brain and spinal cord injury, epilepsy, brain and spinal cord tumor, migraine, neuroautoimmune disease, Alzheimer's disease, Parkinson's disease, X-linked Charcot-Marie-Tooth disease, Pelizaeus-Merzbacher-like disease, spastic paraplegia and maxillofacial dysplasia. Connexins are considered to be a potential novel target for protecting the central nervous system.

***Key Words:** connexin; gap junction; biosynthetic pathways; biodegradation; brain; central nervous system diseases*

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Introduction

The majority of cell types that compose the neurovascular unit, including neurons, astrocytes and endothelial cells, have been found to express connexins (Cx), which play vital functions in the human central nervous system (CNS). Since Paul (1986) cloned and sequenced the first Cx in 1986, the Cx gene family has been found to constitute 19 members in mouse and 20 in human, 19 of which can be grouped as sequence-orthologous pairs (Willecke et al., 2002). Cxs are commonly named according to their molecular weight (*e.g.*, Cx43 has a molecular weight of 43 kDa) predicted from their primary amino acid sequence (Beyer et al., 1987). All Cxs have a topology composed of four membrane spanning regions, two extracellular loops, a cytoplasmic loop and cytoplasmic termini (Kumar and Gilula, 1996). Their primary function is to form jap junctions (GJs) and directly exchange ions and small molecules between adjacent cells (Leithe et al., 2012). Cxs have a short half-life of only a few hours, which is responsible for responding to physiological requirements to either up- or down-regulate the extent of GJ channel expression (Laird, 2006). This function appears to be essential for Cx-mediated gap junctional intercellular communication (GJIC) (Guo et al., 2003), which implies that modulation of Cx synthesis and degradation rate may be important for control of GJ levels under physiological or pathophysiological conditions (Laird, 2010). The most well established function of Cxs is to form GJs between adjacent cells. This network determines how easily small molecules diffuse by GJIC and how far locally initiated signals can be spread (Anders et al., 2014). Cxs can also form hemichannels (HCs) to exchange ions and signaling molecules between the intra- and extra-cellular environments. HCs can serve as autocrine/paracrine cellular communication pathways (Vega et al., 2013). Furthermore, Cxs have critical effects on cell adhesion, motility and migration that do not involve intercellular channels (Matsuuchi and Naus, 2013). Thus, Cxs/HCs/GJs have complex functions depending on the differing environments. Despite the fact that these functions remain to be fully elucidated, emerging evidence suggests a critical role of their dysfunction in the pathogenesis of CNS diseases (Seifert et al., 2006). In this review, we will discuss the function of Cxs/HCs/GJs under physiological conditions. Additionally, we will describe recent findings of diseases associated with Cxs/HCs/GJs in the CNS. An improved understanding of the function of Cxs/HCs/GJs in CNS pathogenesis offers the potential for development of novel strategies to treat neurological disorders.

Biosynthesis and Degradation of Cxs

Cx biosynthesis

Similar to other plasma membrane proteins, Cxs are delivered to the cell surface through the secretory pathway (Berthoud et al., 2004). Cxs are thought to be co-translationally thread into the rough endoplasmic reticulum (ER) *via* the

translocon and encoded start and stop transfer sequences (Laird, 2006). The majority of Cxs are subsequently delivered through the Golgi compartment. On their transit from the ER through the trans-Golgi network, Cxs are oligomerized into hexamers (or connexons). Proper folding and oligomerization of Cxs appear to be important steps for quality control systems. If newly synthesized Cxs are not correctly folded, they may be expelled in a proteasome-dependent manner *via* a process termed ER-associated degradation (Berthoud et al., 2004). At least 40% of newly synthesized wild-type Cx43 is estimated to undergo this process, which has been hypothesized to be a mechanism for regulating GJIC under physiological and pathological conditions (VanSlyke and Musil, 2002; Kelly et al., 2007). Connexon-containing vesicles are transported from the trans-Golgi network to the plasma membrane. Once they reach and fuse with the plasma membrane, some connexons dock with each other in the adjacent cells and become parts of GJ plaques (Berthoud et al., 2004; Laird, 2006). Cxs can also form HCs that communicate with the extracellular space and maintain the integrity of the lumens of these intracellular compartments (Froger et al., 2010). There is strong evidence to suggest that microtubules facilitate Cx trafficking to improve the efficiency of the delivery process, although they do not appear to be essential (Johnson et al., 2002; Lauf et al., 2002).

Cx degradation

The half-life of Cxs is very short, which indicates a rapid rate of metabolism. Gaietta et al. (2002) first reported that newly synthesized Cxs were transported to the plasma membrane and incorporated at the periphery of existing GJs, whereas old Cxs were removed to the central core. These finding suggest that the older Cxs in the center of GJs were likely to be destined for internalization and degradation in the cytoplasm. Approximately two decades earlier, researchers discovered that the entire GJs or fragments may form double-membrane circular structures. These structures named "annular junctions" were bound to be degraded (Severs et al., 1989). A later study revealed that annular junctions originated from pre-existing GJ plaques between contacting cells (Jordan et al., 2001), and they were thus renamed 'connexosomes' to reflect their Cx-rich status (Laird, 2006). The connexosomes internalize into one of two opposing cells to form double-membrane GJ vesicles, and are quite large and structurally different from typical endocytic organelles. Therefore, the internalization mechanism is likely to be distinct from conventional endocytic processes such as classical endosomes orphagosomes (Hesketh et al., 2010). It is now known that Cxs are internalized by at least two distinct pathways. Piehl et al. (2007) reported that complete or large portions of GJ plaques were subdivided into large cytoplasmic vesicles (0.5–5 μm in diameter) that were slowly degraded by endo/lysosomal pathways, which occurred over a period of 20–60 minutes. Another continuous and fast (few seconds) internalization mechanism was reported for small GJ vesicles (0.18–0.27 μm in diameter), which bud from the central regions of plaques and translocate much more rapidly (within minutes) into cells for degradation (Falk et al., 2009). After entering the early endosome, endocytosed proteins can be transported further downstream in the degradation pathway to the lysosome, to undergo recycling to the plasma membrane, or are transported to the trans-Golgi network (Scita and Di Fiore, 2010).

The Function of Cxs/HCs/GJs under Physiological Conditions

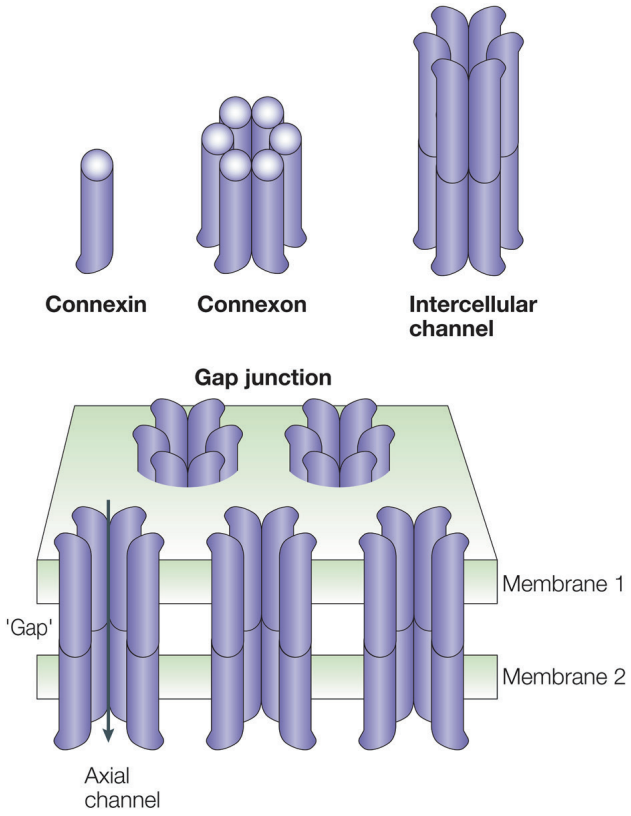

Figure 1 Connexin (Cx), connexon/hemichannel (HC) and intercellular channel and gap junction (GJ).
Schematic showing the relationships between the Cx monomer, the hexameric assembly of Cxs into an HC and the two HCs forming an intercellular channel. Clusters of intercellular channels are known as GJs, which provide an axial channel (arrow) that allows exchange of ions and small metabolites between adjacent cells. This figure is reproduced from *Nature Reviews Molecular Cell Biology* (Goodenough and Paul, 2003) with permission.

Channel-dependent function

The most well established function of Cxs is the formation of GJs (**Figure 1**). The term 'GJ' comes from histological studies, where they were first described by electron microscopy using heavy metal incubation (Revel and Karnovsky, 1967). GJ channels are large, poorly selective, aqueous pores that are assembled by the head-to-head docking of two half GJ channels or HCs, each delivered by one of the partner cells (De Bock et al., 2014a). Freeze-fracture analysis revealed that GJs can form GJ plaques that contain less than a dozen

to up to 200,000 units, and which extend from several nanometers to a few micrometers in diameter (Bruzzone et al., 1997; Falk, 2000). GJ plaques composed of multiple GJ channels spanning the two plasma membranes are found in almost all tissues (Sosinsky and Nicholson, 2005). Hydrophilic molecules smaller than 1 kDa are able to travel through the GJ channels, which is critical for direct exchange of ions and small metabolites between adjacent cells (Alexander and Goldberg, 2003). It is widely believed that connexons also play critical functions in the exchange of ions and signaling molecules between the intra- and extracellular environment, which are often termed HCs (Stout et al., 2004; Spray et al., 2013). HCs are not incorporated into GJs, and they mainly allow direct contact between the cytosol and extracellular space. Under normal conditions, most HCs are primarily in a closed state, while they can open following stimuli such as mechanical stress, ischemia, inflammation and high extracellular calcium (Goodenough and Paul, 2003; Burra et al., 2010; Eugenin et al., 2012; Fiori et al., 2014). HC opening allows the free exchange of ions and small metabolic or signaling molecules with a molecular weight < 1–2 kDa in a bidirectional manner, which may contribute to paracrine signaling (Wang et al., 2013a).

Channel-independent function

In addition to the established role of GJs and HCs, there are many channel-independent roles in cytoskeletal changes. These processes include alterations in cell morphology, the regulation of cell polarity and an influence on cell motility (Kameritsch et al., 2012; Matsuuchi and Naus, 2013). The underlying mechanism involved in these processes includes the initial breakdown of the existing actin cytoskeletal network matrix. Although the exact mechanistic details remain unclear, the C-terminal region of Cxs is thought to contain the majority of the regulatory and protein-protein interaction domains (Cina et al., 2009; Solan and Lampe, 2009). Extensive evidence also suggests that Cxs regulate cell growth *via* a variety of processes, which plays an important role in the control of gene expression and tumor formation (Kardami et al., 2007; Cronier et al., 2009). Although some of these effects may be linked to GJIC, increasing evidence indicates that at least some of these changes are the results of the interaction between Cxs and other scaffold or signaling molecules (Olbina and Eckhart, 2003; Fu et al., 2004).

Both Cx47 and Cx32 knock-out mice were found to develop profound CNS demyelination associated with gross tremors and tonic seizures that cause death by postnatal weeks 5–6 (Menichella et al., 2003). This study provided the first evidence that GJIC was crucial for normal CNS myelination. In addition, a recent study supported that Cx was a rather unexplored but promising target for influencing CNS barrier function (De Bock et al., 2014a). Nevertheless, the mechanisms underlying non-channel activities remain largely unknown, but likely reflect cell-type, tissue-type and Cx-type specificity of responses, as well as multiple protein-protein interactions.

The Function of Cxs/HCs/GJs in the Human CNS Disorders

Brain ischemia

Brain ischemia remains a leading cause of morbidity and mortality in the developed world. Neuronal cell death can occur in the core region within seconds to minutes after ischemia. If blood flow is not restored quickly, the progression of cell injury and death will last for several hours to days, thereby expanding the infarct region to the surrounding area, termed the penumbra (Turner et al., 2013). Cxs have been suggested to contribute to the wave of delayed secondary injury that spreads from the core region to the penumbra (De Bock et al., 2014b). Ischemia-related injury is mostly associated with changes of astrocyte GJIC and an aberrant opening of HCs (Retamal et al., 2007; Wang et al., 2013b). The opening HCs leads to cell dysfunction *via* the collapse of membrane potential and entry of Ca^{2+}, and also promotes release of glutamate and ATP that may further cell death (Decrock et al., 2011). Additionally, GJs may contribute to the propagation of cortical spreading depression (CSD), a wave of tissue depolarization followed by neuronal inactivation associated with increased infarct volume after cerebral ischemia (Theis et al., 2003).

Traumatic injury

Traumatic brain and spinal cord injury may cause immediate neuronal and glial cell death, followed by a cascade of secondary events leading to on-going spread of tissue damage. This secondary injury includes the inflammatory response, free radical formation and excitotoxicity, which lead to demyelination, axonal degeneration, glial scarring and neovascularization surrounding the area of initial damage (Hausmann, 2003; Norenberg et al., 2004). In the rodent, the level of Cx43 protein in astrocytic GJs is up-regulated after traumatic injury of the CNS (Cronin et al., 2008). Treatment with a Cx43 mimetic peptide also reduces secondary tissue damage after spinal cord injury by reducing gliosis and cytokine release (O'Carroll et al., 2013). Cx36 plays a detrimental role in injury-mediated neuronal death, and elimination of Cx36 and/or inactivation of the mechanisms for increased Cx36 expression is neuroprotective (Wang et al., 2012). In addition, the expression of Cxs (Cx29 and Cx32) on oligodendrocytes is increased in the narrow band border between the penumbra and the core regions of injury (Moon et al., 2010).

Epilepsy

Epilepsy is a CNS condition characterized by the periodic and unpredictable occurrence of seizures. Enhanced GJIC between neurons is considered a major factor involved in direct intercellular cytoplasmic connections and the promotion of hypersynchronous neuronal activity associated with seizures (Seifert et al., 2010). Interestingly, neurosurgical specimens from patients with temporal lobe epilepsy typically demonstrate marked reactive gliosis (Seifert et al., 2010). The expression of astrocytic Cx mRNAs (Cx30 and Cx43) is several fold higher than that of neuronal Cx mRNAs (Cx36

and Cx45), and glial cells outnumber neuronal cells in mammalian hippocampal and cortical tissues (Mylvaganam et al., 2014). GJs in astrocytes appear to play a dual role: on one hand they counteract the generation of hyperactivity by facilitating clearance of elevated extracellular K^+ levels, while on the other hand they constitute a pathway for energetic substrate delivery to fuel neuronal hyper-activity (Steinhäuser et al., 2012). Although there is no doubt that Cx-based GJs and HCs are related to epilepsy, the specific details of their involvement remain to be elucidated.

Tumors

Tumors of the CNS are relatively rare compared with tumors of other tissues, accounting for less than 2% of all malignancies (Parkin et al., 2001). However, brain tumors are often lethal and the average survival rate of patients is low after diagnosis. Glioma is one of the most frequent primary brain tumors in adults, which is a space-occupying mass in the brain that causes a high intracranial pressure, vessel occlusion and brain edema (Behin et al., 2003). Glioma has been traditionally classified by the World Health Organization into four grades, of which grade IV is the most malignant (Sin et al., 2012). In the last decade, Cx has been shown to participate in diverse cellular processes including development, differentiation, homeostasis and survival. Interestingly, Cx43 was identified at areas of cell-to-cell contact between co-cultured glioma cells and astrocytes, and this cellular coupling had profound effects on the phenotypic transformation of astrocytes and the susceptibility of surrounding tissue to glioma invasion (Zhang et al., 1999). However, the potential role of Cxs in tumor growth and expansion remains controversial (Schalper et al., 2014). For example, although it is widely recognized that Cxs exhibit tumor suppressive properties, recent evidence suggests a role in promoting tumor growth and metastasis under some circumstances (Naus and Laird, 2010). In general, decreasing Cx43 expression is associated with increasing proliferation and higher tumor grades. In a screen of 18 human samples in which Cx43 expression was examined in different glioma stages, a reduction of Cx43 was found to be associated with glioma progression (Huang et al., 1999). A similar result was also obtained by other groups (Soroceanu et al., 2001; Pu et al., 2004). However, a recent study of 32 human samples reported elevated levels of Cx43 mRNA, but reduced levels of Cx43 protein, in high-grade glioma, suggesting an alteration of post-transcriptional mechanisms (Caltabiano et al., 2010). Therefore, it is clear that Cx43 expression is highly heterogeneous, and it may perform different functions depending on local microenvironment of the tumor (Naus and Laird, 2010).

Migraine

Migraine is a complex familial disorder of the brain characterized by recurrent unilateral headache, usually accompanied by nausea, vomiting, photophobia and/or phonophobia (Edvinsson and Uddman, 2005). Although several hypotheses have been proposed for the initiation of migraine, its mechanism remains unclear (Goadsby et al., 2009). A mutation in a calcium gene channel has been suggested to sensitize individual neurons to environmental factors, resulting in a wave of CSD when the attack occurs (Edvinsson and Uddman, 2005). Neuronal activity is thought to be the main cause of migraine initiation. However, it has also been shown that neuronal-glial communication *via* GJs and paracrine signaling was involved in CSD activity and migraine pathology (Thalakoti et al., 2007). Tonabersat was first identified as an anti-epileptic drug and showed significant efficacy as a novel GJ modulator for the treatment of migraine (Silberstein, 2009). Tonabersat can inhibit Cx26 GJIC between glial cells and neurons in the sensory part of the trigeminal nerve and prevent CSD, resulting in reduced migraine attacks in animal models and in humans (Damodaram et al., 2009). Nevertheless, further studies are required to determine the exact roles of Cxs in migraine.

Neuroautoimmune disease

Multiple sclerosis is a chronic inflammatory demyelinating disease of the CNS, characterized by degeneration of oligodendrocytes, demyelination of neurons and consequently axonal loss and neurological deficits (Compston and Coles, 2002). Similarly, demyelination occurs in neuromyelitis optica, a variant of multiple sclerosis, although with a different pathophysiology and localization. In an experimental autoimmune encephalomyelitis model of multiple sclerosis, demyelinating lesions were found to exhibit significantly decreased Cx43 expression, while recovering lesions had markedly increased Cx43 expression (Roscoe et al., 2007). Masaki et al. (2013) also reported that oligodendrocyte Cx32/Cx47 expression was decreased in most active and chronic lesions from multiple sclerosis and neuromyelitis optica patients. Interestingly, Cx43-specific antibodies were absent in all samples in that study. Further, Cx43 loss was significantly associated with a rapidly progressive disease course of multiple sclerosis and neuromyelitis optica. Therefore, the differential expression of Cx43 in active and chronic lesions implies differing roles at different stages in multiple sclerosis and neuromyelitis optica (Moinfar et al., 2014). However, the underlying mechanisms of Cx43 signaling in these diseases remains poorly understood (Kielian, 2008).

Alzheimer's disease (AD)

AD is one of the most prevalent forms of dementia in humans. The major clinical manifestations are cognitive deficits such as memory and learning impairment. AD is histologically characterized by substantial neuronal and synaptic loss associated with extracellular β-amyloid (Aβ) accumulation in the form of senile plaques (Selkoe, 2001). Several studies have shown that Aβ can stimulate astrogliosis, which is also observed in the human AD brain (Nagele et al., 2004; Olabarria et al., 2010). The expression of Cx43 and Cx30, the two main Cxs in astrocytes, was reported to increase in the immediate vicinity of the majority of Aβ plaques in AD mouse models (Mei et al., 2010). Further, brain samples of AD patients displayed an increase in Cx43 expression in senile plaques (Nagy et al., 1996). Moreover, Aβ peptide has been shown

to promote astrocyte activation and the release of glutamate and ATP through Cx43 HCs, resulting in neuronal death by opening of Cx36 HCs in neurons (Orellana et al., 2011).

Parkinson's disease (PD)

PD is an adult-onset neurodegenerative disease characterized by the loss of dopaminergic neurons in the substantia nigra-striatum. The clinical symptoms of PD include progressive tremor, muscle rigidity and gait disturbance. Rufer et al. (1996) reported that immunoreactive Cx43 protein was increased in the striatum of the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of PD, although no evidence of increased functional coupling was observed. Kawasaki et al. (2009) also reported that Cx43 expression was enhanced in a rat PD model induced by rotenone and in vitro in cultured astrocytes stimulated with rotenome. These data suggest that modulation of Cx43 in astrocytes with a participation of GJIC may play an important role in PD pathology (Kawasaki et al., 2009). However, the mechanism linking enhanced astrocyte GJIC and dopaminergic neuron death requires urgent clarification. Tremors are also commonly observed in PD patients. Although the causes of these tremors remains unclear, the inferior olive is thought to play a key role (Loewenstein, 2002). Interestingly, there is some evidence for a role of GJIC in inferior olive neurons (Loewenstein, 2002), although no difference in the severity of harmaline-induced tremors was observed between Cx36 knockout mice and wild-type mice (Long et al., 2002). Further experiments are required to clarify the mechanism of tremors in PD patients.

X-linked Charcot-Marie-Tooth disease (CMTX)

CMTX is an inherited motor and sensory neurological disorder that causes progressive distal muscle weakness and atrophy, sensory loss and decreased or absent tendon reflexes (Scherer and Kleopa, 2012); cognitive impairment can also occur (Stancanelli et al., 2012). Over the last two decades, there have been rapid advances in understanding the molecular basis for CMTX, with more than 400 different mutations in GJB1 described (Scherer and Kleopa, 2012). GJB1 can encode Cx32, a GJ protein expressed by many cell types, including oligodendrocytes and Schwann cells. Cx32 forms reflexive channels that allow the passage of ions and signaling molecules across the myelin sheath in Schwann cells (Bicego et al., 2006). Nevertheless, despite this broad expression pattern of Cx32, peripheral neuropathy is usually the sole clinical manifestation of CMTX. It is possible that co-expression of other Cxs may offset some damage caused by the loss of Cx32 (Scherer and Kleopa, 2012). A GJB1 gene replacement strategy may be useful treatment of patients with CMTX (Shy et al., 2007).

Pelizaeus-Merzbacher-like disease (PMLD)

PMLD is an autosomal recessive inherited severe leukoencephalopathy in humans caused by mutations in GJA12 (or GJC2) gene encoding for Cx47, and is characterized by nystagmus, progressive spasticity, ataxia and hypomyelination on MRI (Uhlenberg et al., 2004). Cx47 is primarily expressed in the cell bodies of oligodendrocytes, and mutant proteins appear to accumulate partially in the endoplasmic reticulum. Mutations associated with PMLD disrupt Cx47/Cx47 and Cx47/Cx43 GJIC between oligodendrocytes and astrocytes, impede the passage of molecules between cells and results in loss of function (Orthmann-Murphy et al., 2007; Mi et al., 2013). These changes result in a decreased number of cells coupled within glial networks, rather than detrimental function of the mutated Cx47 protein (Tress et al., 2011).

Spastic paraplegia

Spastic paraplegia is a syndrome describing inherited disorders in which lower extremity weakness and spasticity are the predominant symptoms. There are more than 50 genetic types of spastic paraplegia (Fink, 2013). Orthmann-Murphy et al. (2009) described three cases of SPG44 from one family with a homozygous 133M mutation of GJA12/GJC2. This phenotype caused a milder symptom: late onset (first and second decades), cognitive impairment, slowly progressive, spastic paraplegia, dysarthria and upper extremity involvement. The mutant proteins form GJ plaques at cell borders similar to Cx47, but fail to form functional homotypic channels. This is particularly interesting because the spastic paraplegia phenotype is thought to represent a length-dependent axonopathy, and suggests that mutant forms of Cx47 may directly interfere with interactions between oligodendrocytes and their associated axons (Abrams and Scherer, 2012).

Maxillofacial dysplasia

Oculodentodigital dysplasia is a clinically variable genetic disorder caused by mutations of the GJA1 gene at chromosome 6q22–23, predominantly inherited in an autosomal dominant fashion (Paznekas et al., 2003). The genetic mutations affect highly conserved amino acid residues located in different portions of the Cx43 protein (Jamsheer et al., 2014). The phenotype of oculodentodigital dysplasia comprises craniofacial, dental and digital abnormalities (Paznekas et al., 2003). Hallermann-Streiff syndrome is a rare disorder, mostly reported in case studies. Hallermann-Streiff syndrome is an autosomal recessive or sporadic syndrome that shows substantial overlap with oculodentodigital dysplasia, and is characterized by dyscephalia, and facial and dental abnormalities (Thomas et al., 2013). However, while oculodentodigital dysplasia is a dominantly inherited disorder due to mutations in GJA1, the inheritance pattern of Hallermann-Streiff syndrome remains controversial (Pizzuti et al., 2004). As noted, some patients with a 'full blown' Hallermann-Streiff syndrome phenotype had no mutations in the GJA1 coding region (Abrams and Scherer, 2012).

Conclusion/Perspective

It is now clear that Cxs can form GJs and syncytial networks, and play a key role in the direct exchange of ions and small molecules between adjacent cells. Cxs can also form HCs that provide large, relatively nonselective conductances in single plasma membranes. In addition, the function of Cxs

is independent from HCs in the regulation of growth, development and differentiation of the CNS. Although the molecular basis of Cxs/HCs/GJs function remains to be fully determined, novel physiological functions are continuing to be found. The combination of specific Cxs/HCs/GJs blockers with gene ablation approaches would provide the most compelling evidence for their function. The discovery of Cx-linked human diseases has provided further interest in the biology of HCs/GJs. The roles of Cxs in human CNS diseases appear to be dependent on the particular role of the Cxs and the interaction with other proteins in a given tissue. Clearly, the establishment of additional animal models of Cx-linked diseases will help to elucidate the roles of Cxs in normal and pathological conditions in the human CNS, although the methods for separating the intercellular channel, hemichannel and non-channel activities of Cxs need to be developed. A complete understanding of these communication systems will provide essential information for the development of novel therapeutic approaches.

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