

# Connexin and Pannexin Based Channels in the Nervous System

## Gap Junctions and More

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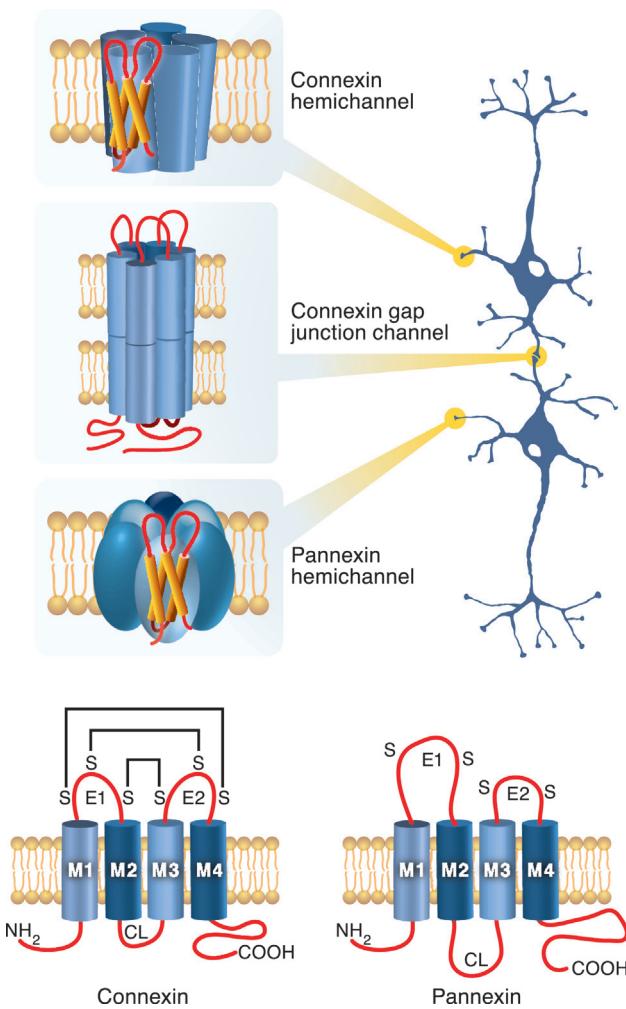
### CELL INTERACTIONS IN THE NERVOUS SYSTEM—THE LARGER PICTURE

The coordination of cell functions is a challenge for all organ systems of the body. This is achieved in part through a variety of exocrine and endocrine mechanisms that involve the complex release of signals from cells, their detection by receptors in other cells, and the ultimate generation of an intracellular response in the target cell. However, there is also an ancient mechanism that evolved with the earliest of multicellular organisms whereby signals, nutrients and other metabolites under about 1,000 in MW are exchanged directly between adjacent cells without dilution through the extracellular environment. This occurs through structures called gap junctions. The nervous system is no exception to this. Gap junctions are present at homocellular and heterocellular contacts between most cells in the CNS and PNS, including astrocytes, oligodendrocytes, microglia, endothelial and ependymal cells and some neurons. Between neurons, gap junctions have a unique role in that they form electrical synapses, which contrast with, and complement, the role of the more extensively studied chemical synapses that predominate in the nervous systems of most animals above the Coelenterates. This chapter will discuss the functional significance of electrical synapses in the nervous system, as well as other roles of gap junctions associated with the intercellular transport of metabolites and signals between not only neurons, but many cell types within the CNS. The significance of hemichannels, which form away from sites of cell-cell contact and allow exchanges of similar repertoire of molecules with

the extracellular environment, will also be considered, along with roles of the gap junction as a nexus for signaling and an adhesive structure between cells of the nervous system. Misregulated or abnormal hemichannels and/or gap junction channels contribute to both acquired and genetic pathologies, and their roles in specific disease states, including knockout phenotypes, will be discussed.

### GENERAL PROPERTIES AND STRUCTURE OF GAP JUNCTION CHANNELS AND HEMICHANNELS

Gap junctions are specialized membrane structures between closely apposed cells that permit limited cytoplasmic continuity between cells. A gap junctional plaque contains up to thousands of gap junction channels that span the plasma membranes of the two cells and the 2-nm wide extracellular “gap” that separates them, and gives them their name (Revel and Karnovsky, 1967). Each channel is formed by two hemichannels, or connexons, found at the appositional membrane of adjacent cells (Fig. 9.1). The hemichannel is composed of six protein subunits called connexins in vertebrates, or innexins in invertebrates. While these two groups of proteins have similar topologies and form analogous structures, they are unrelated in sequence. In fact, distant relatives of the innexins, called pannexins, are found in the vertebrates, including man, but as we will discuss in the following section, their function as gap junctions has been replaced by connexins. A comprehensive summary



**FIGURE 9.1** Schematic diagram of the distribution of gap junctions and hemichannels on a neuron. Gap junctions, representing large parallel arrays of intercellular channels composed of connexin proteins (two hexameric ‘hemichannels docked head-to-head—top left insert), form electrical synapses between neurons. Hemichannels can also form on unopposed cell surfaces of neurons and most other cells in the CNS, and are composed of either connexin (top right insert) or pannexin proteins (middle right insert). The topology of pannexins and connexins (bottom), is very similar, and while each have conserved cystines forming intramolecular disulfides in the extracellular loops (Foote et al., 1998), there are three per loop in connexins and two per loop in innexins. There is no homology in their primary sequence, as pannexins evolved from the invertebrate gap junction protein family of innexins.

of both connexin and pannexin/innexin gene families can be found in [Abascal and Zardoya \(2013\)](#).

## Connexins

Connexins form a highly conserved protein family encoded by 21 different genes in the human and 20 in the mouse genome (Willecke et al., 2002) that are

widely expressed in mammalian tissues. Each connexin is commonly referred to according to the predicted molecular weight of the human ortholog (e.g., connexin43 (Cx43) has a molecular weight of ~43 kDa), with species other than human designated by a prefix (e.g., mCx43 for mouse, XeCx37 for *Xenopus*, etc.) A different nomenclature has been adopted for the genes, using the prefix GJ and grouping the connexins into four classes (A, B, C and E) based on sequence homology. Within each class, individual genes are assigned an Arabic numeral that largely reflects their order of discovery (i.e., Cx43 is GJA1). A list of connexins found in the nervous system, along with both protein and gene names, and the cell types in which they are expressed, is provided in [Table 9.1](#).

## Pannexins and Innexins

By contrast, only three pannexin genes have been identified in mammals (designated Panx 1–3) ([Litvin et al., 2006](#)). However, in invertebrates, innexins display a diversity comparable to the connexins, with 24 genes in *C. elegans* and 8 in *D. melanogaster*. While an innexin naming system has been adopted for the genes, the proteins are often referred to by complex names that relate to the mutant phenotype with which they are associated (e.g., in *C. elegans*, the Unc proteins associated with uncoordinated movement, and the Eat proteins associated with digestive tract problems, and in *Drosophila*, the Ogre protein associated with head and eye deformities and the ShakB protein associated with muscle tremors).

## Channel Structure

Connexins and innexins are both tetra-membrane spanning proteins with N- and C-termini located in the cytoplasm. While both families have highly conserved cysteines in their extracellular loops, there are only two per loop in innexins, and three in connexins. These have been shown to form intramolecular disulfides in connexins that are essential for docking of the hemichannels to form gap junctions (Foote et al., 1998). Higher resolution structures of the pore, based on electron diffraction (Unger et al., 1999), cryo-electron microscopy (Oshima et al., 2007) and X-ray diffraction (Maeda et al., 2009) implicate two of the four transmembrane helices of each subunit as contributing to the pore (Fig. 9.2B), identified as the first and second transmembrane spans in X-ray models. A systematic screening of cysteine mutations for exposure to the pore had also implicated the first transmembrane span as lining the pore in hemichannels of Cx46 (Zhou et al., 1997; Kronengold et al., 2003), but similar scans of gap junction

**TABLE 9.1** Connexin and Pannexin Expression in the Nervous System

Cell Type	Protein	Cell Type/Stage	Gene Name
Neurons	Cx36	broadly expressed	<i>Gjd2</i>
	Cx30.2	broadly expressed	<i>Gjc1</i>
	Cx45	retina/olfactory bulb	<i>Gjd3</i>
	Cx57	horizontal cells	<i>Gja10</i>
	Cx26	horizontal cells/ hemichannels	<i>Gjb2</i>
	Px1	broadly expressed	<i>Px1</i>
	Px2	broadly expressed	<i>Px2</i>
Neurons (early devp't.)	Cx43	stomach and intestine	<i>Gja1</i>
	Cx26	radial glia	<i>Gjb2</i>
	Cx32		<i>Gjb1</i>
Astrocytes	Cx43		<i>Gja1</i>
	Cx30		<i>Gjb6</i>
	Cx26		<i>Gjb2</i>
Oligodendrocytes	Cx32	also in Schwann cells	<i>Gjb1</i>
	Cx47		<i>Gjc2</i>
	Cx29		<i>Gjc3</i>
Microglia	Cx43	when activated	<i>Gja1</i>
	Cx32	when activated	<i>Gjb1</i>
	Px1		<i>Px1</i>
Vasculature (BBB)	Cx43	endothelium and myo-endothelium	<i>Gja1</i>
	Cx40	endothelium	<i>Gja5</i>
	Cx37	myo-endothelium	<i>Gja4</i>
Leptomeninges	Cx26		<i>Gjb2</i>
	Cx43		<i>Gja1</i>
	Cx30		<i>Gjb6</i>

**Bold Proteins** indicate the most abundant or widely expressed in that cell type

channels indicated that the second and third transmembrane segments form the pore in this configuration of the channel (Skerrett et al., 2002). This raises the possibility that gap junctions and hemichannels may have different folding configurations.

The most variable domains between members of the family are those located in the cytoplasm, with the central loop and C-terminus varying significantly in size and sequence within the family. The C-terminal domain, in particular, contains many regulatory and binding sites, which have been well characterized in the case of Cx43 (Fig. 9.2C). The C-terminus has not only been shown to modulate junctional activity

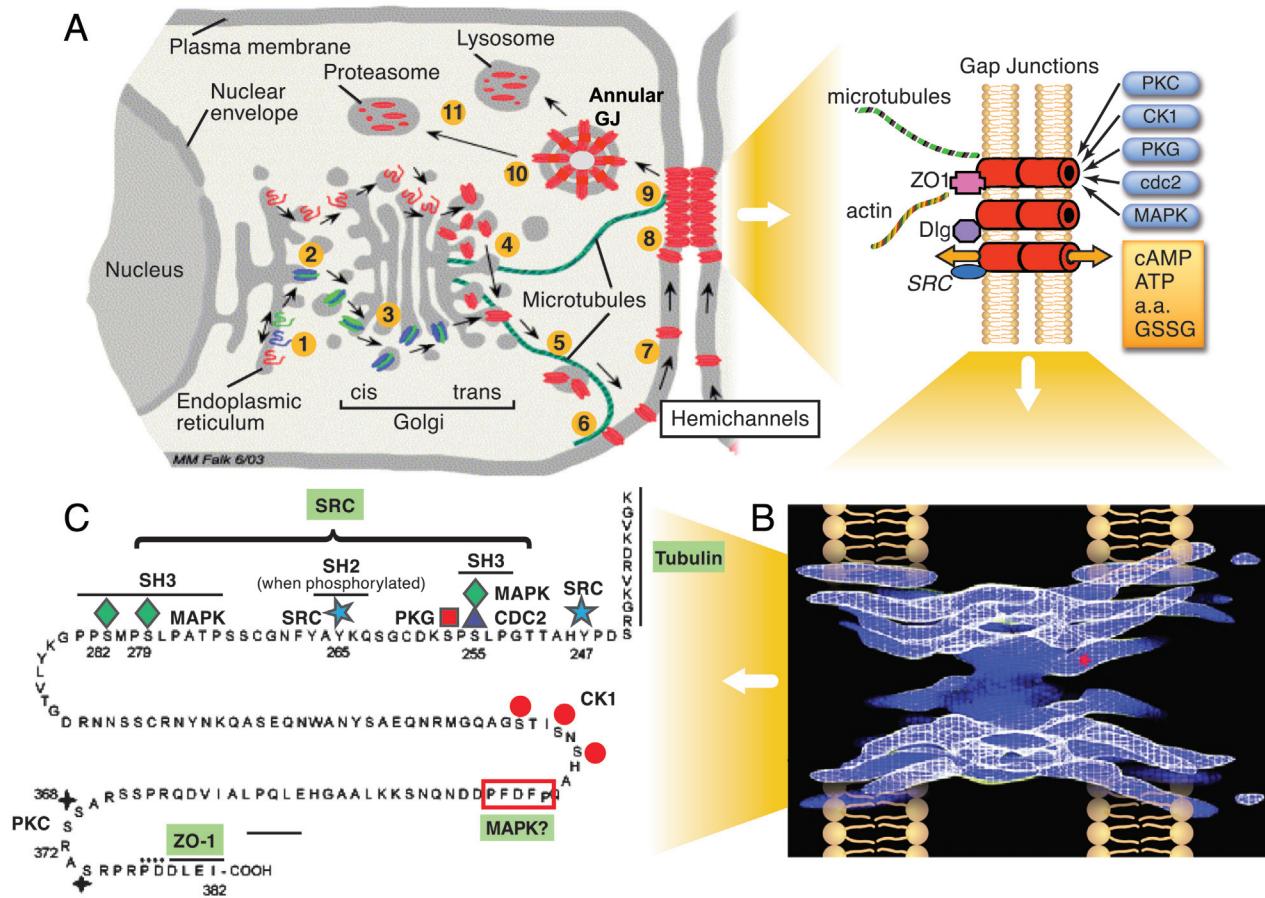
through gating, degradation, or changes in biosynthesis, it has also been implicated in mediating some of the effects of connexins through binding of signaling molecules and proteins that mediate links to the cytoskeleton or other junctional complexes, such as tight and adhesion junctions (Hervé et al., 2012). There have even been reports that the C-terminus can be cleaved, move to the nucleus, and potentially affect transcription of other genes (Jiang and Gu, 2005).

## Lifecycle of a Gap Junction

Depending on the connexin type, newly synthesized connexins are assembled in the ER (e.g., in the case of Cx26 and Cx32) or Golgi apparatus (e.g., in the case of Cx43) to form hemichannels (Martin et al., 2001; Sarma et al., 2002). The hemichannels are then transported in vesicles to the cell surface via a microtubules-dependent system (George et al., 1999; Martin et al., 2001; Shaw et al., 2007). Once inserted into the membrane at sites of cell-cell apposition, hemichannels diffuse laterally towards the center of a plaque to find and dock in series with hemichannels from an adjacent cell, thus forming gap junction channels (Fig. 9.2A). In time, new channels are formed at the periphery of a gap junction plaque while existing channels are removed from the central region by internalization into one of the two contacting cells. The fact that gap junction channels are not split into two hemichannels before internalization indicates that interaction between hemichannels provided by two contacting cells is very strong, so that connexins may play relevant functional roles as adhesion proteins. During the internalization process, cytoplasm from the adjacent cell is captured, (Gaietta et al., 2002) presumably resulting in a small amount of intercellular trafficking of macromolecules. Internalized junctions are then degraded via proteosomes and/or lysosomes (Laing et al., 1997; Jordan et al., 2001). Hemichannels forming cell-cell gap junction channels do not seem to be reused. The half-life of several rodent connexins has been measured as 2 to 5 h (Laird, 2006).

## Channel Properties of Gap Junctions

The rapid turnover of gap junctions indicates that intercellular coupling could be modulated by changes in synthesis and/or degradation rate of connexins, at least within reasonable time-frames of development. More rapid regulation can be achieved by gating of the gap junction channels, of which only a small fraction (~10%) are open under resting conditions (Bukauskas et al., 2000; Palacios-Prado et al., 2009, 2010). These channels can be induced to close by changes in transjunctional ( $V_j$ ) and sometimes



**FIGURE 9.2** (A) Schematic diagram of the life cycle of connexins. Different connexin subunits (red (e.g., Cx43), blue (e.g., Cx32) and green (e.g., Cx26)) are inserted into the ER, where some oligomerize (Cx32 and 26), while others only do so in the Golgi (Cx43). Hexamers are transported to the surface on microtubules to sites of close cell apposition. These “hemichannels” then diffuse to points of cell contact where they dock with a hemichannel from the apposing cell and assemble laterally into a gap junction. Gap junctions are removed by invagination of the whole structure into one cell to form an “annular gap junction” in the cell that is then targeted for proteasomal or lysosomal degradation. This whole process has a half-life of 2–5 hours. The insert at the right shows how gap junctions, which serve as conduits for exchange of ions and other metabolites (yellow box), provide anchoring points for both cytoskeletal and signaling elements within the cell, and are targets of regulation by several kinases. Modified from [Yeager et al., 1998](#).

(B) Detailed structure of a gap junction channel in profile derived from electron diffraction studies of isolated Cx43 gap junctions at 7.5 Å resolution. From [Unger et al., 1999](#).

(C) Diagram of the C-terminal cytoplasmic domain of Cx43 that illustrates the number of phosphorylation sites (PKC, CKI, ERK, PKG, cdc2 and v-src) and binding sites for cytoskeletal (tubulin and ZO1 for actin binding) and signaling (src, MAPK) molecules that can potentially regulate these channels.

transmembrane voltage ( $V_m$ ), elevated cytoplasmic  $H^+$ ,  $Ca^{2+}$  levels, certain lipophilic agents, and protein phosphorylation (Harris, 2001).

Gap junctions allow coordination of cellular responses by permitting the exchange of metabolites (e.g., ATP, ADP, glucose, glutamate and glutathione) and second messengers (e.g., cAMP,  $Ca^{2+}$  and inositol 1,4,5-triphosphate ( $IP_3$ )) (Sáez et al., 2003). They also coordinate electrical activity within cell communities through the passive spread of electronic potentials. These functions depend, in part, on channel conductance and permeability properties. Some gap junction

channels are slightly more permeable to anions, whereas most show a mild preference for cations or exhibit little charge selectivity (Sáez et al., 2003). However, their selectivity for larger molecules is more marked (size selectivity or exclusion size). Some connexins form highly restrictive pores with size cut-offs below 400 Da, while others allow the passage of molecules well above 1,000 Da (Harris, 2001). In addition, there is growing evidence that gap junctions composed of different connexins can show distinct selectivities for endogenous metabolites and signaling molecules (Goldberg et al., 1999; Bedner et al., 2006). These

channels have also been shown to pass significantly larger molecules if in a linear configuration, specifically peptides up to 6 or 8 amino acids in length during cross-presentation of antigens (Neijssen et al., 2005), and small interfering and micro-RNAs (Valiunas et al., 2005; Kizana et al., 2009). The latter has broad relevance to how gene regulation may be propagated between cells in close contact. Therefore, the physiological role of gap junctions is determined by their total expression levels, regulatory properties, as well as the specific signals and/or metabolites that each connexin isotype transmits.

### Hemichannels—More than Half of a Gap Junction

The presence of hemichannels on the cell surface (Fig. 9.1 and 9.2A) has been demonstrated using morphological, biochemical, electrophysiological, and functional criteria (Bennett et al., 2003). Several connexins (e.g., Cx46, 50, 46, 45, 43, 32, 26 and XeCx38) expressed in exogenous systems have been shown to generate relatively nonselective currents in the plasma membrane that have been attributed to hemichannel opening (Retamal et al., 2007a). These hemichannels are permeable to fluorescent permeability tracers (carboxyfluorescein, cascade blue, Lucifer Yellow and calcein) as well as large ions like ethidium bromide. Physiological concentrations of extracellular  $\text{Ca}^{2+}$  (1–2 mM) and membrane depolarization maintain these hemichannels closed to prevent leak of metabolites from cells. Hemichannels have, however, been induced to open in the presence of basal  $\text{Ca}^{2+}$  concentrations, in response to mechanical stress on the membrane (Bao et al., 2004a; Batra et al., 2012), treatment with FGF-1 in proliferating cells, with glucose in tanicytes (Orellana et al., 2012), following metabolic inhibition, hypoxia-reoxygenation, or treatment with inflammatory cytokines in astrocytes (Contreras et al., 2002; Retamal et al., 2007b; Orellana et al., 2010, 2011).

Intracellular conditions can also induce hemichannels opening, such as a rise in intracellular free  $\text{Ca}^{2+}$  levels up to ~500 nM in the case of Cx43 (De Vuyst et al., 2009). Cx26 and Cx43, both expressed in astrocytes and leptomeningeal cells, form hemichannels permeable to  $\text{Ca}^{2+}$  (Sánchez et al., 2010; Schalper et al., 2010; Fiori et al., 2012) that play relevant roles to control second messengers under physiological and pathological conditions. Particularly, Cx26 hemichannels seem less sensitive to voltage and been shown to open in normal  $\text{Ca}^{2+}$  conditions without compromising the cell viability (González et al., 2006). Astroglial Cx43 hemichannels are activated by nitrosylation, a response that is reversed by antioxidant compounds (Retamal et al., 2006). The permeability of Cx43 hemichannels to positively charged

molecules is saturable, competitive and can vary significantly depending on the permeant tracer (Orellana et al., 2011).

It was mentioned above that pannexins may have lost the ability of their ancestors, the innexins, to form gap junction channels, although Panx1 has been reported to do so when expressed in *Xenopus* oocytes (Bruzzone et al., 2003). However, there is ample evidence that Panx1 can form hemichannels. As both Cx43 and Panx1 are widely expressed, and coexpressed in many cell types (Barbe et al., 2006; Laird, 2006; Boassa et al., 2007; Shestopalov and Panchin, 2008), so careful dissection of their roles in different physiological responses is often required. Like connexins, Panx1 hemichannels are activated by membrane depolarization and mechanical stimulation. In addition, they are also activated by enhanced intracellular  $[\text{Ca}^{2+}]$  and ATP-mediated purinergic receptor (i.e., P2X) transactivation (Bao et al., 2004b; Locovei et al., 2006a; Locovei et al., 2006b; Pelegrin and Surprenant, 2006; Locovei et al., 2007), and are not sensitive to extracellular  $\text{Ca}^{2+}$  concentrations. While they can be blocked by some of the lipophilic agents that close connexin channels, they are insensitive to  $\text{La}^{3+}$  and heptanol (Retamal et al., 2007a). Panx1 also forms an anion selective hemichannel of conductance (~68 pS) (Ma et al., 2012) within the range of most connexins, with the possible exception of Cx37. In contrast, S-nitrosylation opens Cx43 hemichannels whereas it closes Panx1 hemichannels (Retamal et al., 2006; Lohman et al., 2012).

## CONNEXINS IN CNS ONTOGENY

The distribution of connexins varies according to the developmental stage, cell type and brain region (Dermietzel et al., 1989; Batter et al., 1992; Nadarajah et al., 1997; Leung et al., 2002; Maxeiner et al., 2003; Nagy et al., 2004; Söhl et al., 2005; Van Bockstaele et al., 2004; Orthmann-Murphy et al., 2007). It is believed that gap junctions mediate the intercellular transfer of signals (e.g., second messengers and/or morphogenic agents) generating intracellular gradients that control ontogeny. Accordingly, neuroblasts, progenitor cells of neurons and macroglia (astrocytes, oligodendrocytes and ependymal cells), are well coupled through gap junctions that contain Cx43, and possibly other connexins. Coordinated rises in intracellular free  $\text{Ca}^{2+}$  concentration occur early in neurogenesis in the ventricular zone of the embryonic neocortex, and it has also been proposed that connexins may help in the propagation of  $\text{Ca}^{2+}$  waves (Lo Turco and Kriegstein, 1991). The specific role of connexins in  $\text{Ca}^{2+}$  waves will be discussed in considering the role of gap junctions in astrocytes.

During advanced neuronal differentiation, gap junctional communication is progressively downregulated (Nadarajah et al., 1998; Leung et al., 2002), suggesting that a high degree of coupling is required for other functions beside electrical coupling, and perhaps decreased intercellular communication favors neuronal proliferation and differentiation. These findings are replicated in primary cultures of neuronal and glial progenitor cells. Neuronal phenotypic changes are associated with loss of Cx43, coupling reduction, and selective expression of Cx36, and possibly Cx45. In other progenitor populations acquiring the astrocytic lineage, cells remain well coupled through primarily Cx43 channels (Rozental et al., 2000), although Cx30, and in some cases Cx26 expression, have also been reported (Theis et al., 2005).

The functional significance of these changes in connexin expression in different cell types has been demonstrated through several *in vivo* studies. The deletion of Cx43 in GFAP positive cells led to disorganization in the development of several brain regions, including the cortex, hippocampus and cerebellum (Wiencken-Barger et al., 2007). These results are consistent with the effect of less specific pharmacological inhibition of gap junctions during neuronal and astrogliar differentiation (Bani-Yaghoub et al., 1999).

However, caution needs to be used in interpreting all these developmental effects as reflective of losses in intercellular exchanges of ions or signals. In a recent elegant study on the embryonic development of the mouse cortex, the ablation of Cx26 or 43 in radial glia, the neuronal stem cells of the cerebral cortex, led to a lack of migration of neurons to the outer layers of the cortex. The expression of selected point mutants of Cx26 and 43 demonstrated, however, that it was not the channel function of these proteins that was important, but their adhesive properties that were needed to promote neuronal migration along the radial glial tracks (Elias et al., 2007). A functional role of hemichannels in neuronal differentiation has also been documented. The NGF induced differentiation of PC12 cells overexpressing Cx43 or Cx32 is accompanied by process outgrowth due to enhanced ATP released through hemichannels in the absence of significant changes in gap junctional communication between cells (Belliveau et al., 2006).

## CONNEXINS IN NEURONS OF THE ADULT CNS

### Electrical Synapses vs. Chemical Synapses

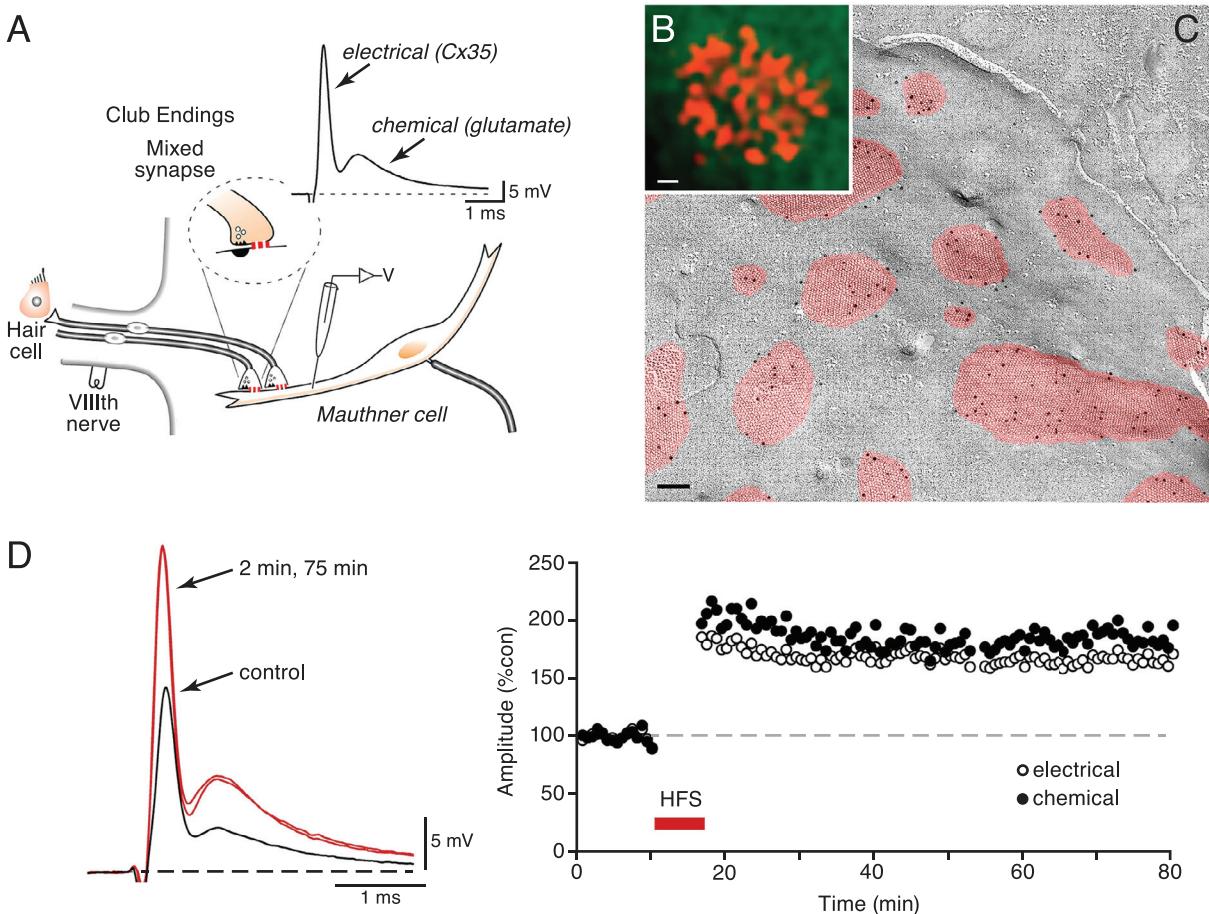
During the last half-century, chemical synapses in all animal phyla have received particular attention.

Numerous properties of the pre- as well as postsynaptic organelles, their molecular components, physiological events and functional roles of chemical synapses are described in Chapters 8–11, 16 and 19. Moreover, the involvement of chemical synapses in pathological conditions is well documented and frequently applied in therapies for humans as discussed in Chapter 20.

Chemical synapses were thought of as the only pathway for functional cell-cell propagation of electrical signals around the late 1950s, until the work of Furshpan and Potter (1959) that demonstrated direct passive electrical transmission in segments of giant fibers in crustaceans. All early studies of electrical synapses were done in lower-order vertebrates and invertebrates. However, after the discovery of their molecular constituents in mammals 22 years ago (Paul, 1986; Kumar and Gilula, 1986), the tools became available to demonstrate that the components of electrical synapses were expressed in most cellular elements of the nervous system in higher vertebrates as well. The presence of functional coupling of cells was first demonstrated by electrophysiological and dye transfer recordings but later this was correlated with the structures we now recognize as gap junctions in electron microscopy of thin sections and freeze fracture replicas (for review see Bennett and Zukin, 2004).

Several functional properties of electrical synapses distinguish them from chemical synapses, and allow them to play a complementary role in both central and peripheral nervous system function. Firstly, due to their complex structure, chemical synapses are typically unidirectional, the one exception being in the nerve net of the jellyfish *Cyanea* (Anderson, 1985; Anderson and Grunert, 1988), while electrical synapses are generally bidirectional, as they allow passive ion flux in both directions. The exceptions to the latter are the few cases, all reported in invertebrates, where the  $V_m$  sensitivity of the connexins produces a rectifying electrical conductor (e.g., the crayfish giant axon – Furshpan and Potter, 1959). This bidirectional property is well suited to ensuring synchronous firing of neurons as seen in various regions of the mammalian brain. In general, electrical synapses play roles in propagation of excitatory impulses. However, the hyperpolarizing chemical PSPs, as well as the hyperpolarizing phase of the action potential, can be electrotonically spread through gap junctions (e.g., in Mauthner neuron – Furukawa and Furshpan, 1963).

Secondly, due to the simple conductance pathway that does not require any vesicular release mechanisms, diffusion of neurotransmitter and activation of a receptor, electrical synapses propagate potentials rapidly without the “pause” seen in chemical transmission. They effectively serve as a low-pass filter between neurons (Bennett and Zukin, 2004; Galarreta



**FIGURE 9.3** (A) Club endings exhibit mixed synaptic transmission. Typical experimental arrangement showing VIIIth nerve auditory primary afferents (which contact saccular hair cells; “hair cell”) terminating as Club endings on the ipsilateral M-cell lateral dendrite. Inset represents a Club ending, at which both mechanisms of synaptic transmission, electrical (gap junction) and chemical, coexist. VIIIth nerve stimulation evokes a mixed electrical and chemical synaptic potential. The trace represents the average of 20 individual responses. (B–C) Cx35 is found at Club ending gap junctions. (B) Laser scanning confocal immunofluorescence (average of 3 z-sections) showing Cx35 at a club ending. There are multiple puncta immunoreactive for Cx35. Calibration: 1 μm. (C) FRIL image from a club ending showing Cx35 localization at the gap junctions. All 14 gap junctions in this image are labeled with 10-nm gold beads. Calibration: 0.1 μm. (D) Following a 7 min 500 Hz burst of neuronal firing, both the electrical (first tall peak) and chemical (second, lower peak) post synaptic potentials in the Mauthner neuron show enhanced amplitude (red traces) compared to before the stimulus (black trace). Following the peak amplitudes over time show that this potentiation of both electrical and chemical components is long lived (right panel). *Figure was kindly provided by Dr. Alberto Pereda.*

and Hestrin, 1999; Gibson et al., 1999). For this reason, electrical synapses feature heavily in escape responses, including the jump response of *Drosophila* (see review by Phelan, 2005), and the escape response of crayfish (see previous) and teleost fishes (i.e., Mauthner neuron—Fig. 9.3).

Thirdly, the complexity of chemical synapses allows for multiple levels of regulation that lead to synaptic plasticity, a critical property of even the most primitive nervous systems to allow adaptation to the environment and ultimately “learning” (Chapter 19). The simple structure of gap junctions suggests that they are passive conductors that do not show modulation. However, we now know this view to be too simplistic.

The efficacy of electrical synapses can be modulated over time through the rapid turnover of the proteins that allows for transcriptional driven changes in channel number within hours. Much more rapid modulation can also be achieved when the conductance changes in the surrounding membrane shunt the conductance and decrease the effectiveness of electrical coupling. This happens frequently in mixed chemical and electrical synapses (Fig. 9.3A–C), which are common in vertebrates, where the two types of synapse are located adjacent to one another (Llinás, 1988). However, probably the most important, regulation of electrical synapses is via activity-dependent phosphorylation of the connexins. In the mixed synapse club endings of the 8<sup>th</sup>

nerve inputs to the Mauthner neuron of goldfish, the efficacy of electrical transmission was increased substantially, and over a prolonged period, following repetitive patterns of neuronal activity (Fig 9.3D). This post-tetanic potentiation (PTP) was previously thought to be unique to chemical synapses. Two different mechanisms have been implicated, depending on the frequency and patterns of stimulation. PTP following trains of 100-Hz stimuli (Yang et al., 1990) was caused by release of endocannabinoids from the Mauthner neuron which induces dopamine release from neighboring neurons, resulting in activation of dopamine 1,5 receptors and cAMP dependent protein kinase (PKA), leading to phosphorylation of Cx35 in the Mauthner cell (Cachope et al., 2007). Bursts of higher frequency (500 Hz) cause enhanced electrical activity through activation of NMDA receptors and CaM Kinase II, which also induces phosphorylation of Cx35 (Pereda et al., 1998). Intriguingly, this regulation of connexins is spatially regulated and is specific to each different gap junction, so that facilitated and nonfacilitated synapses can co-exist in the same cell. This is seen in both the Mauthner neuron (Smith and Pereda, 2003) and in the mammalian CNS (e.g., in the inferior olive – Hoge et al., 2011).

The same two pathways have also been observed to contribute to the responses of the mammalian retina to different light conditions via adjustments of the coupling levels of Amacrine II cells. Glutamate-driven NMDA receptor activation of CaMKII increases coupling levels between cells by phosphorylating Cx36 (Kothmann et al., 2012). This effect is opposed by dopamine, through type 1 receptors, which activate PKA, and subsequently protein phosphatase 2, which removes phosphates from Cx36 (Kothmann et al., 2009). Note that this dopamine effect is the reverse of the Mauthner neuron, where PKA directly phosphorylates Cx36, and emphasizes the importance of the location of the various kinases and phosphatases in the cell. Activity-dependent modulation of Cx36 coupling of neurons through glutamate receptors is also seen in the hippocampus (Hamzei-Sichanni et al., 2012; Vivar et al., 2012).

## Connexin Expression Patterns

Connexins are broadly expressed in the CNS (Table 9.1) with Cx26, 29, 31.1, 32, 37, 43, 40, 47 and 57 transcripts being detected in diverse neuronal types at different developmental stages (Nadarajah et al., 1998; Leung et al., 2002; Maxeiner et al., 2003; Nagy et al., 2004; Sohl, et al., 2005; Van Bockstaele et al., 2004). However, only Cx36, 45, 57, and most recently Cx30.2, have been reproducibly identified at the ultrastructural level in neuronal gap junction structures of the adult rat brain (Rash et al., 2000; Rash et al., 2004; Rash

et al., 2005; Rash et al., 2007a; 2007b). Cx36 is the most widely expressed neuronal gap junction protein and immunogold labeling of freeze-fracture replicas has demonstrated its localization between neurons in the inferior olive, spinal cord, retina, olfactory bulbs, visual cortex, suprachiasmatic nucleus and locus caeruleus. Cx30.2 is frequently co-expressed with Cx36 in interneurons (Kreuzberg et al., 2008). Cx45 neuronal gap junctions have been located in the retina and olfactory bulb (Rash et al., 2000; Rash et al., 2005; Kamasawa et al., 2005), although expression of the Cx45 gene has also been detected in neurons of the cerebral cortex, hippocampus and thalamus, as well as basket and stellate cells of the cerebellum (Maxeiner et al., 2003). Cx57 expression appears restricted exclusively to Horizontal cells in the retina. Cx26 and Cx32 are expressed in pre-Bötziinger neurons of neonatal and adult rats, but their localization to gap junction structures has not been confirmed (Solomon et al., 2001). In addition to connexins, it should also be noted that Panx 1 and 2 have both been found expressed in the CNS, particularly in the hippocampus (Bruzzone et al., 2003).

Cells of CNS glands also express connexins. Rat pinealocytes express both Cx26 and Cx36 (Sáez et al., 1991; Belluardo et al., 2000), anterior pituitary cells express Cx36 (Belluardo et al., 2000), while a small percentage of cells containing luteinizing hormone present Cx43 immunoreactivity (Yamamoto et al., 1993), as do foliculostellate cells (Shirasawa et al., 2004).

Numerous cells of the peripheral nervous system express connexins and form functional gap junctions that are highly regulated by physiological conditions. For example, the expression of Cxs 32, 36, and 43 is developmentally regulated in the trigeminal motor nucleus, while Cx26 expression remains high throughout postnatal development. In the mesencephalic trigeminal nucleus, Cx26, 32, and 43 expression is intense throughout development, with only Cx36 showing a developmental regulation (Honma et al., 2004). Connexins and gap junctions are also found in neurons of the enteric nervous system. In the stomach and intestines, Cx43 gap junctions are found between interstitial cells of Cajal (ICC) as well as between ICC and adjacent muscle layers of each tissue (Daniel and Wang, 1999; Seki and Komuro, 2002).

Studies on gap junctions in neuronal primary cultures have been limited by the loss of connexin expression, most likely due to the lack of regulatory factors in cultured cells that lead to a level of dedifferentiation. One regulator of connexin expression in primary cultures is basic FGF. It increases gap junctional communication and Cx43 levels in cortical progenitor cells (Nadarajah et al., 1998). Moreover, basic FGF enhances cell-cell communication via gap

junctions, and levels of Cx43, in rat embryonic day 14 midbrain cultures (Siu et al., 2001).

The physiological significance of the specialized expression of connexins in different regions remains obscure. We do know that some connexins will form heterotypic channels with other connexins expressed in neighboring cells, but this is a selective process, the rules of which we still do not fully understand, although generally connexin within the same genetic groupings (A – E) prefer to interact. Regulatory properties of connexins vary significantly, including their sensitivity to voltage gating. However, the time course of gating in response to voltage differences between cells is slow (hundreds of milliseconds) compared to the time course of action potentials in neurons (tens of milliseconds). Permeability properties of different connexin channels also vary, as noted above, but the understanding of this is in its infancy and provides little physiological insight at this time. It is notable that the major connexin between neurons in the CNS is Cx36, which has the smallest single channel conductance and size cut-off for larger molecules of any connexin studied, indicating that it is largely specialized for electrical conductance rather than metabolite transfer.

## Functional Roles

The roles of gap junction coupling of neurons in the CNS are likely to be primarily at the level of fine tuning of oscillatory networks. This is both suggested by extensive modeling of electrical networks within the brain, and tested empirically through Cx36 genetic knockouts in mice (Buhl et al., 2003). Cx36 is primarily expressed between interneurons at dendro-dendritic connections. Modeling studies have suggested that axo-axonal electrical coupling may also play a critical role in synchronization of neuronal firing patterns. It is likely that these gap junctions are composed of Cx45, but functional testing of their role has not been possible, as no CNS specific knock-out has been developed, and the whole animal knock-out is lethal embryonically.

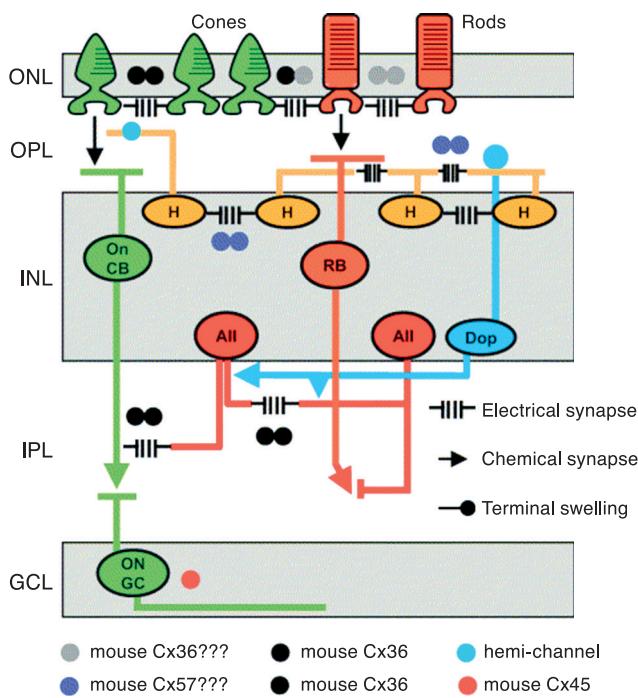
The major effects of loss of Cx36 expression in the CNS is a loss of synchronicity of selected oscillatory patterns mediated by interneurons in the inferior olive of the cerebellum (2–10 Hz subthreshold oscillations), the hippocampus (12–90 Hz gamma oscillations, but not the slower theta or the fast high-frequency oscillations) and the cortex (supra- and subthreshold oscillations, particularly among GABA interneurons). Typically the basic oscillatory behaviors are retained in the absence of Cx36, but their power and synchrony is reduced or lost. In the cerebellum, it has been proposed that this would result in the loss of 10–20 ms of precision in the coordination of neuronal activity. Hence, it is not surprising that these mice show no major loss of motor skills or

coordination, yet do show more subtle defects in object recognition and memory and other behavioral impairments, and disrupted circadian rhythms (Hormuzdi et al., 2004; Frisch et al., 2005). The role of co-expression of Cx30.2 in regulating these oscillations is unclear, as no defects in Cx30.2<sup>-/-</sup> mice have been detected (Kreuzberg et al., 2008).

Electrical coupling between pyramidal neurons has been shown to play a significant role in oscillatory network activity, spatial exploration and learning and memory (Mercer, 2012). Electrical coupling between inhibitory neurons are frequent in the hippocampus, cerebral cortex, striatum, amygdala, the inferior olive, cerebellum, olfactory bulb and suprachiasmatic nucleus (Bennett and Zukin, 2004). Cx36 deficient mice show reduced CaMKII levels in the striatum and behavioral changes in open-field activity, anxiety-related behavior in the light-dark box and one-trial object-place recognition (Zlomuzica et al., 2012). Cx36 also appears to be required for normal spatial coding in the hippocampus and short-term spatial memory (Allen et al., 2011) and fear learning and memory (Bissiere et al., 2011). Plasticity of the electrical synapse is also evident at the LTP level (Wang and Belousov, 2011) and is regulated by chemical synapses, including noradrenergic, glutamatergic and GABAergic activity (Zsiros and Maccaferri, 2008; Colwell, 2000). However, electrical synapses are present as very small gap junctions between contacts of hippocampal neurons (Hamzei-Sichanni et al., 2012; Vivar et al., 2012).

## The Retina—A Case Study of Diversity

A particularly intriguing model for the study of diverse roles of connexins in regulating neuronal function is the retina (see Fig. 9.4 for overall circuitry). Cx36 is the most widely expressed, being found in cone cells, OFF cone bipolar cells, AII amacrine cells and ganglion cells. Cx45 is expressed by ON cone bipolar cells, and a small subset of amacrine cells and ganglion cells, while Cx57 is found exclusively in horizontal cells, which in the fish also express Cx26, but probably only in the hemichannel form (summarized in Sohl et al., 2005). Initially, the coupling of the photoreceptor cells (cones are coupled, not only to one another by Cx36, but also to rod cells through an unknown connexin) seems counterintuitive, as it might be expected to reduce the ability to distinguish between closely spaced stimuli. However, it appears that the largest problem in retinal detection is the background noise generated by spontaneous firings of receptors in the absence of specific stimuli, and the coupling of cells greatly mutes background noise, thereby increasing the signal to noise ratio.



**FIGURE 9.4** Simplified diagram of the circuitry of the retina illustrates the dependence of rod signaling on Cx36 gap junctions. Cones and rods are coupled to themselves (by Cx36) and one another (through unknown heterotypic channels). Cones signal through chemical synapses to ON Ganglion cells (GC) via ON Cone Bipolar cells (CB) (green pathway). Rod cells, however, chemically innervate Rod Bipolar cells (RB), which innervate AII Amacrine cells (AC) (red pathway). These are electrically coupled to one another, and to ON CB cells. Thus, rods can signal to ganglion cells through either the RB/AII system, or via cones, but both circuits have at least one connection that is fully reliant on electrical coupling. Both AII and Horizontal cells (H) are coupled to one another electrically, forming a network parallel to the surface of the retina that is important in adjusting receptive field size. The coupling of both of these networks is regulated by light sensitive dopaminergic neurons (Dop) (blue). From Hormuzdi et al., 2004.

The coupling between horizontal cells and AII amacrine cells has always made more intuitive sense, as these cells have been implicated in the regulation of the receptive field size in the retina, through center surround inhibition effects (where responses around the central area of stimulation are suppressed) and integration of signals from multiple receptors. These effects are clearly important for balancing maximal acuity in bright light (photopic) conditions (minimized receptive fields) with maximal sensitivity in low light (mesopic and scotopic) conditions (integrating signals over a wider area). Thus, one might expect there to be a diurnal/nocturnal regulation of coupling to modulate receptive field size. Indeed, this is seen in both horizontal and AII amacrine cells, where light induces a dopaminergic response that elevates cAMP levels which reduces coupling between these cells (Bloomfield et al., 1997). In the case of Cx36, this has

been associated with PKA sensitive sites on the cytoplasmic loop (Mitropoulou and Bruzzone, 2003).

Horizontal cell connexins are also involved in a rather unusual means of influencing the electrical responsiveness of cone cells through a mechanism called ephaptic transmission (summarized by Kamermans and Fahrenfort, 2004). In turtle retinas, Cx26 has been identified as being expressed in the complex “ribbon” synapse of cones, where the presynaptic cone synapse with the ON bipolar cells also involves postsynaptic contacts with horizontal cells. Cx26 is expressed by the horizontal cells at this site, but no gap junctions can be detected, indicating that they are likely to exist exclusively in hemichannel form. Cones are typically depolarized in the dark, resulting in release of glutamate and depolarization of horizontal cells. Light causes hyperpolarization of cone cells, and the loss of glutamate release results in a shift of the horizontal cell membrane potential to more negative values.

The problem the retina faces is how to adjust the gain in response to allow detection to small changes in light, but over many orders of magnitude differences in the ambient light conditions. This requires some kind of positive feedback loop that can reset the threshold for glutamate release at different light levels, and appears to occur through a change in the response levels of the  $\text{Ca}^{2+}$  channels that trigger glutamate release. The proposed mechanism relates to extracellular currents that are generated in the very narrow synaptic cleft of the cone-horizontal cell interaction. As the horizontal cells become more hyperpolarized in high light conditions, there is a stronger driving force for current across the Cx26 hemichannels clustered in this postsynaptic region (these channels are insensitive to  $V_m$ , and remain open). This current flow results in a drop in the potential of the extracellular space, which is sensed by the cone cell membrane as a depolarization (i.e., the potential difference between intracellular and extracellular environments decreases). This results in activation of  $\text{Ca}^{2+}$  channels and release of glutamate, despite the fact that the absolute internal potential of the cone cells did not change. Connexin hemichannels are uniquely adapted to this role, although as of yet, Cx26 has not been detected in mammalian cone-horizontal cell synapses, which may mean other channels can also mediate this novel method for adjusting the gain of a synapse.

Perhaps the most surprising role of Cx36 coupling in the retina was revealed when it was ablated genetically in mice (Deans et al., 2002). This resulted in a loss of all rod signaling to ganglion cells under low light (scotopic) conditions. The reason for this is that rods do not make direct contact with rod-specific ganglion cells, but rather connect to rod bipolar cells that innervate AII amacrine cells, which in turn synapse with

ON cone bipolar cells, leading to activation of ganglion cells (Fig. 9.4). While much of this pathway is mediated by standard chemical synapses, the AII amacrine to ON cone bipolar cell connection is exclusively mediated by electrical synapses composed of Cx36. An alternative route for rod activation of ganglion cells does exist through their coupling to cone cells directly, which also activate ON cone bipolar cells, however, this coupling also requires Cx36 expression in the cone cells (Fig. 9.4). Hence, in the absence of Cx36, all connectivity between rod cells and ganglion cells is lost. An interesting side effect of the loss of AII amacrine cell – ON bipolar cell coupling was also observed that serves to remind us that gap junction can serve roles beyond that of an electrical synapse. Cone cells and amacrine cells are glycinergic transmitters, yet the cone cells do not express a glycine transporter for recapturing the neurotransmitter. In the Cx36<sup>-/-</sup> mice, glycine levels in the bipolar cells are significantly reduced, supporting a previous proposal (Cohen and Sterling, 1986 and Vaney et al., 1998) that they receive their glycine through gap junctions from AII amacrine cells that express the glycine transporter.

## ASTROGLIAL CONNEXINS

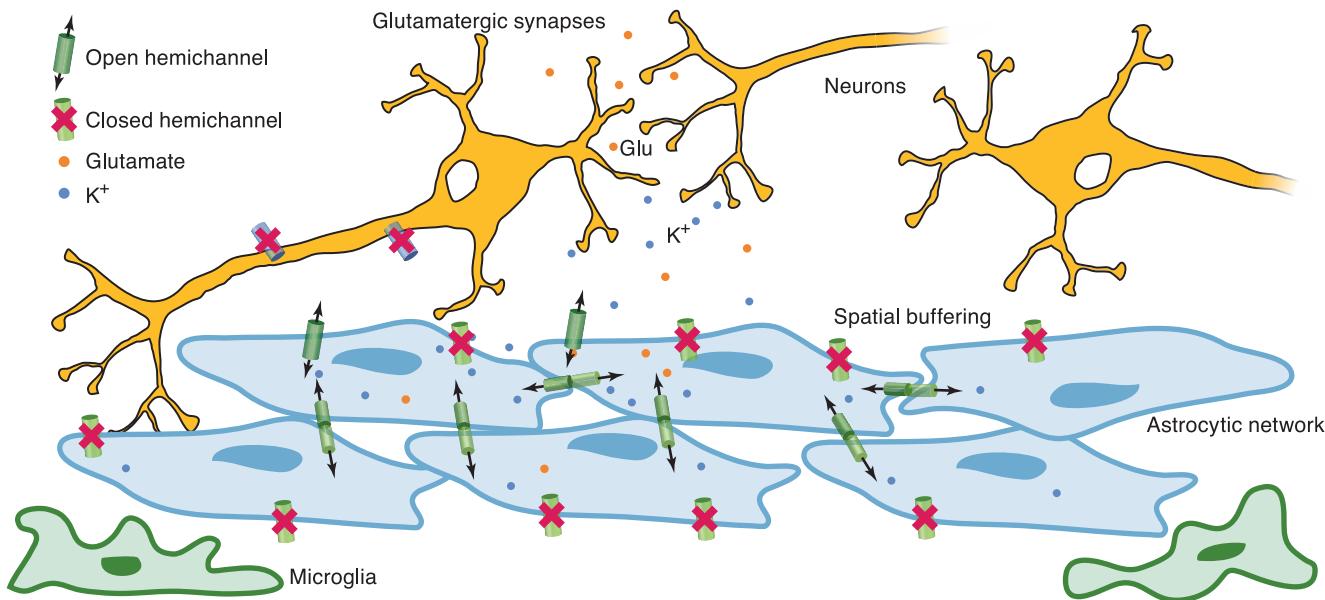
### Connexin Expression Patterns

Cortical astrocytes present immunoreactivity for Cxs 26, 30, 40, 43 and 45 (Nagy et al., 1997; 1999; Dermietzel et al., 2000; Rash et al., 2000) but only Cxs 26, 30 and 43 show colocalization at ultrastructurally defined astroglial gap junctions (Rash et al., 2000; Nagy et al., 2001). Although Cx43 and Cx30 are both present in astrocytes of the visual cortex (Rochefort et al., 2005), characterization of unitary currents of gap junctions in cultured astrocytes are consistent with only homotypic Cx43 gap junction channels (Dermietzel et al., 1991; Giaume et al., 1991; Moreno et al., 1994; Kwak et al., 1995; Bukauskas et al., 2001). Consistent with this, cultured astrocytes from Cx43 deficient mice do not form functional gap junctions (Naus et al., 1997). Cultured cortical astrocytes also express Cx26, but it has been difficult to detect its expression at either cell–cell contacts (Martínez and Sáez, 1999) or unapposed cell membranes, where only Cx43 hemichannels are detected (Retamal et al., 2007b). Thus, Cx43 is thought to be the main functional connexin in cultured astrocytes. However, astrocytes co-cultured with neurons with active chemical synapses also express Cx30 (Rouach et al., 2000). In terms of function, Cx30 expressed by astrocytes of the olfactory glomeruli sense changes in extracellular K<sup>+</sup> due to neuronal activity (Roux et al.,

2011). In general, astroglial networks formed by Cx30 and Cx43 gap junctions tone down hippocampal synaptic transmission in CA1 pyramidal neurons since they facilitate extracellular glutamate and potassium removal during synaptic activity through modulation of astroglial clearance rate and extracellular space volume. This regulation limits neuronal excitability, release probability, and insertion of postsynaptic AMPA receptors, silencing synapses (Pannasch et al., 2011).

Gap junctions between astrocytes are dynamically regulated and play diverse functions. Levels of astrocytic Cx43 are upregulated at about the time that the rat pineal gland becomes innervated (Berthoud and Sáez, 1993), suggesting that Cx43 expression is also regulated by neuronal activity. Because Cx43 presents multiple phosphorylation sites, astrocytic gap junctional communication can be rapidly increased or decreased upon activation of protein phosphatases or kinases. Accordingly, glutamate or high extracellular K<sup>+</sup> concentration enhance astrocyte gap junctional communication (Enkvist and McCarthy, 1994) through a mechanism linked to increases in Cx43 phosphorylation mediated by calmodulin kinase (De Pina-Benabou et al., 2001). Moreover, astroglial gap junctions are downregulated by pro-inflammatory conditions including hypoxia-reoxygenation and pro-inflammatory cytokines and bacterial compounds that activate microglia (Retamal et al., 2007b; Karpuk et al., 2011; Orellana et al., 2010, 2011).

Heterocellular gap junctions between astrocytes and neurons have been described, with both dye transfer and electrical coupling of astrocytes and neurons being demonstrated in the locus coeruleus (Alvarez-Maubecin et al., 2000; Van Bockstaele et al., 2004) and astrocyte/neuron co-cultures (Fróes and de Carvalho, 1998; Fróes et al., 1999; Rozental et al., 2001). Nevertheless, *in vivo* ultrastructurally defined neuron-astroglial gap junctions have not been observed (Rash et al., 2007a, 2007b), and it is likely that these would have to be restricted in order to prevent undue shunting of current from active neurons into the large population of glia. Electrical coupling mediated by Cx36 between neurons and microglia has been observed in cell co-cultures (Dobrenis et al., 2005), but the physiological significance of this coupling is as yet undefined. However, there is growing evidence of gap junction formation between oligodendrocytes and astrocytes that would create a pan-glial syncytium (Tress et al., 2012). Cx32 and Cx47 serve to connect oligodendrocytes (Maglione et al., 2010) whereas compatibility studies performed in exogenous expression systems suggest that Cx43/47 and/or Cx30/32 pairings of connexins mediate the heterotypic gap junctions between astrocytes and oligodendrocytes (listed as astrocyte/oligodendrocyte side).



**FIGURE 9.5** Scheme showing functional roles of gap junction channels between astrocytes. Regions of highly active neurons release into the extracellular fluid that can induce hyperexcitability and neuronal apoptosis. Surrounding Astroglia take up both the  $K^+$  (blue dots) and glutamate (orange dots) either through open hemichannels or specific  $K^+$  channels or glutamate transporters, and distribute them through gap junctions throughout the astrocytic syncytium before releasing them at a remote site. It has been proposed that in cases of myelinated neurons (not shown), the oligodendrocytes that are in closest proximity to the axons may be the first site for taking up  $K^+$ , which is then passed to the astrocytic population through heterotypic Cx32/30 or Cx47/43 channels (see text).

## Functional Roles

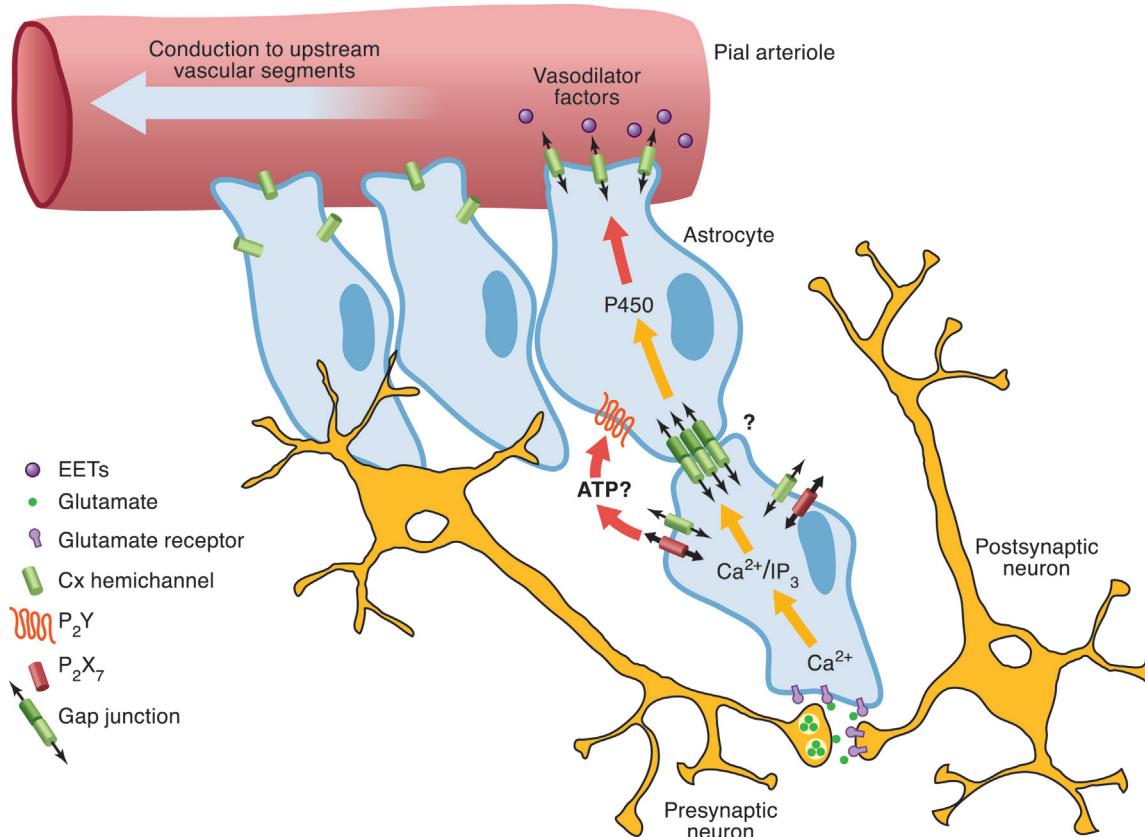
Cortical astrocytes have been considered as “nursing cells” because they maintain the extracellular homeostasis and provide metabolic support to neurons for their normal functioning.

The “spatial buffering” of  $K^+$  and small molecules like neurotransmitters (e.g., glutamate) is a very important function of glial networks, which form extensive syncytia through gap junction coupling (Fig. 9.5).  $K^+$  and glutamate, which accumulate in the extracellular milieu surrounding foci of high neuronal activity, are taken up, either passively through  $K^+$  channels, or actively via transporters, by astrocytes located in the vicinity. The potassium ions are diluted intracellularly to regions distal to the area of high neuronal activity while the glutamate is converted to glutamine, which recycles to neurons where it can be modified to glutamate and used as a neurochemical transmitter (Fig. 9.5). This function may be facilitated by the recently reported Cx43-induced regulation of astrocytic glutamate transporters (Figiel et al., 2007).

The importance of this spatial buffering of  $K^+$  and glutamate stems from the fact that exposure of neurons to high levels of these components in the extracellular fluid leads to hyperexcitability of the neurons, which is ultimately toxic. A phenomenon known as spreading depression (SD) is likely a product of this, where propagating waves of depolarization of neurons are

followed by neuronal inactivation. It had been proposed, based on the application of pharmacological inhibitors of gap junctions, that astrocytic coupling may exacerbate this process (Nakase and Naus, 2004). However, studies on Cx43<sup>-/-</sup> mice have shown that loss of Cx43 leads to an enhanced rate of SD propagation (Theis et al., 2003). The discrepancy in the results could either stem from the relatively nonspecific nature of gap junction pharmacological agents that could be toxic in their own right, or the involvement of connexins other than Cx43 in aspects of SD. In concert with its role in inhibiting SD, Cx43 has also been shown to serve a protective role in focal brain ischemia by reducing stroke volume (Nakase et al., 2003).

Related to their role in spatial buffering, gap junctional coupling of astrocytes is also likely to be important in the distribution of energy resources throughout the brain. Under normal conditions, the main energy substrate of the brain is glucose metabolized via glycolysis in astrocytes and by oxidative phosphorylation in neurons (Kasischke et al., 2004). The latter is favored by a tight metabolic balance between neurons and astrocytes, termed “neurometabolic coupling.” The glucose in this balance is provided by glycogen stored in astrocytes that can be mobilized over a period of seconds during periods of high neuronal activity. Immediately following the initial burst of glycogen-derived glucose, blood-borne glucose is shuttled over



**FIGURE 9.6** Scheme showing the proposed astrocyte signaling function in the neurovascular unit. Astrocytes have been shown to be essential for mediating vascular responses to high neuronal activity. One model is that a subset of astrocytes known to express glutamate receptors (instead of transporters) responds in the vicinity of the elevated neuronal activity and released glutamate by taking up  $\text{Ca}^{2+}$ . This is then propagated between astrocytes in a  $\text{Ca}^{2+}$  wave, either directly through Cx43 gap junctions, or via extracellular release of ATP through Cx43 hemichannels and activation of P<sub>2</sub>Y purine receptors on adjacent cells, which initiate the  $\text{Ca}^{2+}$  response all over again. Ultimately, astrocytic end feet must release vasoactive factors to the arteriole, a step that could also be mediated by Cx43 hemichannels.

the blood-brain barrier through a mechanism termed “neurobarrier coupling” (Leybaert, 2005). These metabolic responses occur with a close spatial and temporal correlation between high neuronal activity and hyperemia (Roy and Sherrington, 1890).

Astrocytes release gliotransmitters such as glutamate and ATP via Cx43 hemichannels, which play a relevant role in the establishment of fear memory (Stehberg et al., 2012). Opening of hemichannels also serve as a key element in the transduction system activated by glucose in tanocytes, through activation of several glucose-sensing proteins (Orellana et al., 2012).

Astroglial cells have been proposed as the mediators of changes in brain microvascular tone in response to neuronal activity, causing rapid and localized changes in cerebral blood flow upon demand (Fig. 9.6) (Ye et al., 2003; Metea and Newman, 2006; Takano et al., 2006). *In situ* studies, using chemical cell-type-specific ablations, demonstrated that arterial dilation in response to elevated neuronal activity (in either pathogenic or normal range) required astrocytes, but

not endothelial cells (Xu et al., 2007). This supports the contention that astrocytes act as a conduit of forward signals during neurovascular coupling. That this also required longer distance propagation of the response through gap junctions was demonstrated by employing connexin-mimetic peptides specific to the extracellular domains of Cx43 that selectively prevent formation of Cx43 gap junctions through competition, and also induce closure of Cx43 hemichannels (Evans and Leybaert, 2007).

The propagation of signals through astrocytes that mediate changes in microvascular tone has been linked to intracellular  $\text{Ca}^{2+}$  concentration, and likely involves propagation of  $\text{Ca}^{2+}$  waves through the astrocytes (Ye et al., 2003). This is a well-established behavior in astrocytes and many other cells in culture, and can be mediated via two routes—the passage of IP<sub>3</sub> between cells through gap junctions, thus propagating a regenerating wave of  $\text{Ca}^{2+}$  release from intracellular stores, or via an extracellular pathway involving ATP release (summarized in Iadecola and Nedergaard,

2007). The ATP release in the latter mechanism may also be connexin dependent, and has been proposed to occur through Cx43 hemichannels (Stout et al., 2002). The released ATP then diffuses to adjacent cells, binds to purinergic P2Y receptors, leading to activation of PLC, production of IP<sub>3</sub>, and subsequent Ca<sup>2+</sup> release from internal stores. In brain slices, extracellular ATP mediates the propagation of Ca<sup>2+</sup> waves (Schipke et al., 2002) that seem to coordinate neurovascular responses (Zonta et al., 2003). Cx43 hemichannels may also play a role in the release of vasoactive signals at the glial end feet, as they remain separated from the arterioles by ~20 nm (Nagelhus et al., 2004), and the signaling can be ablated through extracellular perfusion (Xu et al., 2007).

## CONNEXINS IN OLIGODENDROCYTES

### Connexin Expression Patterns

Cx29, 32 and 47 have been detected in oligodendrocytes (Dermietzel et al., 1997; Sohl et al., 2001; Li et al., 2004; Kamasawa et al., 2005) and gap junction communication has been demonstrated between oligodendrocytes (Kettenmann et al., 1983; Kettenmann and Ransom 1988), and with their astrocytic neighbors (Orthmann-Murphy et al., 2007). Electrophysiological recordings and immunolabeling experiments demonstrated that oligodendrocyte-astrocyte gap junctions are most likely composed of Cx47/Cx43 heterotypic channels at the stroma of the oligodendrocytes and Cx32/Cx30 heterotypic channels in the area of the myelin sheath (Orthmann-Murphy et al., 2007).

### Functional Roles

Gap junctions between oligodendrocytes and with astrocytes, linking into a larger panglial syncytium, contribute to spatial buffering of potassium ion released during neuronal activity, as this will be particularly concentrated near nodes of Ranvier where the myelin sheath is the first site for uptake of excess K<sup>+</sup> (Menichella et al., 2006). In addition to their proposed neuroprotective role in spatial buffering, there is direct genetic evidence for a requirement for connexins in the maintenance and/or development of myelination of both peripheral and central neurons (Abrams et al., 2003). This began with the first link of connexins to an inheritable disease by Fischbeck and colleagues, who found that mutations of the Cx32 gene caused the X-linked version of Charcot-Marie-Tooth disease (CMTX) in which myelination of peripheral nerves fails in early adulthood, leading to partial leg paralysis (Bergoffen et al., 1993). This is discussed in detail following, but is thought to arise from loss of gap junctions

that form between the myelin wrappings to shorten diffusion of signals and nutrients between the soma and the inner-most myelin wrapping of Schwann cells (Oh et al., 1997; Abrams et al., 2000). In oligodendrocytes, these “reflexive” gap junctions have not been confirmed, and only occasional CNS involvement is noted in CMTX patients (Taylor et al., 2003). However, Cx47<sup>-/-</sup> mice show vacuolation of central nerve fibers, particularly in the optic nerve, consistent with failure of myelination (Odermatt et al., 2003), and Cx47 defects have been linked to abnormal CNS myelin in Pelizaeus-Merzbacher-like disease (Uhlenberg et al., 2004). Most dramatically, mice with double knockouts of Cx32 and Cx47 die at 6 weeks and appear to never develop appropriate myelination, suffer oligodendrocyte death and axonal loss (Menichella et al., 2003). Thus, while the role of connexins in forming a reduced diffusion pathway through myelin that is required for maintenance of peripheral myelin may not be so critical in the CNS, connexins are required for normal oligodendrocyte development and particularly Cx47 has been shown to be essential for myelinization in the CNS (Tress et al., 2012).

## CONNEXINS IN MICROGLIA

In the adult rat brain, microglia are sparse and a few express low levels of Cx43 (Eugenín et al., 2001). Under CNS threatening conditions, microglia migrate to the affected area forming clusters where they then express Cx43. In primary cultures, microglia are partially activated, and express low levels of Cx32, Cx36, Cx43 and Cx45 but they do not form functional gap junctions (Eugenín et al., 2001; Dobrenis et al., 2005; Takeuchi et al., 2006). After treatment with proinflammatory compounds (e.g., peptidoglycans or IFN-γ with bacterial endotoxin or TNF-α), microglia express increased levels of Cx32 and Cx43 (Eugenín et al., 2001; Takeuchi et al., 2006; Garg et al., 2005), resulting in functionally detectable coupling of the microglia (Eugenín et al., 2001; Garg et al., 2005). This effect may be linked to increases in the intracellular free Ca<sup>2+</sup> concentration (Martínez et al., 2002).

## CONNEXINS IN THE BLOOD-BRAIN BARRIER

The blood-brain barrier (BBB) is formed by endothelial cells that line all cerebral microvessels. The permeability of this endothelium is unique in the vasculature, and is critical for regulating the access of compounds, including intravenously delivered drugs, to the brain. *In vivo*, these endothelial cells express Cx43 and Cx40 (Little et al., 1995). Cell lines derived from the BBB are

well coupled, and  $IP_3$  induces  $Ca^{2+}$  waves that depend on extracellular ATP released through Cx hemichannels (Braet et al., 2003). Other functional interactions of Cx40 and Cx43 gap junctions have been observed in primary culture of porcine BBB endothelial cells, which interact via tight-junctions to establish the barrier function of this endothelium (Nagasawa et al., 2006). Gap junction blockers oleamide and glycyrrhetic acid inhibit the established of these tight junctions, as determined by measurement of paracellular flux of manitol and insulin and ions (Nagasawa et al., 2006), indicating an interaction between gap and tight junction structures. This is consistent with reported associations between Cx43 and cytosolic components of tight junctions like ZO-1 and Discs Large (Fig. 9.2; reviewed in Giepmans, 2004; Laird, 2006). Cx37 and Cx43 are also expressed by myoendothelial cells (Haddock et al., 2006), which allows for possible electrical coupling between the muscle and endothelial layers of the blood vessels that could facilitate the synchronization of vasomotor activity along the vessel length through  $Ca^{2+}$  waves (Haddock et al., 2006). Although there is no *in vivo* evidence of astrocyte-endothelial cell coupling, which remain separated by a basal lamina, *in vitro* studies have documented weak electrical coupling (but no dye spread) between astrocytes and BBB endothelial cells associated with the spread of both gap junction dependent and independent calcium waves (Braet et al., 2001).

## CONNEXINS IN EPENDIMAL CELLS AND LEPTOMENINGEAL CELLS

Beyond the major cell types, connexin expression has also been reported in the meninges lining the brain and its ventricles. The ependymocytes, glial cells that line the ventricles of the brain and the central canal of the spinal cord, are highly coupled through gap junctions, identified at the ultrastructural level (Rash et al., 1997), composed of Cx26 and Cx43 (Dermietzel et al., 1989; Yamamoto et al., 1990; Yamamoto et al., 1992), and possibly Cx30 (Kunzelmann et al., 1999; Nagy et al., 1999). These junctional channels allow the synchronization of rhythmic ciliary beating. Ependymocytes also form Cx43-based gap junctions with astrocytes *in situ* (Rash et al., 1997), although the functional significance of this remains unclear.

There are three layers of meninges around the brain and spinal cord: the outer layer (dura mater), the middle layer (arachnoid) and an inner layer (pia mater), connected to the arachnoid by numerous threadlike strands. The space under the arachnoid, the subarachnoid space, is filled with cerebrospinal fluid and contains blood vessels. Gap junction communication is widely extended in the developing and adult meninges, and strong coupling is seen in cultured

leptomeningeal cells (Spray et al., 1991; Dermietzel and Krause, 1991). In cultured cells, coupling probably occurs through Cx26, Cx30 and Cx43 which are expressed at high levels in these cells (Mercier and Hatton, 2001; Nagy et al., 1999).

## PATTERN OF PANNEXIN LOCALIZATION IN BRAIN CELLS

In addition to the connexin family, three distant relatives of the invertebrate gap junction proteins (innexins), called pannexins (Panx), have been cloned in mammals (Bruzzone et al., 2003; Baranova et al., 2004). While pannexins and innexins share a similar membrane topology to connexins (Fig. 9.1), their sequences are unrelated (Panchin, 2005). Panx1 is also glycosylated during targeting to the plasma membrane (Boassa et al., 2007), unlike connexins.

### Expression Patterns of Pannexins in Mammalian CNS

Panx1 is ubiquitously expressed in mammalian tissues, including the brain (Baranova et al., 2004). It can be detected in several regions of the CNS including cortex, striatum, olfactory bulb, hippocampus, thalamus, inferior olive, inferior colliculus, amygdala, spinal cord, retina and cerebellum (Bruzzone et al., 2003; Ray et al., 2005; Zappalà et al., 2006). At the cellular level, Panx1 is localized in different neuronal types, including olfactory bulb mitral cells, Purkinje cells, dopaminergic neurons, cholinergic neurons and glutamatergic neurons (Bruzzone et al., 2003; Ray et al., 2005; Zappalà et al., 2006). In the cerebral cortex and hippocampus, Panx1 is localized in the postsynaptic cells (Zoidl et al., 2007). Although Panx1 has been detected in Bergmann glia in the cerebellum, it has not been found in other astroglial cells *in situ* (Ray et al., 2005; Vogt et al., 2005; Zappalà et al., 2006), although its expression has been reported in cultured microglia, astrocytes, immature oligodendrocytes and neurons under resting conditions. Panx1 is also present in microglia (Shestopalov and Panchin, 2008; Orellana et al., 2011).

Panx2 is expressed preferentially in the brain (Panchin, 2005), including olfactory bulb, hippocampus, amygdala, superior colliculus, substantia nigra, cerebellum, hypothalamus and spinal cord (Bruzzone et al., 2003; Zappalà et al., 2006). Under normal conditions hippocampal astrocytes do not express Panx2. However, a transient expression of Panx2 occurs in hippocampal astrocytes several hours after ischemia/reperfusion (Zappalà et al., 2006), the functional significance of which is unknown.

## Functional Roles

In exogenous expression systems (e.g., *Xenopus* oocytes, LNCaP or C6 cells) the overexpression of Panx1, but not Panx2 or Panx3, leads to gap junction formation (Bruzzone et al., 2003; Vanden Abeele et al., 2006; Lai et al., 2007). However, there is no evidence that endogenously expressed pannexins form functional gap junctions in any system *in situ*. At unapposed plasma membranes, functional Panx1, Panx1/Panx2 and Panx3 hemichannels have been demonstrated in exogenous expression systems (Bruzzone et al., 2003; Bao et al., 2004b; Bruzzone et al., 2005; Barbe et al., 2006; Locovei et al., 2006a; Pelegrin and Surprenant, 2006; Peñuela et al., 2007). Moreover, Panx1 hemichannels have been recorded in hippocampal neurons exposed to oxygen-glucose deprivation (Thompson et al., 2006), although not in cortical astrocytes under either resting conditions, or after treatment with pro-inflammatory cytokines or FGFs (Huang et al., 2007).

Panx1 hemichannels are permeable to  $\text{Ca}^{2+}$  and small molecules such as ATP and ethidium (Locovei et al., 2006b; Pelegrin and Surprenant, 2006; Pelegrin and Surprenant, 2007), calcein (Thompson et al., 2006), sulforhodamine (Thompson et al., 2006; Peñuela et al., 2007) and carboxyfluorescein (Locovei et al., 2006a). The activity of Panx hemichannels is inhibited by low intracellular pH and some connexin blockers such as low concentrations of carbenoxolone and, to a lesser extent by flufenamic acid. However, they are insensitive to other known blockers of connexin hemichannels like 1-heptanol, 1-octanol,  $\text{La}^{3+}$  and  $\text{Gd}^{3+}$  (Pelegrin and Surprenant, 2006; Retamal et al., 2007a). This, and their single-channel conductances (550 pS (Bao et al., 2004b) or 68 pS (Ma et al., 2012)), allows their function to be distinguished from connexin-based hemichannels. However, in most cases the specific contribution of connexins and pannexins to hemichannel related activity in neurons and glial cells has not been resolved.

Microglia contain Panx1 hemichannels that are upregulated by proinflammatory conditions such as peptide  $\beta$  amyloid (Orellana et al., 2011). In macrophages, activation of  $\text{P2} \times 7$  receptors by ATP has long been associated with the opening of large pores. It has been proposed that this may occur through a conformational change of the  $\text{P2} \times 7$  channel that is normally small ion permeant, to allow movement of larger molecules (North, 2002). However, strong evidence has suggested recently that the release of interleukin-1 $\beta$  from macrophages requires functional Panx1 hemichannels that are activated through  $\text{P2} \times 7$  (Pelegrin and Surprenant, 2006) by a mechanism that remains to be elucidated, but may involve a heteromeric protein complex.

## GAP JUNCTION CHANNELS AND HEMICHANNELS IN ACQUIRED AND GENETIC PATHOLOGIES OF THE CNS

Changes in neuronal and glial gap junction channels and hemichannels, have been described in diverse pathological conditions, including epilepsy, schizophrenia and drug addiction (McCracken et al., 2005; Nilsen et al., 2006; Aleksic et al., 2007), but herein we described those in which molecular mechanisms involving gap junction channels and/or hemichannels are better understood.

### Ischemia-Reperfusion, Trauma and Inflammatory Response

The inflammatory response is a common pathophysiological process of most if not all acute and chronic diseases. Under threatening conditions, microglia and astrocytes are activated and show phenotypic changes related to the intensity, duration and quality of the threat. At moderate "threat levels," ATP is released from glia via Cx43 hemichannels, leading to an increase in extracellular levels of adenosine that mediates a preconditioning response (Lin et al., 2008) conferring tissue resistance to successive deleterious insults within a limited period of time. At higher threat levels, both microglia and astrocytes manifest rapid phenotypic changes *ad hoc* to either neutralize the threatening agents or condition and/or to quickly adapt to the new microenvironment (e.g., high extracellular concentrations of  $\text{K}^+$  and proinflammatory molecules). In contrast, neurons cannot adapt quickly and show a progressive increase in electrical activity leading to excitotoxicity. As more cell debris is generated, more microglia become activated and more proinflammatory molecules are released, generating a positive feedback mechanism. A similar mechanism can be triggered by pathogens or foreign molecules detected by microglia. Free radicals and pro-inflammatory cytokines generated at injured loci enhance the gap junctional communication in astroglial cells (Contreras et al., 2002; Même et al., 2006; Retamal et al., 2007b). These changes deprive neurons of numerous glial protective functions and they become more susceptible to changes in their environment and die.

In chronic diseases, this inflammatory mechanism can be activated by anomalous molecules such as  $\text{A}\beta$ ,  $\alpha$ -synuclein and Huntingtin in Alzheimer's disease, Down syndrome, Parkinson's and Huntington's diseases, by long-lasting infections such as multiple sclerosis and HIV, or by oxidizing environments in ALS (Dangond et al., 2004) and diabetes (Münch et al., 2003; Raza and John, 2004). The inflammation-induced edema

also reduces tissue perfusion leading to an ischemic event that worsens with the inflammatory response. Edema causes pressure that might reduce astroglial expression of Cx43 (Malone et al., 2007). However, after mechanical stress, such as occurs after brain trauma, the extracellular medium becomes hypertonic, which in turn induces glutamate release through Cx43 hemichannels (Jiang et al., 2011). This response could be mediated by integrin  $\alpha 5\beta 1$  (Batra et al., 2012), or the RhoA GTPase and contractile system (Ponsaerts et al., 2012). Hemichannel opening may be associated with an interaction between negatively charged amino acid residues of the C-terminus (Asp 278 and Asp 279) and a domain of the intracellular loop (D'hondt et al., 2013).

The role of connexins in ischemic insult is likely to be closely linked to the phenomenon of spreading depression (SD) discussed previously under the section "Astroglial Connexins: Functional Roles." While pharmacological blockers of connexin function had been reported to exacerbate damage caused by spreading depression, or ischemia, genetic ablation of Cx43 in mice indicated that Cx43 serves a protective role. Elevations in extracellular  $K^+$  shift the activation potential of Panx1 hemichannels to a more physiological range. Panx1 hemichannels expressed in astrocytes might serve as  $K^+$  sensors for changes in the extracellular milieu, such as those occurring under pathological conditions (Suadicani et al., 2012), or physiologic conditions of high neuronal activity. Elevations in extracellular  $K^+$  of the magnitude occurring during periods of high neuronal activity have been proposed to affect the intercellular signaling among astrocytes (Scemes and Spray, 2012). Gliotransmitter release in the hippocampus in response to high neuronal activity has also been found to be sensitive to P2 receptor and Panx1 hemichannel blockers (Heinrich et al., 2012). The possible regulation of Cx expression in glial cells by  $K^+$  concentration remains unknown.

## Genetic Pathologies

Mutations of different connexins have been directly linked to a diverse array of human diseases, some of which have been associated with the nervous system, including peripheral nerve paralysis and the most common genetically inherited form of nonsyndromic deafness. Expression of mutated connexins in cells and mice have confirmed the causative role of many of these connexin defects in producing specific phenotypes associated with the disease (White and Paul, 1999; Gerido and White, 2004). Exogenous expression of the mutated connexins has revealed a variety of problems, from failures in biosynthesis, to changes in permeability or gating (e.g., failure to open, or shift in the

gating profile to voltage, etc.). When the effects are specific to one function of the channels (e.g., the ability to form gap junctions or hemichannels, or permeability to specific molecules), these mutations can provide valuable insights into the underlying cause of the disease. Co-expression of mutant and wild-type connexins has also been shown, in some cases, to reproduce the dominant or recessive nature of the disease (Skerrett et al., 2004). However, when considering the function of these connexins *in situ*, it is important to remember that many tissues express more than one connexin type, so that only cells without the ability to compensate for the specific connexin defect might be affected by the mutation.

## Cx32 and X-Linked Charcot-Marie-Tooth Disease

Bergoffen et al. (1993) described the first definitive link of connexins to human disease by tracing Cx32 mutations to being the causative factor behind the X-linked form of Charcot-Marie-Tooth (CMTX) disease, a demyelinating neuropathy predominantly affecting the peripheral nervous system (PNS), with occasional CNS involvement (Taylor et al., 2003). Subsequently, Cx47 mutations have been associated with abnormal CNS myelin in Pelizaeus-Merzbacher-like disease (Uhlenberg et al., 2004). CMT occurs with a frequency of 1:3000 and is the most common type of inherited nerve disorder in children. With a frequency of about 10%, the X-chromosome-linked form of CMT is the second most common inherited neuropathy, and is genetically defined by over 260 distinct mutations in the *GJB1* gene encoding Cx32 (Nave et al., 2007; Shy et al., 2007). While many of the mutations in Cx32 cause accumulation of the protein within the cell (Deschênes et al., 1997), others form functional channels with gating abnormalities (Oh et al., 1997; Ressot et al., 1998; Abrams et al., 2001, 2002) or permeability changes (Oh et al., 1997; Ressot et al., 1998; Bicego et al., 2006). Overall, the evidence supports that the channel function of Cx32 is important for normal myelin structure.

Cx32 is located at the paranodes and Schmidt-Lantermann incisures of myelinating Schwann cells in peripheral nerves (Scherer et al., 1995), where "reflexive" gap junctions (connecting cytoplasmic domains of the same cell) between myelin layers are believed to reduce the diffusion distance between the Schwann cell nucleus and the myelin wrap closest to the axon (Oh et al., 1997; Abrams et al., 2000) that may be critical to the normal function of the axon-Schwann cell unit. While a role for these reflexive gap junctions is certainly consistent with most or all of the disease-linked mutants studied to date, it should be

noted that several CMTX-linked mutations of Cx32 (S85C, D178Y and F235C) have been associated with altered hemichannel properties, typically resulting in much higher open probabilities under physiological conditions (Castro et al., 1999; Abrams et al., 2002; Gómez-Hernández et al., 2003; Liang et al., 2005). Such an increase in Cx32 hemichannel activity in myelinating Schwann cells may induce damage through loss of ionic gradients and small metabolites, and increased  $\text{Ca}^{2+}$  influx, thus providing a mechanism by which some Cx32 mutants may damage cells in which they are expressed.

### Cx43 and Oculodentodigital Dysplasia

Oculodentodigital dysplasia (ODDD) is a rare autosomal dominant disorder characterized by craniofacial anomalies involving the teeth and skull, as well as fusion of the digits, as the name would suggest. Patients also manifest neurological symptoms, such as mental retardation, ataxia, neurogenic bladder, seizures, spasticity and hearing impairment (Loddenkemper et al., 2002; Kjaer et al., 2004; Vitiello et al., 2005). *GJA1*, encoding Cx43, is the only gene in which mutations have been found in patients or families affected with ODDD. So far, over 35 distinct mutations causing ODDD have been identified, involving most domains of Cx43, all with a negative influence on gap junction channel function (Paznekas et al., 2003). The correlation of disease phenotype with altered hemichannel function is less clear, as in the cases that have been tested, some mutants cause loss of both gap junction and hemichannel activity (Lai et al., 2006), while in others an increase in hemichannel activity was noted, correlated with an extended half-life of the mutant, in the absence of any detectable gap junction function (Dobrowolski et al., 2007). The latter study concluded that the presence of more open hemichannels than normal could aggravate the disease.

Patients with ODDD also present hearing defects, yet Cx43 is not the predominant connexin in the adult ear. This apparent contradiction might be explained if Cx43 is required for normal ear ontogeny. Since Cx43 is not expressed by most neurons, this suggests that the presence of central nervous system symptoms in ODDD patients is related to the altered function of other cell types, most likely astrocytes and microglia, or in neural progenitor cells that still express Cx43.

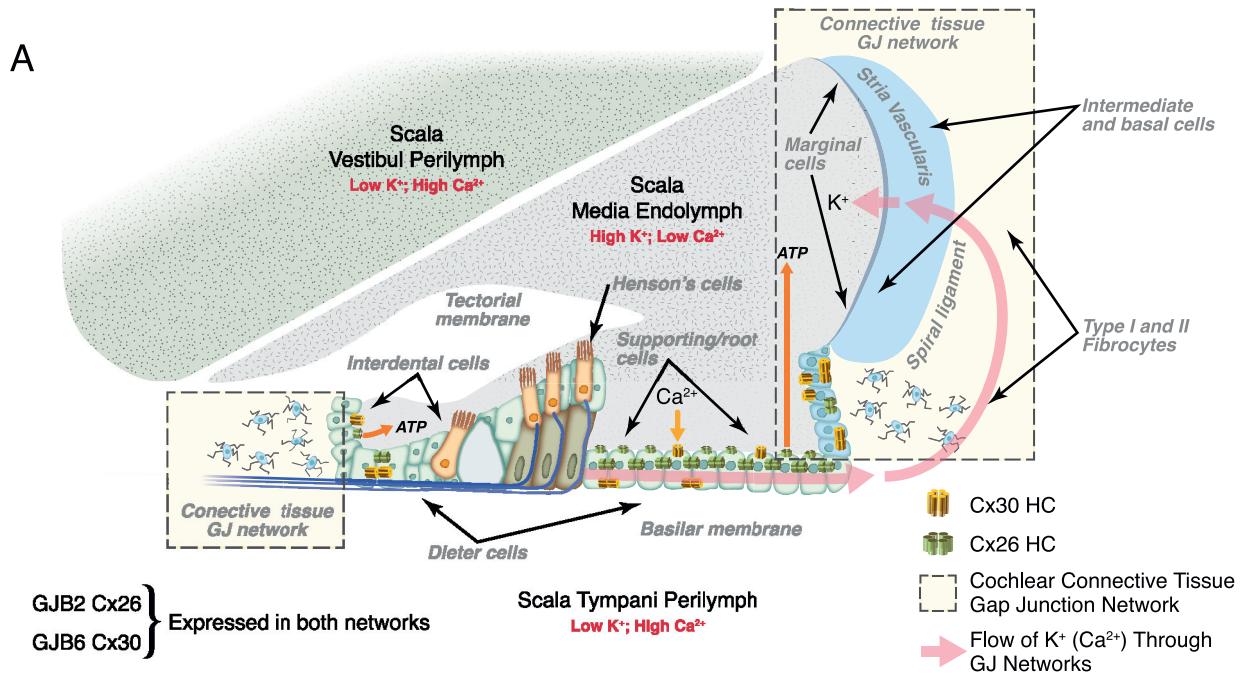
### Cx26 (also Cx30 and 31) and Nonsyndromic Deafness

Mutations of *GJB2* gene, encoding Cx26, are the most common cause of hereditary deafness, with more than 90 different mutations associated with

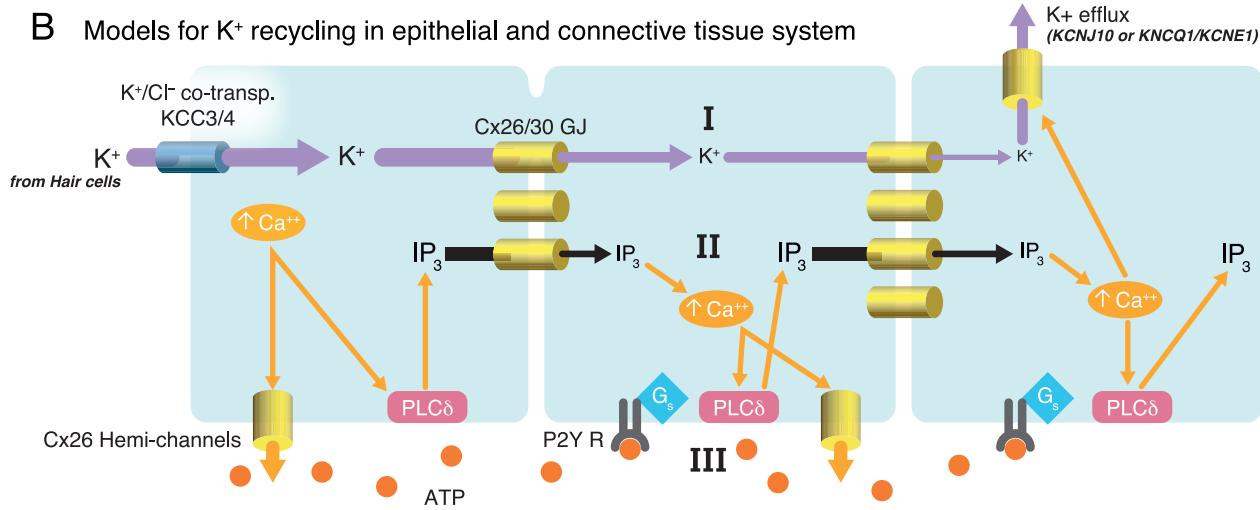
recessive forms of nonsyndromic hearing loss (Oguchi et al., 2005). In fact *GJB1* is one of the most highly mutated genes in the human genome, for largely the same reason that CFTR is heavily mutated—there is a string of Gs at the beginning of the gene that is prone to errors during replication. Among other tissues, the cochlea expresses Cx26 (Sohl and Willecke, 2004) in the nonsensory epithelial cells in the organ of Corti, stria vascularis and type II fibrocytes of the connective tissue beneath the epithelial layer (Kikuchi et al., 1995). Connexin30 (Cx30) is also present in this tissue and co-localizes with Cx26, presumably forming heteromeric hemichannels (Forge et al., 1999, 2003).

In the inner ear, Cx26 is thought to contribute to maintaining cochlear homeostasis by offering an intercellular pathway between the supporting cells of the organ of Corti for recycling the endolymphatic  $\text{K}^+$  that is expelled from the sensory cells during auditory transduction (Fig. 9.7A) (Kikuchi et al., 1995; Zhao et al., 2006; Zhang et al., 2005). This is likely to be important for “spatial buffering” of  $\text{K}^+$  as described for astrocytes in the cortex, and consistent with this, mice with conditional knockout of Cx26 in the ear show significantly enhanced death of the hair cells, consistent with hyperexcitability-induced apoptosis (Aarts and Tymianski, 2004). However, there is also a specialized need for  $\text{K}^+$  recycling in the ear, as  $\text{K}^+$  must diffuse intercellularly from its release from the hair cells through the supporting cells, to the spiral ligament of the stria vascularis, until it is ultimately released into the endolymph by  $\text{Na}^+/\text{Cl}^-/\text{K}^+$  co-transporters or  $\text{Na}^+/\text{K}^+$  antiporters (Fig. 9.7B). Gap junctions comprise an important part of this “circuit.” This is required for normal hearing, as the endolymph maintains a high  $\text{K}^+$  concentration, and hence potential ( $\sim +80 \text{ mV}$ ) (Fig. 9.7A), that provides an enhanced driving force across the hair cell membrane ( $V_m$  of  $\sim 160 \text{ mV}$ ), resulting in an enhanced signal upon activation of the hair cells (translating into an amplification of almost 90 decibels in effective hearing).

Although many deafness mutants are recessive, some do display dominant phenotypes (Kelsell et al., 1997), and this has been correlated with dominant-negative effects when co-expressed with wild type Cx26 in exogenous systems like the *Xenopus* oocyte (Skerrett et al., 2004; Laird, 2008). Dominant mutants are also often found associated with phenotypes in the skin (reviewed in Xu and Nicholson, 2013). As with the previous two diseases discussed, while disease-causing mutants are dispersed throughout the protein, there is a strong correlation of disease phenotype with channel function (Xu and Nicholson, 2013). Some effects of these mutants on hemichannels have been reported (Stong et al., 2006), but in at least one case the dominant nature of the disease correlated better with the effects of the



### B Models for K<sup>+</sup> recycling in epithelial and connective tissue system



**FIGURE 9.7** A. Diagram of the cochlea and its various compartments, showing the proposed return of K<sup>+</sup> (possibly mediated by a propagated Ca<sup>2+</sup> wave) from the hair cells to the endolymph (red arrows) through the epithelial (green color) and connective tissue (dotted box) gap junction networks. The former is comprised of Dieters' cells and supporting cells, while the latter is comprised of types I and II fibrocytes in the spiral ligament and basal and intermediate cells of the stria vascularis. Ultimately, released K<sup>+</sup> is taken up by the marginal cells of the stria vascularis and released into the endolymph, thus maintaining the elevated K<sup>+</sup> levels (and high resting potential of ~ + 100 mV) in this compartment. This is essential for amplifying the responses of hair cells to sound, by increasing the driving potential for K<sup>+</sup> flux across the hair cell membrane when it is activated. B. Possible models to explain how K<sup>+</sup> is recirculated in the ear are illustrated as: I: passive K<sup>+</sup> flux through gap junctions. This seems unlikely, as it would decay over a relatively short distance; II: a regenerative Ca<sup>2+</sup> wave that is propagated by IP<sub>3</sub> flux between cells. IP<sub>3</sub> is regenerated in each cell by IP<sub>3</sub> induced Ca<sup>2+</sup> release from intracellular stores that activates phospholipase C (PLC). In the stria vascularis, K<sup>+</sup> is released into the intrastrial space or the endolymph through KCNJ10 (Kir4.1) or PIP<sub>2</sub> regulated (KNCQ1/KCNE1) K<sup>+</sup> channels; III: A regenerative Ca<sup>2+</sup> wave that is propagated extracellularly by Ca<sup>2+</sup> activated release of ATP through connexin hemichannels which then activates P2Y receptors in adjacent cells. These in turn can activate PLC $\delta$ , which will generate IP<sub>3</sub> and re-initiate the Ca<sup>2+</sup> response. Figure from Xu and Nicholson *Biochim et Biophys Acta – Biomembranes* 1828: 167–178 (2013).

mutant on gap junctions rather than on hemichannels, where it showed recessive characteristics (Chen et al., 2005). One particularly instructive deafness mutant (V85L) studied by Mammano and colleagues showed normal gap junction characteristics in terms of gating, conductance, and even passage of dyes between cells. Only when it was specifically tested for IP<sub>3</sub> permeability was its defect apparent (Beltramello et al., 2005), suggesting that recycling of K<sup>+</sup> may not occur directly, but through a regenerative mechanism analogous to Ca<sup>2+</sup> waves (Fig. 9.7B). Of course, interpretation of all of these findings is complicated by the co-expression of at least two other connexins in these tissues, each of which, when mutated, can lead to deafness.

## SUMMARY AND PERSPECTIVE

In this chapter we have concentrated on the roles of connexins in vertebrates, yet invertebrate systems use electrical systems to control activity to an even greater extent. Composed of innexins, electrical synapses in invertebrates control coordination of muscle contractions as required in *C. elegans* for locomotion (unc7 and 9) or rhythmic contractions in the digestive system (eat5 and inx3 and 6), and escape responses such as the jump reflex in *D. melanogaster* (shakB lethal), and the tail flip of crustaceans, or transmission at the giant synapse of the squid. All of these responses utilize the reduced synaptic time constant at electrical compared to chemical synapses to achieve rapid response times. Of what is known so far, electrical synapses in the adult nervous systems of vertebrates seem to be more restricted in scope, but similarly tend to be involved in rapid and bidirectional transmission as seen in escape reflexes (Mauthner neuron in teleost fish) or coordination of oscillatory activity in various brain regions like the hippocampus, cerebellum and cortex. These oscillations are likely to serve in the fine-tuning of various behaviors and memories, as has been recently demonstrated in studies of knockout mouse models, but more studies are required to establish their specific functional roles. The role of neural coupling is better understood in the retina, which serves as a model system to illustrate multiple connexin roles involved in direct transmission, and in the tuning of neuronal responsiveness under different light conditions, or the size of receptive fields. In vertebrates, the frequent co-existence of chemical and electrical synapses at the same cell contact allows for optimal flexibility in neuronal responses, including activity dependent potentiation of electrical synapses that has usually been considered to only occur with chemical transmission.

While neuronal electrical synapses may be less common in vertebrate nervous systems, gap junctions form

between many other cell types, and mediate a variety of functions related to intercellular transfer of not only ions, but also larger metabolites. Primary among these is the supporting role of astrocytes and oligodendrocytes in maintenance of neuronal health at sites of high neuronal activity by spatial buffering of K<sup>+</sup> and glutamate. Oligodendrocytes, and their PNS cousins, Schwann cells, also require connexin expression for the formation and maintenance of myelination. Several diseases of the PNS and CNS associated with defects in Cx32, 47, 26 and 30 illustrate these functions. This chapter also emphasizes the functions of connexins beyond gap junctions, including their roles as hemichannels, composed of either connexins or the descendants of innexins in vertebrates, Pnx1 and possibly Pnx2. A role for connexins in adhesion during neuronal migration in early development is also described.

Compared to other ion channel classes and receptors, the study of connexins, and particularly pannexins, in the nervous system is in its infancy. We have already learned that they play very diverse roles in regulating CNS and PNS function, but a true understanding of the mechanisms underlying these roles lies ahead as we begin to understand the permeability and regulatory features of each connexin, which functions are redundant and unique, and how to more selectively probe their physiological relevance through more targeted genetic ablations. The next decade offers great prospects for a deeper understanding of the integrative effects of gap junctions on the function of the nervous system.

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