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Bridging the Gap – Understanding the Role of Gap Junctions in Seizures

Logan J. Voss, Gregory Jacobson and Jamie W. Sleight
*Waikato Hospital Department of Anaesthesia and Waikato University
 New Zealand*

1. Introduction

Epilepsy is a major cause of ongoing disability and a significant cause of hospital admission. According to the World Health Organisation, the disease affects approximately 50 million people worldwide and current pharmacological treatment regimes are ineffective in approximately 30% of cases (<http://www.who.int/mediacentre/factsheets/fs999/en/index.html>). Clearly, there is a need for more effective and targeted pharmacological epilepsy treatments.

In simplistic terms, seizures are said to result from an imbalance in the regulation of brain activity, such that too much excitation or too little inhibition tips the balance towards hyperexcitability (seizure). The reality is almost certainly more complicated, as evidenced by common reports of low efficacy of some currently used antiepileptic drugs, whose primary mechanisms of action are either depression or enhancement of brain excitatory and inhibitory pathways, respectively. The complexity of this area of neurobiology is further highlighted by the observation that seizures can be triggered by anaesthetic drugs, many of which are themselves effective anticonvulsants. Clearly, there are subtleties to the mechanisms of seizure generation and regulation, the complexity of which we are only beginning to understand. A greater understanding of these underlying mechanisms will inevitably lead to more targeted and effective treatment options.

One area that has become the focus of considerable amounts of research in the last 10 years is the role of gap junctions in seizure mechanisms. Gap junctions form direct cytoplasmic connections between cells, providing electrical continuity and allowing passage of low molecular weight molecules from one cell to another. Gap junctions are created from the assembly of six connexin proteins in the cell membrane into a hemichannel; and the association of two hemichannels on neighbouring cells forms a mature gap junction. Acting as electrical synapses, it has been hypothesised that gap junctions could promote seizure activity by facilitating the spread and synchronisation of electrical activity in the brain. In support of this, there are a growing number of experimental studies showing a reduction in seizure severity following pharmacological gap junction blockade (Bostanci and Bagirici 2007; Nassiri-Asl et al. 2009). Intriguingly, several studies point to gap junction blockade having the opposite effect (Voss et al. 2009; Jacobson et al. 2010), suggesting that the relationship between gap junction regulation and seizure propensity is multifaceted.

The apparent discrepancies in the literature surrounding the role of gap junctions in seizurogenesis probably reflect two main complicating factors. Firstly, there are no known

drugs that selectively regulate gap junctions; all have off-target effects which could confound experiments utilising these agents (Juszczak and Swiergiel 2009). Secondly, the effect of gap junction regulation on seizure activity will almost certainly depend on the specific gap junction subtype manipulated. The reason for this is that gap junctions of a given subtype tend to be restricted to a particular class of cells; meaning that targeted modulation of a specific gap junction subtype effectively restricts the effect to a specific cell population. It should not be surprising therefore, that blockade of gap junctions linking inhibitory interneurons for example, will have a different effect to blockade of those between excitatory pyramidal cells.

The central tenet of this chapter is that we cannot begin to fully understand the role that gap junctions play in seizure mechanisms until we appreciate the need for a targeted approach to gap junction modulation; in terms of both off-target, non-gap junctional side-effects and gap junction subtype specificity. These ideas are also important for the development of more effective epilepsy treatment options based on gap junction modulation, which will depend upon the targeted modulation of gap junction subtypes (Song and Tanouye 2006). Achieving targeted gap junction modulation is a major challenge for experimental biologists; however, there are new techniques and approaches that offer some hope for future research. For example, mimetic peptides (short polymers of amino acids) have shown promise as a tool for blocking gap junction formation (Evans and Boitano 2001). The specificity of effect of mimetic peptides comes from the sequence of amino acids, which are chosen to mimic a portion of the extracellularly exposed gap junction connexin protein. The introduced peptide binds to the native protein and interferes with the cell-cell docking process required for mature gap junction formation. In this way, formation of new gap junctions of the targeted subtype is prevented. Experimental approaches can also be supplemented by mathematical modelling studies, which have the enviable advantage that model parameters can be manipulated with absolute specificity. Clearly, no computer model developed to date comes close to representing the brain in all its complexity or functionality; but when aligned with (and refined by) experimental data, computer models can provide an informative adjunct to experimental biology.

In this chapter, we provide a detailed review of the current knowledge around the aforementioned topics. Our discussion focuses on the relationship between gap junctions and seizurogenesis in the mature, adult brain. Gap junction expression is highly dynamic early in development and understanding the contribution of changing levels of different gap junction subtypes to seizures is beyond the scope of this discussion. To this end, the chapter will be structured into three sections. Firstly, we will review the neurobiology of gap junctions and the distribution of gap junction subtypes across cell populations and cerebral locations in the adult brain. Our focus will be on brain regions known to be involved in seizurogenesis; principally the hippocampus and the cerebral cortex. We will then give an overview of recent as well as potential novel approaches to manipulating gap junctions that may provide more specificity of effect, such as mimetic peptides and siRNA technologies; and may also provide the basis of new therapeutic approaches to treating epilepsy. Finally, we will discuss how differentiation between gap junction-linked astrocytic, interneuronal and pyramidal cell networks may help us to understand the nature of gap junction regulation of seizure processes. This research is in its infancy, but there are clues from recent experimental and mathematical modelling studies that provide a solid foundation from which to explore this topic.

2. Gap junction structure and subtype distribution

Gap junctions are proteinaceous structures that form connections between adjacent cells, directly linking the cytoplasm of one cell to another. The basic structural unit is the connexin protein, of which there are 21 known subtypes in the human (Sohl and Willecke 2004). Connexins associate within the cytoplasmic membrane into a hexameric structure known as a connexon, or hemichannel; the association of two hemichannels on adjacent cells forms a mature, functional gap junction (see Fig 1). The junction so-formed allows direct electrical communication between cells and the passage of small molecular weight chemicals; and also serve intracellular signalling roles independent of their channel-forming function (see (Goodenough and Paul 2003; Jiang and Gu 2005) for reviews).

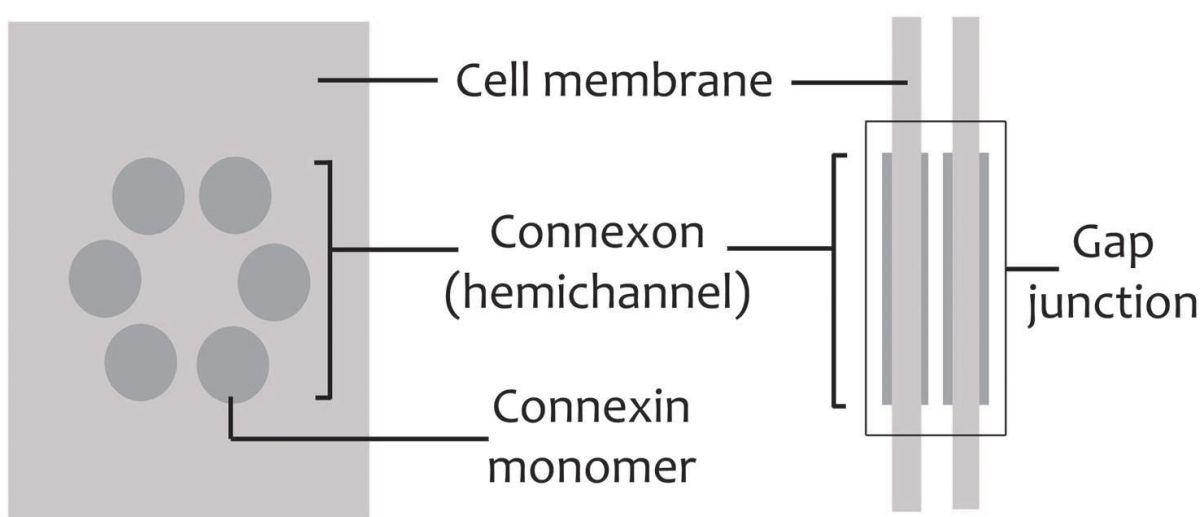


Fig. 1. Schematic representation of gap junction structural organisation

Gap junctions are found in most animal tissues and are widely expressed throughout the mammalian brain (Condorelli et al. 2003). However, expression patterns are not uniform and the distribution of different gap junction subtypes is dependent upon location and developmental maturity. For simplicity and in order to focus on the regions of greatest clinical relevance for human epilepsy (Hauser and Kurland 1975; Wiebe 2000), we will limit our discussion to the distribution of gap junction subtypes within the mature cerebral cortex and hippocampus.

2.1 Cortical and hippocampal gap junction expression

Only a limited number of connexins have been shown unequivocally to be expressed in the mature cerebral cortex. Connexin30, 32, 43, 45 and 47 gap junctions are expressed by cortical glial cells (Dermietzel et al. 1991; Dermietzel et al. 1997; Condorelli et al. 2002; Condorelli et al. 2003). The most common of these are connexin30 and connexin43, which are expressed by astrocytes (Dermietzel et al. 1991; Condorelli et al. 2002). Oligodendrocytes express connexin32 and connexin45 (Dermietzel et al. 1997), but constitute a small fraction of glial cells in the CNS (5-10% compared to 50-60% for astrocytes (Singh et al. 2003)). Connexin32 expression has also been localised to CNS neurons, but only in subcortical structures (thalamus and basal ganglia) (Dermietzel et al. 1989). Central nervous system neurons

express connexin36, 45 and 57, with the latter restricted to horizontal cells of the retina (Hombach et al. 2004). Connexin36 is the most common neuronal gap junction in the mature cerebral cortex, and its expression is restricted primarily to inhibitory interneurons (Deans et al. 2001). Connexin45 is expressed neuronally (Condorelli et al. 2003; Maxeiner et al. 2003), however adult cortical expression of connexin45 is low and restricted to parieto-occipital and entorhinal cortical regions (Maxeiner et al. 2003). Thus, in the mature cerebral cortex, the most prominent gap junctions are those between inhibitory neurons (connexin36) and those between astrocytes (connexin30 and 43).

Connexin distribution patterns in the hippocampus are similar to those in the cerebral cortex; astrocytic gap junctions are formed predominantly by connexin30 (Condorelli et al. 2002; Rouach et al. 2008), and connexin43 (Rouach et al. 2008) and GABAergic interneuronal junctions by connexin36 (Deans et al. 2001). There is evidence that connexin36 may also be sparsely expressed by pyramidal cells in the hippocampus where they are thought to form axo-axonal junctions (Schmitz et al. 2001; Hamzei-Sichani et al. 2007).

3. Genetic experimental techniques for targeting gap junction subtypes

Determining the exact role of gap junctions in seizurogenesis has been challenging because all known gap junction blocking drugs lack specificity (for an excellent review see (Juszczak and Swiergiel 2009)). For example, the gap junction blockers quinine and mefloquine have anti- and pro-seizurogenic properties independent of their gap junction-blocking effects. Quinine, at modest concentrations (~20 μ M), is well known to block a variety of neuronal ion channels; in particular quinine's use-dependent blockade of sodium channels is a very similar action to the antiepileptic drug phenytoin (Lin et al. 1998). In vitro, quinine is convulsive at an intraperitoneal dose of 250 mg/kg (Amabeoku and Chikuni 1992) and mefloquine at 150mg/kg (Amabeoku and Farmer 2005) in mice. This effect is via a GABA_A antagonist action (Thompson and Lummis 2008) and is seen in *in vitro* slices at 100 μ M and 400 μ M for mefloquine and quinine, respectively (Thompson and Lummis 2008). Mefloquine may also enhance neuronal excitability in cultures via a disruption to calcium homeostasis at greater than 30 μ M (Dow et al. 2003). Mefloquine also inhibits 5-HT₃ receptors at 10 μ M (Thompson and Lummis 2008). 5-HT₃ receptors are a ligand-gated Na/K channel and blockade would tend to have an inhibitory effect on neuronal excitability.

The lack of specificity of pharmacological gap junction blockers has made it very difficult to interpret studies utilising these agents. Not only do they have non-gap-junctional off-target effects, they also affect multiple gap junction subtypes. Perhaps the exception to this is mefloquin (Cruikshank et al. 2004), which is reasonably specific for connexin36 gap junctions when delivered at an appropriately low dose (this is particularly so for cell cultures, where low doses that are more specific for connexin36 can be utilised (Cruikshank et al. 2004)). To fully understand the role of gap junctions in seizure processes, it is imperative to differentiate between gap junction subtypes. That is, it would be naïve to assume that blocking connexin43 gap junctions linking astrocytes would have the same functional effect as blocking connexin36 gap junctions linking inhibitory interneurons.

Genetic approaches to controlling connexin subtype expression and function (reviewed recently by Giaume and Theis (2010)) have potential to greatly increase our understanding of gap junction function. By targeting the subtype-specific connexin sequences at either the DNA, RNA or protein level, it may be possible to regulate gap junction function with unparalleled precision. In the section that follows we will outline the genetic techniques that

offer the greatest promise as experimental tools in this regard, including mimetic peptides, transgenic manipulation and DNA/RNA interference. In section four we will look at functional studies that have utilised some of these techniques for the purpose of understanding gap junction regulation of seizure activity.

3.1 Mimetic peptides

Connexin mimetic peptides are short amino acid polymers corresponding to extracellular regions of the connexin protein. These molecules have shown promise as specific blockers of gap junction activity (Evans and Boitano 2001; Herve and Dhein 2010). It is thought that by binding to the connexin protein, connexin mimetics prevent connexon docking and block formation of the fully functional gap junction (Evans and Boitano 2001). In theory it should be possible to effect blockade of a specific gap junction subtype by designing mimetic peptide sequences specific to the connexin subtype in question. While promising in principle, there are some obstacles to the use of mimetics to effectively block gap junctions.

Firstly, there is considerable overlap in the amino acid sequences in the extracellular domains of connexin proteins. For example, GAP27 is a mimetic peptide that was developed to target the second extracellular loop of the connexin43 protein. The amino acid sequence SRPTEK within GAP27 is also present in the extracellular domains of most other connexin subtypes; thus, GAP27 may block gap junctions universally (Evans and Boitano 2001). Specificity to connexin43 can be enhanced by restricting the age range of animals used. Cortical grey matter expression of connexin30 does not develop until three weeks after birth, peaks at four weeks and remains high into adulthood (Kunzelmann et al. 1999). In contrast, connexin43 is already expressed in neonatal tissue and also remains high into adulthood (Kunzelmann et al. 1999). Thus, in one to three week old rodents, connexin43 is the predominant astrocytic gap junction. Expression of the other main cortical connexin subtype, interneuronal connexin36, develops between that of connexin43 and connexin30, peaking at two weeks after birth and declining during the third week (Belluardo et al. 2000). Expression of connexin43 and connexin30 in astrocytic cultures show similar developmental time-courses, with connexin43 present in the first days of culture and connexin30 expression not detected until the cultures are five to six weeks old (Kunzelmann et al. 1999).

Secondly, connexin mimetic peptides also block connexin hemichannels, albeit at different concentrations and over a different time course compared to their effect on gap junctions. Hemichannel effects occur at lower concentrations (5 μ M compared to 500-1000 μ M) (O'Carroll et al. 2008; Samoilova et al. 2008) and more rapidly (<30mins compared to >10 hours, cultures and hippocampal slices, respectively) (Leybaert et al. 2003; Samoilova et al. 2008). Although under normal physiological conditions the open probability of connexon hemichannels is low due to the blocking effect of divalent extracellular ions (magnesium and calcium) (Ebihara 2003; Ebihara et al. 2003), the open probability can be enhanced when extracellular magnesium and/or calcium levels are reduced (Ebihara et al. 2003). Thus, a possible effect of mimetic peptides on hemichannels must be considered likely when utilising the low-magnesium seizure model and at the higher mimetic concentrations required for gap junction blockade.

Thirdly, the brain also expresses channels formed from pannexins, a group of proteins that share basic structural similarities to the connexin family at the level of the mature protein but are otherwise unrelated. Pannexins are expressed by inhibitory neurons in the hippocampus (Bruzzone et al. 2003); however, the specific cellular expression in the cerebral cortex is not known. Pannexins form hemichannels *in vivo*, not complete cell-cell

gap junctions (MacVicar and Thompson 2009). Because pannexins do not share sequence homology with connexins (Baranova et al. 2004; Locovei et al. 2006), in theory they should not be blocked by connexin mimetic peptides. However, there is evidence showing that pannexin hemichannels are blocked by connexin mimetic peptides via a physical steric hindrance mechanism (Dahl 2007; Wang et al. 2007). Also, unlike connexon hemichannels, pannexin channels can be activated under normal physiological conditions (Bao et al. 2004). Thus, a possible effect of mimetic peptides on pannexin channels cannot be overlooked. Furthermore, pannexins themselves may be involved in seizurogenic processes, as shown by mimetic peptide blockade of Pannexin-1 in hippocampal slices (Thompson et al. 2008).

3.2 Connexin transgenic knockout animals

Gene knockout is a widely used technique for studying the function of a gene by removing that gene in an otherwise normal animal. This prevents the expression of the gene and its protein product. With conventional knockout techniques, embryonic stem cells are genetically manipulated by recombining a DNA cassette in place of the gene of interest to disrupt that locus. The manipulated stem cells are transferred into a developing embryo at the blastocyst stage and offspring homozygous for the altered loci are produced through a series of breeding crosses. Thus, the knockout animals develop in the absence of the gene under investigation. While this can be a powerful tool for investigating the function of the absent gene, it suffers from the limitation that developmental compensation can confound functional studies. Furthermore, because the gene is rendered non-functional in all tissues from which it would normally be expressed, it can be difficult to isolate any functional effects observed to a specific tissue or organ.

To circumvent these problems, these techniques have been refined to allow for conditional knockout of a gene of interest in a site- and time-specific manner. The most common of these techniques utilises the viral-derived Cre/loxP recombinase system (see Kuhn and Torres (2002) for review). Cre is a recombinase enzyme that catalyses the removal of DNA segments flanked by loxP; which are specific 34 base-pair DNA sequences. The technique involves crossing two transgenic strains of animals, one expressing Cre and the other engineered with LoxP sites flanking a gene of interest (i.e. a *floxed* allele) to create a new strain in which Cre-mediated recombination removes the floxed gene. Tissue-specific gene knockout is achieved by restricting Cre expression to a particular tissue, for example by placing the enzyme under the control of a tissue-specific promoter (Kuhn et al. 1995). Time-specific gene knockout can be achieved by using an inducible Cre promoter to control the expression of the Cre recombinase transgene (Kuhn et al. 1995). This conditional knockout approach thereby theoretically allows any gene of interest to be deleted in a tissue- and time-specific manner during development. An example of an application of this approach is the conditional removal of connexin36 in neurons by crossing NESTIN-Cre animals with connexin36 floxed animals (Wellershaus et al. 2008).

Deletion of various members of the connexin family has revealed some detail of the role of these proteins in regulating normal brain behaviour and thereby points to the contribution that these proteins may make to various normal and pathological neurological states. Examples include observations that Connexin36 knockout mice have reduced frequency hippocampal gamma oscillations (Buhl et al. 2003) and display an increased sensitivity to the seizures induced by the proconvulsant drug pentylenetetrazol (Jacobson et al. 2010).

3.3 Antisense oligodeoxynucleotide (AsODN)

Antisense oligonucleotide (AsODN) directed destruction of messenger RNA (mRNA) molecules is a method by which to reduce gene expression at the level of the expressed transcript. AsODNs are short DNA sequences that are complementary to a targeted single stranded mRNA. For association of an AsODN with its target sequence, mRNA binding must occur. This involves binding of matched nucleotides along the length of both the AsODN and targeted mRNA sequence - with purine bases (guanine or adenine) associating with pyrimidine bases (cytosine or thymine or uracil); accordingly, very specific binding to a matched target sequence can be achieved. The AsODN-mRNA hybrid molecule formed by this association is digested by the endogenous enzyme RNase H1, effectively eliminating the targeted AsODN-transcript hybrid and reducing levels of the associated mature protein due to lower levels of translation. There are a number of reports of AsODNs being used to experimentally reduce levels of connexin protein (e.g. Moore and Burt (1994); Law et al. (2006)) although none of these studies have addressed the effects of connexin depletion on seizure phenotype.

3.4 Small interfering RNA (siRNA) targeting of connexin gene expression

Another approach to studying the function of a protein is to reduce levels (knockdown) of its messenger RNA (mRNA) transcripts by the use of small interfering RNA. The effect on phenotype (the physical manifestation) of such a knockdown provides clues as to the role of the targeted transcript, and thereby its protein product, in normal physiology.

Small interfering RNA are short RNA duplexes (i.e. double stranded) molecules 21-28 nucleotides long, which can recruit cellular protein machinery - i.e. the RNAi silencing complex or RISC - to detect a specific transcript and to degrade it in the cytoplasm. As a result, the mRNA transcript is no longer available to the translation protein machinery, thereby reducing levels of the mature protein (reviewed in (Karkare et al. 2004)). They can be introduced *in vitro* to cells in culture, *in vivo* into various target tissues by injection (e.g. intrathecally) and by viral transfection.

There are a number of reports of successful application of siRNA to knockdown connexin levels (e.g. (Nakano et al. 2008; Schock et al. 2008)) although, similarly to the AsODN approach, to date this methodology has not been used *in vivo* to study the effect of depletion of any of the connexin protein family on seizures.

3.5 Other methods for reduction of connexin based cell-to-cell connectivity

Other methods for selective reduction or elimination of cell-to-cell connectivity via gap junctions is via the use of antibodies directed to regions of the connexon extracellular loop. This approach was used by Lin and colleagues (2002) to study the effect of connexin43 blockade on glioma cells. In that study the authors raised an antibody (namely EL1-46) to an epitope (peptide) corresponding to amino acid positions 46-76 in the first external loop (EL1) of the mature protein. The resultant antibodies were shown to produce up to 70% blockade of gap junctions when used at 60mg/mL against cultured astrocytes. In another study, antibodies (EL2-186) raised to an epitope corresponding to amino acids 186-206 in the second extracellular loop of connexin43 were shown to give up to 50% reduction in cell-to-cell coupling in cultured astrocytes (Hofer and Dermietzel 1998).

While antibodies have not been widely used to study seizure by *in vivo* application, the rapid turnover of the connexin family of proteins would likely make them good targets for such an approach.

4. Do gap junctions promote or hinder seizure activity?

Interest in the subject of gap junction involvement in the generation of seizure activity has been driven largely from two complementary ideas: that seizures result from hypersynchronous activation of neuronal populations within the central nervous system; and that direct electrical communication between neurons (via open gap junctions) ought to promote hypersynchronous activity because of the near instantaneous propagation of electrical activity between gap junction-linked cells. The idea that open gap junctions promote seizure activity has been fuelled by a large number of studies showing that gap junction blockade with pharmacological agents is almost universally anticonvulsant (Xiong et al. 2000; Kohling et al. 2001; Bostanci and Bagirici 2007a; Bostanci and Bagirici 2007b; Medina-Ceja et al. 2008; Nassiri-Asl et al. 2008; Nassiri-Asl et al. 2009). However, taking these studies at face value belies the complexity of the nervous system and underestimates the bluntness of many of the tools with which it has been examined.

As we have already proposed, we do not believe it is possible to approach the question of gap junction involvement in seizure mechanisms without considering the distribution of gap junction subtypes within specific cell populations. In the following section we will review the theoretical and experimental basis for pro- and/or anti-convulsant effects of gap junction subtypes on seizures. Because connexin36 and connexin43 subtypes have been most extensively studied, the discussion which follows will focus separately on the role of connexin36-linked neuronal and connexin43-linked astrocytic populations. This does not preclude the possibility that other less well studied gap junction subtypes may also be important.

4.1 Gap junction-linked interneuronal networks

In the mature brain, neuronal gap junction expression is restricted largely to inhibitory interneurons and are almost exclusively of the connexin36 subtype. Connexin36 gap junctions are not expressed uniformly across all interneuron classes. Rather, two main expression patterns predominate: that between same-class parvalbumin-containing multipolar-bursting (MB) cells (Deans et al. 2001; Liu and Jones 2003; Markram et al. 2004; Baude et al. 2007), which synapse onto pyramidal cells in the region of the soma or proximal dendrites (Deans et al. 2001; Liu and Jones 2003; Markram et al. 2004); and that between multipolar calretinin-positive (MCR) and MB interneurons (see Fig 2).

The effect on global brain dynamics of a disruption to gap junction connectivity within these inhibitory networks is not intuitively obvious. Figure 2 is a simplified wiring diagram showing the main synaptic and gap junction connections within the cerebral cortex. According to this schema, blockade of direct electrical communication between inhibitory cells could have *excitatory* effects at the level of pyramidal cell activity via two mechanisms. Firstly, blocking gap junctions between same-class MB interneurons (I_2 in figure 2) will result in a reduction in synchronous firing within this population (Deans et al. 2001) and cause a disruption to inhibitory timing at the pyramidal cell soma. Inhibitory timing is a critical element in maintaining stability in pyramidal cell networks and small inhibitory delays provide a powerful seizurogenic stimulus (Steyn-Ross et al. 2004). Secondly, open gap junctions between MCR and MB cells (I_1 and I_2 , respectively in figure 2) provide an “excitation” path between MCR and MB cells, effectively enhancing the inhibitory effect of MB cells at the pyramidal cell soma. Thus, closing these gaps will effectively reduce MB activity and release MB inhibition of the pyramidal cell population.

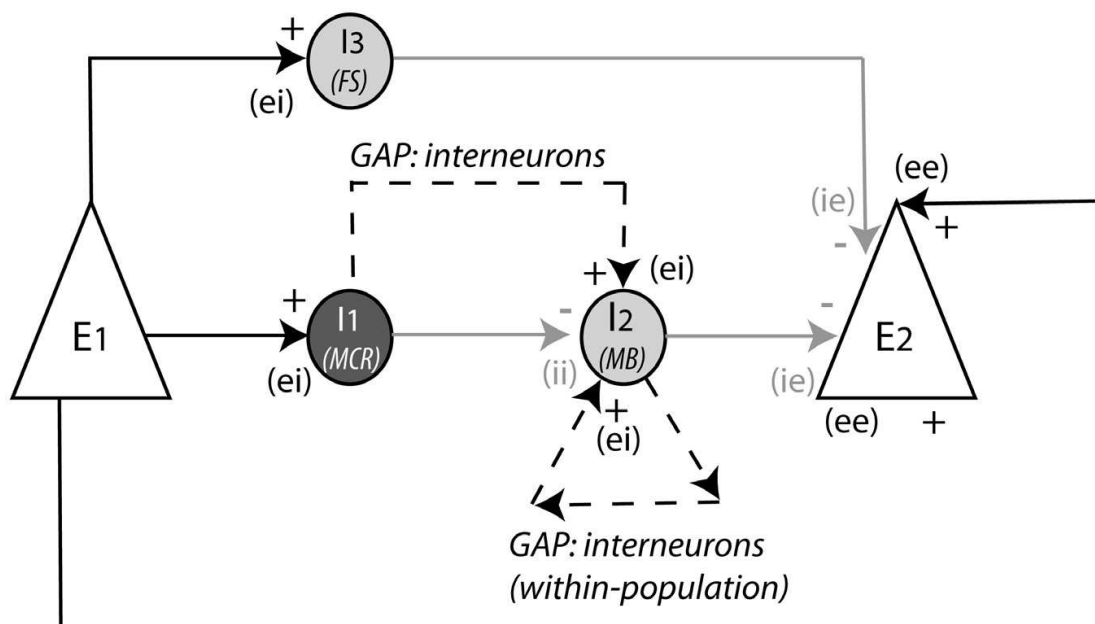


Fig. 2. Schematic showing possible connections involved in seizure spread. Two excitatory (triangles, E₁ and E₂) and two inhibitory (circles, I₁ and I₂) neurons are shown. Chemical synaptic pathways are shown in solid lines and gap-junction mediated pathways are shown in dashed lines. Excitatory pathways are indicated by a "+" and inhibitory pathways by a "-".

The prediction is that connexin36 gap junction blockade will tend to have a pro-seizure effect and there is accumulating experimental evidence to support this hypothesis. Yang and Ling (2007) have shown an increase in excitatory post synaptic potential amplitude following uncoupling of (GABAergic) inhibitory interneurons with carbenoxolone. Carbenoxolone is a broad spectrum gap junction blocker (Gajda et al. 2005; Nilsen et al. 2006) and would have likely blocked all gap junctions in this study. Enhancement of seizure-like event (SLE) frequency has been shown in hippocampal slices following application of carbenoxolone and quinine (Kraglund et al. 2010). The seizure models used in this study (Cs⁺-induced SLE and low-Ca SLE activity) are non-synaptic in origin, confirming that the excitation effect is not via a synaptic mechanism. Similar excitatory effects have been observed in neocortical slices with mefloquin, which blocks connexin36 gap junctions with greater specificity than carbenoxolone (Voss et al. 2009). This effect is eliminated in connexin36 knockout animals (Voss et al. 2009). Furthermore, connexin36 knockout mice have a greatly enhanced propensity for pentylenetetrazol seizures (Jacobson et al. 2010) and increased hippocampal inter-ictal discharges (Pais et al. 2003) compared to wild-type animals.

Interestingly, connexin32 knockout mice also exhibit neocortical neuronal excitability (Sutor et al. 2000). One of the explanations for this given by the authors is a desynchronisation of inhibitory interneuronal networks; although this is based on the speculation that connexin32 gaps are expressed by interneurons in the cerebral cortex. Currently, there is no evidence that cortical neurons express connexin32 gaps (Dermietzel et al. 1989), although neurons from subcortical nuclei such as the thalamus and basal ganglia show a low level of neuronal expression (Dermietzel et al. 1989).

Contrary to the above findings, hippocampal slices from connexin36 knockout mice show a reduction in ongoing seizure-like activity in response to the convulsant 4-aminopyridine (Maier et al. 2002) and a reduction in fast “ripple” (100-200Hz) oscillations (Maier et al. 2002). Ripples are partly of inhibitory origin (Grenier et al. 2001) and have been implicated in the initiation of seizures (Grenier et al. 2003). The implication is that connexin36 blockade inhibits seizure initiation by disrupting ripple formation.

The discrepancies in experimental findings clearly illustrates that the role of connexin36 gap junctions in seizureogenesis remains to be unequivocally resolved. Many of the studies mentioned above suffer from the limitations already discussed, particularly in terms of non-specificity of pharmacological drug action. In those studies where connexin36 knockout animals have been studied, compensatory effects may also confound the interpretation of results (Voss et al. 2010). Furthermore, the utilisation of different seizure models, analysis methods and choice of tissue between research groups adds further complexity that may account for the apparent contradictions in some results.

4.2 Mathematical models of gap junction effects

A further avenue of investigation that may help to untangle some of this complexity is computer-aided mathematical modelling. Modelling studies have the enviable advantage that selected parameters can be manipulated with absolute specificity. As mentioned in the introduction, no computer model developed to date comes close to representing the brain in all its complexity or functionality; but when aligned with (and refined by) experimental data, computer models can provide an informative adjunct to experimental biology.

Clearly a generalised seizure is the manifestation of a dramatic change in the mode of activity of neuronal populations. It can be most accurately described as a change in the dynamics of a neural mass. The most important conclusion from modelling studies is that the seizure state is principally a transition from a stable steady mode of operation to an oscillatory mode. We would emphasize that the dynamic signature of a seizure is *oscillation*, rather than simple *hyperexcitation* – although often hyperexcitation (manifest clinically as the tonic phase of a generalised seizure) will precipitate a secondary oscillation (manifest clinically as the clonic phase of a generalised seizure). The tendency for this transition to occur depends on both the intrinsic properties of each of the neurons, and also on how they are connected together into networks. The strength and time-course of the interneuronal connections are critical in whether the behaviour of the system will be stable or unstable (oscillatory). The synapses may be chemical or electrical, and are modulated by a variety of glial activities (as mentioned previously). We emphasize that the electrical connections between neurons differ from the chemical synapses in three critical ways:

- i. a chemical synapse between inhibitory neurons is inhibitory – i.e. it effectively *reduces* the activity of the downstream neuron, thus allowing downstream excitatory neuronal activation. In contrast an electrical synapse between inhibitory neurons has similar dynamic effects as an *excitatory* glutamatergic synapse – i.e. *increasing* the activity of the second neuron, which in turn dampens the excitatory cells and the system as a whole (see fig 2). Dynamically this is equivalent to an increase in the strength of the basket cells, which tend to control seizure spread.
- ii. if the interneuronal gap junctions are open, the inhibitory neurons become a form of *syncitium*, which supports spatial demarcation of areas of high-firing in the neocortex and reduces the tendency of the cortex to become oscillatory.

- iii. open gap junctions will reduce input resistance of the neuron, and hence act to *shunt* both excitatory and inhibitory synaptic input, which effectively results in a weakening of chemical synaptic connectivity.

Quantitative modelling of the influences of electrotonic synapses is still at an early stage. There are a number of papers which model the effects of gap junctions on other oscillatory behavior in the brain (gamma rhythms); and quite a few papers that model seizures of various types – but relatively few papers that look at both seizures and gap junctions. Broadly, there are two approaches to quantitative modelling of gap junctions and their modulation of neural dynamics. One is to model, in detail, modest (typically $\sim 4\,000$) numbers of inhibitory and excitatory neurons. We refer to these as “neuron-by-neuron” models. These models try to include the various multicompartmental ion channel conductances within neurons, and chemical and electrotonic synaptic connections between different neuronal subtypes. Whilst these approaches present a seductive verisimilitude with real brain connections, they have significant, and under-appreciated, disadvantages. They are computationally very expensive, and – whilst it is easy to replicate experimentally-derived EEG or ECoG signals in the model output – it is difficult to generalise the results beyond the immediate outputs; and thus achieve some sort of broader analytic understanding of the dynamics of the neuronal populations. The other approach is to use some form of “mean-field” or “neural mass” model. These models quantify the behavior of a ‘typical’ neuron, and are unable to distinguish individual neurons. Therefore the accurate correspondence of the parameters in the model with neurobiological measurements is more difficult – but the mean-field models are computationally very tractable, and allow a more general understanding of processes that influence the dynamics of the brain. They also allow the full mathematical arsenal of statistical physics to be applied to neurobiology. The seizure state is usually identified as an extreme oscillation in neuronal dynamics. This being the case, the problem of whether the brain will enter a seizure can be rephrased in the language of dynamical systems theory as: “whether the state of the various parameters that control the brain dynamics is such that the brain can enter a region of instability?” If the problem is linearised it simplifies to the question of: “whether the largest real eigenvalue in the system is greater than zero?” We can look at ranges of values for various parameters, that we have deemed to be important in controlling the behaviour of the model system. Certain combinations of parameter ranges will result in a stable neuronal activity (the system will evolve towards a fixed point); and at other ranges the system will be unstable (it will oscillate or undergo irregular chaotic behaviour). The boundary between these two modes of behavior is called a “basin boundary”. If the system is close to a basin boundary that encloses an unstable state, minor noise-induced changes in parameter values are more likely to precipitate the neuronal population into a seizure. Typically the choice of parameter values are constrained by experimental estimates from different neuronal populations of such factors as the number of neuronal connections, neuronal membrane potential, and synaptic gain.

4.2.1 Neuron-by-neuron models

Using very detailed neuron-by-neuron models, Traub and co-workers (Traub 2003) have published a number of papers in which they investigate various aspects of the effects of both inhibitory-inhibitory gap junctions and excitatory-excitatory axonal gap junctions. They found that inhibitory-inhibitory gap junctions were not necessary for the presence of gamma oscillations, but that the presence of open gap junctions increased the strength and precision of

these oscillations. This has been a recurring theme in almost all experimental and modelling studies of the contribution of gap junctions to neural activity. Namely, that the role of the gap junctions is a secondary one; in which they interact with chemical synaptic function to sculpture and augment existing neuronal rhythms. In these papers this group were primarily interested in testing whether pyramidal cell axo-axonal gap junctions were necessary for the expression of very fast oscillations – which are believed to be important in the process of seizure development (see section 4.3 below). The role of the much more common inhibitory-inhibitory gap junctions was relegated to a few sentences in which they stated “in addition to gamma oscillations, synchronized epileptiform bursts also occur in the connexin36 knockout (but not wild-type) in the presence of kainate (Pais et al. 2003).” (Traub 2005).

The other important attempts at neuron-by-neuron modelling of interneuronal gap junction effects were by van Drongelen (2004) and Di Garbo (2004). Both papers had similar results. They found that inhibitory-inhibitory gap junctions acted to synchronize the inhibitory cell populations, but the actual effects depended strongly on pre-existing activity. If this activity was already strongly synchronous, then whether gaps junctions were open or closed had little influence.

4.2.2 Mean-field models

In mean-field models seizures are usually conceived of as the result of a so-called “Hopf bifurcation” in the dynamics of the brain (Breakspear et al. 2006). There is mathematical precision and complexity behind this statement, but in simple descriptive terms, the dynamics of the brain change from a fixed point to a widespread oscillation between zero firing and high firing states. To date there are no publications of mean field models of the effects of gap junctions in seizure generation, but preliminary communication by Steyn-Ross et al. in general agree with experimental results and the neuron-by-neuron models – i.e. that open inhibitory-inhibitory gap junctions tend to stabilise the brain, whereas excitatory-excitatory gap junctions have the opposite effect. The effects are not very large but may be clinically important. As an example of the sort of output from the mean-field approach, figure 3 shows the regions that are associated with oscillatory behaviour in the presence of open or closed inhibitory-inhibitory gap junctions. The white area is the region of seizure behavior if the gap junctions are open, and the grey area is the *increased area* of oscillation if the gap junctions are closed. It can be seen that closure of these gap junctions increases the size of the basin boundary by about 20%. This means that the range over which combinations of the magnitude of the inhibitory post-synaptic potential and the degree of resting membrane depolarisation result in seizures is modestly greater if the inhibitory-inhibitory gap junctions are closed.

4.3 Gap junction-linked pyramidal axo-axonal networks

A discussion on neuronal gap junctions and seizures would not be complete without considering pyramidal cell axoaxonal junctions. Axoaxonal gap junctions have been demonstrated between pyramidal cells in the hippocampus (Schmitz et al. 2001; Hamzei-Sichani et al. 2007) and are probably of the connexin36 subtype (Hamzei-Sichani et al. 2007). Modeling studies have implicated these junctions in the generation of fast ripple oscillations (Traub et al. 2001) and epileptogenesis (Traub et al. 2002); and blockade is theorised to have an anticonvulsant effect. The finding that neither pharmacological nor genetic connexin36 blockade has an anticonvulsant effect in neocortical slices (Voss et al. 2010) suggest that

either these gap junctions are not present in the cortex or are so few as to have minimal impact on seizure processes. If initial findings that axoaxonal gap junctions are composed of connexin36 subunits (Hamzei-Sichani et al. 2007) prove to be correct, this may explain some of the discrepancy in connexin36 gap junction blocking studies. That is, it would provide a rational basis for connexin36 blockade potentially having both pro- or anti-convulsant effects, depending on whether effects on interneuronal or pyramidal cell populations predominated.

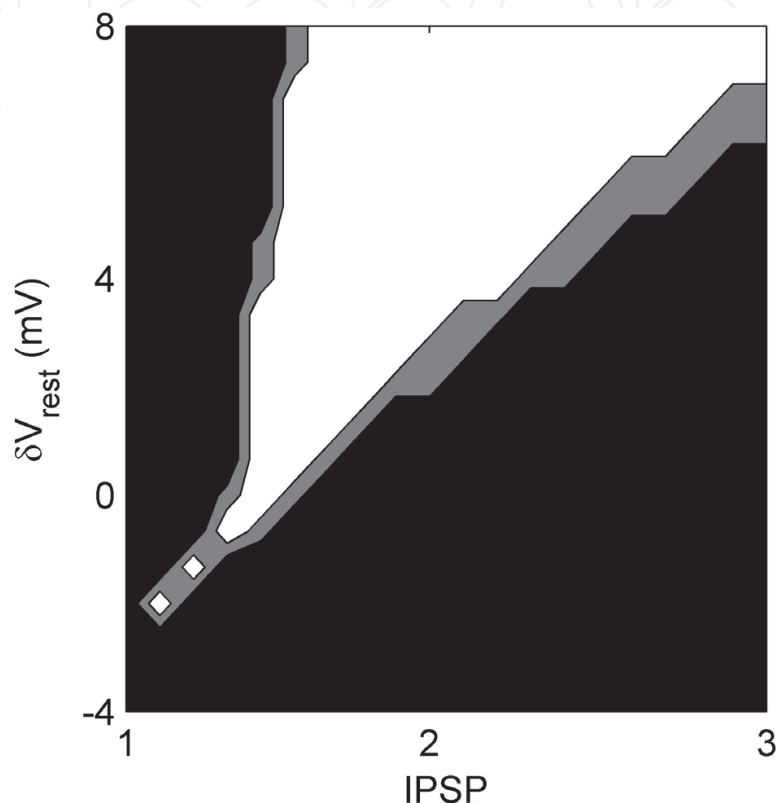


Fig. 3. Size of areas where the model shows seizure-like oscillatory behavior. We have chosen a parameter space with axes of synaptic gain (Inhibitory Postsynaptic Potential (IPSP)) and intrinsic neuronal excitability (change in resting membrane potential (δV_{rest})). The white area is the region of parameter space in which the model cortex is unstable (positive real eigenvalues) when the inhibitory-inhibitory gap junctions are open. The grey area is the region of instability with closed inhibitory-inhibitory gap junctions. The black area is the region of stable cortical dynamics.

4.4 Gap junction-linked astrocytic networks

Increasingly important and diverse roles for glial cells in cortical neurophysiological function and dysfunction are being reported. In particular, the role of astrocytes in epilepsy has been (Penfield 1929) and remains a subject of considerable interest (see Steinhäuser and Seifert (2002) for review). Astrocytes are extensively linked by gap junctions (primarily connexin43 and connexin30) and connexin43 is upregulated at epileptic foci in vivo (Fonseca et al. 2002), implicating a role for this gap junction in the seizure process. The nature of this role however is not clearly understood. Two divergent possibilities present themselves from the literature.

Firstly, gap junction-linked astrocytic networks could *contribute* to seizure genesis by facilitating the spread of neuronal activity via propagating calcium waves (Nedergaard 1994) and glutamate release. Astrocytes produce spontaneous slow calcium transients during seizure-like activity in many in vitro models of epilepsy (Tashiro et al. 2002; Stout and Charles 2003; Tian et al. 2005); these events occur independent of neuronal activity (Parri et al. 2001; Wang et al. 2006) and are causally linked to astrocytic release of glutamate (Parpura et al. 1994; Tian et al. 2005) and an increase in neuronal excitability (Fellin et al. 2006) and synchronicity (Fellin et al. 2004). Furthermore, astrocytic release of glutamate can induce epileptiform activity in pyramidal cells independent of synaptic activity (Kang et al. 2005; Tian et al. 2005) and can enhance synaptically driven seizure-like events (Fellin et al. 2006). There is also evidence that gap junction-coupled astrocytes may support epileptiform activity by supplying glucose to neuronal networks (Rouach et al. 2008). Together, these data build a strong case for an important function for gap-junction-linked astrocytes in promoting seizure activity. There is experimental support for this from hippocampal slice studies, where connexin43 gap junction blockade with GAP27 has been shown to attenuate seizure-like activity (Samoilova et al. 2008). An important caveat is that this study may be confounded by effects of the mimetic peptide on pannexin and hemichannels. Indeed, pannexin1 hemichannel blockade with “panx” (100 μ M, sequence WRQAAFVDSY) has antiepileptic effects in hippocampal slices (Thompson et al. 2008). Pannexin1 hemichannels augment synaptic function by providing an NMDA-linked depolarizing current during intense synaptic activity (Thompson et al. 2008).

Alternatively, gap junction-linked astrocytic networks could *limit* seizure activity by acting as a sink for extracellular potassium ions (Orkand 1986) and/or excitatory neurotransmitters such as glutamate. The effect of an elevation in extracellular potassium is to shift the equilibrium potential for potassium to a more depolarised level; the flow-on effect of which is resting membrane depolarisation and enhanced cell excitability. A similar sequestering role for gap-junction-linked astrocytic networks has been proposed for the excitatory neurotransmitter glutamate (Tanaka et al. 2008). Uncoupling astrocytic connexin43 gap junctions has also been shown to directly reduce the expression of the glutamate transporter GLT-1, resulting in reduced glutamate uptake by astrocytes (Figiel et al. 2007). Astrocytic networks could also limit seizure activity through the coordinated release of ATP (see Halassa and Haydon (2010) for review), the conversion of which to adenosine has an inhibitory effect on neuronal activity. Experimental support for seizure-limiting effects of astrocytic coupling comes from hippocampal slice studies showing that conditional deletion of astrocytic connexin43 and unrestricted deletion of connexin30 results in impaired potassium clearance and reduced seizure threshold (Wallraff et al. 2006). While this study has the advantage of targeted genetic manipulation, one cannot rule out the possibility of confounding compensatory developmental effects in the transgenic animals.

Connexin30 is the other main connexin subtype expressed by astrocytes in the mature CNS. Functional effects of targeted manipulation of connexin30 gap junctions have not been investigated. However, connexin30 has been shown to be up-regulated following kainate-induced seizures in rats (Condorelli et al. 2002). While this implicates connexin30 in the seizure process, an inherent problem with this and similar studies is that changes in connexin or gap junction expression do not necessarily indicate whether these modifications are a cause *or* a consequence of the seizure process. Thus, while there are a growing number

of papers documenting alterations in connexin subtype expression (including connexin43 (Fonseca et al. 2002)) during or after seizures (see (Rouach et al. 2002) and tables 2 and 3), studies such as these are generally not helpful in determining the functional role of the subtype in question.

In summary, the role of astrocytic gap junctions in seizureogenesis has not been unequivocally resolved; with theoretical and experimental grounds for both pro- and anticonvulsant effects. It may be that both hypotheses will hold true and that the functional expression of astrocytic gap junction manipulation will be shown to depend upon secondary factors such as the genetic background of the animals (Wiencken-Barger et al. 2007) and/or physiological factors underlying the regulation of astrocytic function.

<i>Gene</i>	<i>Seizure model</i>	<i>Expression change</i>	<i>Reference</i>
Connexin30	Kainate or kindling	No significant changes found	(Sohl et al. 2000)
	Kindling	Upregulated with increased apoptosis	(Condorelli et al. 2002)
Connexin32	In vitro bicuculline (hippocampal slices)	mRNA increased 2-3 fold within 6h and protein increased after 6h	(Li et al. 2001)
	4-aminopyridine	Significantly increased	(Samoilova et al. 2003)
Connexin36	Kainate or kindling	mRNA up 44% reduced by wk4 post application, protein level only slightly reduced (apoptosis linked?)	(Sohl et al. 2000)
	4-aminopyridine Kindling	Significantly increased Upregulated during focal seizures then back to basal levels with onset of generalized seizures	(Samoilova et al. 2003) (Beheshiti et al. 2010)
	4-aminopyridine	Gradual decrease up to 8h post injection (I.P.)	(Zappala et al. 2006)
Connexin43	4-aminopyridine	Significantly increased	(Samoilova et al. 2003)

Table 1. Connexin expression changes associated with experimentally-induced epilepsy in rodents

<i>Gene</i>	<i>Epilepsy condition/type</i>	<i>Expression change</i>	<i>Reference</i>
Connexin32	Temporal lobe epilepsy	Decreased (hippocampus)	(Collignon et al. 2006)
Connexin36	Temporal lobe epilepsy	Unchanged (hippocampus)	(Collignon et al. 2006)
Connexin43	Intractable seizure disorder	Increased	(Naus et al. 1991)
	Complex partial seizure disorder	No significant change	(Elisevich et al. 1997)
	Temporal lobe epilepsy	Increased (hippocampus)	(Collignon et al. 2006)

Table 2. Connexin expression changes associated with clinical epilepsy

5. Conclusion

In this chapter we have sought to bring together research from a wide range of disciplines encompassing electrophysiology, molecular biology and mathematical modelling, with the aim of addressing the role of gap junctions in the mechanism of seizures. The prevailing notion that open gap junctions promote seizure activity is overly simplistic and does not do justice to a growing body of literature showing that the opposite may be true in certain situations. There is also no evidence to support the idea that gap junctions either cause or ablate seizures *per se*; rather, they perform a modulatory role that is dependent upon the prior activity of the system and upon gap junction subtype. In any discussion it is essential to be precise about the type of experimental manipulation used, and exactly which gap junction subtype is under consideration. Thus, excitatory effects may be expected when pyramidal cell axo-axonal gap junctions are opened, while the opposite is likely with opening of inhibitory interneuronal gap junctions. Electrophysiological and modelling studies support this delineation. However, there is still some way to go before we will fully understand this complex area of neurobiology. The role of astrocytic gap junctions in particular remains an open question. Astrocytes are increasingly being recognised for their complex neuroregulatory functions and gap junctions are well suited for this role. Whether astrocytic gap junctions promote or hinder seizure activity is likely to depend upon prevailing neurophysiological factors governing the state of ongoing neuronal activity. Furthermore, many gap junction subtypes have been poorly studied to date and their possible role in seizure processes is undetermined. Greater understanding of these matters rests upon development and application of experimental techniques and pharmacological tools for targeted modulation of specific gap junction subtypes.

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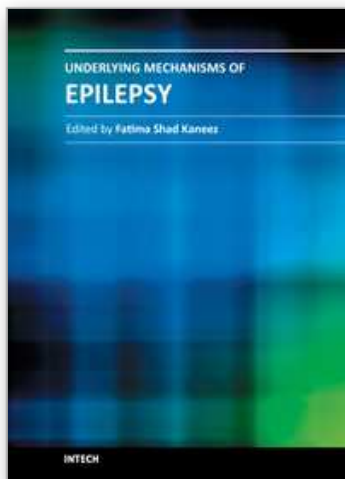
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Underlying Mechanisms of Epilepsy

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This book is a very provocative and interesting addition to the literature on Epilepsy. It offers a lot of appealing and stimulating work to offer food of thought to the readers from different disciplines. Around 5% of the total world population have seizures but only 0.9% is diagnosed with epilepsy, so it is very important to understand the differences between seizures and epilepsy, and also to identify the factors responsible for its etiology so as to have more effective therapeutic regime. In this book we have twenty chapters ranging from causes and underlying mechanisms to the treatment and side effects of epilepsy. This book contains a variety of chapters which will stimulate the readers to think about the complex interplay of epigenetics and epilepsy.

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InTech China

Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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