Dedicated to Prof. P.T. Frangopol on the occasion of his 75th anniversary

Gap Junctions Involvement in Brain Hyperexcitability Phenomena

DORU-GEORG MARGINEANU*
UCB Pharma SA, CNS Research, 1420 Braine-l'Alleud, Belgium

Hyperexcitability pathologies, epitomized by epilepsy, are a largely unmet medical need, asking for conceptual developments on the functioning of networks of inter-communicating neurons and glia. Intercellular communication via gap junction (GJ) channels is largely present in mammalian brain. The GJ channels are made of proteins, essentially the connexins (Cxs) widely expressed in brain and in peripheral organs, the most abundant being Cx43. Expression level of Cx43 appears elevated in epileptic brains. Many different compounds actually modify the strength of GJ intercellular communication, though none is specific for GJs. Reference GJ blockers have anticonvulsant effects in numerous experimental models of epilepsy and some data suggest that GJ blockade might specifically act on epileptic hyper-synchrony, a feature hardly targeted by current antiepileptic drugs. The involvement of GJs in migraine is also suggested by recent results.

Keywords: connexin, gap junction blockers, Cx36 channel blockers, epilepsy, cortical spreading depression

Human brain, the information processing organ distinctively human, is an intricate web of some 10^{11} neurons of at least a thousand different types, and of more than 10^{12} glial cells. The neurons are the main signaling units of the nervous system, each of them functioning as a full-fledged computer connected chemo-electrically with an average of some 10^4 other neurons and with surrounding glial cells (see, e.g. [1]). Glia, particularly the astrocytes (that make contact with the vasculature, having important roles in metabolic support and in conveying the level of neuronal activity to the vasculature to regulate local blood flow) and the oligodendrocytes (providing myelination to neurons) are active communication elements [2], deeply connected between them and with the neurones. Thus, human brain – that epitomizes the features of mammalian brains in general and crowns their evolution – is certainly a most complex system, arguably the most complex one in the Universe.

The metabolic processes in the brain are so intense that brain utilizes 20 % of the whole oxygen consumed by the body, though its mass does not exceed 2 % of body mass (e.g. [3]). Its bewildering complexity and metabolic intensity make possible that human brain supports the most elaborate biological function – human thinking. Conversely, it is also that very complexity that entails a substantial risk of "over-racing", as clearly expressed in the hyper-excitability phenomena characteristic for several brain pathologies, including the severe and frequent epilepsy and migraine [4, 5] but also pathological pain states, essential tremor and psychiatric disorders with neuronal hyperactivity. Epilepsy denotes a family of chronic functional disorders of the brain characterized by recurrent and unpredictable occurrence of symptoms due to abnormally excessive and synchronous neuronal activity in the brain – the epileptic seizures. Epilepsy is widely considered the prototypic hyperexcitability pathology since it exaggerates the basic features of brain functionality (see below). Hyperexcitability pathologies are a largely unmet medical need, asking for conceptual developments on brain operation, particularly in what concerns the functioning of networks of inter-communicating neurons and glia. This short review is devoted to such an example, namely the involvement of gap junctions (GJs) in brain hyperexcitability.

Gap junctional intercellular communication

Brain functionality as body's informational center relies on the integration of the intrinsic neuronal excitability, a cellular-level feature, into synchronized responses of multi-cellular ensembles. Neuronal excitability essentially derives from conductance transitions of the ionic channels, specialized membrane proteins operated by trans-membrane voltage changes [6]. The functioning of supra-cellular networks of neurons and glia relies on their intercellular communication that occurs via two types of specialized structures, the synapses and the GJs. More usual in mammalian brain is the chemical neuro-mediation occurring at synapses, where neurotransmitters and/or neuromodulators released from the pre-synaptic cell act as ligands on specialized receptors in the post-synaptic cell, inducing there a specific response. However, a direct electrical communication between some central neurons was also long demonstrated, it underlying a rapid synchronization of neuronal firing within brain nuclei, in either normal rhythmic activity or burst discharges during epileptic seizures [7]. Moreover, it was shown that some neocortical neurons that communicate by chemical synapses may also be electrically coupled.

The direct electrical inter-neuronal communication occurs via junctions termed "gap" since they do not seal together the membranes of the two adjacent cells, in contrast to the "tight" junctions. The GJs are clusters of packed inter-cellular GJ channels, formed by juxtaposition of hemi-channels (or "connexons") in the adjacent cells that dock to each other (fig. 1). GJs are present in nearly all mammalian tissues, except for mobile cells (e.g. sperm and erythrocytes) and adult skeletal muscle. Each hemi-channel is made of 6 monomers of a class of proteins termed connexins[1] and noted "Cx" followed by MW designation. The Cxs have low MW, usually 26 – 60 kDa, and a specific trans-membrane topology, with both C- and N- termini on the cytoplasmic side, four trans-membrane

* *email: Doru.Margineanu@skynet.be*

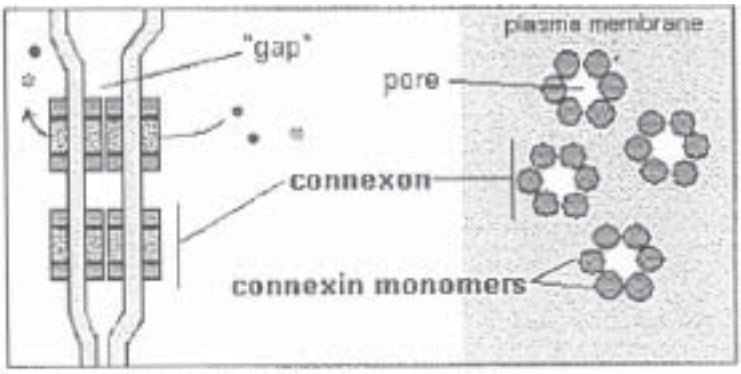

Fig. 1. Scheme of a gap junction, in transversal (at left) and top (at right) views. A more suggestive color image can be freely accessed on: http://en.wikipedia.org/wiki/Gap_junction

passes, two extra-cellular loops and a cytoplasmic loop. The Cxs undergo a rapid turnover, allowing a fine regulation of the physiological processes to which they participate [9].

Connexin expression in the brain and epilepsy-associated changes

In the human genome are identified 21 Cx genes, most ubiquitously expressed being Cx43. At least 11 of the 21 human Cx genes, namely: Cx26, Cx29, Cx30, Cx32, Cx36, Cx37, Cx40, Cx43, C45, Cx46 and Cx47 are expressed in the nervous system, with differing cell specificity [10], as illustrated in figure 2. One can notice that the most abundant in astrocytes is the ubiquitous Cx43 that is present in brain neurons too. Cx36 is considered neuronal "specific" since in the nervous system it is expressed only in neurons, but Cx36 is also present in insulin-secreting pancreas cells [11].

It has long been reported that the expression level of Cx43 (the one which is most prominent in astrocytes) is elevated in samples of human temporal lobe neocortex, resected for surgical treatment of drug-resistant epilepsy, while much lower expression levels were detectable in peri-tumoral temporal lobe tissue samples obtained during removal of cerebral tumours [12]. Remarkably, however, in cases where the tumours had induced acute seizures, Cx43 expression was higher than that detected in epileptic samples. Similar changes, but with less dramatic differences were observed for Cx32, suggesting that severe epilepsy is associated with an increase in the synthesis of GJ proteins in the temporal cortex of patients, which presumably leads to an increase in intercellular coupling. Confirming that early finding a significant increase in astrocytic Cx43 protein levels in human hippocampal tissue resected from patients with drug-resistant temporal lobe epilepsy, and duly hypothesised that the large up-regulation in Cx43 may exacerbate generalised seizures in the progression of epilepsy. was noticed [13]. On the other hand, conflicting data have also been reported [11], a situation fairly common for any evolving field! Nevertheless, the prevailing view is that increased GJ communication (particularly between glia, but not restricted to them) plays an intrinsic role in the epileptogenic process, conceivably promoting the hyper-synchrony.

Modulation of gap junction communication: relevance for brain hyperexcitability

The GJ channels can be closed or open, subject to voltage and/or chemical gating. When open, the GJ channels readily pass between neighboring cells all molecules with MW ≤ 1 kDa, i.e. they allow the direct intercellular exchange of all inorganic ions, cAMP, amino acids, sugars and nucleotides. The extent of GJ intercellular communication is expressed by the gap-junctional conductance $\mathbf{g_{GJ}}$. Obviously, $\mathbf{g_{GJ}}$ is the product of single-channel conductance (**j**) and the average number of open GJ channels, which in turn is the product of the existing number of GJ channels (**N**) and of the probability (**P**) of the channels to be in open configuration. Thus: $\mathbf{g_{GJ} = j\ x\ N\ x\ P}$. This simple relationship has the merit to underpin the two principle modalities of pharmacological modulation of GJs, either by drugs that modulate the synthesis, trafficking and degradation of connexins, i.e. **N**, or by drugs that acutely close or open the channels, i.e. decrease or increase **P** and **j**.

A wealth of different compounds actually modify the strength of GJ intercellular communication [14]. Thus:

- among the miscellaneous compounds that affect Cx synthesis, trafficking and degradation are cholesterol, ethanol, nicotine, some hormones (e.g. estradiol, FSH, T3), carotenoids (e.g. all-trans retinoic acid) etc;
- the inorganic cations H^+, Na^+, Ca^{2+}, Mg^{2+}, as well as various organic compounds: higher alcohols (heptanol, octanol), fatty acids (e.g. oleic acid), narcotics (e.g. halothane, isoflurane) cardiac glycosides (ouabain), glycyrrhizic acid metabolites (carbenoxolone), quinine derivatives, fenamates (meclofenamic acid) etc acutely block GJs, while
- cAMP-enhancing drugs (e.g. forskolin), several anti-arrhythmic peptides (e.g. AAP10), serotonin, histamine etc acutely enhance GJ communication.

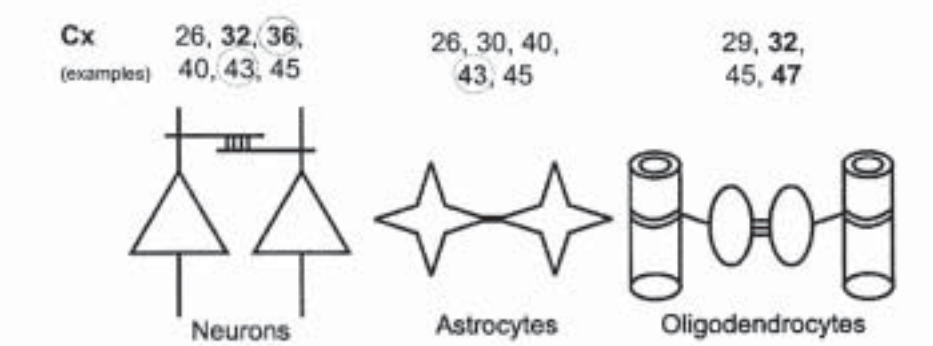

Fig. 2. Illustrative distribution of the different connexins in the major types of brain cells. The highest and typical expressions are highlighted

However, it is sobering to notice that among the numerous classes of compounds that alter GJ communication none is acting specifically on GJs. In particular, either traditional aliphatic alcohol GJ blockers heptanol and octanol, or the synthetic analog of 18a-glycyrrhetinic acid, carbenoxolone, which are frequently used as reference GJ blockers (fig. 3), do not act exclusively on GJs and have no clear preference among various GJ-forming Cxs [15]. On the other hand, it was shown that the antimalarial drugs quinine [16]S, its stereoisomer quinidine, and the derivative mefloquine [17] specifically block Cx36 and (with lesser potency) Cx50, while other Cxs were either not affected, or only affected at 10 – 100-fold higher concentrations. These Cx36-prefering quinones are thus remarkable since Cx36 is exclusively expressed in neurons, while Cx50 is not expressed in the mammalian brain [10], but they have non-GJ targets, too (e.g. [18]).

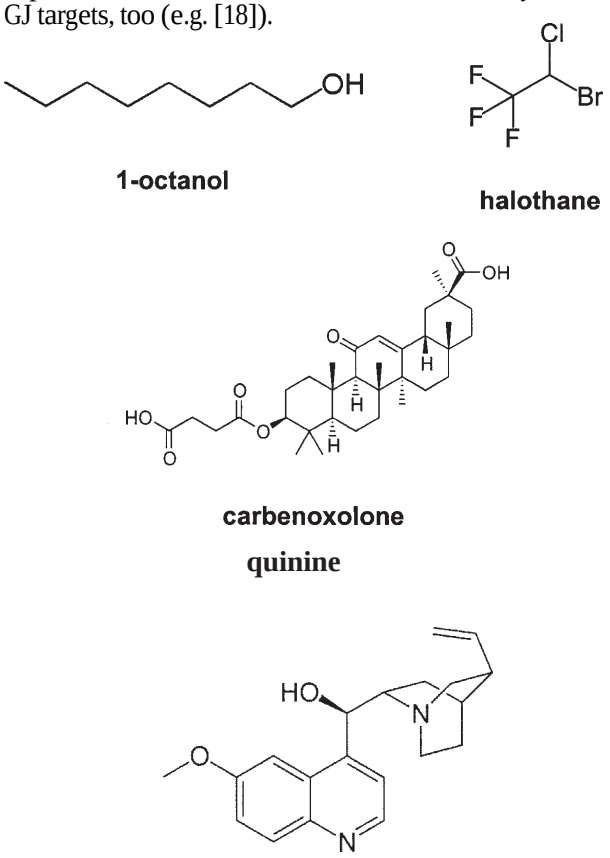

Fig. 3. Frequently used reference gap-junction blockers include compounds of very different classes; see text for details

A considerable number of publications report significant anticonvulsant effects of reference GJ blockers in various *in vivo* animal models of epilepsy, both induced (e.g. [19]) and genetic (e.g. [20]), as well as in several *in vitro* brain slice models of epileptiform discharges [20, 21], suggesting that GJ blockade might have a general effect of opposing epileptiform activities, irrespectively of the mechanism by which they are induced. Of some interest might be the possibility that GJ blockade could offer a specific handle on epileptic hyper-synchrony, as some data have suggested [22]. Thus, in a rat hippocampal slice model, in which perfusion with a high-K^+, low-Ca^{2+} fluid induces epileptiform activity *in vitro*, the reference GJ blocker carbenoxolone inhibited spontaneous bursting (fig.4A), a putative marker of excessive synchronization of spontaneous firing of a vast population of neurons, while not inhibiting the epileptiform repetitive responses evoked by single stimuli (fig. 4B), that likely express the excessive neuronal excitability. This specific effect of carbenoxolone was fairly similar to that exerted in the same

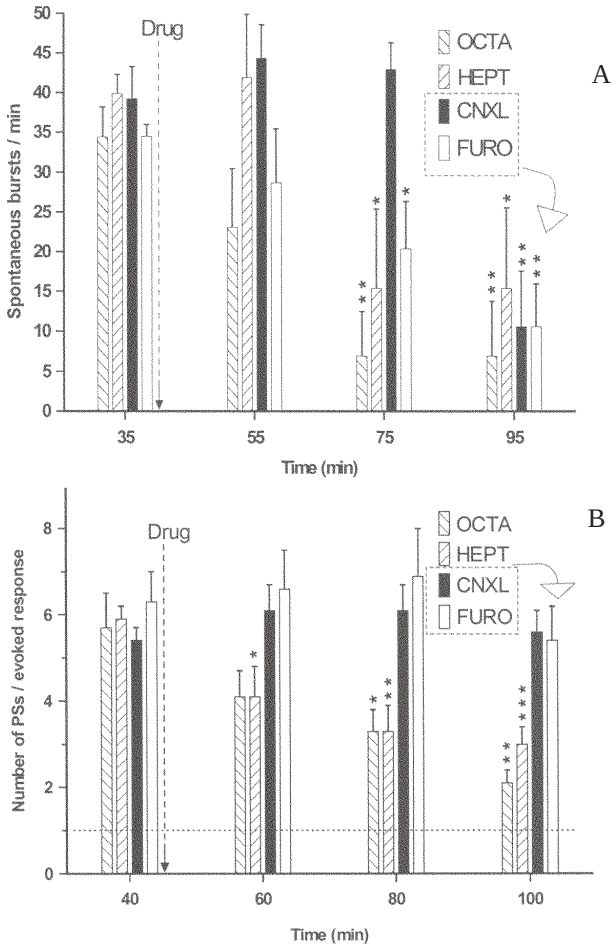

Fig. 4 (adapted from [22]). Carbenoxolone (CNXL), a more GJ-preferring blocker appears to distinguish between epileptiform hyper-synchrony and hyperexcitability. **A**: CNXL, along with the non-specific GJ blockers 1-octanol (OCTA) and 1-heptanol (HEPT), and the diuretic furosemide (FURO) inhibited spontaneous bursting induced upon perfusion with high-K^+, low-Ca^{2+} fluid (HKLCF) in rat hippocampal slices. **B**: CNXL did not inhibit HKLCF- induced epileptiform repetitive responses (PSs) in rat hippocampal slices, similarly to FURO, but in contrast to OCTA and HEPT

in vitro model by furosemide, acknowledged to differentiate synchronization from neuronal excitability in models of epilepsy *in vitro* [23]. As hyper-synchrony is an intrinsic feature of epileptic seizures, which is not, however, specifically targeted by any current antiepileptic drug [22, 24], the above mentioned possibility appears bearing a therapeutic promise.

Apart the epileptiform activity, another phenomenon typical for brain hyper-excitability is the cortical spreading depression (CSD), a wave of bulk neuronal and glial depolarization, associated with a deep but transient suppression of brain bioelectric activity, which propagates in the mammalian brain at ~mm/min velocity. This phenomenon is experimentally produced in the whole brain of anesthetized animals, as well as in rodent and human neocortical slices *in vitro*, most often by local application of depolarizing chemicals (usually, concentrated KCl), but also by various other noxious maneuvers (for review of CSD mechanisms, see [25]). Though it was described more than 60 years ago, CSD enjoys a maintained high interest, as it underlies the aura of migraine and is the most probable primary event in migraine without aura too [26], thus being a key event in a severe painful brain pathology that affects around 10% of human population [27].

We recently put forward an experimental model in which CSD can be regularly induced and quantified on rat

neocortical slices *in vitro*, and showed that the putatively specific Cx36 blockers quinine, its stereoisomer quinidine and the related quinone mefloquine, consistently inhibited the CSD episodes [28]. These results reflect an involvement of neuronal Cx36 channels in CSD generation and propagation and bear potential drug-discovery relevance for migraine therapy.

Conclusions

The extended occurrence of GJs in mammalian (particularly human) brain is definitely established and the distribution in the brain of the GJ-forming proteins (essentially Cxs) is largely described.

The involvement of GJs in the prototypic brain hyper-excitability pathology, the epilepsy is firmly attested by numerous studies reporting anticonvulsant effects of various GJ-blocking compounds in a wealth of models in vitro and in vivo, in which epileptiform phenomena derive from different mechanisms. The involvement of GJs in other brain hyper-excitability pathology, the highly prevalent migraine is suggested by some recent results.

Multiple pathways to pharmacologically modulate intercellular GJ communication, acting upon either turnover or conductance, are already identified, suggesting that GJs might provide future targets for the therapy of brain hyperexcitability disorders. That prospect requires, however, overcoming several major hindrances, chiefly relating to the spread distribution of GJ-forming Cxs both in the brain and in peripheral organs, and to the lack of specificity of the currently existing GJ-modulating compounds.

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