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Review

Emerging role of gap junctions in epilepsy

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Summary. This review highlights the contribution of gap junctions to the pathophysiology of epilepsy. The tissue expression and spatiotemporal regulation of connexins is discussed, and the phenotypes of specific connexin knockouts are considered. Electrophysiologic studies have implicated gap junctions in the generation of very fast oscillations preceding seizures. Gap junction inhibitors have shown powerful anticonvulsant effects, to date primarily in *in vitro* studies. Specific inhibition of gap junctions *in vivo* along with more detailed human tissue studies are needed to understand more fully the role of gap junctions in epileptogenesis.

Key words: Epilepsy, Gap junctions, Connexins, Very fast oscillations, Seizures

Introduction

Evidence for direct electrical transmission between neurons was first described in invertebrate systems in the late 1950s (Watanabe, 1958; Furshpan and Potter, 1959) and in vertebrate tissue in the early 1960s (Bennett et al., 1963). Later, direct electrotonic coupling was found in various areas of rat brainstem (Baker and Llinás, 1971; Korn et al., 1973). In 1981, MacVicar and Dudek were the first to demonstrate, using dual intracellular recordings, direct electrotonic coupling of neurons in rat hippocampal slices (MacVicar and Dudek, 1981). This led to the hypothesis that electrotonic coupling may contribute to the synchronization of neurons into seizure activity (Dudek et al., 1986, 1998, 1999; Jefferys, 1995a,b; Bennett and Zukin, 2004). However, it was not until recently that the role of gap junctions in specific *in vitro* and *in vivo* models of epilepsy has been examined in greater detail.

Gap junctions were originally described by electron microscopy as structures connecting adjacent cells with the extracellular space reduced to a narrow gap (Revel and Karnovsky, 1967). Structural analyses have revealed that a complete gap junction is formed by the approximation of two hemichannels, or connexons, each of which consists of a hexameric structure consisting of six connexin proteins with a 1.2-nm-diameter hydrophilic pore at its center. This large-diameter pore allows intercellular transfer of ions, second messengers, metabolites, and other small molecules (Bennett and Zukin, 2004; Nakase and Naus, 2004), thereby subserving not only electrical coupling between cells but also chemical and metabolic coupling.

Gap junctions are thought to play an important role in processes that require rapid intercellular communication, such as brain development, morphogenesis, and pattern formation (Bennett and Zukin, 2004). In addition, new experimental evidence implicates gap junctions in the pathogenesis of several diseases, including epilepsy, stroke, neurodegenerative disease, and brain tumors (Nakase and Naus, 2004). In this review, we specifically assess the role of gap junctions in the hyperexcitability associated with epilepsy. First, we describe the expression of connexins in specific central nervous system (CNS) cell types. Second, we summarize the phenotypes of connexin knockout mice. Third, we examine the evidence that gap junctions contribute to very fast oscillations which precede seizures *in vivo*. Fourth, we review the functional effects of pharmacologic gap junction inhibition. Lastly, we review the evidence for increased gap junctional coupling in human epilepsy.

Connexins: cloning and tissue expression

While the existence of electrical synapses in the mammalian CNS has been known for a long time, the cloning and characterization of connexin genes has greatly facilitated functional analysis of gap junctions. The first connexin was isolated from a cDNA expression library using an antibody against rat liver gap junctions (Paul, 1986). Since that time, the connexin gene family has grown to at least 20 members in mammals (Simon and Goodenough, 1998; Willecke et al., 2002). Connexins have conserved sequences and similar three-dimensional structure; however, they can associate as

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homohexamers or heterohexamers to form channels with unique biophysical properties. This diversity allows gap junctions to assume distinct physiological roles in various tissues (Bruzzone et al., 1996; Cao et al., 1998; Harris, 2001; Saez et al., 2003).

In the CNS, gap junctions are expressed in almost all of the major cell types, including neurons, astrocytes, oligodendrocytes, microglia, and ependymal cells (Nakase and Naus, 2004). In addition to homocellular connections (e.g. neuron-neuron and astrocyte-astrocyte), heterocellular connections have also been found (e.g. neuron-astrocyte and astrocyte-oligodendrocyte) (Nedergaard, 1994; Nagy and Rash, 2000). It is thought that these interconnections facilitate rapid communication between different cell types, allowing metabolic coupling.

Connexins 26, 29, 30, 32, 36, 37, 40, 43, 45, 46, and 47 are synthesized in the CNS, and each is regulated differently with respect to cell type and developmental stage of expression (Nakase and Naus, 2004). During CNS development, radial glia express Cx26 and Cx43, allowing them to couple with neuronal precursors during early stages of cortical development (Bittman et al., 1997; Bittman and LoTurco, 1999). Cx26, Cx32, Cx36, and Cx45 are also expressed during brain development, and Cx37 and Cx40 are expressed in the developing spinal cord (Maxeiner et al., 2003; Nakase and Naus, 2004).

Glial cells, and specifically astrocytes, are more extensively coupled through gap junctions than are neurons in the adult (Cotrina et al., 2001). The major astrocytic connexin is Cx43 (Giaume et al., 1991; Dermietzel and Spray, 1998). Other connexins expressed in astrocytes include Cx26, Cx30, Cx40, Cx45, Cx46, and Cx47 (Nakase and Naus, 2004). Astrocytic gap junctions have many potential roles, including signaling by calcium waves (Nedergaard, 1994; Nedergaard et al., 2003), clearance of extracellular potassium after neuronal activity (Ransom, 1996; Nedergaard et al., 2003), cell-cell communication (Smith, 1994), and distribution of metabolic substrates to neighboring cells (Tabernero et al., 1996).

In adults, neuron-neuron gap junctional coupling occurs in the cortex and the hippocampus via Cx36 (Rash et al., 2000, 2001) and Cx45 (Maxeiner et al., 2003). In particular, Cx36 appears to electrically couple hippocampal and cortical GABAergic interneurons (Venance et al., 2000).

Of the other cell types in the CNS, resting microglia are not extensively coupled but express Cx43 upon activation (Eugenin et al., 2001). Oligodendrocytes express several different connexins, including Cx29, Cx32, Cx36, and Cx47 (Nagy and Rash, 2000). The role of gap junctions in oligodendrocytes is thought to be to allow passage of ions and nutrients from the cell body throughout the myelin sheath (Paul, 1995). This function is further supported by the finding that mutations in Cx32 are associated with X-linked Charcot-Marie-Tooth disease, a form of hereditary neuropathy with demyelination (Bergoffen et al., 1993).

Regulation of connexins by seizure activity

Several studies have examined regulation of connexins by seizures in animal models of epilepsy. Sohl et al. studied expression and localization of Cx30, Cx32, Cx36, and Cx43 in two rat models of temporal lobe epilepsy (kindling and kainate treatment). While they found a large increase in glial fibrillary acidic protein (GFAP) mRNA and protein associated with gliosis in rats 4 weeks after kainate treatment, no change in Cx30 or Cx43 mRNA or protein was found (Sohl et al., 2000). They did find a 44% decrease in Cx36 mRNA with a smaller reduction in Cx36 protein, effects possibly attributable to neuronal cell death. Following kainate-induced seizures in rats, Condorelli et al. found a region-specific regulation (increase, decrease, or no change) in Cx30 mRNA and protein levels (Condorelli et al., 2002) and unclear regulation of other connexins (Condorelli et al., 2003). In contrast, using an *in vitro* bicuculline model of epilepsy in mouse hippocampal slices, Li et al. found marked increase in Cx32 mRNA and protein (Li et al., 2001). A similar model also showed increases in Cx43 and Cx32 mRNA and protein, but no change in Cx26 and Cx36 (Samoilova et al., 2003). Using a neocortical epilepsy model involving local application of the K^+ channel antagonist 4-aminopyridine (4-AP), Gajda et al. found that mRNA levels for Cx32, Cx36, and Cx43 increased significantly at both the primary site as well as at the mirror focus (Gajda et al., 2003). Further studies are needed to carefully tease out the details of connexin regulation by neuronal activity as distinct from other processes such as cell death.

Functional evidence from connexin knockout studies

Knockout mice lacking specific connexin proteins have been generated. Mice deficient in Cx36, the major neuronal connexin, have visual deficits (Guldenagel et al., 2001) as well as deficient synchronous activity of inhibitory interneuronal networks in neocortex (Deans et al., 2001). Initial studies using hippocampal slices from $Cx36^{-/-}$ mice demonstrated impaired hippocampal gamma (~30-80 Hz) oscillations but relative preservation of very fast (~140-200 Hz) oscillations (VFOs or "ripples") (Hormuzdi et al., 2001; Pais et al., 2003). In contrast, Maier et al. found that spontaneous sharp waves and ripples were less frequent in hippocampal slices from $Cx36^{-/-}$ mice; furthermore, epileptiform discharges induced by 4-AP were attenuated (Maier et al., 2002). In an *in vivo* electrophysiological study of $Cx36^{-/-}$ mice, Buhl et al. found no difference in ripples or hippocampal theta oscillations but a mild reduction in gamma power (Buhl et al., 2003). Behavioral tests of locomotor activity have not revealed any difference between wild-type and $Cx36^{-/-}$ mice (Long et al., 2002).

Other connexin knockout mice display a diversity of

phenotypes. Cx32 knockout mice have myelination defects, display intrinsic neuronal hyperexcitability, have impairments in inhibitory synaptic transmission, and display enhanced vulnerability to ischemia (Anzini et al., 1997; Scherer et al., 1998; Sutor et al., 2000; Oguro et al., 2001). Deficiency of another oligodendrocyte connexin, Cx47, leads to nerve fiber vacuolization (Odermatt et al., 2003); and Cx32/47 double knockout leads to severe myelination deficits (Menichella et al., 2003; Odermatt et al., 2003). Cx26 knockout is embryonic lethal (Gabriel et al., 1998). Mice with targeted postnatal ablation of Cx26 in the ear develop hearing impairment (Cohen-Salmon et al., 2002). Cx30 knockout also causes severe hearing impairment (Teubner et al., 2003). Cx45 deletion is embryonic lethal (Kruger et al., 2000).

Knockout of Cx43, the major astrocytic connexin, is neonatal lethal and causes neural crest migration deficits (Lo et al., 1999; Xu et al., 2001; Fushiki et al., 2003). Heterozygous deletion of Cx43 enhances neuronal injury following ischemia (Siushansian et al., 2001; Nakase et al., 2003). Targeted postnatal inactivation of Cx43 in astrocytes enhances spreading depression (Theis et al., 2003).

While these studies suggest that connexins may be involved in synchronization of certain types of neural activity, a direct role in seizure synchronization has yet to be shown. Interestingly, mutation of the *shak-B*$_2$ gene (a neuron-specific connexin in *Drosophila*) increases seizure threshold and leads to a delay in seizure synchronization (Kuebler et al., 2001). Similar experiments studying connexin knockout mice in established seizure models must be performed. It is important to recognize that the interpretation of these experiments may be confounded by the potential for upregulation of other connexin proteins. Therefore, the recent development of floxed connexin knockouts will be useful to examine adult roles of these proteins.

Role of gap junctions in very fast oscillations and seizure synchronization

Recently, exciting new data have implicated very fast neuronal oscillations (VFOs, 140-200 Hz) in the generation of seizures. These "ripples" have been shown to immediately precede seizure onset in a number of experimental models as well as in human clinical electroencephalograms (EEG) (Allen et al., 1992; Fisher et al., 1992; Alarcon et al., 1995; Traub et al., 2001). Computer models of hippocampal circuitry developed by Traub and colleagues predicted these high-frequency oscillations when axo-axonal gap junctions were included in the simulations (Traub et al., 1999). In fact, only a very small number of gap junctions linking neurons are required to produce the appropriate oscillatory activity (Traub et al., 2004). This prediction was confirmed by electrophysiological detection of these axo-axonal gap junctions together with evidence of direct dye-coupling between CA1 pyramidal neurons (Schmitz et al., 2001). In addition, rat hippocampal slices displayed VFOs before epileptiform bursts, the VFOs were abolished by the gap junction inhibitor carbenoxolone, and they were not dependent on chemical synaptic transmission (Draguhn et al., 1998; Traub et al., 2004). Similar findings have been described *in vivo* in cat neocortex, in which ripples are observed at seizure onset and halothane (another gap junction inhibitor) blocks ripples and subsequent seizure activity (Grenier et al., 2003; Timofeev and Steriade, 2004).

Gap junction inhibition: a potential novel anticonvulsant mechanism?

There is striking pharmacologic evidence that gap junction inhibitors may be effective anticonvulsants. The standard compounds that have been used to study gap junctions include carbenoxolone, glycyrrhetinic acid, halothane, and octanol. Glycyrrhetinic acid, a natural constituent of licorice, is a known inhibitor of 11ß-hydroxysteroid dehydrogenase with additional effects on vascular smooth muscle (Dobbins and Saul, 2000). Carbenoxolone, a synthetic derivative of glycyrrhetinic acid, is a drug that was first used in the treatment of gastric ulcers, duodenal ulcers, and recurrent aphthous oral ulcers (Pinder et al., 1976). Halothane is a commonly used inhaled anesthetic.

In vitro studies using gap junction inhibitors

In the last few years, the specific effects of gap junction inhibitors have been studied in *in vitro* models of epilepsy. Using a calcium-free model of epilepsy in hippocampal slices (in which chemical synaptic transmission is blocked), Perez-Velazquez et al. demonstrated suppression of epileptiform activity in CA1 under conditions that suppressed gap junctional conductance (acidification, halothane, and octanol); conversely, intracellular alkalinization increased epileptiform activity (Perez-Velazquez et al., 1994). Carbenoxolone reduced spontaneous burst activity in a zero-Mg^{+2}/4-AP model in hippocampal slices (Ross et al., 2000). In the high-K^+/low Ca^{+2} model, gap junction inhibitors (heptanol, octanol, and carbenoxolone) inhibited spontaneous field bursts in CA3 (Margineanu and Klitgaard, 2001) and dentate gyrus (Schweitzer et al., 2000). In a zero-Mg^{+2} model, halothane, carbenoxolone, and octanol were able to block secondary epileptiform discharges in CA3 of rat hippocampal slices; conversely, the gap junction opener TMA (trimethylamine) reversibly induced epileptiform discharges (Kohling et al., 2001). In an electrical stimulation model in rat hippocampal slices, Jahromi et al. demonstrated that carbenoxolone reduced the duration of primary afterdischarges (Jahromi et al., 2002). In a chronic model of bicuculline exposure in organotypic hippocampal slice cultures, carbenoxolone reversibly inhibited spontaneous and evoked epileptiform discharges (Samoilova et al., 2003).

In vivo studies using gap junction inhibitors

Despite the rapidly accumulating evidence from the *in vitro* studies summarized above, few studies have yet examined gap junction inhibition *in vivo*. Using a rat focal seizure model in which 4-AP is applied to the cortex, Szente et al. demonstrated that carbenoxolone caused a significant decrease in seizure activity at both the primary and mirror foci (Szente et al., 2002). In a follow-up study, the same group confirmed the anticonvulsant effect of carbenoxolone and also found that TMA increased seizure duration (Gajda et al., 2003).

Gap junction inhibitors have yet to be used clinically to treat seizure disorders; however, these experiments support the concept of gap junction inhibition as a novel anticonvulsant strategy for medically-intractable epilepsy. Carbenoxolone has already been used clinically for several decades in the United Kingdom for treatment of peptic ulcer disease and has been well tolerated (Pinder et al., 1976). One main limitation of current inhibitors is lack of specificity (Rozental et al., 2001). More specific inhibitors of connexins as well as distinct methods (such as RNA inhibition) will allow more rigorous determination of the role of particular connexins in the generation of seizure activity. Further experiments using both current and future gap junction inhibitors *in vivo* will provide vital information on their potential role in the treatment of epilepsy.

Human tissue studies

Studies of gap junctional coupling in human epileptic tissue have been limited. The first report examining the link between gap junctions and epilepsy was of a series of 8 patients undergoing temporal lobe resection for the management of intractable epilepsy (Naus et al., 1991). They found that mRNA levels of Cx43 and Cx32 were elevated in epileptic temporal lobe compared to peritumoral temporal cortex obtained during tumor resection. Interestingly, in the subset of patients with tumors that were associated with acute seizures, they observed higher levels of Cx43 mRNA. These results provided the first evidence for the upregulation of gap junctions in human epilepsy tissue. In contrast, Elisevich et al. found no upregulation of Cx43 mRNA or protein in 15 patients undergoing temporal lobectomy for intractable epilepsy when compared to 5 nonepileptic patients undergoing temporal lobectomy for emergent causes (Elisevich et al., 1997). These authors suggested that epileptogenicity may be related to a change in the dynamic properties of the existing channels (open vs. closed) rather than an upregulation in the absolute number of channels. Another study claimed to show upregulation of Cx43 protein expression in temporal lobectomy specimens in comparison to autopsy controls (Fonseca et al., 2002).

In another human tissue study, Lee and colleagues in 1995 studied primary astrocytes derived *ex vivo* from 8 patients who underwent temporal lobectomies for intractable epilepsy and compared them to primary astrocytes derived from 8 patients who underwent craniotomy for astrocytoma resection (Lee et al., 1995). In the epilepsy group, tissue was obtained from the hippocampus, the surrounding hyperexcitable parahippocampus, and "normal" cortex. In the tumor patients, tissue was obtained from the astrocytoma itself, from cortical margins with normal EEG activity, and from surrounding hyperexcitable cortex. Using the optical technique fluorescence recovery after photobleaching (FRAP), Lee et al. showed that astrocytes derived from hyperexcitable tissue in the parahippocampus and in cortex surrounding astrocytomas demonstrated greater gap junctional coupling (faster fluorescence recovery) when compared to controls. While provocative, this study utilized primary astrocyte cultures that spent 2-3 weeks *in vitro* following tissue explantation prior to FRAP analysis; future studies using FRAP analysis of fresh *ex vivo* human tissue slices would more closely approximate *in vivo* gap junctional coupling.

One general disadvantage of human tissue studies to date has been the lack of appropriate controls. In most studies, the control for epilepsy tissue is tumor tissue (and vice versa). Therefore, it is still unclear whether there really is a functional upregulation of gap junctions and gap junctional coupling in human epilepsy tissue or whether there is a downregulation in control (tumor) tissue. In fact, several studies have shown downregulation of gap junction proteins in tumor specimens. Huang et al. demonstrated downregulation of Cx43 in high-grade gliomas (Huang et al., 1999). Laird et al. showed downregulation of Cx43 in a variety of breast tumors (Laird et al., 1999). Soroceanu et al. found downregulation of Cx43 in glioma specimens and also found decreased gap junctional coupling by FRAP in primary astrocyte cultures derived from glioma specimens compared to epilepsy tissue (Soroceanu et al., 2001). Interestingly, while Aronica et al. found decreased Cx43 immunoreactivity in high-grade gliomas as well, they also found upregulation of Cx43 protein in peritumoral reactive astrocytes surrounding low-grade tumors, suggesting that they may contribute to tumor-related seizures (Aronica et al., 2001).

Conclusions

While electrotonic coupling of cells in the nervous system has been known for several decades, the specific role of gap junctions in the neuronal hypersynchrony of epilepsy is only beginning to be investigated. Converging evidence from neural network models, *in vivo* electrophysiologic studies, and human clinical EEG has highlighted the role of gap junctions in very fast oscillations that may play a role in initiating or facilitating seizure activity. Pharmacologic studies have demonstrated powerful anticonvulsant effects of gap junction inhibition. Further experiments using selective

inhibitors in animal models of epilepsy will be required to determine the role of glial vs. neuronal gap junctions in the pathophysiology of epilepsy. Lastly, more detailed studies will be required to understand alterations in connexin expression as well as changes in gap junctional coupling in tissue from patients with epilepsy.

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