CLINICAL AND GENETIC ASPECTS OF EPILEPSY

Edited by **Zaid Afawi**

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Preface

This book on Epilepsy was conceived and produced as a source of information on wide range of issues in epilepsy. We hope that it will help health care providers in daily practices and increase their understanding on diagnosis and treatment of epilepsies.

The book was designed as an update for neuroscientists who are interested in epilepsy, primary care physicians and students in health care professions.

This epilepsy book is the result of a collaborative effort of investigators who have used a wide range of experimental preparations and recording techniques. Authors from a variety of backgrounds have contributed significantly with papers from their respective fields. I believe they have provided a comprehensive description of key issues and important developments within each filed of research.

The studies included in this book are drawn from several disciplines of modern neuroscience and include various Chapters distributed in sections on Mechanisms Underlying Epileptic Seizures, Molecular Genetics of Epilepsy, Animal Models, Ion and Channels and New Treatments

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Part 1

Mechanisms Underlying Epileptic Seizures

1

A Functional Role for Microglia in Epilepsy

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1. Introduction

Microglia are the immune competent cells of the CNS and comprise the major mechanism of self-defense against brain injury, infections and disease. Activation of microglia occur as a response to these insults, and both neurotoxic and neuroprotective factors can be released (Streit et al., 1999, Streit, 2002, Schwartz, 2003, Schwartz et al., 2006). There is a great deal of evidence suggesting that microglia have a role in neurodegenerative diseases, either to promote the pathology, or to counter it. However in the case of epilepsy, specific questions still remain including how, why, and when microglia are activated. In this first section, we will provide a general introduction to the role of activated microglia in the central nervous system (CNS). In subsequent sections, we will specifically discuss evidence of a functional role for microglia in epilepsy.

In the normal brain, the majority of microglia are in the resting, or quiescent ramified state. This shape with their long processes, allows them to quickly assess and respond to CNS injury or pathogens. During excitotoxic insult or inflammation, microglia become activated. This activation is manifested morphologically by retraction of their processes and changing to a rounded amoeboid morphology. Microglia also become proliferative and migratory. Their electrophysiological characteristics are altered and the expression of potassium, proton, sodium, calcium and chloride currents changes (Eder, 1998, Ducharme et al., 2007, Averaimo et al., 2010, Skaper, 2011). Once activated, the primary function of microglia is to return injured tissue to homeostasis (Streit and Xue, 2009), but this is a double edged sword, as their presence can be both 'good' and 'bad' for neurons. These effects are complex and overlapping, and not necessarily mutually exclusive.

In an effort to protect surviving cells during pathological conditions, microglia have been shown to 'execute' damaged or dying neurons injured from excitotoxicity in order to protect nearby cells from lytic release of toxic intracellular contents. Thus, microglia will facilitate local tissue repair by phagocytosing these cells and cell debris. This occurs through initial mobilization of cells near the site of injury and recruitment of distant microglia into the damaged area, which release proinflammatory mediators. At appropriately minimal and transient microglial activation, this process is ultimately neuroprotective (Vilhardt, 2005). However unregulated hyperactivation and release of toxic factors, such as nitric oxide (NO),

leading to over production of excess peroxynitrite, and reactive oxygen and nitrogen species (ROS, RNS), may result in an unmanageable level of oxidative stress causing degeneration in nearby 'bystander' cells. Oxidative stress can cause further neuroinflammation as well by recruitment of peripheral immune cells into the damaged brain. This could occur through a compromised blood-brain-barrier (BBB) as can be the case in stroke, Alzheimer's disease, amyotropic lateral sclerosis, and epilepsy (del Zoppo et al., 2000, Mhatre et al., 2004, van Vliet et al., 2007).

Microglia can also be modulatory by secreting a host of inflammatory mediators upon activation, including cycloxygenase-2 (Cox-2), interleukin-3 (Il-3), interleukin-6 (Il-6), interleukin-1beta (IL-1β), tumor necrosis factor-alpha (TNF-α), prostaglandins (PGs), tissue plasminogen activator (tPA), monocyte chemoattractant protein-1 (MCP-1), vascular endothelial growth factor (VEGF), lymph toxin, matrix metalloproteinases (MMPs), and macrophage inflammatory protein-1alpha (MIP-1α). The identity, quantity and duration of release of these factors can vary widely based on the specific injury. Some of these molecules can be toxic in high amounts, and certainly can have a direct impact on neuronal function. For example, we have studied the protease tissue plasminogen activator (tPA), which along with other trophic factors released by microglia, has been demonstrated to be critical in the sprouting of mossy fibers emanating from the dentate gyrus (DG) (Wu et al., 2000, Ferrer, 2002, Zhang et al., 2004) and may potentially facilitate seizure recruitment and the chronic maintenance of convulsions (Sloviter et al., 1996, Buckmaster et al., 2002, Shibley and Smith, 2002, Winokur et al., 2004). Indeed a number of studies including our own, have suggested that tPA from neurons, and potentially also microglia, plays a role in mediating seizure development (Qian et al., 1993, Schmoll et al., 2003, Yepes and Lawrence, 2004, Pawlak et al., 2005, Mirrione et al., 2007).

There is as well, a good side to this double-edged 'microglia' sword. They can also release several neuroprotective factors, such as neurotrophins including brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF), which can in fact promote neuronal stability (Elkabes et al., 1996, Miwa et al., 1997, Elkabes et al., 1998, Heese et al., 1998, Nakajima et al., 2001a). Maximizing this neuroprotective function can become particularly important in the aging brain as microglia cells enter a phase of senescence, and aged neurons require greater neuroprotection (Streit et al., 2008). Microglia can also positively influence pathological conditions by facilitating the recovery of injured neurons through release of trophic factors as well as extracellular matrix molecules, such as thrombospondin, important for sustaining neuronal function (Chamak et al., 1994). Additionally, application of microglia to the site of spinal cord injury has been shown to improve regenerative neurite outgrowth (Rabchevsky and Streit, 1997).

During CNS injury and glutamate excitotoxicity, microglia can act as both antigen presenting cells (Neumann et al., 1996, O'Keefe et al., 2001), and as cells that can remove harmful materials, including glutamate. Activated microglia express the high affinity glutamate transporter GLT-1, and thus can contribute approximately 10% of glutamate recycling (Nakajima et al., 2001b, Shaked et al., 2005, Persson et al., 2006), which may become more important under pathological conditions especially if astrocytes are overburdened or impaired. In addition, the cerebellum bergman glia have been shown to be involved in tonic GABA release through the ion channel Best1 (Lee et al., 2010), which may also be expressed and function in microglia in other brain regions (Ducharme et al., 2007).

While this remains to be explored, tonic GABA release could also be a mechanism for microglia to modulate seizures.

Other ways in which microglia may be beneficial involves the emerging notion of protective autoimmunity (Shaked et al., 2005). During CNS injury such as glutamate toxicity, a proposed mechanism to minimize the destruction would involve building a tolerance to autoimmune self-antigens, and could involve priming or preconditioning of microglia cells (Schwartz et al., 2003). This may be accomplished in several ways, but one experimental way is to expose microglia to lipopolysaccharide (LPS) prior to the injury or pathology. There are a few examples in the literature where preconditioning microglia with LPS modulates seizures including our own, and this will be discussed in more detail in the next section (Sayyah et al., 2003, Akarsu et al., 2006, Arican et al., 2006, Dmowska et al., 2010, Mirrione et al., 2010, Yang et al., 2010a). One mechanism that may contribute to the positive effects attributed to LPS preconditioning is through enhanced neurotrophin release (Nakajima et al., 2001a). While activated rat microglia have indeed been shown to release BNDF, this release is significantly enhanced when they are stimulated with LPS. Furthermore, these activated microglia also released NGF, suggesting that in the context of LPS preconditioning, microglia could be neuroprotective through release of neurotrophins.

A careful balance needs to be maintained in dosage and timing of LPS application, as of course it also activates release of potentially toxic factors. Therefore, new strategies to precondition microglia in more refined ways to promote neuroprotective functions, and minimize neurotoxic factor release, may prove to be therapeutically beneficial. In fact one study showed that application of ceramide increased the release of protective factors, but not other potentially dangerous molecules, through specific signaling mechanisms involving protein kinase C (PKC) (Nakajima et al., 2002). As well, interferon-gamma (INF-gamma) administration causes microglia to significantly increase their ability to remove glutamate, also without causing an inflammatory response (Shaked et al., 2005). These strategies should be explored further in the context of seizures and potentially exploited as neuroprotective modulators.

2. A functional role for microglia in seizures: The 'bad' and the 'good'

A growing body of evidence suggests that microglia have a functional role in the pathology and symptoms of CNS diseases including ischemia and Alzheimer's disease. In this section, we discuss the evidence linking microglia to seizures and epilepsy. We will focus on examples from the literature suggesting both 'bad' neurotoxic, and 'good' neuroprotective, contributions. However, while it is clear from these data that a functional role for microglia exists, these effects are certainly complex and even contradictory in different literature reports.

Are microglia activated concurrent with seizure pathology, and if so what is the timing, duration, location and extent of this activation? There is growing evidence suggesting that yes, extensive microglial activation known as 'microgliosis' occurs in the brain parenchama of patients with recurrent seizure episodes (as well as reactive astrogliosis), and in animal models of epilepsy particularly in the hippocampus (Beach et al., 1995, Drage et al., 2002). Activated microglia are observed in the same hippocampal regions associated with seizure induced neuronal death clinically and in animal models, owing them a reputation for facilitating neuronal malfunction (Beach et al., 1995, Taniwaki et al., 1996, Tooyama et al., 2002, Borges et al., 2003). In patients, microglia activation was found in the sclerotic hippocampus, suggesting that neuronal degeneration continues to occur as a result of

ongoing seizure activity (Beach et al., 1995). A recent study confirmed the presence both of activated microglial and also immunoreactive leukocytes in tissue resected from patents with intractable medial temporal lobe epilepsy, and in kainic acid (KA) treated mice, either associated with blood vessels or distributed intraparenchymally in the CA1-CA3, hillus, and to a lesser extent, dentate gyrus (Zattoni et al., 2011). Following pilocarpine induced seizures in mice, microgliosis persists for at least 3-31 days in regions of neuronal loss such as the hippocampus and amygdala (Borges et al., 2003, Yang et al., 2010a). However, we have shown that during KA excitotoxicity, microglia activation is associated with tPA release from injured neurons, and thus is a consequence rather than a cause of neurodegeneration, but overall this microglia activation can further exacerbate the injury (Siao et al., 2003). Gliomas have also been associated with epilepsy, and histological examination of patients' tissue following surgical removal has demonstrated that the numbers of activated microglia correlated with the duration of epilepsy, as well as with the frequency of seizures prior to surgical resection (Aronica et al., 2005). Overall, this evidence would suggest that microglia activation is 'bad' for the epileptic brain and is associated with neurodegenerative pathology.

Knowing that microgial activation is associated with seizures and epilepsy is the first step, but what exactly are these cells doing? Given the plethora of molecules microglia release, one can easily imagine numerous potential outcomes. For instance, it has been proposed that microglia activation may contribute to spontaneous recurrent seizures (SRS) by facilitating aberrant migration of newborn neurons in the DG (Yang et al., 2010a). This study showed that LPS injected directly into the DG promoted the development of ectopic hilar basal dendrites in the hippocampus, while addition of minocycline that blocked microglia activation, prevented it. Neurotrophin and cytokine release can contribute to this aberrant granule cell neurogenesis (Scharfman, 2005). The expression of pro-inflammatory transcripts changes after pilocarpine-induced seizures in mice, and includes upregulation of toll-like receptor type 2 (TLR2, a microglia/macrophage marker) and I kappa B alpha (IκBα, index of NF–kappa B activation), particularly in areas undergoing neurodenegeration or demyelination (Turrin and Rivest, 2004). Increased microglial activation is also found in adult animals given KA when they have already been exposed to it during adolescence (Somera-Molina et al., 2009). In this model, early-life seizures created an increased susceptibility to seizures later in life ('two-hit seizure' model), when KA is given at postnatal day 5 (P5), and then again at P45. The expression of proinflammatory cytokines IL-1 beta, TNF-alpha, S100B, and the chemokine CCL2 was found to be enhanced, corresponding to increased susceptibility to seizures in the adults.

Recent evidence strengthens a strong implication of cytokine involvement in modulating acute seizures. mRNA expression of TNF-alpha and IL-6 correlated with seizure development in a viral infection model, and knockout mice lacking the receptors for these cytokines showed reduced seizure frequency (Kirkman et al., 2010). These results suggest the innate immune response to viral infection contributes to seizures through cytokine expression, and can potentially occur through modulation of glutamate receptors/transporters on astrocytes. Such modulation can eventually lead to excessive extrasynaptic/extracellular glutamate levels, which could also be detrimental (Choi and Koh, 2008). Another study supports the role of cytokines showing that in brain regions associated with seizure damage from the nerve agent soman, there was an increased production of IL-1alpha and IL-1beta (neurotoxic cytokines by activated microglia) as well as IL-6 (neuroprotective factor released from neurons and astrocytes) at 72 hrs, in the

piriform cortex, hippocampus, and thalamus (Johnson and Kan, 2010). However in this same model, neurotoxic COX-2 expression, which mediates the production of prostaglandins, was found expressed in neurons, but not in microglia or astrocytes (Angoa-Perez et al., 2010). In the mouse kindling model, COX-1 from microglia was enhanced in the hippocampus during progression of seizures and the administration of SC-560 (a selective COX-1 inhibitor) or indomethacine (a non-selective COX inhibitor) reduced the progress of seizures (Tanaka et al., 2009). These two studies suggest that in addition to cytokines, cyclooxygenase enzymes derived from neurons and microglia may also play an important role in mediating seizures.

In terms of electrophysiological changes, one study showed that the cortical innate immune response to LPS application actually increased local neuronal excitability. Furthermore, in a subset of animals, this also produced motor seizures (Rodgers et al., 2009). Based on their experimental evidence, the authors suggest that microglial activation may therefore be a potential precursor to seizures, and *not* a consequence of them. Specifically, they showed that when LPS is applied to the cortex *in vivo,* the evoked field potential amplitudes were acutely enhanced (as measured within 5min-1hr) and produced focal epileptiform discharges, which were prevented by pre-application of an interlukin-1 receptor antagonist. They suggested that this rapid response may result in increased glutamate and noradrenaline release within 10 min, potentially causing the rapid increases of neuronal excitability (Wang and White, 1999). While this study showed that LPS induced microglial activation produces increases in neuronal excitability, they only measured short time points, and so further experiments are necessary to determine whether this effect is long lasting and can truly facilitate seizure symptoms.

Although the aforementioned data suggest a strict neurotoxic role of microglia, it must be kept in mind however, that this activation is a response to ongoing injury and abnormal circuitry rewiring. Little affirmative evidence is available suggesting that microglia are causing neuronal malfunction directly (with the exception of the acute study by Rogers et. al. described above) especially since this evidence comes from rodent models where chemoconvulsants are typically used to trigger the symptoms. Therefore it is important to establish whether microgliosis is a consequence of recurrent seizure episodes or a direct early contributor to symptoms. This is inherently complex however, based on their multiple functions as described in the first section.

The most obvious way to probe these questions is to pharmacologically activate and inhibit microglial activation before, during, and after experimental seizure induction and monitor differences in acute seizure symptoms and pathology. Another way to study this is to examine seizures in knockout mice missing integral components of the inflammatory response. Zattoni et al (Zattoni et al., 2011) showed that selective pharmacological ablation of peripheral macrophages prior to kainate injection actually increased dentate granule cell degeneration. This result suggested that the F4/80 (microglia/macrophage marker) positive cells, specifically of peripheral origin, appear to be required for long-term survival of granule cells. The same investigators also showed enhanced neurodegeneration in mutant mice lacking B or T cells, also suggesting a strong impact of immune-mediated responses on network excitability. Overall, these findings support the idea that lymphocytes and macrophages infiltrating the epileptic focal area may have a neuroprotective role.

Several other groups including ours have used LPS to activate microglia at different time points prior to seizures. An interesting result that may be counterintuitive given all the studies described above, is that preconditioning microglia before seizures has shown to be

potentially protective. This is akin to reports that sublethal stress stimuli induce tolerance in ischemia (Marsh et al., 2009). In one study, LPS administration prior to pentylenetetrazole (PTZ)-induced seizures was beneficial by increasing plasma levels of NO and IL-6, which reduced blood brain barrier permeability (Arican et al., 2006). In kindling, LPS was inhibitory but only when it was administered daily for 16 days, as it blunted the acquisition of kindled behavioral seizures (Sayyah et al., 2003). Neuronal protection from cell death in hippocampal CA1, CA3 and DG was observed with LPS preconditioning 72 hours prior to seizure induction, although seizure behavior was not affected at this low dose of LPS (Dmowska et al., 2010). Importantly, one study addressed the time course of LPS delivery prior to seizure induction and demonstrated complex effects (Akarsu et al., 2006). This group showed that LPS given 4 hr before PTZ-induced seizures was pro-epileptic, but when given 18 hr before, it conferred anticonvulsant effects attributable to the expression of COX-1 and -2. It is possible that LPS stimulated release of cytokines concurrent with PTZ drives the pro-convulsant effect, but how protection is conferred by delaying seizure induction for hours to days after LPS administration is less obvious. Tolerance to excitatory input, depletion of intracellular stores of proinflammatory mediators, and upregulation of glutamate recycling in anticipation of the next insult, are all plausible explanations which need further study.

Important questions that remain include what are the circumstances that cause microglia activation during seizures specifically, and are microglia strictly necessary for seizure development? We were surprised recently in studying these questions to find that the activation state of microglia in the hippocampus has a direct impact on the sensitivity of acutely induced seizures. In our experiment, we asked whether conditional microglia/macrophage ablation could affect epileptogenesis using genetically modified mice (Mirrione et al., 2010). These mice express the herpes simplex virus thymidine kinase (HSVTK) gene under the CD11b macrophage/microglia promoter (CD11b-HSVTK+/-). As shown in the schematic of Figure 1a, activated microglia are present throughout hippocampal subfields associated with seizures. Ganciclovir (GCV) is administered intra-hippocampally through an implanted osmotic mini-pump. Over time, GCV can be taken up by cells in the hippocampus near the infusion site, which extends throughout the ipsilateral hippocampus. Subsequently, LPS and pilocarpine can be administered i.p. during the experiment. Figure 1b describes the effect of the HSVTK transgene which is under the control of the Cd11b promoter, and thus expressed exclusively in microglia and macrophages. Cd11b is expressed in resting, ramified microglial cells at low baseline levels, which results in some moderate HSVTK expression. However, cells that are activated (by LPS or pilocarpine) begin to upregulate Cd11b, and thus will express more of the HSVTK suicide gene. HSVTK by itself does not harm the cell, similar to GCV, which by itself is harmless. However, cells that contain both HSVTK and GCV will be susceptible to ablation. HSVTK will phosphorylate GCV, which is further phosphorylated by intracellular kinases. This toxic triphosphate then competes with thymine for DNA synthesis, and causes DNA replication failure killing the cell (Heppner et al., 2005). Therefore, one can selectively target microglia/macrophages for ablation as the expression of Cd11b is restricted to these cells, and increased in activated microglia. This drives increased expression of the HSVTK gene, which causes apoptosis. Microglia/machrophage cells that are in S phase of cellular division are particularly susceptible to ablation using this method, however non-dividing cells are also susceptible, albeit at reduced levels, which may be due to interference with mitochondrial DNA synthesis (Herraiz et al., 2003).

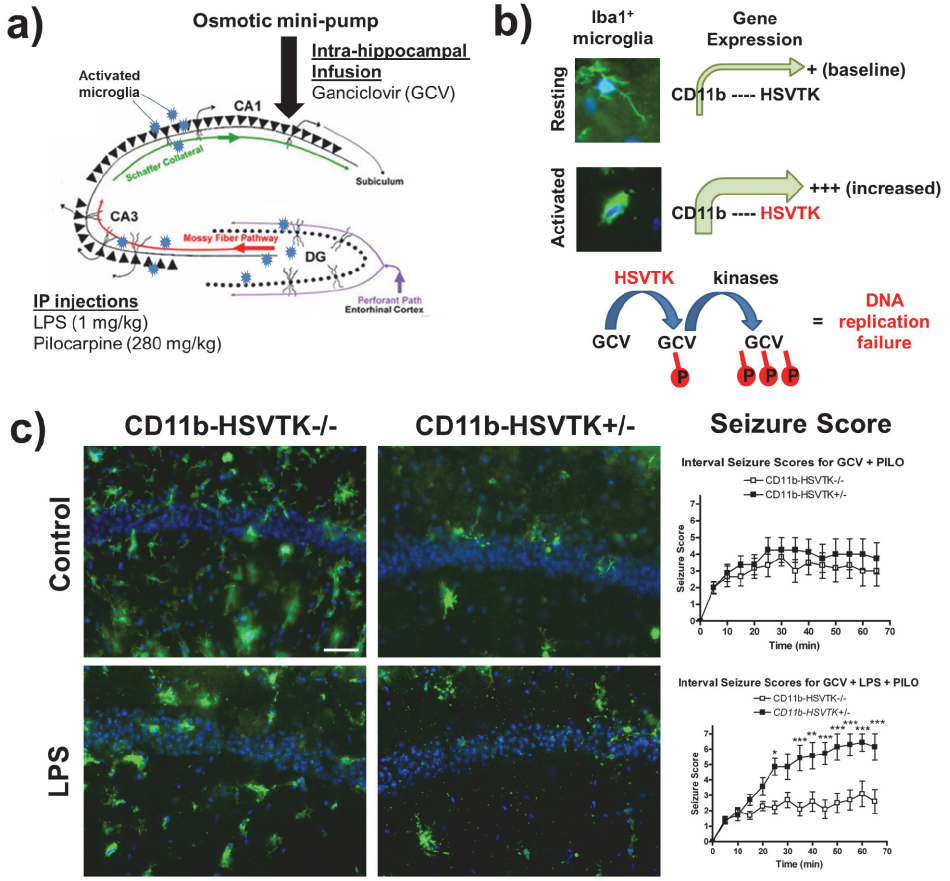

Fig. 1. Microglial ablation and lipopolysaccharide preconditioning modulates pilocarpine-induced seizures in mice. A) Activated microglia are present in hippocampal subfields following LPS and/or pilocarpine induced seizures, Neuronal circuitry in the hippocampus consists of a mainly unidirectional loop of excitatory and inhibitory signals known as the 'trisynaptic circuit', although additional connections between subfields also exist. These neurons are vulnerable to neurodegeneration in seizure models, and DG neurons specifically undergo circuitry rewiring propagating seizure activity. We have shown that that the activation state of microglia in the hippocampus can have a direct impact on the sensitivity of acutely induced seizures. In the experiment, the hippocampus is targeted with intra-hippocampal infusion of ganciclovir (GCV) through an osmotic mini-pump. LPS and pilocarpine are given intraperitoneally (i.p.) and seizure symptoms are recorded. B) Mechanism of cell ablation via GCV and HSVTK. The morphology of resting and activated microglia are shown. The microglia/machrophage specific integrin protein, Cd11b (the alpha chain of the Mac-1 integrin), has low expression in resting cells, and is increased in activated cells. The HSVTK suicide gene is placed under the control of the promotor CD11b. When microglia are activated, CD11b expression increases and HSVTK is highly expressed. In a cell which has taken up GCV, and is expressing HSVTK, GCV is phosphorylated by

HSVTK and endogenous kinases (phosphorylation represented by red circle's labeled 'P'). This turns GCV into a toxic triphosphate which competes with endogenous thymine for DNA synthesis. Ultimately, DNA replication is disrupted and the cell undergoes apoptosis. C) Data showing CD11b-HSVTK-/- (wild type, left) mice compared to CD11b-HSVTK-/- (transgenic mice, right) under control and LPS preconditioning (CA1 subfield, DAPI (blue) and Iba1 (green), scale bar 50 μm) conditions. There were significantly fewer Iba1 positive cells (microglia/macrophages) in the transgenic mice following GCV in both conditions; however seizure symptoms (right panels) only showed a significant divergence, when LPS was administered 24 hr before the pilocarpine injection. In this group, seizure scores were dramatically increased over the first 60 min following pilocarpine injection. Thus in the absence of LPS activated microglia/macrophages, seizure symptoms were significantly elevated. Additionally, LPS preconditioning in CD11b-HSVTK-/- (wild type) mice also showed a trend toward reducing seizure scores. (Part C, adapted and partially reproduced from (Mirrione et al., 2010)).

Our results showed that unilateral ablation of quiescent (non-activated) microglia from the dorsal hippocampus did not necessarily alter acute seizure sensitivity (Figure 1 c, top panel), but ablation of activated microglia (by LPS given 24 hrs prior to pilocarpine) did in fact significantly enhance seizure symptoms (Figure 1c, bottom panel, and summarized in Table 1). Furthermore, we observed a trend toward reduced seizure activity in LPS preconditioned mice, similarly to what other groups have reported (Sayyah et al., 2003, Akarsu et al., 2006, Arican et al., 2006, Dmowska et al., 2010). These data would suggest that LPS activated microglia were providing some protection to the hippocampus, perhaps by controlling the spread of seizure activity. Additional studies are required to identify the chemical mediators released by microglia that may underlie this protective effect, and whether bilateral microglia/macrophage ablation would be further protective As well, it would be interesting to further evaluate whether neurodegeneration patterns are altered at chronic time points. In regards to how LPS activated microglia upregulate Cd11b, one group showed this was mediated by nitric oxide (NO) (Roy et al., 2006, Roy et al., 2008). Therefore, LPS may be enhancing inducible nitric-oxide synthase (iNOS) in microglia, which produces NO, upregulating Cd11b, and concurrently the HSVTK gene. This would increase the percentage of cells ablated by GCV over time, potentiating the response to pilocarpine (Table 1). Furthermore, there is evidence as well that ablation of macrophages, and neutrophils which also express CD11b, may certainly play an important role, and shouldn't be discounted as potential mediators (Zattoni et al., 2011). In the future, these transgenic mice can be extremely useful for in depth examination of conditional microglial/macrophage ablation in other brain regions such as the amygdala, cortex, or more specifically targeted subfield regions of the hippocampus.

Microglia in transgenic mice (CD11b-HSVTK +/-)	Expression level		# in S phase (dividing)	GCV	Cell death	Response to PILO (compared to CD11b-HSVTK-/-)
	CD11b	HSVTK				
Naïve (resting or in S phase)	+	+	+	No	no effect	n.s.
				Yes	+	n.s.
LPS Activated	+++	+++	+++	No	no effect	n.s.
				Yes	+++	potentiated

Note: plus signs (+) represent relative amounts; (PILO) pilocarpine; (n.s.) not significantly different (data from Mirrione et al., 2010)

Table 1. Expression of transgene based on activaction state of microglia.

In our study, we also explored whether changes in glial function were a component of metabolic changes in these mice following pilocarpine using small animal imaging with 2-deoxy-2[(^{18})F]fluoro-d-glucose (^{18}FDG) and positron emission tomography (PET). Our goal was to find the brain regions involved in the behavioral divergence we observed in CD11b-HSVTK+/- mice, compared to wild type mice, using a systems approach. In pilocarpine treated animals of both genotypes, we found increased ^{18}FDG uptake during seizures in the septum, thalamus, hippocampus, midbrain, and cerebellum, along with decreases in the striatum, which was consistent with our previous findings (Mirrione et al., 2007). In addition, we observed reduced metabolic activation in the ipsilateral hippocampus corresponding to the location of unilateral microglial ablation. While we interpreted this result with caution, we concluded that activated microglia may contribute a small, but potentially important, proportion of the metabolic signal. It is possible then, that changes in glial function could be a component of metabolic abnormalities which are observed in epileptic patients (Lamusuo et al., 2001, Goffin et al., 2008b) and rodent seizure models (Kornblum et al., 2000, Mirrione et al., 2006, Mirrione et al., 2007, Goffin et al., 2009). Therefore, in the next section we will discuss how neuroimaging approaches using specific radiotracers which target microglia, can facilitate our understanding of the pathology underlying epileptogenesis.

3. Neuroimaging as a potential tool for exploring *in vivo* function of microglia during epileptogenesis

We and others have taken advantage of the unique systems level, *in vivo* neuroimaging modalities to probe questions related to seizure circuitry and malfunction. These techniques have become increasingly utilized both clinically and in basic research to understand epileptogenesis. Metabolic neuroimaging with positron emission tomography (PET) is the most widely used diagnostic, (for review (Mirrione and Tsirka, 2011). A handful of recent studies in animal models have also begun to utilize specific radioligands studying GABA, serotonin, dopamine, and the cannabinoid systems (Werhahn et al., 2006, Goffin et al., 2008a, Liefaard et al., 2009, Liew et al., 2009, Assem-Hilger et al., 2010). There are also a few pilot studies in the literature examining the acetylcholine (Mohamed et al., 2005) and opioid systems (Theodore et al., 1992) in epilepsy patients. While the availability of these radioligands is still somewhat limited in clinical settings, and requires further validation, they may prove to be valuable tools for future research and diagnosis.

PET imaging utilizes a radiotracer, a radiolabeled molecule, that when injected intravenously into the subject will bind specifically to the location of interest. For microglia, the radiotracer should be a ligand which binds to a receptor or transporter expressed specifically on activated microglia. The ligand needs to be chosen carefully, as it would need to exhibit the necessary pharmacokinetic properties to make it a good radiotracer. Specifically, it should effectively enter the central nervous system through the blood stream by crossing the blood brain barrier (lipophilicity is expressed as log D, or log P), and it should bind to activated microglia (known as the affinity, or dissociation constant Kd) with enough strength to be injected in very low amounts, but still located by the PET camera. Other helpful properties of these radiotracers (radioligands) are that they have low non-specific binding (that they do not bind randomly to other molecules), and are they are not converted into 'active' metabolites, which can bind to some other unwanted target in the brain and perturb the analysis.

The first major radioligand that has been used to study activated microglia is carbon-11 labeled PK11195, a compound that binds to the peripheral benzodiazepine receptor (PBR). The PBR is a mitochondrial protein expressed on both microglia and macrophages. Its expression is increased after brain injury or neuroinflammation. [^{11}C]PK11195 was used in a pilot study comparing 4 normal human subjects, 3 patients with clinically stable hippocampal sclerosis and low seizure frequency, and 2 patients with histologically confirmed Rasmussen's encephalitis (RE) (Banati et al., 1999). This initial study showed that specific binding of the radiotracer the RE patients showed a focal and diffuse increase in binding throughout the seizure-affected hemisphere. Interestingly, the clinically stable hippocampal sclerosis group was similar to normal controls, suggesting that at this stage for these few patients, activated microglia were no longer contributing to the symptoms or pathology. Furthermore, a case of cerebral vasculitis in refractory epilepsy was cured with [^{11}C]PK11195 imaging (Goerres et al., 2001). This adult female patient showed an area of increased radiotracer signal in the left occipital lobe. Biopsy showed that this was positive for activated microglia, vascular and perivascular accumulations of mononuclear cells. The patient was then treated with prednisolone and azathiorprine (immune suppressing drugs) which subsequently reduced her seizures.

Based on these initial studies, a recent case report using [^{11}C]PK11195 of encephalitis facilitated treatment of a 5 year old patient who did not respond to medication (Kumar et al., 2008). Using ^{18}FDG was not particularly informative, as this patient showed diffuse glucose hypometabolism without any focal abnormality. As the physicians suspected that underlying brain inflammation was the cause, a [^{11}C]PK11195 scan was conducted which showed an area of increased uptake in the left temporal-occipital cortex. This area was subsequently resected and the patient fully recovered. They concluded that focal areas of neuroinflammation play an important role in refractory seizures associated with encephalitis, and using this imaging approach highlighted the region for surgical removal, and led to a successful recovery. This approach was used again and resulted in the treatment of another female patient with intractable epilepsy due to focal cortical dysplasia (Butler et al., 2011).

One can easily imagine from these case reports how examining the time course of microglia activation over the development and progression of epileptogenesis in patients and animal models can truly shed light on the microglial contribution to pathology. While PK1195 has proven to be a successful indicator of microglia activation in seizures, it has limited uptake in the brain and thus has limited quantitative capacity. Much effort has been put into finding alternative ligands for the PBR which have higher affinity's and better pharmacological properties as radiotracers. The list of these experimental compounds is actually quite long, and the available ligands for studying microglia were recently reviewed (Leung, 2004-2010, Schweitzer et al., 2010). One potential tracer [^{11}C]DAA1106 has been shown to have a higher affinity to the PBR compared to PK1195 and may be more suitable for measuring subtle changes in microglia activation during disease (Maeda et al., 2004). Several other tracers have similar enhanced potential including [^{125}I]DPA-713 (a ligand for SPECT imaging, which also has a slightly higher affinity) (Wang et al., 2009), and [^{11}C]CLINME (Boutin et al., 2007) which has lower non-specific uptake.

Thus far, the most popular target to image microglia remains as the PBR, however other targets for activated microglia should also be explored. New tracers may be significantly useful for examining the impact and progression of microgliosis during different stages of

epileptogenesis, first in animal models and eventually in patients. We urge the continued development of such radiotracers specifically for this purpose.

4. Optogenetics as a potential tool for exploring *in vivo* function of microglia during epileptogenesis

Optogenetics is an exponentially growing field in neuroscience where light responsive ion channels called opsins are genetically introduced into neurons and astrocytes (Deisseroth, 2011). Thus far, no experimental evidence is available that suggests that microglia could also be targeted with opsins, however this is certainly plausible. Channel rhodopsin-2 can activate neurons with blue light by allowing influx of sodium, but can also allow for influx of calcium, which has been exploited for activating astrocytes (Gradinaru et al., 2009, Figueiredo et al., 2011). Astrocytes in fact, could also be explored in the context of seizures using these methods. Microglia are unique however, in that once activated they express an inward chloride conductance (Averaimo et al., 2010). This could also theoretically be mimicked with expression of halorhodopsin, which when activated with yellow light, allows passage of chloride ions. These swelling-activated chloride channels I (Clswell) are naturally expressed on microglia (Ducharme et al., 2007), and contribute to their phagocytic capacity (Furtner et al., 2007), and to the production of nitric oxide (Kjaer et al., 2009). Similar to experiments on astrocytes, transgenic mouse lines can be created where these channels are expressed under microglia/macrophage specific promoters. Cre-dependent viruses could also be used to target microglia specifically. Implanted optical fibers could then deliver light stimulation to specific brain regions *in vivo* under various experimental conditions.

This method may be advantageous over pharmacological approaches, as these channels respond very quickly to light on and off by opening and closing, thus allowing for a variety of experimental manipulations *in vivo*, in real time. Potentially this could allow for fast activation and deactivation of microglia. However, the real kinetics that returns the cells to a resting state once activated is of course very different in glia than neurons. This could potentially be a limitation of this approach, since once microglia are activated, it takes them time to naturally return to a resting state. Despite this caveat, optogenetics may provide an interesting basic research avenue in the future for studying channel properties of microglia.

5. Microglia as a therapeutic target for epilepsy

Can microglia be therapeutically targeted to benefit epilepsy patients? While this question has yet to be systematically explored, a few case studies and basic research experiments may suggest a tentative yes. However, given the complexity of microglia-neuronal interactions, a systematic analysis of activated microglia function at various stages during epileptogenesis is necessary.

There are several drugs used experimentally in animal models to modulate the activation state of microglia, which are effective as anti-inflammatory agents. One recent example showed that erythropoietin administered to rats reduced a rise in inflammatory genes, edema, and the numbers of activated microglia after febrile seizures, thereby reducing the risk of subsequent spontaneous seizures (Jung et al., 2011). Another report showed that levetiracetam reduced the harmful spread of excitation during seizures by restoring gap junctions in astrocytes co-cultured with microglia (Stienen et al., 2011). The investigators

found that TGFbeta1 expression was increased, which normalized the impaired astrocyte membrane resting potentials via modification of inward and outward rectifier currents. TGFbeta1 has also been shown to be a potent anti-inflammatory and neuroprotective agent by either preventing the classical activation of microglia, or by enhancing the presence of alternatively activated microglia, and reducing oxidative stress (Qian et al., 2008).

Inflammatory blockade has also been accomplished with celecoxib, a selective cyclooxygenase-2 inhibitor (Jung et al., 2006). When given during the latent period, it prevented neuronal death and microglia activation in the hilus and CA1. It also inhibited ectopic granule cell formation in the hilus and new glia infiltrating the CA1. Interestingly, COX-2 was shown to be neuroprotective as SC-58236, another selective COX-2 inhibitor, reversed the anti-seizure activity of LPS given 18 hr before PTZ (Akarsu et al., 2006). In the same study, a COX-1 selective inhibitor, valeryl salicylate, facilitated PTZ-induced seizures. However, another group showed that in the mouse kindling model, the administration of SC-560 (a selective COX-1 inhibitor) or indomethacine (a non-selective COX inhibitor) actually reduced the progress of seizures (Tanaka et al., 2009).

Carbamazepine was also anti-inflammatory as it inhibited the production of NO and prostaglandin E2 production (Matoth et al., 2000). Resveratrol, a naturally occurring nonflavonoid polyphenol found in grapes, has been shown to inhibit microglia activation, proinflammatory factor release, and NADPH oxidase which protected neurons (Zhang et al., 2010a, Zhang et al., 2010b). Ghrelin attenuates KA induced cell death in the hippocampus of mice by preventing the activation of microglia and astrocytes and their expression of cytokines TNF-alpha, IL-1b, and COX-2 (Lee et al., 2010a). Minozac, another small molecule that reduces proinflammatory cytokine production, was given following early life seizure induction. Minozac attenuated the enhanced microglial responses that increased vulnerability to seizures in adulthood (Somera-Molina et al., 2009). Similarly, naloxone is also anti-inflammatory, reducing IL-1beta synthesis and microglial activation following early life seizure induction. It also lowered the vulnerability of immature brains to a second seizure episode in adulthood (Yang et al., 2010b).

In terms of cytokines and inflammatory mediators, IL-1 beta has been shown to be proconvulsant, and IL-1 receptor antagonism (via IL-1Ra) has proven to be a powerful inhibitor of seizures (Vezzani et al., 1999, Vezzani et al., 2000). A recent study reported findings that supported this notion, as IL-1 receptor antagonism blocked the effects of LPS induced enhancement of evoked field potential amplitudes and focal epileptiform discharges in the first minutes to hour of exposure (Rodgers et al., 2009). However, in this case, further study at longer time points should be evaluated to determine if this effect is lasting, or whether it changes, as we and others have shown is the case for LPS on seizure symptoms. Other inflammatory mediators, interleukin-18 (IL-18) and interferon-gamma (INF-gamma) have also proven to be protective against neuronal damage following status epilepticus (SE) (Ryu et al., 2010). Valproic acid, a standard clinical therapy for seizures, was shown to inhibit the production of NF-kappaB, which blocked the release of TNF-alpha and IL-6 by microglia (Ichiyama et al., 2000). It has also been shown to cause microglial apoptosis through the p38 MAPK and mitochondrial apoptosis pathways (Xie et al., 2010).

A recent review highlights the potential for targeting ion channels on microglia to modulate microglial activity (Skaper, 2011). For example, chloride intracellular channel 1 translocation to the activated plasma membrane generates ROS which mediates amyloid beta-peptide

clearance (Milton et al., 2008, Paradisi et al., 2008). Also, inhibiting the Ca^{2+}/calmoduline-activated K^+ channel expressed on microglia actually reduced LPS induced toxicity (Li et al., 2008).

Several studies have been published suggesting positive regulation of microglia can also have beneficial effects for seizure therapy. An intriguing concept is to activate or 'program' microglia with certain drugs to harness their neuroprotective capabilities. Ceramide is one such compound, that when applied in culture, increased the release of protective BDNF, but not other dangerous molecules, TNF-alpha, IL-1beta, or NO (Nakajima et al., 2002). Interferon-gamma (INF-gamma) has been shown to drive microglia to significantly increase their ability to remove glutamate, without causing an inflammatory response (Shaked et al., 2005). Also, insulin-like growth factor-1 (IGF-1) from microglia and astrocytes was shown to promote hippocampal CA1 neuronal survival following injury to dentate granule cells (Wine et al., 2009).

While there is overwhelming evidence that microglia should be inhibited as a means to treat epilepsy, it is important to keep in mind the context of this experimental evidence as it mostly originates from chemoconvulsant models and *in vitro* measurements. *In-vivo* situations need to be further explored to fully validate these hypotheses and potential therapeutic approaches. Overall however, the strategies outlined in this section should be assessed in the context of seizures. Of particular interest clinically, is the concept of pharmacologically harnessing the neuroprotective qualities of microglia, while inhibiting their neurotoxic properties.

6. Summary

Overall, a growing body of evidence suggests that microglia have a functional role in the pathology and symptoms of epilepsy. Future studies should focus on elucidating the time course of microglial activation during seizure progression, as well as specific factors released at these various times. Furthermore, novel approaches such as *in vivo* neuroimaging and optogenetics will provide additional avenues to examine these questions. Fully exploiting the therapeutic benefit of modulating microglia for patients with epilepsy has great potential.

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2

The Blood-Brain Barrier in Epilepsy

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1. Introduction

The blood-brain barrier is altered in epilepsy. This includes altered expression of transporters and metabolic enzymes as well as barrier leakage that have been linked to antiepileptic drug resistance and seizure genesis, respectively. Here we highlight current understanding of these pathological changes. Three critical components of barrier function - 1) tight junctions, 2) metabolising enzymes and 3) transporter proteins - are introduced and we describe how they are changed in epilepsy and affected by epilepsy treatment. Recent efforts in blood-brain barrier research to overcome drug-resistant epilepsy are also discussed.

2. The blood-brain barrier

The History of Blood-Brain Barrier Discovery. First experiments contributing to the discovery of the blood-brain barrier were performed by Paul Ehrlich in 1885 (**Figure 1**). Ehrlich observed that water-soluble "vital dyes" injected into the blood of rats did not stain the brain (Ehrlich, 1885). In 1900, Lewandowsky made similar observations and coined the term "blood-brain barrier" ("Bluthirnschranke") to explain this phenomenon (Lewandowsky, 1900). Ehrlich's student, Edwin Goldmann, injected the same dyes Ehrlich had used into the subarachnoid space, and found the opposite: intense staining of the brain but no staining of peripheral tissues (Goldmann, 1909; 1913). Goldmann concluded that a barrier had to exist between the brain and the periphery, thus the concept of a vascular barrier was born. In 1923, Spatz postulated that the brain capillary endothelium had to be the structure responsible for barrier function, which initiated a debate that lasted for decades (Spatz, 1933). It was Reese and Karnovsky, and Brightman and Reese who solved the mystery of the blood-brain barrier in the late 1960s. Using electron microscopy, they discovered that tight junctions connect adjacent capillary endothelial cells and seal the intercellular space (Brightman & Reese, 1969; Reese & Karnovsky, 1967). With this, the molecular structure responsible for barrier function was identified and the barrier was localized to the brain capillary endothelium.

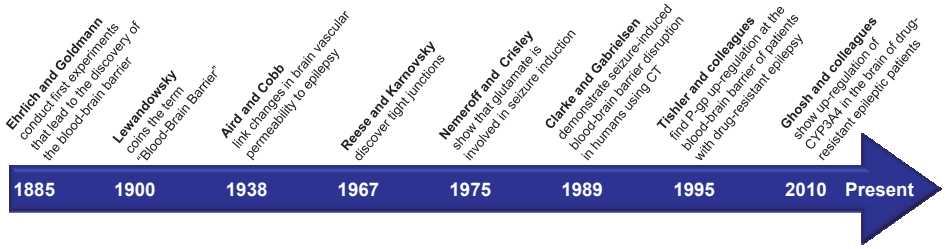

Fig. 1. Evolution of Blood-Brain Barrier Methodology/History

The History of the Blood-Brain Barrier in Epilepsy. In the 1930s, Aird and Cobb discovered that brain uptake of "vital dyes" was increased in epileptic mice. Based on their observation, they suggested that the brain vasculature may be a barrier between the central nervous system (CNS) and the periphery and that altered brain vascular permeability may be a factor contributing to epilepsy (Aird, 1939; Cobb et al., 1938). In the mid 1950s, Bercel used diuretics in patients to increase brain uptake of antiepileptic drugs (AEDs (Bercel, 1955)). Co-administration with diuretics reduced AED doses below toxic levels in ten of ten patients and in seven of these ten patients seizure control was improved (Bercel, 1955). Nemeroff and Crisley made a critical discovery in 1975 when they found that glutamate is involved in seizure induction and increases cerebrovascular permeability in rats (Nemeroff & Crisley, 1975). Further, blood-brain barrier dysfunction was shown to go along with an increase in blood pressure and cerebral vasodilation during seizures (Bolwig et al., 1977; Petito et al., 1977). In 1989, Clarke and Gabrielsen demonstrated seizure-induced blood-brain barrier leakage in humans using computed tomography (Clarke & Gabrielsen, 1989). In 1995, Tishler et al. made the observation that mRNA of *MDR1* (*ABCB1*), the gene encoding the efflux transporter P-glycoprotein (P-gp) is increased at the blood-brain barrier of patients with drug-resistant epilepsy (Tishler et al., 1995). This was a critical finding because P-gp acts as a "gatekeeper" that limits therapeutic drugs from crossing the blood-brain barrier and from entering the brain (Miller et al., 2008). Research in this field initially focused on P-gp, but other transporters such as multidrug resistance proteins (MRPs) and breast cancer resistance protein (BCRP) are also increased in epilepsy animal models or patients (Awasthi et al., 2005; Dombrowski et al., 2001; Sisodiya et al., 2006; Van Vliet et al., 2005).

Today, the role of some of these transporters in epilepsy is still unclear. It has been discussed that P-gp could be involved in seizure generation (Marchi et al., 2004) and that multiple transporters may act in concert to limit brain uptake of a broad range of AEDs (Lazarowski et al., 2007). Recent studies show that AED-metabolizing enzymes such as cytochromes (CYP) 3A4, 2C8, and glutathione sulfotransferase (GST) μ and π are also upregulated in the brain of epileptic patients forming a metabolic barrier that contributes to AED resistance (Ghosh et al., 2010; Shang et al., 2008; Ueda et al., 2007).

2.1 Blood-brain barrier anatomy

Numbers and Facts. The blood-brain barrier is a network of brain capillaries (microvessels). With a diameter of 3-7 μm, brain capillaries are the smallest vessels of the vascular system (**Figure 2A**) (Rodriguez-Baeza et al., 2003). The microvasculature in the human brain is comprised of about 100 billion capillaries forming a highly branched vascular network

(Zlokovic & Apuzzo, 1998). Due to the high capillary density in the brain, capillaries are about 40 µm apart from each other, a distance short enough for small molecules to diffuse within 1 second (Rodriguez-Baeza et al., 2003). This ensures that every neuron (about 100 billion in human brain) is in contact with and perfused by its own capillary, which allows efficient nutrient and oxygen supply. Despite the huge number of 100 billion brain capillaries, the total capillary lumen occupies only about 1% of total brain volume, or about 12-15 ml in an adult human brain of about 1,400 ml (Pardridge, 2003b). Thus, at any given time, about 8-10% (about 10 ml) of total cerebral blood (about 150 ml) is in the lumen of brain capillaries. Not taking the capillary lumen into account, it is estimated that the brain capillary endothelium occupies only about 0.1% of total brain volume (~ 1-1.5 ml) (Pardridge, 2003b). Lastly, the total length of the capillary network is about 600-650 km in an adult human brain with a total surface area of about 20 m^2. This makes the blood-brain barrier the third largest surface area for drug exchange after intestine and lung (Pardridge, 2003a).

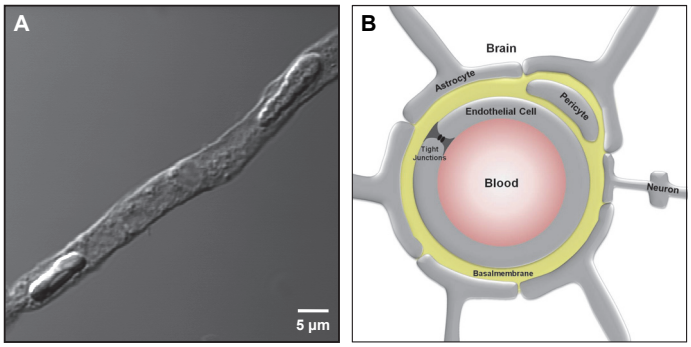

Fig. 2. (A). DIC image of isolated brain capillary. (B) Neurovascular unit.

Morphology and Anatomy. Brain capillaries are the next higher level of organisation from endothelial cells that are the smallest anatomical unit of microvessels. Brain capillary endothelial cells are flat, thin, spindle-shaped, polarized cells. Their apical membrane faces the blood (luminal), and their basolateral membrane faces the brain parenchyma (abluminal; (Betz et al., 1980)). It is through the basement membrane that brain capillary endothelial cells are in contact with pericytes, astrocytes, and neurons (**Figure 2B**; (Goldstein & Betz, 1983)). This 4-cell structure is referred to as "*Neurovascular Unit*" and is responsible for maintaining and regulating blood flow, and for controlling barrier function (Begley, 2004).
One fundamental characteristic of endothelial barrier function is a complex, multi-protein structure called a *tight junction*, which is unique in the vascular system (Nagy et al., 1984). Brain capillary endothelial cells also lack intercellular clefts and have low pinocytotic activity, which limits solute exchange between blood and brain. Lastly, to meet the large energy demand of ATP-consuming processes like metabolism and active efflux transport, brain capillary endothelial cells possess a large number of mitochondria (Goldstein & Betz, 1983).

2.2 Blood-brain barrier physiology

Blood-brain barrier functions include CNS protection, and regulation and maintenance of CNS homeostasis. Three components determine barrier function: *1. Tight Junctions, 2.*

Transporters and *3. Metabolising Enzymes*. The following paragraphs describe these components in more detail.

1. Tight Junctions

Tight junctions are cell-cell contacts that seal the intercellular space between adjacent endothelial cells, thereby creating a non-fenestrated endothelium and limiting hydrophilic molecules from paracellular diffusion (Nag, 2003). Tight junctions are multi-protein complexes composed of transmembrane proteins like occludins, claudins, e-cadherins and junctional adhesion molecules as well as adaptor and regulatory proteins (Matter & Balda, 2003a; Vorbrodt & Dobrogowska, 2003). Adaptor proteins include zonula occludens proteins, cingulin, catenin and membrane-associated guanylate kinase inverted proteins that connect junctional transmembrane proteins with cytoskeletal actin filaments (Matter & Balda, 2003b). Regulatory proteins include G proteins, atypical protein kinase C isoforms, and symplektin that are involved in signalling (Matter & Balda, 2003b; Wolburg & Lippoldt, 2002). Together, tight junctions guarantee a tight barrier, and thus, protection of the CNS (Kniesel & Wolburg, 2000). However, under pathological conditions such as epilepsy, tight junctions can be dysfunctional or disrupted, leading to barrier leakage, impaired neuronal function, and brain damage (Huber et al., 2001).

2. Metabolic Enzymes

The concept of a "metabolic barrier" is widely accepted but little information is available on metabolising enzymes at the blood-brain barrier. Early studies focused on phase I enzymes in whole brain tissue but later studies differentiated between different brain cell types. Walther et al. showed that CYP P450 enzymes are located in the inner mitochondrial membrane of neurons and glia from rat, guinea pig, rabbit, and pig brain. This is in contrast to liver, where most CYP isoforms are located at the endoplasmic reticulum (Walther et al., 1986). Consistent with this, Ghersi-Egea et al. found CYP P450 protein expression in mitochondria from rat brain tissue (Ghersi-Egea et al., 1987), and demonstrated CYP activity in various brain regions and isolated human microvessels. They found low 1-naphthol-UDP-glucuronosyltransferase and NADPH-CYP P450 reductase activity, and high GST and epoxide hydrolase activity (Ghersi-Egea et al., 1993). The same group also found Cyp P450 activity in rat brain microvessels (Ghersi-Egea et al., 1994).

Dauchy et al. used isolated microvessels from resected human brain and found mRNA expression of CYP1A1, 1B1, 2B6, 2C8, 2D6, 2E1, 2J2, 2R1, 2S1, and 2U1, and detected CYP1B1 by Western blotting (Dauchy et al., 2008). Immunohistological studies by Rieder et al. confirmed localisation of CYP1B1 in human brain capillaries (Rieder et al., 2000). In a follow up study, Dauchy et al. showed CYP2U1 and CYP2S1 mRNA expression in the human cerebral microvascular endothelial cell line hCMEC/D3 (Dauchy et al., 2009). CYPs with low mRNA expression included CYP2R1, 2B6, 2E1, 1A1, 2D6, 2C18, 1B1, 2J2, 1A2 and 2C8. Except for CYP2C18, all CYP genes found in hCMEC/D3 cells were also detected in isolated human brain microvessels. A novel CYP P450, Cyp4x1, was identified in 2006 by Al-Aznizy et al. in mouse brain (Al-Anizy et al., 2006). Immunohistochemical staining showed strong Cyp4x1 protein expression in neurons, choroid plexus epithelial cells, and brain microvessel endothelial cells. In 2010, mRNA and protein expression of CYP3A4, the most prominent enzyme involved in xenobiotic metabolism in the liver, was found by Ghosh et al. in human brain endothelial cells (Ghosh et al., 2010).

While most blood-brain barrier enzymes have been detected at the mRNA level, protein expression and activity of only few enzymes have been demonstrated. These include

gamma-glutamyl transpeptidase (Beuckmann et al., 1995), alkaline phosphatase (Beuckmann et al., 1995), aromatic L-amino acid decarboxylase (Betz et al., 1980; Matter & Balda, 2003b), the phase I metabolising enzymes CYP1A1 (Filbrandt et al., 2004), CYP1B1 (Filbrandt et al., 2004), CYP3A4 (Ghosh et al., 2010; Ghosh et al., 2011), and Cyp4x1 (Al-Anizy et al., 2006), NADPH-CYP P450 reductase (Chat et al., 1998; Ghersi-Egea et al., 1988; Minn et al., 1991; Ravindranath et al., 1990), epoxide hydrolase (Ghersi-Egea et al., 1988; Minn et al., 1991), and the phase II enzymes, 1-naphthol-UDP-glucuronosyltransferase (Ghersi-Egea et al., 1988) and GSTμ (Shang et al., 2008), and GSTπ (Bauer et al., 2008; Shang et al., 2008).

The presence of these enzymes in the brain microvasculature indicates the existence of a metabolic barrier. However, more studies are needed to better define the role metabolising enzymes play at the blood-brain barrier under physiological and pathophysiological conditions and whether these enzymes can indeed limit AED delivery to the brain.

3. Transporters

The blood-brain barrier is an active, dynamic and selective interface that responds to signals from both the periphery and brain. Key components of barrier function include influx and efflux transporters that are responsible for brain homeostasis, nutrient supply, and protection of the brain from endogenous and exogenous toxins.

Influx transporters that maintain CNS homeostasis and nutrient supply include A-and N-system amino acid transporters (Betz et al., 1980; O'kane & Hawkins, 2003), excitatory amino acid carriers 1, 2, and 3 (O'kane & Hawkins, 2003; O'kane et al., 1999), alanine/serine/cysteine/threonine (ASCT) transporters for neutral amino acids (Boado et al., 2004; Tayarani et al., 1987), glucose transporters GLUT1 and GLUT3/14 (Pardridge, 1991; Simpson et al., 2007), monocarboxylate transporters MCT1 and MCT8 (Braun et al., 2011; Ito et al., 2011; Simpson et al., 2007), and the equilibrative nucleoside transporter ENT1 (Kitano et al., 2002), as well as Na^+-K^+-ATPase (Betz et al., 1980). These transporters belong to the solute carrier (SLC) superfamily. Prominent SLC transporters that have been detected at the blood-brain barrier also include the organic anion transporter Oat3, organic anion transporting polypeptides Oatp1a4, 1b1, 1c1, 2b1, 14, and organic cation transporters OCT1, OCT2 (Ito et al., 2011; Lin et al., 2010). Of these SLCs, Oat3, Oatps, and Octs are involved in drug transport. However, it is currently not known if these SLC transporters can handle AEDs.

An interesting blood-brain barrier transporter is the large neutral amino acid transporter LAT that transports the amino acids valine, leucine, isoleucine, tryptophan, and tyrosine. LAT1 mediates brain uptake of L-DOPA that is used in Parkinson's disease (Del Amo et al., 2008). LAT1 has also been reported to transport the AEDs gabapentin and pregabalin across the blood-brain barrier into the brain (Del Amo et al., 2008; Liu et al., 2008; Su et al., 1995). Whether LATs are affected in epilepsy is unknown.

In total, 21 transporters have been detected at the protein level in brain capillaries and brain capillary endothelial cells from various species by immunohistochemistry, Western blotting, or quantitative LC/MS/MS (Kamiie et al., 2008; Neuwelt et al., 2011). Seven of these transporters belong to the ABC (ATP-binding cassette) transporter family and include P-glycoprotein (P-gp, *MDR1*, *ABCB1*), the multidrug resistance proteins 1, 2, 3, 4, and 5 (MRPs, *ABCC1-5*) and breast cancer resistance protein (BCRP, *ABCG2*). These transporters are ATP-driven and mainly located at the luminal membrane of the brain capillary endothelium (Mrp1 and Mrp4 are also in the abluminal membrane). This "first line of defence" protects the brain from neurotoxicants and limits CNS drugs from entering the brain, and thus, is an obstacle for CNS pharmacotherapy.

Together, transporters ensure proper CNS nutrient supply and mediate efflux of metabolic wastes from the brain, thus, helping maintain CNS homeostasis. The following section describes the role of transporters, metabolic enzymes, and barrier leakage in epilepsy.

3. Blood-brain barrier function in epilepsy

Epilepsy affects more than 60 million people worldwide. The majority of patients respond to treatment with AEDs, but up to 40% of patients are drug-resistant (Kwan & Brodie, 2003; Loscher & Potschka, 2005). Patients with AED resistance suffer from uncontrolled seizures, which elevates their risk of brain damage and mortality (Sperling et al., 1999). These patients experience a low quality of life and, despite advances in pharmacotherapy and neurosurgery, drug-resistant epilepsy remains a major clinical problem (Devinsky, 1999).
Evidence indicates that the blood-brain barrier is altered in patients with epilepsy. Changes in the brain capillary endothelium include upregulation of efflux transporters and metabolic enzymes as well as barrier leakage that have been linked to AED resistance and seizure genesis (Bauer et al., 2008; Ghosh et al., 2010; Marchi et al., 2007). The following section describes the role of transporters, metabolic enzymes, and barrier leakage in epilepsy.

3.1 Transporters in epilepsy

One factor underlying AED resistance is, at least in part, seizure-induced over-expression of drug efflux transporters at the blood-brain barrier (Bauer et al., 2008). Some of these transporters, such as P-gp, Mrp2, and BCRP have been implicated with AED resistance. The first evidence for involvement of efflux transporters in epilepsy goes back to studies by Tishler and co-workers in 1995. These researchers observed increased P-gp mRNA in the brain and protein expression in the capillary endothelium of patients with drug-resistant epilepsy (Tishler et al., 1995). The findings by Tishler et al. were confirmed by other groups (Dombrowski et al., 2001; Lazarowski et al., 1999; Sisodiya et al., 2002) and it was suggested that this phenomenon could prevent AEDs from entering the brain and cause AED resistance. However, studies in cell lines of non-brain endothelial origin showed that some AEDs such as vigabatrin, gabapentin, phenobarbitone, lamotrigine, carbamazepine, and phenytoin are not, or are only weak, P-gp substrates, questioning whether P-gp could be the primary reason for AED resistance (Crowe & Teoh, 2006; Maines et al., 2005; Owen et al., 2001; Weiss et al., 2003). In contrast, Cucullo et al., compared phenytoin permeation in brain capillary endothelial cells from drug-resistant epileptic human brain tissue with that of commercially available human brain microvascular endothelial cells (Cucullo et al., 2007). They demonstrated that phenytoin permeation was 10-fold lower in endothelial cells from AED-resistant patients compared to purchased human endothelial cells. Although this comparison is flawed, inhibiting P-gp increased phenytoin permeation in the AED-resistant cells. Moreover, recent *in vivo* data, including our own studies, demonstrate that P-gp does limit AEDs from entering the brain (Brandt et al., 2006; Liu et al., 2007; Van Vliet et al., 2007). Using a drug-resistant epilepsy rat model, Potschka et al. showed that animals not responding to phenytoin exhibited 2-fold higher P-gp expression levels in brain capillaries compared to animals responding to treatment (Potschka et al., 2004). van Vliet et al. demonstrated that inhibiting P-gp counteracted phenytoin resistance, which reduced seizure occurrence in rats (Van Vliet et al., 2006). Marchi et al. supported these findings showing that patients with high blood-brain barrier P-gp expression had low brain levels of

oxcarbazepine (Marchi et al., 2005). These studies demonstrate that, in drug-resistant epilepsy, certain, but not all AEDs have restricted access to the brain due to increased blood-brain barrier P-gp, and that modulation of P-gp can enhance brain distribution of some AEDs such as phenytoin (Potschka & Loscher, 2001; Van Vliet et al., 2006; Van Vliet et al., 2007).

In addition to P-gp, data indicate that BCRP plays a significant role in drug efflux at the blood-brain barrier. Recent studies show that both transporters, P-gp and BCRP, "team up" and work together to limit chemotherapeutic drugs from permeating across the blood-brain barrier and penetrating into the brain (Chen et al., 2009; De Vries et al., 2007). However, little information is available on the extent to which BCRP contributes to AED resistance and if P-gp and BCRP work in concert in AED efflux from the brain. Some studies found no upregulation of BCRP in human epileptogenic brain tissue and no evidence for BCRP-mediated AED transport *in vitro* (Cerveny et al., 2006; Sisodiya et al., 2003), but other studies reported upregulation of BCRP expression in the microvasculature of epileptogenic brain tumors (Aronica et al., 2005; Vogelgesang et al., 2004) and in chronic epilepsy animal models (Van Vliet et al., 2005). More studies are needed to unequivocally clarify the role of BCRP, especially in conjunction with P-gp, in AED-resistant epilepsy.

Only little information is available on the multidrug resistance proteins (Mrps) in epilepsy. van Vliet et al. used the pilocarpine status epilepticus model in rats and found by immunohistochemistry and Western blotting that Mrp1 and Mrp2 protein expression was upregulated in astrocytes within several limbic structures including the hippocampus (Van Vliet et al., 2005). These findings were confirmed by Hoffmann et al., who also demonstrated Mrp2 upregulation in brain capillaries by immunohistochemistry following pilocarpine-induced status epilepticus (Hoffmann et al., 2006). In control rats, Mrp2 was barely detectable in the brain capillary endothelium, but in status epilepticus rats, Mrp2 staining was evident in brain capillary endothelial cells. MRP2 has also been found to be over-expressed in sclerotic hippocampal tissue of AED-resistant patients with mesial temporal lobe epilepsy (Aronica et al., 2004). In the same patient population, MRP1 expression was upregulated in glial endfoot processes around cerebral blood vessels. Observations of chronic epileptic rats showed that protein levels of Mrp1 and Mrp2 were also upregulated in blood vessels and this over-expression correlated with seizure frequency and reduced brain uptake of phenytoin (Van Vliet et al., 2005). However, phenytoin brain uptake was enhanced by the MRP inhibitor probenecid. While upregulation of mRNA was observed for Mrp1, 5, and 6, increased protein expression was only found for MRP1 and 2 in isolated capillary endothelial cells from patients with drug-resistant epilepsy (Dombrowski et al., 2001; Kubota et al., 2006). A time-course study revealed that 6-24 h after onset of a pilocarpine-induced status epilepticus in rats, mRNA of P-gp, Mrp1, and Mrp5 was decreased in hippocampus, amygdala, and the piriform cotex. This initial decrease in mRNA levels was followed by a 24h period of normal mRNA expression and then increased mRNA levels about 4 days after status epilepticus (Kuteykin-Teplyakov et al., 2009). These findings are in contrast to an earlier study where P-gp mRNA levels in mouse hippocampus were increased by 85% 3-24 h after kainic acid-induced limbic seizures, but returned to control levels after 72 h (Rizzi et al., 2002). Treatment with AEDs for 7 days did not change P-gp mRNA expression (Rizzi et al., 2002). In the same study, the authors also used rats with spontaneous recurrent seizures 3 months after electrically induced status epilepticus. P-gp mRNA levels were increased 1.8- and 5-fold in the hippocampus and entorhinal cortex,

respectively. Thus, changes in P-gp mRNA levels occur after both acute and chronic epileptic activity. The same authors (Rizzi et al., 2002) also used microdialysis and demonstrated that AED brain levels were significantly reduced. While a direct connection between blood-brain barrier P-gp levels and AED brain levels was not shown, it was concluded that seizure-induced changes in P-gp could contribute to AED resistance in epilepsy. Note that none of these studies provided data on transporter protein expression or activity.

3.1.1 Transporter inhibition

The discovery that drug efflux transporters are upregulated at the blood-brain barrier in AED-resistant patients suggested that transporter inhibition could overcome AED resistance in epilepsy. This notion was encouraged by studies that showed enhanced brain uptake of AEDs when co-administered with transporter inhibitors. Using verapamil and probenecid, Potschka et al. used microdialysis and demonstrated in healthy rats that P-gp and Mrp limit carbamazepine brain uptake (Potschka & Loscher, 2001). A follow-up study showed that administration of the metabolic inhibitor sodium cyanide and the P-gp inhibitors verapamil and PSC833 into the frontal cortex significantly increased extracellular fluid concentrations of phenytoin. This indicated that P-gp limits phenytoin distribution into the brain under physiological conditions (Potschka & Loscher, 2001). Similar observations were made with phenobarbital, lamotrigine, and felbamate (Potschka et al., 2002). Verapamil has also been used in case studies with AED-resistant patients (Iannetti et al., 2005; Summers et al., 2004). For example, the status epilepticus in an 11-year old boy who was first unresponsive to conventional AEDs disappeared after administration of verapamil i.v. (Iannetti et al., 2005). However, this anticonvulsive response could have been due to verapamil directly blocking neuronal calcium channels instead of inhibiting P-gp at the blood-brain barrier.

In 2005, tariquidar (XR9576), a non-competitive P-gp inhibitor was first used to block P-gp function. (Martin et al., 1999; Mistry et al., 2001). Tariquidar has a good oral bioavailability, long duration of action and low potential for toxic side effects, all of which make this a favourable P-gp inhibitor. For example, van Vliet et al. demonstrated that inhibiting P-gp with tariquidar significantly reduced seizure duration, frequency and severity, which improved phenytoin efficacy in a rat model for temporal lobe epilepsy. This suggested that combination of AEDs with a transporter inhibitor may be a promising therapeutic strategy for AED-resistant patients (Van Vliet et al., 2006). The same researchers also found that P-gp over-expression in the temporal hippocampus and parahippocampal cortex of chronic epileptic rats reduced phenytoin levels by about 30% in these brain regions. Treating animals with tariquidar significantly increased phenytoin brain levels in regions with over-expressed P-gp (Van Vliet et al., 2007). Another group found that tariquidar restored the anticonvulsive activity of phenobarbital in drug-resistant rats (Brandt et al., 2006). These animal studies demonstrate that transporter inhibition increases AED blood and brain levels and improves seizure control.

Encouraged by animal studies and case reports that suggested transporter inhibition can be used to overcome AED resistance in epilepsy, clinical trials employing P-gp inhibitors were initiated. Currently, two trials using carvediol and verapamil to inhibit P-gp in AED refractory patients are ongoing (www.clinicaltrials.gov, #NCT00524134, #NCT01126307). However, while both carvedilol and verapamil are FDA-approved and readily available, neither drug is a highly specific nor potent P-gp inhibitor (Arboix et al., 1997; Takara et al.,

2004). In addition, to effectively inhibit over-expressed P-gp, high inhibitor plasma concentrations will be needed, which carries the risk of drug-drug interactions and toxic side effects (Pennock et al., 1991). In a recent study, add-on treatment with verapamil to improve seizure control in dogs with phenobarbital-resistant epilepsy had to be discontinued due to detrimental effects (Jambroszyk et al., 2011). Thus, since potent and specific inhibitors that can be safely given to patients are currently not available, transporter inhibition does not seem to be a viable treatment option in drug-resistant epilepsy at this point in time.

3.1.2 Modulation of transporter regulation

Targeting signalling pathways that regulate drug efflux transporters is another strategy to overcome transporter-mediated AED resistance. The advantages of this approach are three-fold. First, modulating transporter regulation to increase AED brain delivery may allow fine tuning of the transporter. For example, modulating the molecular switches of a transporter may allow turning it off for a short, controlled period of time to deliver drugs into the brain, after which it can be turned on again. Second, preventing or blocking seizure-induced upregulation of transporters may normalise transporter expression and functional activity, and thus, prevent or block development of transporter-mediated AED resistance. Third, since transporter upregulation in epilepsy has been linked to increased seizure occurrence, prevention of transporter upregulation holds the promise of better seizure control. Thus, mapping the signalling pathways involved in efflux transporter upregulation at the blood-brain barrier in epilepsy can help identify new targets that may potentially be used to overcome transporter-mediated AED resistance and improve seizure treatment.

Several signalling pathways have been identified that regulate P-gp, BCRP, and Mrp2 at the blood-brain barrier. For BCRP, the most recent signalling mechanisms include Nrf2, NfkB, COX-2, Pim-1 kinase, and the nuclear receptors CAR and AhR (Kalalinia et al., 2011; Singh et al., 2010; Tan et al., 2010; Wang et al., 2010). Of those, CAR and AhR have been shown to upregulate BCRP at the blood-brain barrier (Tan et al., 2010; Wang et al., 2010), which is the opposite of what one would want to improve AED delivery into the brain. Whether targeting any of the other pathways could be used as a therapeutic strategy in AED-resistant epilepsy is unknown and remains to be determined. Mrp2 is also regulated through nuclear receptors (PXR, FXR, CAR; (Bauer et al., 2008; Kast et al., 2002)), but the signalling that upregulates Mrp2 in epilepsy is unknown.

Most information on transporter regulation is available for P-gp, where signalling pathways have been shown to be present in various tissues (liver, kidney, intestine; (Ho & Piquette-Miller, 2006; Nawa et al., 2010; Thevenod et al., 2000). They also involve various signalling molecules: inflammatory mediators including TNF-α, ET-1, IL1-β, IL-6, NO; COX-2 (Dixit et al., 2005; Goralski et al., 2003; Nawa et al., 2010; Patel et al., 2002; Poller et al., 2010; Sukhai et al., 2001; Von Wedel-Parlow et al., 2009), nuclear receptors PXR, CAR, AhR, and GR (Bauer et al., 2004; Bauer et al., 2007; Geick et al., 2001; Narang et al., 2008; Wang et al., 2011; Wang et al., 2010), protein kinase C (Bauer et al., 2007; Chambers et al., 1990a; Chambers et al., 1990b; Hartz et al., 2004; Miller et al., 1998; Rigor et al., 2010), and NFkB (Bauer et al., 2007; Bentires-Alj et al., 2003; Kim et al., 2011; Liu et al., 2008; Thevenod et al., 2000; Yu et al., 2008). These pathways have been found in several diseases including Alzheimer's disease, HIV, and diabetes (Hartz et al., 2010; Hayashi et al., 2006; Nawa et al., 2010).

One pathway that involves glutamate signalling through the NMDA receptor (NMDAR) followed by cyclooxygenase -2 (COX-2) and prostaglandin E receptor 1 (EP1) activation seems to be critical for seizure-induced upregulation of P-gp (**Figure 3**, (Bankstahl et al., 2008; Bauer et al., 2008; Pekcec et al., 2009; Zhu & Liu, 2004)). During seizures, neurons release high amounts of the excitatory neurotransmitter glutamate, which can reach interstitial brain concentrations of 10-100 µM for a short period of time (Ronne-Engstrom et al., 1992; Ueda & Tsuru, 1995). Zhu and Liu were the first to connect glutamate with P-gp upregulation at the blood-brain barrier. They found that glutamate increased P-gp expression and activity in rat brain microvessel endothelial cells and suggested that activation of the NMDAR plays a critical role in glutamate-mediated P-gp upregulation (Zhu & Liu, 2004). Consistent with this, Bauer et al. demonstrated that exposing isolated rat and mouse brain capillaries to glutamate increased P-gp expression and activity (Bauer et al., 2008). It was shown that glutamate signalling through NMDAR and COX-2 upregulates blood-brain barrier P-gp, and that COX inhibition prevented P-gp upregulation suggesting that AED brain uptake can be enhanced by COX inhibition. Bankstahl et al. confirmed glutamate involvement in seizure-induced P-gp over-expression and that blocking NMDAR prevents P-gp upregulation and neuronal damage *in vivo* (Bankstahl et al., 2008). Another study showed that pre-treatment with celecoxib, a specific COX-2 inhibitor, prevented seizure-induced P-gp upregulation in rat brain capillaries (Zibell et al., 2009), and yet another study demonstrated that pre-treatment with celecoxib for 6 days followed by administration of phenobarbital for 16 days reduced the frequency of spontaneous recurrent seizures and restored the anticonvulsant effect of phenobarbital in AED-resistant epileptic rats (Schlichtiger et al., 2010). van Vliet et al. evaluated the use of the COX-2 inhibitors SC-58236 and NS-398 in rats with recurrent spontaneous seizures. They found that 2-week treatment with these COX-2 inhibitors prevented P-gp upregulation and enhanced phenytoin brain uptake in chronic epileptic rats (Van Vliet et al., 2010). While these studies are promising, COX-2 inhibitors bear the risk of severe cardio- and cerebrovascular side effects (Mukherjee, 2001; Stollberger & Finsterer, 2003). In addition, it has been demonstrated that COX-2 inhibition can lead to increased seizure frequency and mortality in epileptic rats (Holtman et al., 2010). Thus, although COX-2 inhibition may reduce AED resistance in animal models, it may not be a valid target in the clinic over the long term.

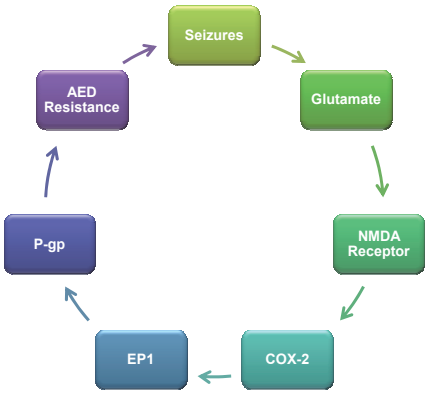

Fig. 3. Glutamate – NMDAR1 – COX-2 – EP1 Signaling Pathway

Another signalling protein involved in glutamate-mediated P-gp upregulation at the blood-brain barrier is the EP1 receptor. EP1 is activated by prostaglandin E2, the main product of COX-2, and was tested as potential target to prevent transporter upregulation in epilepsy. Pekcec et al. found that EP1 is a key signalling protein in the pathway that drives P-gp upregulation during seizures (Pekcec et al., 2009). Studies using the EP1 inhibitors SC-51089 and misoprostol showed that SC-51089 decreased seizure severity in rats when given prior to electrical kindling, but it also prolonged seizure duration at higher doses, whereas misoprostol decreased duration of motor seizure activity (Fischborn et al., 2010).
Together, these studies show that glutamate released during seizures mediates P-gp upregulation through NMDAR, COX-2, and EP1 and that these signalling proteins could potentially be used as therapeutic targets to reduce AED-resistance. Whether this pathway also signals upregulation of other blood-brain barrier proteins is unknown at this time.

3.2 The metabolic blood-brain barrier

Xenobiotic metabolism is a 3-phase process during which low polar molecules (e.g., drugs) are enzymatically converted to polar molecules that are then excreted from the body mostly through bile, faeces, or urine. Most chemicals are pharmacologically or toxicologically inactivated during metabolism, only some are transformed into active metabolites. The liver is recognized as the main site of biotransformation, but extrahepatic tissues such as the kidney, lung, intestine, skin, and brain also contribute to drug metabolism.
The processes involved in the biotransformation of drugs are classified into phase I (functionalisation) and phase II (conjugation) reactions that are followed by phase III excretion of the metabolite (**Figure 4**). Substrates of phase I enzymes are in general lipophilic and undergo functionalisation reactions such as monooxygenation, dealkylation, reduction, aromatisation, or hydrolysis. The modified molecules are substrates for phase II enzymes, which conjugate the functional group with a polar compound, such as an amino acid, sulphate, glutathione, or a sugar (Minn et al., 1991). In the last phase III step, functionalised and conjugated xenobiotics are excreted from cells by efflux transporters.

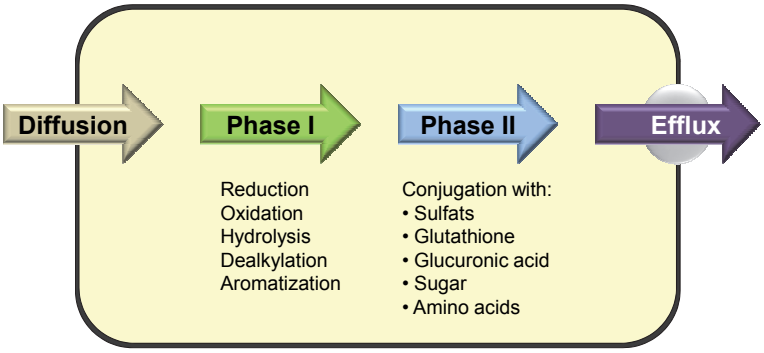

Fig. 4. Schematic of 3-phase drug metabolism and excretion

Most CNS drugs, including AEDs, have to cross the blood-brain barrier and penetrate into the brain parenchyma to reach their target sites. En passage across the barrier and within the brain, drugs can undergo inactivation and elimination comparable to hepatic drug

metabolism. In the brain, the following phase I enzymes have been identified: monoamine oxidases, CYP P450, NADPH-CYP P450 reductase, and epoxide hydrolases (Chat et al., 1998; Dutheil et al., 2010; Ghersi-Egea et al., 1998; Ghersi-Egea et al., 1988; Ghersi-Egea et al., 1993; Minn et al., 1991; Ravindranath et al., 1990). Phase II enzymes identified in the brain include UDP-glucuronosyltransferase (UGT), phenol sulfotransferase (PST), and GST (Dutheil et al., 2010; Ghersi-Egea et al., 1998; Ghersi-Egea et al., 1988; Ghersi-Egea et al., 1993; Minn et al., 1991). Several phase I and II enzymes have been found in the brain capillary endothelium, where they possibly form a metabolic barrier for drugs en route into the brain (Dutheil et al., 2010; Ghersi-Egea et al., 1998; Ghersi-Egea et al., 1993; Minn et al., 1991; Stamatovic et al., 2008). Several reports on metabolism-coupled efflux transport (phase III) suggest that biotransformation of drugs and efflux of the metabolites are part of barrier function. Together, the rodent and human brain, including brain microvessels forming the blood-brain barrier, express enzymes and transporters that are part of the detoxification pathways that affect metabolism of therapeutic drugs. In addition, AED elimination by coupling of two important biological processes – metabolism and efflux transport - could contribute to AED resistance and intractable epilepsy.

3.2.1 Metabolism of AEDs and the metabolic barrier in epilepsy

Phase I. CYP P450 enzymes are responsible for most phase I metabolic reactions and have the greatest impact on the biotransformation of therapeutic drugs. CYPs form a large and functionally diverse superfamily of enzymes that are found throughout various species ranging from bacteria to humans. In humans, the majority of CYPs are expressed along the inner plasma membranes of mitochondria and the endoplasmic reticulum. Although a distinct group of CYPs (CYP11A1, 11B1, 11B2, 17A1, 21A2) is involved in steroid hormone synthesis in humans (Hrycay & Bandiera, 2009), most CYP enzymes contribute primarily to the elimination of endogenous and exogenous substrates through oxidation to enhance their excretion from the body. By-in-large, CYP-mediated metabolism occurs in the liver and contributes to the "first pass effect" of orally administered drugs. In the liver, several CYPs exist as allelic or genetic variants and such CYP polymorphisms have been shown to influence the plasma concentration of some AEDs. CYPs are also expressed in the kidney and renal excretion is an important elimination route, particularly for most novel AEDs.

With regard to epilepsy it is noteworthy that many AEDs are metabolised by CYPs (**Table 1**). CYP2C9 and CYP2C19 are the two major enzymes involved in AED metabolism including diazepam, phenobarbital, phenytoin, and valproic acid (**Table 1**; (Klotz, 2007)). Several studies demonstrated differences in the biotransformation of these AEDs depending on the underlying CYP genotype. For example: phenytoin metabolism depends on the allelic composition of the gene encoding for CYP2C19 and CYP2C9. Several mutated alleles of these genes are known. Thus, based on genotype, poor phenytoin metabolisers can be distinguished from phenytoin hypermetabolisers and their frequency distributions vary between different ethnic populations (Klotz, 2007).

Regarding CYPs in the brain, *in vitro* and *in vivo* functional expression of CYPs has been detected in various CNS cell types from different species. CYP expression in distinct CNS cell populations is variable but can be as high as in the liver (Bhagwat et al., 2000). Importantly, it has been shown that CYPs are functionally active at the blood-brain barrier (Dutheil et al., 2010).

DRUG	ENZYME/PATHWAY
Carbamazepine	**CYP3A4, mEH1 for carbamazepinee-10, 11-epoxide**
Ethosuxemid	**CYP3A4?, 10-20% renal**
Phenobarbital	**CYP2C19, hydroxylation, glucuronidation, 25% renal**
Phenytoin	**CYP2C9, CYP2C19**
Valpoate	**CYP2C9, glucuronidation, oxidation**
Diazepam	**CYP2C19, CYP3A4**
Felbamate	**40-60% renal, hydroxylation, glucuronidation**
Gabapentin	**Renal**
Lamotrogine	**Glucuronidation, 8% renal**
Levetirazepam	**66% renal, hydrolyse**
Oxcabazepine	**Reduction to active metabolite that is glucuronidated**
Pregabalin	**98% renal**
Tiagabine	**CYP3A4, 25% renal**
Topiramate	**60-80% renal, oxidation, hydrolysis, glucuronidation**
Vigabatrin	**60-80% renal**
Zonizamide	**CYP3A4, N-acetylation, glucuronidation, 30% renal**

Table 1. Antiepileptic drugs and their route of biotransfomation (modified from Klotz, 2007)

In the human brain, 20 CYP isoforms have been identified so far: CYP1A1, 1A2, 1B1, 2B6, 2C8, 2D6, 2E1, 3A4, 3A5, 8A1, 11A1, 11B1, 11B2, 17A1, 19A1, 21A2, 26A1, 26B1, 27B1, and 46A1 (Dutheil et al., 2010). The exact expression pattern within the CNS depends on the particular CYP and greatly varies between different brain cells. In a recent study, CYP mRNA expression levels were measured in a human brain microvessel cell line and in human microvessels isolated from surgically removed brain tissue from epileptic patients or patients with brain tumours (Dauchy et al., 2008). The authors found mRNA expression of CYP2U1, CYP2S1, CYP2R1, CYP2B6, CYP2E1, CYP1A1, CYP2D6, CYP1B1, CYP2J2, CYP1A2, and CYP2C8. In another study, Gosh et al. used commercially available human microvascular cerebral endothelial cells and found mRNA expression of CYP1A1, 1B1, 2A6, 2B6 2C, 2C9, 2E1, 2J2, 3A4, 4A11, 11b, CYP3A5, 4B1, C1, 21A, and 51A1 (Ghosh et al., 2010). The cells that were used for this study originated from surgically resected brain specimens of drug-resistant epileptic patients, brain specimens resected from aneurism domes, commercially available human microvascular cerebral endothelial cells (used as control), and from human umbilical vein endothelial cells (used as control). mRNA expression from 11 of the 16 CYPs was increased in endothelial cells from epileptic tissue compared to microvascular cerebral endothelial cells. Although this comparison may be flawed due to the different nature of these cells, mRNA expression in endothelial cells from drug-resistant epileptic patients was not different compared to brain specimens resected from aneurism domes without seizures (Ghosh et al., 2010). This result argues against a seizure effect on the regulation of blood-brain barrier CYPs. However, it is clear from these findings that more detailed and accurate studies with appropriate controls are needed.

Overall, expression profiles, tissue and cellular distribution, and relative expression of CYP enzymes seem to depend on study design and the models used. Therefore, additional research is needed to clarify the importance of individual CYPs at the blood-brain barrier under both physiological and epileptic conditions.

CYP Regulation in the CNS

Carbamazepine induces CYP3A4 protein and mRNA expression in human brain endothelial cells and hepatocytes (Ghosh et al., 2010; Luo et al., 2002). But carbamazepine is metabolized by CYP3A4 (, **Table 1**), and high expression of CYP3A4 protein was found in endothelial cells isolated from surgically resected epileptic brain tissue (Ghosh et al., 2011). Neuronal CYP3A4 expression has also been demonstrated in brain sections by immunostaining from patients with temporal lobe epilepsy, tuberous sclerosis, or cavernous angioma, all of whom had intractable epilepsy. In these samples, CYP3A4 was rarely co-localised with the astrocytic marker GFAP (Ghosh et al., 2011). Carbamazepine given to cells that derived from resected epileptic brain tissue underwent metabolism at an extent similar to what was observed in hepatocytes. Thus, increased CYP3A4 expression and metabolic function could be characteristic for endothelial cells in epilepsy, which could contribute to AED resistance.
As mentioned before, AEDs such as carbamazepine act as strong inducers of hepatic and blood-brain barrier CYP expression, thereby influencing the pharmacokinetics of other drugs. Other AEDs have also been reported to increase CYP expression at the blood-brain barrier and exposure of primary rat brain astrocytic cultures to phenytoin increased Cyp2c29 levels (Volk et al., 1995). Moreover, phenytoin was metabolised by the microsomal fraction of astrocyte cultures and chronic treatment of mice with phenytoin resulted in increased levels of phenytoin metabolites in the brain (Volk et al., 1988). These findings support the idea of dynamic CYP regulation at the blood-brain barrier by AED exposure. In general, regulation of CYPs at the blood-brain barrier could be independent from that in the liver. Support for this comes from studies in alcoholics. Levels of CYP2D6 protein were elevated in the brains of alcoholics compared to non-alcoholics (Dutheil et al., 2010; Miksys & Tyndale, 2004). Particularly high CYP2D6 levels were detected in the putamen, globus pallidus, and substantia nigra, but interestingly, CYP2D6 was not elevated in the liver (Dutheil et al., 2010). Nuclear receptors that act as transcription factors control regulation of CYPs and ABC transporters in the CNS. In the human brain, several nuclear receptors have been detected that could control CYP regulation, including AhR, PXR, FXR, CAR, LXRß, RXRα and β, PPAR-α, -δ, and –γ (Dutheil et al., 2009; Nishimura et al., 2004). For example, Dauchy et al. showed that the AhR agonist TCDD increased mRNA expression of CYP1A1 and CYP1B1 in the human cell line hCMEC/D3 (Dauchy et al., 2008). Thus, it is possible that nuclear receptors could be involved in the regulation of CYPs at the blood-brain barrier in epilepsy (Dauchy et al., 2008; Dauchy et al., 2009; Ghosh et al., 2010). This idea is supported by the fact that metabolites of CYP2J2, which is expressed in brain endothelial cells in epilepsy, activate the nuclear receptors PPAR-α (*NR1C1*) and PPAR-γ (*NR1C3*). Future studies are required to address CYP regulation at the blood-brain barrier in health, disease (e.g., epilepsy), and during pharmacotherapy (e.g., AED treatment).
Phase II & III. Metabolism-driven efflux transport, i.e., coupling of phase II and III, has been demonstrated in the liver. Analogous findings from studies at the blood-CSF-barrier show coupling of metabolism and efflux transport also in the CNS. Using cultured rat choroid plexus epithelial cells *in vitro*, Strazielle and Ghersi-Egea demonstrated the presence of a metabolism-driven efflux mechanism for 1-naphthol, a cytotoxic, lipophilic model compound (Strazielle & Ghersi-Egea, 1999). The authors showed that UGT metabolised 1-naphthol *in situ* into a glucurono-conjugate (phase II) that was excreted by an efflux transporter (phase III). In this regard, MRPs have been implicated in cellular export of various glutathione, glucuronide, and sulfate conjugates compounds, and several other

endogenous and xenobiotic compounds (Gerk & Vore, 2002; Jedlitschky et al., 1996; Loe et al., 1996; Oude Elferink & Jansen, 1994). Although Mrp involvement has not directly been shown in Strazielle and Ghersi-Egea's study, Mrp-mediated efflux of the 1-naphthol glucurono-conjugate seems likely as the export was sensitive to the Mrp inhibitor probenecid. Thus, this study demonstrated the biological relevance of metabolism-driven efflux in the brain (Strazielle & Ghersi-Egea, 1999). However, in the human brain, UGT-mediated metabolism of 1-naphthol is less prominent compared to rat brain, and therefore, species differences should be considered (Ghersi-Egea et al., 1993). Activity of another phase II enzyme, GST, seems to be more relevant for human metabolism (Ghersi-Egea et al., 1993). Metabolism-driven transport was shown for GSTπ and Mrp1 by Leslie et al., who demonstrated plasma membrane co-localisation of Mrp1 and GSTπ in H69AR cells and found that functional GSTπ was required for Mrp1-mediated transport (Leslie et al., 2004). mRNA and protein expression of the GST isoform π has been demonstrated in isolated rat and mouse brain capillaries, where GSTπ is predominantly localized in the cytoplasm and the luminal plasma membrane of brain capillary endothelial cells, and to a large extent, co-localises with Mrp2 in the membrane (Bauer et al., 2008). Consistent with regulation by the nuclear receptor PXR, GSTπ protein expression increased in membranes from rat brain capillaries exposed to PCN or dexamethasone, and in capillary membranes from rats dosed with PCN. Immunoblotting of the capillary membrane fraction from hPXR transgenic mice dosed with rifampin further showed enhanced GSTπ expression. GSTπ and Mrp2 upregulation occurred in parallel, suggesting coordinated regulation of phase-II metabolism and phase-III efflux, i.e. Mrp2-mediated transport (Bauer et al., 2008). While these studies provide first insight into the regulation of both GSTπ and Mrp2 in brain capillaries, direct proof of metabolism-coupled efflux transport of chemicals at the blood-brain barrier remains to be shown. Thus, metabolism-coupled excretion of CNS drugs, such as AEDs, by efflux transporters seems likely, but requires further studies. Such studies would have to take into consideration the specific conditions at the human blood-brain barrier. For example, human cerebral microvessels show absence of glucuronidation, low NADPH CYP reductase activity, high GST activity, and pronounced epoxide hydrolase activity (Ghersi-Egea et al., 1993). Understanding metabolising enzymes, specifically at the human blood-brain barrier with respect to their physiological function, regulation in health and disease, and interplay with efflux transporters will allow assessing their impact on drug delivery to the brain, particularly in epilepsy.

3.3 Blood-brain barrier leakage in epilepsy

Seizures are accompanied by impaired blood-brain barrier integrity. This has been observed before, during and after seizures in both experimentally induced seizures in animals as well as in epileptic patients (Cornford & Oldendorf, 1986; Horowitz et al., 1992; Mihaly & Bozoky, 1984; Nitsch & Klatzo, 1983; Padou et al., 1995). As a consequence, impaired blood-brain barrier integrity causes transient barrier leakage, which allows entry of blood borne molecules into the brain (Ndode-Ekane et al., 2010; Seiffert et al., 2004; Sokrab et al., 1989; Van Vliet et al., 2007). It has been shown that seizure duration correlates with reduced barrier function (Cornford & Oldendorf, 1986), and it has been demonstrated that increased blood-brain barrier permeability in epilepsy is limited to anatomically specific brain regions (Bradbury, 1979; Cornford et al., 1998; Nitsch & Klatzo, 1983; Oztas & Sandalci, 1984). Interestingly, brain regions with increased barrier permeability are often anatomically

congruent with the brain regions that are implicated in the development and propagation of seizures. Consistent with this observation, extravasation of blood components into the brain correlates with increased excitability, occurrence of seizures, and epilepsy progression (Friedman et al., 2009; Marchi et al., 2007; Ndode-Ekane et al., 2010; Oby & Janigro, 2006; Seiffert et al., 2004; Tomkins et al., 2008; Van Vliet et al., 2007).

Cause of Blood-Brain Barrier Leakage by Seizures. Studies conducted over the last 3 decades indicate that possibly 3 mechanisms could be involved in causing blood-brain barrier leakage in epilepsy: blood pressure, pinocytosis, and seizure-induced inflammation.

Blood Pressure

The first studies conducted in the 1970s showed that arterial blood pressure is involved in seizure-associated blood-brain barrier leakage (Nitsch & Klatzo, 1983). Several studies unequivocally demonstrated that hypertension has detrimental effects on blood-brain barrier integrity and contributes to barrier leakage (Cornford & Oldendorf, 1986; Lee & Olszewski, 1961; Petito et al., 1977; Westergaard, 1980). Johansson summarized three factors responsible for increased blood-brain barrier permeability: (1) maximal arterial blood pressure, (2) duration of maximal arterial blood pressure, and (3) total increase in blood pressure (Johansson, 1981). The detailed mechanism through which increased blood pressure contributes to barrier leakage is unclear, but a working hypothesis postulates the following (Petito et al., 1977): Neuronal hyperactivity (seizures) leads to increased metabolism, and to an increased nutrient and oxygen demand in the involved brain regions. In turn, cerebral blood flow rises and large cerebral arteries dilate, which leads to increased blood pressure in brain capillaries, small arteries and veins (Ndode-Ekane et al., 2010), and triggers barrier leakage. Consistent with this, extravasation of blood albumin into the brain was found specifically in regions with more EEG spiking activity in humans (Cornford et al., 1998). However, the duration of increased blood pressure is critical and determines the severity of barrier leakage. Thus, more severe seizures that are followed by prolonged blood pressure elevations result in a higher increase in barrier permeability (Oztas & Kaya, 1991; Oztas & Sandalci, 1984). This is also supported by studies where both increased arterial blood pressure and subsequently induced barrier leakage were prevented by cervical cordotomy (Schaefer et al., 1975; Westergaard et al., 1978).

Pinocytosis

It was hypothesized that pinocytosis is involved in transport across the capillary endothelium, thus, affecting barrier permeability (Palade, 1961). The pinocytosis rate at the blood-brain barrier is low, which contributes to a tight barrier endothelium. However, Petito et al. observed that seizure-induced blood-brain barrier leakage correlates with increased micropinocytosis (Petito et al., 1977). In an elegant study using intravenous HRP injections in adult male rats with seizures, the authors made two important findings: (1) Brain capillary vesicles from animals that suffered seizures did contain HRP compared to vesicles from control animals that did not contain HRP; (2) the number of HRP-containing vesicles was higher directly after seizures (within 30 sec of last seizure (Petito et al., 1977)). From these observations the authors concluded that an increased micropinocytosis rate during and shortly after seizures increases blood-brain barrier permeability and counteracts barrier function. Nitsch and Hubauer confirmed these studies and showed in kainic acid-injected rats that blood-brain barrier opening was due to increased transendothelial pinocytosis, while tight junctions stayed intact (Nitsch & Hubauer, 1986).

Seizure-induced Inflammation

Another factor causing barrier leakage in epilepsy is seizure-induced inflammation that could be enhanced by extravasation of blood-borne components into the brain. It has been shown that blood-brain barrier permeability is increased by inflammatory mediators, including histamine, substance P, endothelin-1, bradykinin, VEGF, TGFß, IL1ß, TNFα, INFγ, PGE2, PGF2a, chemokines, free radicals, and other factors such as metalloproteinases, thrombin, amyloid-ß, intracellular calcium, and leukocytes that directly interact with endothelial cells (Stamatovic et al., 2008). Only limited information is available on how these factors alter the blood-brain barrier but some, e.g., IL1ß and chemokines, seem to exclusively affect paracellular permeability (Stamatovic et al., 2008). It is currently unknown if secretion of these factors is a direct consequence of epileptic seizures. In addition, depending on epilepsy aetiopathology, the composition of the inflammatory "cocktail" and the contribution of individual inflammatory mediators to blood-brain barrier damage could vary significantly. The effect of inflammation on barrier permeability is context-dependent, complex and not well understood. It likely depends on the model, dose, time and location of the inflammatory mediators involved.

One hypothesis that could explain some of the phenomena observed at the blood-brain barrier in epilepsy is that seizure-released glutamate activates signalling reducing barrier integrity and increasing permeability. Glutamate release occurs during seizures at sites in the brain with excessive neuronal activity. On the one hand, high glutamate levels are cytotoxic, which contributes to brain damage. On the other hand, subtoxic glutamate levels trigger molecular processes such as local release and activation of matrix-degrading enzymes that breach integrity (Michaluk & Kaczmarek, 2007; Nishijima et al., 2010). It is possible that glutamate-initiated signalling and inflammatory mediators cause barrier leakage and breakdown in epilepsy. Such events would allow extravasation of blood-borne compounds. Whether this scenario is part of seizures remains to be shown.

Consequences of Blood-Brain Barrier Leakage in Epilepsy. Epilepsy is often a consequence of a prior brain insult (e.g., traumatic brain injury, stroke) and seizures are a symptom of an underlying brain disorder (e.g., brain tumour, Alzheimer's disease, brain inflammation; (Marchi et al., 2006; Salazar et al., 1985; Tomkins et al., 2011). Although the factors that are involved in the development of epilepsy remain unclear, impaired barrier function is common after an initial brain insult and likely contributes to epilepsy pathology. This particular topic has recently attracted major interest in the epileptology field.

It has been shown in patients that brain injury, post-ischemic or vascular inflammation often cause seizures and barrier leakage (Stamatovic et al., 2008; Tomkins et al., 2011; Tomkins et al., 2008). The blood-brain barrier is also impaired in epileptic patients and in seizure animal models, and consequently, it has been postulated that barrier leakage is involved in epilepsy aetiology (Friedman et al., 2009; Marchi et al., 2007; Ndode-Ekane et al., 2010; Oby & Janigro, 2006; Seiffert et al., 2004; Tomkins et al., 2011; Tomkins et al., 2008; Van Vliet et al., 2007). In addition, it has been shown that osmotic barrier opening causes seizures (Oby & Janigro, 2006). However, not all implications of barrier opening have been studied. It is known that intra-arterial injection of hyperosmotic mannitol in patients and rodents results in EEG changes and induces seizures (Fieschi et al., 1980). In a recent study, Marchi et al (2007) observed seizures in patients undergoing osmotic barrier opening for delivering chemotherapeutics to treat brain lymphomas. In 25% of patients, seizure onset occurred

immediately after barrier opening. Using a pig model, the authors demonstrated that seizure occurrence correlated with barrier opening and was neither attributed to the existing brain lymphoma nor to chemotherapy (Marchi et al., 2007).

The molecular and cellular events that are triggered by barrier opening and that result in seizures and neuronal hyperactivity are a matter of research. Rigau et al. (2007) demonstrated loss of functional tight junctions and immunoglobulin leakage into the brain in surgically resected hippopcampal tissue from AED-resistant epilepsy patients (Rigau et al., 2007). Additional evidence from rodents and resected epileptogenic human brain tissue shows that extravasation of albumin into the brain triggers epileptogenesis (Friedman et al., 2009; Ivens et al., 2010). It was shown that astrocytes incorporated extravasated albumin, which induced proepileptogenic transformations, including reduced expression of potassium and aquaporin channels and gap junction proteins, impairment of astrocytic glutamate metabolism, and increased release of pro-inflammatory mediators (Friedman et al., 2009). All these changes had detrimental effects on seizure threshold and susceptibility (Friedman et al., 2009). One could postulate that seizures or other factors that induce barrier leakage trigger albumin extravasation with subsequent astrocytic transformation eventually causing seizures. Such a scenario implies a pernicious feedback loop where seizures drive barrier leakage leading to more seizures. Although this hypothesis is a matter of discussion, many epileptologists are convinced that 'seizures beget seizures' and that epilepsy has a progressive nature (Hauser & Lee, 2002). In this regard, alterations of the blood-brain barrier and extravasation of blood-borne compounds could be a critical part of epilepsy pathology that could potentially be a target for new therapies.

4. Conclusions

Research over the last century demonstrated a key role of the blood-brain barrier in the development of epilepsy and AED resistance. Despite the advances that have been made, what we currently know about AED resistance is mostly limited to descriptive observations rather than understanding of the mechanisms underlying the disease. We know that the blood-brain barrier is altered in epilepsy including changes in transporters, metabolic enzymes, and tight junctions. We also know that transporters, enzymes and tight junctions are affected by and/or contribute to epilepsy pathology. Yet, whether each of these molecular players is part of a cause-effect jigsaw puzzle and how each of the pieces fit together is unclear. Thus, AED resistance in epilepsy remains an unsolved clinical problem.

To solve this problem future studies will have to address the mechanism of AED resistance at the molecular level taking all aspects into account in a "big picture approach" rather than focusing on one single piece of the puzzle. The stimuli of blood-brain barrier transporter, enzyme and tight junction regulation in epilepsy will have to be identified and the detailed chain of signalling events will have to be unravelled. Such information will provide novel targets and therapeutic strategies that hold the promise to advance this research field and eventually improve treatment of patients with AED-resistant epilepsy.

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6. References

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3

New Tools for Understanding Epilepsy

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1. Introduction

Epilepsy is one of the most common serious chronic neurological disorders, which is characterized by recurrent seizures with a prevalence rate of 3-6 per 1000 worldwide (Begley et al., 2007). Epilepsy is not a form of mental illness and is not an indicator of intelligence. Epilepsy affects people of any origin or race with high and low intelligence. Some people with mental retardation may have epilepsy, but most people with epilepsy are not mentally retarded (Avoli M et.al, 2001). There are many types of epilepsies and many factors such as neurotransmitters, transporters, granule cells, voltage gated ionic currents and non-neural proliferation may act as major players for this disorder (Berkovic S.F., 1998; Zaid Afawi et al, 2010). According to the literature and our work, both ligand and voltage gated ion channels in neurons and platelets seem to work together during the onset of epilepsy (Fatima Shad K (un published data); Kocsis, J.D. and Mattson, R.H., 1996). We also found similarities in the receptor's profiles of neurons and platelets mainly for serotonin, GABA and glutamate (Fatima Shad K, 2006; Fatima Shad K and Saeed SA, 2009, 2007). Recently, there has been growing evidence that serotonergic neurotransmission modulates experimentally induced seizures and is involved in the enhanced seizure susceptibility observed in some genetically epilepsy-prone animals (Methvin Isaac, 2005). Scientists are now focusing on targets other than brain receptors for the development of anti-epileptic drugs. The brain is an extraordinarily complex organ; there are several concepts about the brain which needed to be cleared when it comes to the understanding of epilepsy (Berkovic S.F., 1998). Firstly, the brain works on electricity and continuously generates tiny electrical impulses in an orderly pattern. Secondly, loss of nerve cells may contribute to the development of epilepsy in some cases. For example, prolonged lack of oxygen may cause a selective loss of cells in the hippocampus, which may lead to epilepsy. According to one theory, epilepsy is caused by an imbalance between excitatory and inhibitory neurotransmitters. If the inhibitory neurotransmitters in the brain are not active enough, or if the excitatory ones are too active, then it's more likely to have seizures. Many of the new medicines being developed to treat epilepsy try to influence these neurotransmitters, by increasing inhibitory and reducing excitatory neurotransmitter's activities. Either way, the idea is to have less uncontrolled electrical activity in the brain, and therefore fewer seizures

(Steinlein, O. K. & Noebels, J. L., 2000). Another concept important in relation with epilepsy is that different areas of the brain control different functions. If seizures arise from a specific area of the brain, then the initial symptoms of the seizure often reflect the functions of that area. The right half of the brain controls the left side of the body, and the left half of the brain controls the right side of the body. So if a seizure starts from the right side of the brain, in the area that controls movement in the thumb, then the attack may begin with jerking of the left thumb or hand. Thus a seizure occurs when the brain's nerve cells misfire and generate a sudden, uncontrolled surge of electrical activity in the brain more precisely in the cerebral cortex. There are three to six layers of neurons found to be present in the cerebral cortex with only three distinct layers; present in the hippocampus, which is located in the medial temporal lobe. The majority of the cortex has 6 distinct cell layers and covers most of the surface of the cerebral hemispheres, the hippocampus which consists of three major regions: subiculum, hippocampus proper (Ammon's horn) and dentate gyrus has three layers in the later two regions and a transitional three to six layers in the subiculum. Given the fact that the basic mechanism of neuronal excitability is the action potential, a hyperexcitable state can result from increased excitatory synaptic neurotransmission, decreased inhibitory neurotransmission, an alteration in voltage-gated ion channels, or an alteration of intra- or extra-cellular ion concentrations in favor of membrane depolarization. A hyperexcitable state can also result when several synchronous subthreshold excitatory stimuli occur, allowing their temporal summation in the post synaptic neurons (Avoli M et.al, 2001).

Action potentials occur due to depolarization of the neuronal membrane, with membrane depolarization propagating down the axon to induce neurotransmitter release at the axon terminal. The action potential occurs in an all-or-none fashion as a result of local changes in membrane potential brought about by net positive inward ion fluxes.

As mentioned earlier hippocampus is involved in epilepsy and a special group of cells known as granule cells (GCs) are the focus of the epilepsy research. These cells are present in dentate gyrus region of hippocampus and are primary gateway of cortical inputs to CA3 region of hippocampus (Crawford I L 1973). GCs act as filters and protect temporal lobe seizures by upregulation of inward rectifier K^+ channels and increase leak conductance in the granule cells of dentate gyrus (Young C et.al, 2009 and Stegan M et al 2009). Repetitive firing of action potential of inter neurons exhibits diverse repetitive firing behaviour depending upon their harboring levels in the granular layer of dentate gyrus (David M.D., et al., 1997). In epileptic patients, most of granule cells display hyperexcitable, non frequency adapted cells (Dietrich D et al. 1990) which are highly susceptible for the generation of temporal lobe epilepsy. Non adapted bursting cells are characterized by their ability to generate action potential without reducing firing frequency and less after-hyperpolarization (AHP). This type of burst firing have also been recorded in inferior colliculus neurons (Tan M L et al, 2007), CA3 pyramidal neurons (Peter H et al 2008) and in spinal cord neurons (Smith M and Perrier J, 2006). The non-adapted bursting firing may depend upon calcium influx (Anthony A G and Benjamin 1984) or due to the presence of persistent sodium currents (Yunru L et al, 2004). In regular frequency adapted neurons, intracellular Ca^{+2} increases (Knopfel T and Gahwiler B H, 1992) which activates the calcium gated potassium channels (Kca). These channels gradually decrease the positivity inside the cell and reduce the firing frequency of action potential in the later stages of Sustained Repetitive firing SRF. Buster cells (type of GCs) have low Kca channel density (Podloger M and Dirk D,2006) due to which they exhibits the continuously same or shorter spike

duration throughout the pulse. Sodium and potassium channels are the main contributors of action potential and underlying cause of repetitive firing pattern (Joshua B C, et al 2000). In buster GCs of dentate gyrus, amplitude and firing frequency of action potential increases in response to number of pulses. Na^+ and K^+ voltage-gated channels change their voltage dependency for activation and inactivation in response to sustained repetitive firing (SRF). Inactivation of sodium channels is significantly modulated by growth factor (Chuan-ju L et al, 2003) and chronic compression (Zhi-Jiang H and Xu-Jun S, 2008). Similarly the potassium channel activation and inactivation are modulated by cAMP (Jian M et al, 1999), potassium channel-interacting proteins KChIPs (Frank A W et al , 2000) growth factor and tyrosine kinases (Mark B, 1997). Literature indicated that sustained repetitive firing can be good electrical tool to find out about the underlying cause of epilepsy. The present study was therefore undertaken to establish the role of underlying currents in SRF as to establish the importance of invitro models in understanding the mechanism of epilepsy. This is our recent unpublished data, exhibiting the interesting findings of the effect of repetitive stimulation on neuronal excitability and on the kinetics of voltage dependent sodium and potassium channels. We used postnatally cultured granule cells from dentate gyrus region of hippocampus from 1-5 day old rat pups to study the role of SRF in regulating voltage gated Na^+ and K^+ currents. These voltage gated monovalent cationic currents were recorded by patch clamping "control" and SRF-induced "epileptic cells" in whole cell configuration. We then measured the changes in the activation and inactivation of both channels before and after SRF in granule cells of dentate gyrus and observe a novel role of "window currents". Overlapping of channel activation and inactivation is referred as window current; a sustained current which is observed if a fraction of channel opens (Popen>0) when the probability for their inactivation is not zero. Here we report that the repetitive depolarizing stimuli alters the kinetics and gating of Na+ and K+ channels and bring the granule cell membrane to more hyper polarized potential. These electrical changes in the membrane of cultured granule cells may partially responsible for the better survival of granule cells in excitotoxic conditions. This data may put some light on the underlying homeostatic mechanism in epilepsy mainly in temporal lobe epilepsy.

2. Material and methods

2.1 Chemicals

All chemicals were purchased from Sigma (USA) and salts from Applichem (Denmark), except D-AP5, TTX, and CNQX disodium salt from Ascent Scientific (Weston-Super-Mare, United Kingdom) and KCl from Merck (Darmstadt, Germany).

2.2 Hippocampal neuronal cell culture

Animals were sacrificed for culturing post natal neurons in accordance with the guide lines of the animal ethic committee at the Panjwani Center for Molecular Medicine and Drug Research (PCMD), University of Karachi, Pakistan after getting animal ethic approval. Hippocampal dentate gyrus cells were cultured as described previously (Fatima Shad and Barry PH, 1998). Briefly, dentate gyri from both hemispheres were isolated under dissecting microscope from 1-5 days old specific pathogen free (SPF) Wister rats and plated at a density of 1×10^5 cells/cm^2 onto three 22 mm rounded cover slips previously coated with poly-L-lysine (0.05 mg/ml), and were placed in a 100 mm cell culture dishes. Cultures were

maintained at 37ºC in a humidified 5% CO_2 incubator and were fed twice a week with DMEM (Dulbecco's Modified Eagle's Medium) plus containing 85ml. DMEM, 1ml. L glutamine, 1ml. Fungizone, 2ml. Streptomycin/Penicillin, 1ml. sodium pyruvate and 10ml. Fetal Bovine Serum. The neuronal cultures were used from the fourth day for the patch clamp experiments.

2.3 Patch clamp recordings

One of the author (FSK) is using patch clamp technique for last more than twenty years, and the present method was a modified version of one of her earlier papers (Fatima Shad K and Barry PH, 1992). Briefly, one polylysinated 22 mm round cover slip with cultured cells grown on it was fitted in the bath chamber (1 ml. capacity) and was mounted on the stage of an inverted microscope (Nikon TE2000-U, Tokyo, Japan). Cells were perfused continuously with Artificial Cerebrospinal Fluid (ACSF) containing (in mM): 126 NaCl, 3.5 KCl, 25 $NaHCO_3$, 1.2, NaH_2PO_4, 2 $CaCl_2$, 1.3 $MgCl_2$, 11 glucose, and pH (7.3), and osmolarity was adjusted to 325 ± 5 mOsm with sucrose. Patch pipettes were pulled from borosilicate glass capillaries using a Brown-Flaming P-97 electrode puller (Sutter Instruments, USA) with a diameter 2-3 µm. After fire polishing using micro forge, pippetes were filled with a solution containing (in mM): 145 KCl, 10 NaCl, 10 EGTA, 1 $MgCl_2$, 2 $CaCl_2$ and 10 HEPES, (pH 7.2) and had a tip resistance of of 4 to 5 MΩ. Osmolality was adjusted to 300±10 mOsm with sucrose. Patch clamp recordings were carried out using HEKA EPC 10 dual pre amplifiers (HEKA Instruments, Inc., New York.) placed on coarse adjustment plates of automated micromanipulators (Scientifica) at either side of the microscope. Data was recorded and analysed using PatchMaster, and temperature was maintained at 25±0.5 by TC-20 temperature controller (ALA Scientific Instruments). Data was filtered with values of 8 and10 kHz and digitized at 10 and 20 kHz for current and voltage clamp respectively. Membrane capacitance (Cm) was recorded by using built in LockIn software after calculating the cell size and getting the current density. The offset potential was canceled before patching the cell and was re-checked after each recording for any drift. Leakage currents, determined by application of small hyperpolarizing commands were substracted automatically from the response.

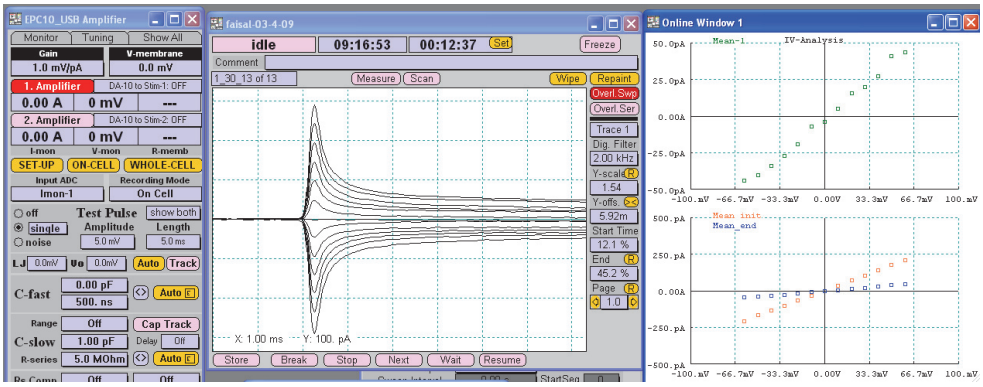
Most pharmacological experiments for the isolation of sodium and potassium currents were conducted in the presence of tetrodotoxin (TTX) and synaptic blockers, that is, D (-)-2-amino-5-phosphonopentanoic acid (D-AP5), 1, 2, 3, 4-tetrahydro-7-nitro-2,3-dioxoquinoxaline-6-carbonitrile disodium (CNQX), and picrotoxin (PTX). In K^+-replacement experiments, cells were first recorded with normal intracellular solution and then with K^+-free intracellular solution. In these cases K+ was replaced with equimolar tetraethyl ammonium (TEA) in the ACSF and the pipette solution contained (in mM): 135 TEACl, 20 CsCl, 0.1 EGTA, 2 $MgCl^2$, and 2 Na^2ATP (pH 7.28 adjusted with TEA hydroxide). Chemicals such as CNQX, D-AP5, TEACl, and TTX were kept in H_2O stocks at 20ºC and PTX was kept in dimethylsulfoxide (DMSO) stocks at 20ºC. Final concentrations were diluted freshly in oxygenated recording ACSF (DMSO 1:1,000) and subsequently kept in syringes of perfusion system (Octaflow, ALA Scientific, U.S.A.) under nitrogen pressurized at 1.3–1.6 bar before bath application.

Whole cell recording of GCs were obtained both in current and voltage clamped mode after checking their resting membrane potential (RMP). Only those cells having RMP more negative than -50 mV were used. Both inward and outward currents were observed in response to + 60 to – 60 mV pulses of 10 mV steps.

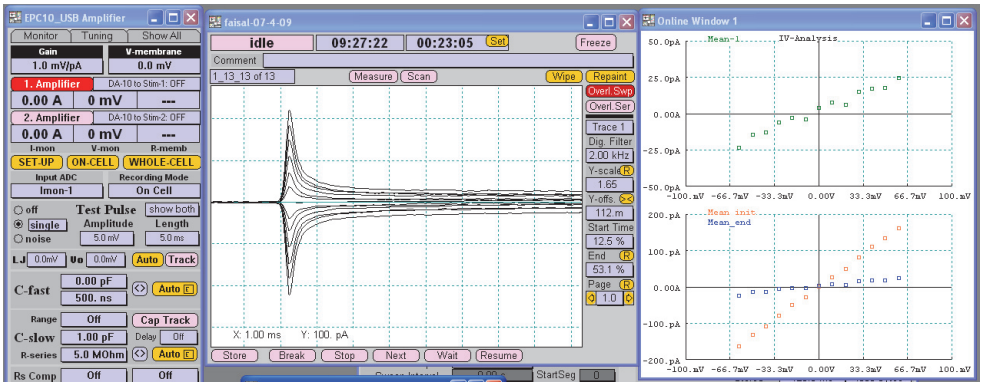
Following diagrams display some raw data to create more understanding about the process of patch clamping and how the windows of the HEKA EPC10 amplifier looks like and what are the parameters constantly regulated by the amplifier itself. Please note the gain values as well as C-fast and C-slow values (fast and slow capacitative transients) automatically adjusted by the amplifier along with liquid junction potential and series resistance.

Symmetrical Sodium solution in response to voltage steps from -60 mV to +60 mV

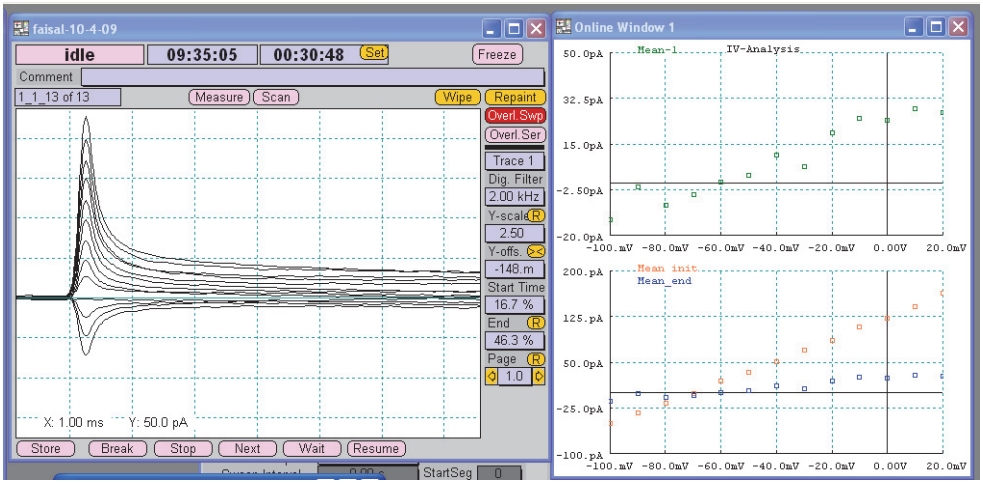
Cell 1



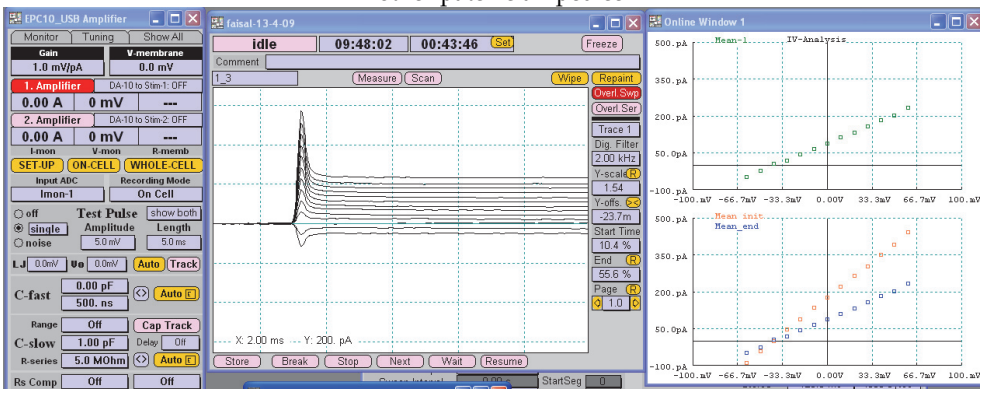
Cell 2

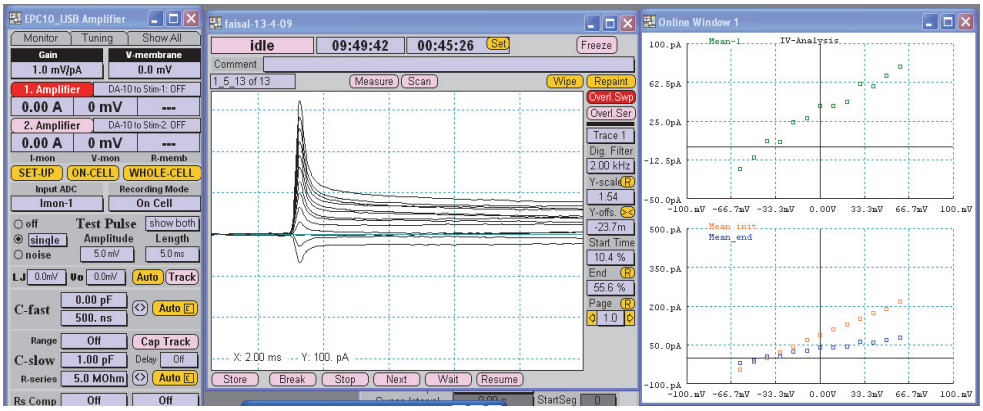


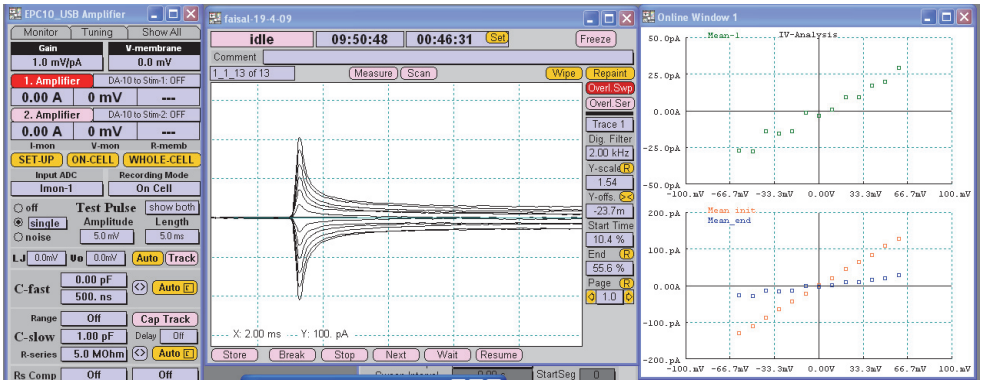
Low sodium



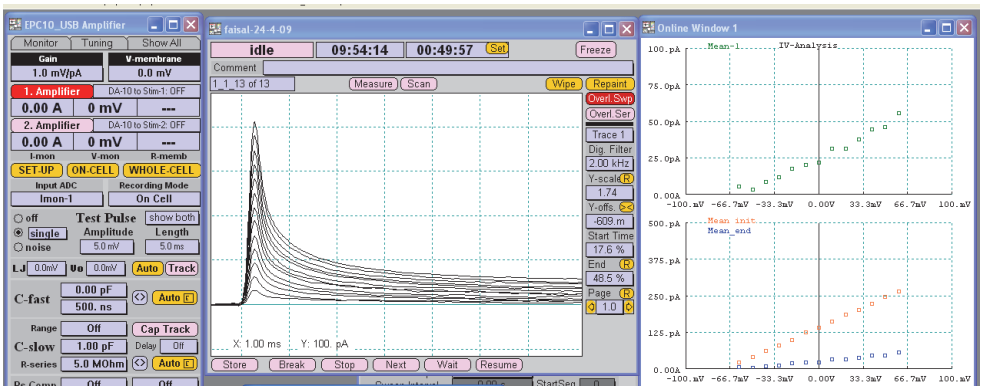
Another patch clamped cell

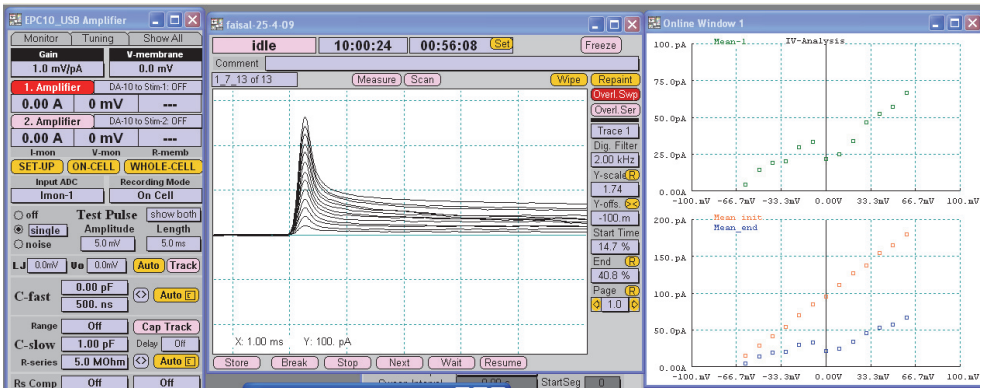






Absence of inward currents





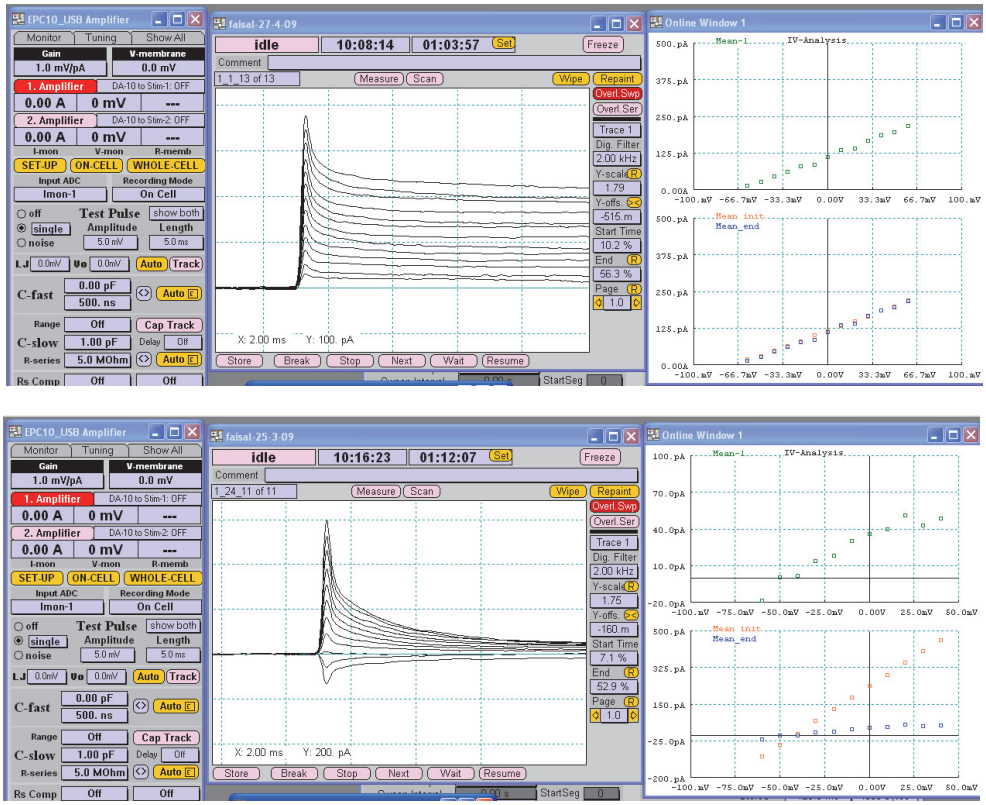
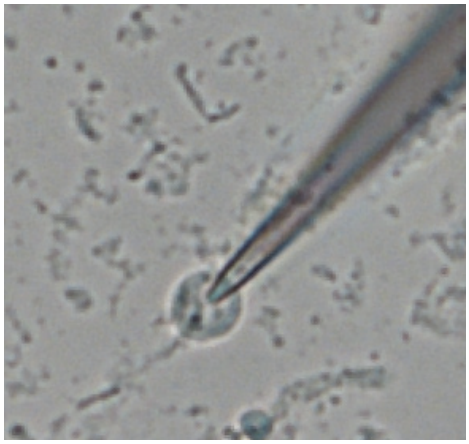


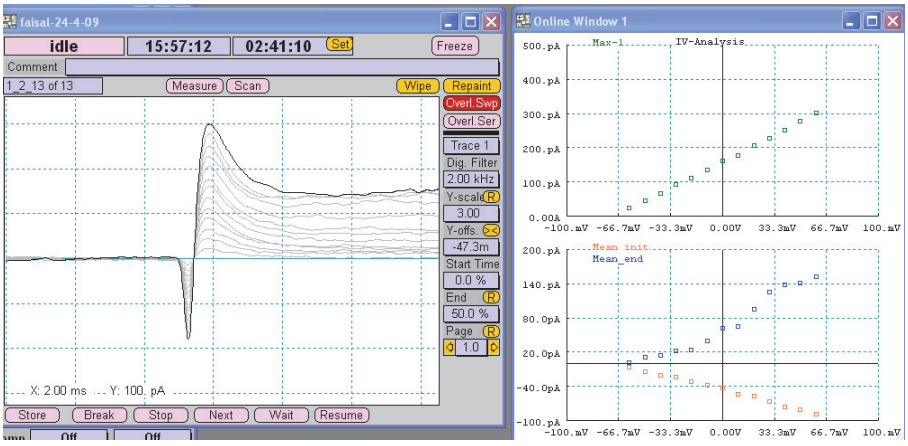
Fig. 1. In the above figures (A-J) presenting raw data (directly from the computer) simply exhibiting that how different cells behave differently and how changes in ionic concentration changes the inward and outward deflection of currents. Literature indicated a variety of approaches for in vitro modeling of epilepsy mainly temporal lobe epilepsy. One can design an epileptic model by using chemical (absence of Mg) or electrical (SRF) tool. These artificial epileptic models are capable of mimicking the silent features of this pathology and can be tested for novel therapeutic agents but with great caution during their interpretation.

We thought it will be beneficial for non expert readers to see how a cell can be patched and how the values of transient and steady state currents look like in response to voltage pulses. One can use the following values to draw a current voltage graph as well as the following equation can be used for practice to see which ion is more permeable for the non selective cation or anion channel. Erev = is the voltage at which the concentration of that particular ion is equal in ECF and ICF and after this voltage the ion will change its direction, z is the valency and $[A]_o$ are the [A]i are the concentration out side and inside. PA and PB are the permeability of ion A and ion B

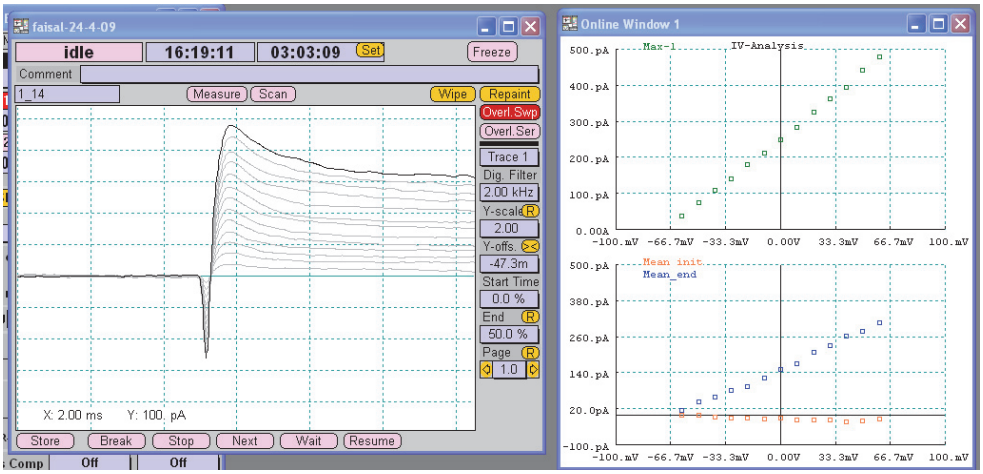
Sodium currents (Number of Sodium channels, permeability of Na ions per unit change in voltage and reversal potential of the sodium or any other ion can be calculated using the following equation)

$$Erev = RT/zF \ln PA [A]_o / PB [A]i$$

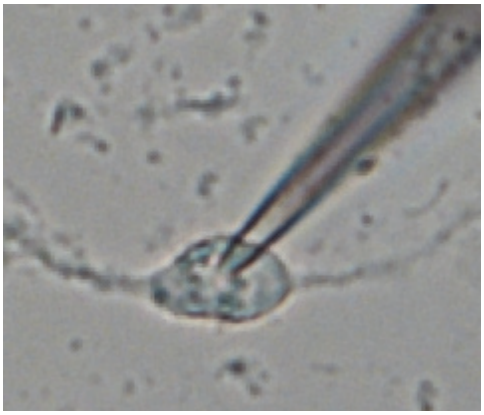


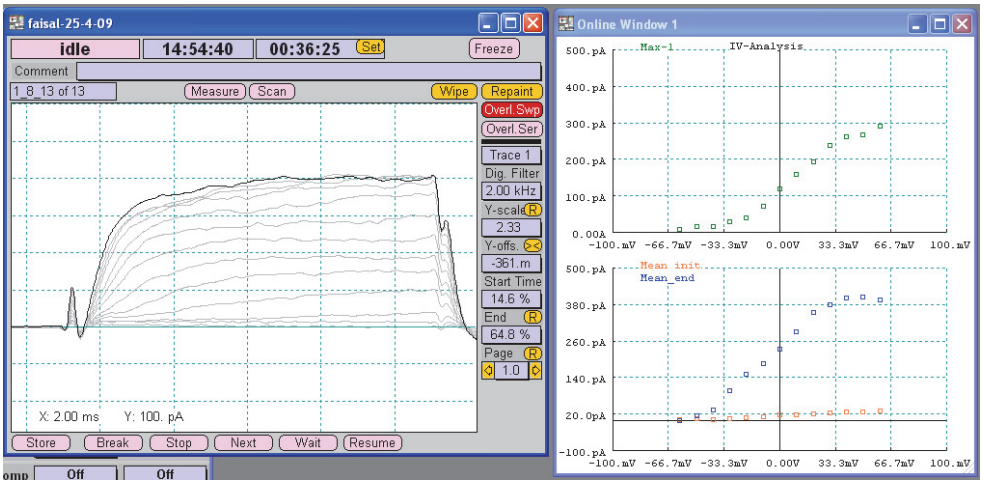


#	Holding Potential (mV)	peak (o) I (pA)	inward I (pA)	Persist (o) I (pA)	Time to peak (i) µ sec.
1	-60	22.567	-6.9942	2.0444	265.21
2	-50	45.241	-13.975	11.293	276.52
3	-40	65.281	-20.338	13.925	275.34
4	-30	91.400	-23.784	22.002	273.24
5	-20	110.64	-31.609	23.278	272.97
6	-10	135.58	-38.035	39.726	275.55
7	0	161.08	-42.521	61.990	274.19
8	10	176.76	-53.981	65.602	275.32
9	20	205.64	-57.132	95.058	278.12
10	30	227.72	-66.613	125.31	275.80
11	40	250.62	-76.349	137.64	277.57
12	50	278.18	-81.710	140.65	277.32
13	60	300.42	-88.700	152.24	276.56



#	Holding Potential (mV)	peak (o) I (pA)	Inward I (pA)	Persist (o) I (pA)	Time to peak (i) sec.
1	-60	37.013	-1.3052	15.280	500.00
2	-50	72.857	-1.6571	41.372	500.00
3	-40	107.55	-7.0724	59.973	500.00
4	-30	140.62	-11.647	80.917	500.00
5	-20	179.49	-9.6198	93.364	500.00
6	-10	211.62	-14.327	122.26	500.00
7	0	249.49	-12.186	149.24	500.00
8	10	282.44	-15.985	170.72	500.00
9	20	324.09	-17.815	207.99	500.00
10	30	363.06	-16.406	230.13	500.00
11	40	394.45	-23.742	261.85	500.00
12	50	442.30	-20.464	277.07	500.00
13	60	478.56	-13.076	306.75	500.00





#	Holding Potential (mV)	peak (o) I (pA)	inward I (pA)	Persist (o) I (pA)	Time to peak (i) sec.
1	-60	7.5033	1.7111	-84.261	249.24
2	-50	15.568	5.0703	13.233	258.98
3	-40	16.076	1.9596	34.109	253.96
4	-30	27.778	5.7529	96.613	252.26
5	-20	39.568	9.0225	149.87	253.74
6	-10	71.728	9.6337	184.22	250.65
7	0	119.43	16.273	231.95	249.77
8	10	158.85	18.046	290.64	248.82
9	20	193.04	21.480	353.00	248.69
10	30	239.25	25.096	379.17	252.02
11	40	261.33	27.338	403.03	251.91
12	50	267.82	26.792	404.47	252.70
13	60	290.25	31.005	395.27	249.12

Fig. 2. In the above table and figures bipolar cells were patch clamped and currents were measured in response to the voltage pulses. Despite of the fact that both cells used were similar and pulse protocol applied was exactly the same but the responses were quite different for both transient and sustained currents, indicating the existence of heterogeneity of the current population in each cell. This again warns us for the presence of significant differences in the same type of cells experiencing the similar electrical stimulus.

Basically these diagrams and tables are given for those who are interested in practicing for making current voltage (I/V) curves for the understanding of the phenomenon of normal impulse as well as the mechanism of epileptic train.

We hope by now its clear that sodium and potassium are the major cations involved in the process of action potential and a high frequency series of action potentials are knows as epileptic episodes. Both Na and K gated currents go through the process of activation and inactivation. These currents when recorded before and after the application of SRF gives two

very different kinetic profiles and this information will be helpful in detecting the cell membrane status its protein channel and their integirity. To analyze the voltage dependence of channel activation, the conductance (G) was calculated by using the equation:

$$G = I/ (Vm-Vrev)$$

Where I is the peak current, Vm is the test pulse voltage and Vrev is the reversal potential of under study ion. Conductance was plotted against Vm and fit to a Boltzmann distribution equation:

$$G = Gmax / (1+exp (V_{1/2}- Vm)/k)$$

Where Gmax is the maximum conductance, $V_{1/2}$ is the potential at which activation is half maximal, and k is the slope of the curve.
For the estimation of channel inactivation, the inactivation parameters were fitted to Boltzmann distribution equation:

$$I / Imax =1 / (1+exp (V_{1/2}- Vpre)/k)$$

Where Imax is the maximum current after the most hyperpolarized prepulse, the Vpre is the prepulse potential, $V_{1/2}$ is the potential at which inactivation is half-maximal.

2.4 Statistics

Significance of differences were tested by Student's *t*-test with $P < 0.05$ considered to be significant.

3. Results

Dentate gyrus granule cells of hippocampus were plated at a concentration of 1×10^5 cells/cm^2 per plate (containing three 22 mm round cover slips). These cells were identified mostly to be granule cells (*Fig.3B right panel). Only shiny healthy intact cells were used for patch clamp experiments. All granule cells of dentate gyrus have similar passive membrane properties (Table. 1) and are of very homogenized morphological properties.
(*Though Fig 3 was suppose to be Fig 1 of the result section)

	Pre SRF	**Post SRF**
Input Resistance (MΩ)	263.1±25.2	192.6±30.6
Resting Membrane Potential, RMP (mV)	-63 ±5.3	-62 ±4.7
Threshold for action potential (mV)	-49±2.6	-42±6.6
Spike Amplitude (mV)	90±10.4	72±12.2
No of Spikes in the burst	15±2	11±3
After hyperpolarization, Amplitude (mV)	1.1±0.2	1.8±0.3

Table 1. Membrane properties of granule cells of mammalian dentate gyrus

After patch clamping the cells for the measurment of their passive membrane properties and their morphological features. We have decided to use them for our in vitro model of epilepsy. For that purpose we have used an electrical tool called Sustained repetitive firing (SRF), a protocol of depolarizing pulses of 0.5 nA amplitude with 500 ms duration and of 0.3 Hz frequency as to mimic epileptic burst in the cultured granule cells. We have used HEKA amplifier pulse protocol program to generate SRF and introduced it into the clamped cell through the recording electrode. In response to SRF pulse, patched cell exhibit regular and continues series of action potentials mimicking an epileptic episode as been observed earlier (DeLorenzo RJ et al, 2000). The duration and frequency of SRF pulses were remains constant for all experiments. Those cells which were patched in whole cell configuration (with out any SRF) were considerd as "control cells". The patch clamped cultured neuronal cells were also tested to confirm that the SRF induced hyperexcitability effect is activity and not time dependent. For testing this phenomenon, the cell was patch clamped in whole cell configuration without any stimulus for a duration of approximately eight seconds (equivqlent to the time of three depolarized pulses) then the SRF pulses were injected (Fig. 3).

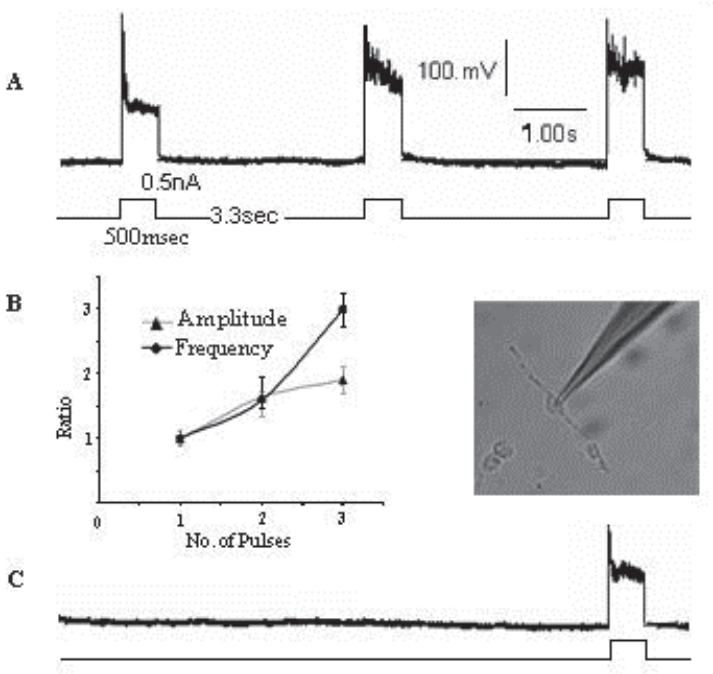


Fig. 3. Effect of SRF on frequency and amplitudes of action potential in granule cells Spike amplitude and firing frequency of action potentials were increased as a result of SRF depolarizing pulses of 0.3 Hz. **A**: In response to sustained repetitive pulses of 0.5 nA, a train of action potential was observed in a patch clamped granule cell in the current clamp mode of whole cell configuration. **B:** exhibits the relationship between the number of pulses with spike amplitude and frequency, inset showing a patch clamped granule cell. **C**: demonstrates the voltage response of SRF after a delay of 8 sec confirming that the increase in amplitude and frequency of action potential is activity not time dependent.

After calculating the activation and inactivation kinetics by using the formulas mentioned earlier, we have found that the value of half maximum activation $V_{1/2}$ for Na+ was -25.8±3.2 mV before and increased to-29±4.1 mV after SRF. Whereas for Ksus (sustained K currents) values were -30 ± 4.3 mV before and was changed to -33 ± 5.1 mV after SRF (Fig. 4A) but transient potassium activation $V_{1/2}$ = -30.4 ± 2.8 mV significantly get changed to -41 ± 5.2 mV after SRF. The transient potassium current (K_t) was not decreased in the presence of 10 mM tetraethylammonium (TEA) in the extra cellular solution but 4AP in the bath reduces this current up to 75%.

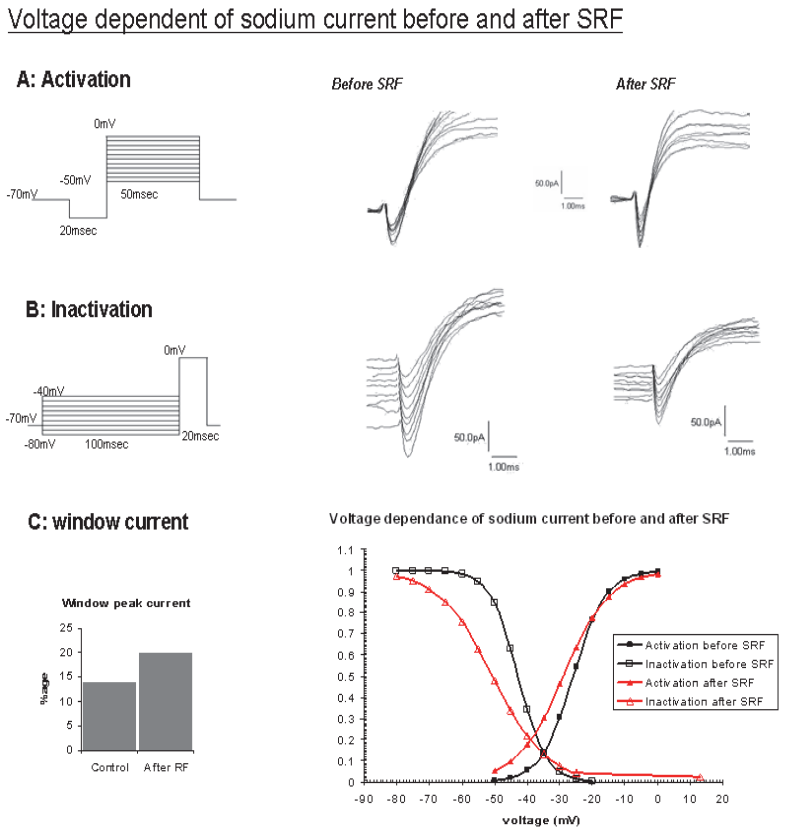


Fig. 4. **A**: Left hand panel exhibits activation pulse protocol (from -50mv to 0mV) and right hand graph shows Na+ activation current response before and after SRF **B:** Steady state inactivation pulse protocol on the left before and after SRF current recordings on the right. **C:** Left panel shows voltage dependency of Na+ activation and inactivation currents. Right panel shows histogram of changes in window currents before and after SRF, drawn from the activation-inactivation curves**.** Peak window current was observed at -35mV which was shifted to -39mV after SRF. $V_{1/2}$ for activation before SRF was calculated to -25.8mV and for inactivation -42.7mV which were also shifted to -29mV and -50.6mV respectively after SRF.

Similarly $V_{1/2}$ mV values of Na+ inactivation = -42.7±6.6; Kt = -68.5±5.6; and Ksus = -58±5.6 are markedly shifted towards –ve potential, Na+ = -50.6±7.4; Kt = -75.6±8.2; Ksus = -63±9.4. These changes in voltage dependency of activation and inactivation currents results in an increase in sodium and decrease in potassium window currents. (Table 2)

	Activation		**Inactivation**				
	$V_{1/2\ (act)}$	**$K_{(activation)}$**	**$V_{1/2\ (inactivation)}$**	**$K_{(inactiva-tion)}$**	**Window Peak-Volt**	**Window range**	**Window Peak**
	(mV)		*(mV)*		*(mV)*	*(mV)*	*%*
Sodium Current							
Before SRF	-25.8±3.2(12)	5±0.5	-42.7±6.6(8)	4.2±1.1	-35	-45 to -25	14
After SRF	-29±4.1(11)	7.2±0.6	-50.6±7.4(6)	8.3±0.9	-39	-54 to -20	20
Potassium Transient							
Before SRF	-30.4±2.8(10)	6.6±0.8	-68.5±5.6(9)	7±1.2	-51	-60 to -40	10
After SRF	-41±5.2(10)	5±1	-75.6±8.2(9)	8±1.3	-55	-60 to -45	8
Potassium Sustained							
Before SRF	-30±4.3(10)	8.9±1.1	-58±5.6(9)	11±1.5	-35	-60 to -10	20
After SRF	-33±5.1(9)	8±0.6	-63±9.4(8)	9.2±1.3	-39	-60 to -20	15

Table 2. Voltage dependence of activation and inactivation before and after SRF in mammalian granule cells.

$V_{1/2(act)}$: is the value of the voltage at which membrane reaches 50% of the maximum activation state
$V_{1/2}$(inact): Is the value of the voltage at which membrane reaches 50% of the maximum inactivation state
K(act): slope of the activation curve
K(inact): slope of the inactivation curve
Window peak volt: Voltage at which window current reaches its peak.
SRF: Sustained Repetitive Firing
The number in the parenthesis indicate the number of experiments
P<0.01 indicate significant differences compared with control group
These cells show non adapting firing frequency in response to superathreshold (0.5nA) current pulses (Fig.5A).

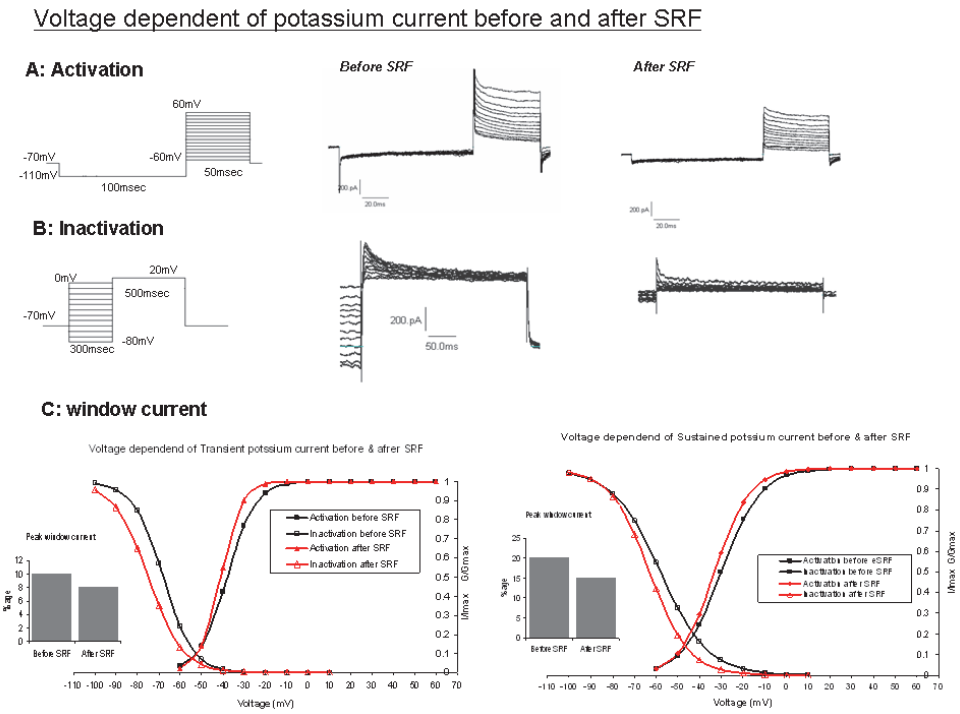


Fig. 5. This figure exhibits both sustained and transient potassium currents **A:** Activation pulse protocol is on the left, control current recording in the middle and after SRF in the right panel **B:** Exhibits (from left to right) inactivation pulse, control and after SRF current responses, for potassium channels. **C:** Voltage dependence of activation and inactivation of transient and sustained potassium currents. Histogram (in the inset) shows percentage of window currents before and after SRF. Amplitude of window current for transient potassium was at -51mV which was shifted to -55mV and sustained peak window current was shifted from -35mV to -39mV after SRF indicating voltage dependency of both type of potassium currents.

For measuring the activity dependent increase in amplitude and firing frequency, we applied three consecutive current pulses at 0.3 Hz (inter pulse duration of 3.3 sec). We observed that the amplitude and frequency of spikes in the next pulse are greater then previous one (Fig. 5C). Amplitude and frequency increased with the ratio of 1.0:1.6:1.9 and 1.0:1.6:3.0 for three pulses respectively. Ratio of amplitude and frequency at the second pulse (after 4 sec) is almost equal but during third pulse (after 8 sec), the ratio for frequency after SRF get increase significantly (Fig. 5B). Amplification of amplitude and number of spikes were not found to be present when only one pulse was given for the same duration of the three pulses (approximately 8 sec) indicating that these changes in spikes were not time but activities dependent (Fig. 5D).

4. Discussion

The present study investigated the electrophysiological profile of granule cells and role of voltage gated Na+ and K+ channels in response to sustained repetitive firing. Amplitude and firing frequency of action potential for cultured granule cells were increased with increasing number of SRF pulses. We observed the voltage dependency of activation and inactivation of both Na+ and K+ currents after repetitive firing indicating both ionic currents have some underlying role in the modulation of action potential train in resposne to SRF. We have observed that both Na^+ and K^+ inactivation traces become less leaky after the exposure of SRF. We also observed that Na^+ channel inactivation and K^+ current activation has been shifted to more negative values indicting the reduce excitabilities in these cells after sustanined repetitive firing. Similar observation was also been made by (Christina C et al 2009 and Stegan M et al 2009) and ascribed that granule cells become leaky with Decreasing severity of temporal lobe epilepsy. Thus similar to our conclusion both authors suggested that seizures triggers expression of different neuroprotective reactions, including upregulation of K^+ channel density.

Literature also indicated that the levels of Ca^{+2} may gradually get increased intracellularly in response to SRF. Increased intracellular Ca^{+2} levels then may triggers the calcium gated potassium channels inducing after-hyperpolarization (AHP) in normal frequency adapting cells. Because of the low density of calcium gated potassium channels in granule cells of dentate gyrus, less adaptation of firing frequency may observed in some cases. Decrease levels of extracellular calcium due to repetitive firing results in sodium persistent current (I_{NaP}) (Hailing S et al 2001). I_{NaP} is capable of amplifying a neuron's response to synaptic input and enhancing its repetitive firing capability (Carl E S 2007). These granule cells are thought to play an important role in electrical signaling, in neuronal synchronization, and rapid information transmission (Adam K et al 2002) and their number found to be increased in the epileptic hippocampus. Similarly, increase intracellular calcium levels results into many changes in the cell such as increased cytosolic cAMP, which inturn significantly increases persistent sodium currents (Bibiane F et al, 1997) as well as inducing prolonged neuronal plasticity (Nikitin ES et al 2006). Literature indicated that sodium inactivation gates are involved in the repetitive firing response and the impairment of the inactivation results in an increase in whole-cell persistent current (Kristopher M.K. et al, 2006). PKA phosphorylate the inactivation gates of sodium channels which in turn prolong their activation state and enhances repetitive firing. β1 subunit of the Na+ channels seems to be involved in the modification of gating properties (Earl PD et al 1994). Similarly β4 subunit of potassium channel observed to be participating in reducing dentate gyrus excitability and

resultant protection against temporal lobe seizures (Robert B et al 2005). When β4 subunits of potassium channel get phosphorylated they alter the channel conductance and decrease the efflux of potassium and hence resultant increased positivity inside the cell. Our findings of shifting of window currents of granule cells to hyperpolarized voltages may mimic in vivo condition during epileptic seizures. Our results also reveals a complex dynamics behind repetitive spike discharge and suggests that a persistent Na^+ current plays an important role in action potential initiation and in the regulation of dentate gyrus granule cells transmission. The resting leak conductance was found to be doubled in epileptic granule cells and roughly 70–80% of this difference was sensitive to K+ replacement. SRF exposed "epileptic granule cells" had strongly enlarged inwardly rectifying currents with a low micro molar Ba^{2+} sensitivity. Further investigations needed to be done in this regard. Beside SRF in conjunction with cultured granule cells, we have other tools in hand as to explore underlying mechanism of epilepsy and its seizure type. Our initial experiments on platelets from epileptic patients suggested that peripheral benzodiazepine receptors of platelets could be a novel and effective therapeutic target.

5. Acknowledgement

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4

Epilepsy: Selenium and Aging

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1. Introduction

Epilepsy affects nearly 50 million people worldwide and is a disease that involves spontaneous frequent seizures caused by electrical disturbances in the brain (Bergen 1998; Meinardi, Scott et al. 2001). Epilepsy is a syndrome, or a collection of symptoms, in which people are predisposed to seizures (Fisher, van Emde Boas et al. 2005; Gomez-Alonso and Giraldez 2007; Jaseja 2009). Epilepsy diagnosis usually occurs after permanent injury to the brain or from inborn errors that cause brain tissue to become abnormally excited (Fisher, van Emde Boas et al. 2005). Health statistics show that children less than 2 and adults older than 65 are the most likely populations to develop epilepsy and that seizure events increase the risk of mortality in the individual (Feng 1978; Tsai 2005; Fountain, Van Ness et al. 2011). It is thought that nearly 4% of the US population will have seizure activity in their lifetimes, with many having seizures and never being aware they occurred.

In some types of epilepsy, the cause of the seizures cannot be clearly defined and does not present with other neurological disorders (Lerman, Sagie et al. 2011). Certain types of epilepsy have a genetic component (Poduri and Lowenstein 2011; Sisodiya and Mefford 2011; Sozmen, Baybas et al. 2011; Wilke, Worrell et al. 2011), but the most common diagnoses of epilepsy occur after neurological damage such as a stroke, transient ischemic attacks, a diagnosis of dementia, traumatic brain injury, brain abscesses, meningitis, encephalitis, neurosyphilis, brain tumors, hematomas, abnormal blood vessels, AIDS, congenital brain defects, metabolic diseases such as phenylketonuria, and liver or kidney failure.

Epileptic seizures range in severity from staring spells to violent convulsions and loss of consciousness (Haut, Lipton et al. 2005; Maillard, Vignal et al. 2009). Given the wide variety of factors that can precipitate epilepsy, it stands to reason that type of seizure is dependent on the location and type of the brain injury. Upon physical examination, many people suffering from epilepsy appear healthy (Maganti, Gerber et al. 2008). Most epileptics will present with clinical abnormal electrical activity as measured by an electroencephalograph (Urbach 2005). Epilepsy is further classified as idiopathic (presumed genetic basis), symptomatic (due to a structural abnormality) or cryptogenic (cause is undetermined) (Kwan and Brodie 2000; Berg and Kelly 2006; Farrell, Wirrell et al. 2006).

Unfortunately, after some epileptic events, there is a risk of neuron destruction. This is especially concerning due to the limited capacity of the brain to regenerate neurons and oftentimes, the damage is permanent. Following a seizure, epileptic's brains often form excitotoxic lesions which destroy neurons by initiating apoptosis. The current treatment for epilepsy is limited to controlling the seizure activity. Anticonvulsants are oral medications used to prevent seizures in patients with epilepsy. The specific drug prescribed to patients

varies based on their past seizure history and any underlying conditions that may have contributed to the epileptic diagnosis. (Prunetti and Perucca 2011; Steinert, Baier et al. 2011; Strzelczyk, Cenusa et al. 2011; Verrotti, Loiacono et al. 2011; Yasuda, Sugiura et al. 2011). In patients with epilepsy resulting from chronic infections, the epileptic symptoms will subside after the course of drug treatment. Also, epilepsy occurring after tumor development or following injury to blood vessels, such as a hematoma, can be successfully managed in some cases after the underlying cause of injury is treated. Nevertheless, it is difficult to treat these types of brain injuries; so many patients use proper medication to prevent the seizures (Sander 1993).

The cases of epilepsy that do not respond to medication (Go and Snead 2008; Berg 2009; Nakken and Tauboll 2009), are often candidates for invasive and risky procedures such as brain surgery. Surgery can remove the damaged cells of the brain which cause the abnormal electrical signals. Other surgical procedures such as implantation of a device, similar to a heart pacemaker, stimulate the vagal nerve and help reduce the frequency of seizures (Landre 2004). Unfortunately, there are also types of epilepsy that cannot be managed well with medication or any other available therapies and the general term for this type of epilepsy is called refractory or intractable epilepsy (Sander 1993; Kwan and Brodie 2000; Lerman, Sagie et al. 2011). Growing lines of evidence have indicated a role of inflammation in causing and exacerbating intractable epilepsy (Vezzani, French et al. 2011). Patients with intractable epilepsy often go on specialized diets which are thought to help reduce the severity and number of seizures. Popular diet choices for epileptic patients are diets low in carbohydrates, such as the ketogenic diet (Kossoff and McGrogan 2005), which is very low in carbohydrates and very high in fat but still calorie balanced for the individual. For undetermined reasons, when the body of an epileptic burns fat for fuel, rather than glucose, it can help control and prevent seizures (Baranano and Hartman 2008; Choragiewicz, Zarnowska et al. 2010; Westmark, Westmark et al. 2010). While the ketogenic diet is effective, long term health implications prevent patients from being on the diet for periods longer than two years (de Kinderen, Lambrechts et al. 2011). Dietary antioxidants are known to protect against seizures possibly by increasing the free radical scavenging capabilities of key antioxidant enzymes in the brain (Garjani, Fathiazad et al. 2009; Mehla, Reeta et al. 2010; Militao, Ferreira et al. 2010; Pages, Maurois et al. 2010; Sharma, Nehru et al. 2010; Tome Ada, Ferreira et al. 2010). Using a nutrigenomics approach to identify compounds with a beneficial effect (Suzuki 2011), researchers can identify genes associated with epilepsy susceptibility and then test whether particular dietary components, in this case selenium, can be used to improve the incidence, frequency, and severity of epilepsy and the resulting complications.

2. Selenium and selenoproteins

Selenium is a non-metal trace element discovered in 1817 as a by-product of sulfuric acid production (Brown and Arthur 2001). Selenium exists in nature in organic (such as selenomethionine and selenocysteine) or inorganic (such as selenate and selenite) forms (Puzanowska-Tarasiewicz, Kuzmicka et al. 2009).

Selenium can be incorporated non-specifically into methionone-rich proteins, such as those in the Brazil nut and sunflower seeds (Kortt, Caldwell et al. 1991). The biological functions of selenium are mainly mediated by selenoproteins, which contain selenocysteine (Allmang, Wurth et al. 2009; Lu and Holmgren 2009). Selenocysteine (Sec) has its own tRNA and its own

codon, UGA (de Jesus, Hoffmann et al. 2006). Sec is inserted into mRNA in response to UGA codons in the genome of all selenoproteins. Selenoprotein mRNA is characterized by selenocysteine insertion sequence element (SECIS) (Howard, Moyle et al. 2007; Mix, Lobanov et al. 2007), and a RNA-binding protein complex (Bock, Forchhammer et al. 1991; Small-Howard and Berry 2005; Allmang and Krol 2006; Squires and Berry 2008). Some selenium-containing proteins such as the liver protein 56K and the liver fatty acid binding protein (Behne, Weiss-Nowak et al. 1994) can sequester Se, but they do not contain selenocysteine.

Selenium was only recognized as a toxicant (Belogorsky and Slaughter 1949; Fels and Cheldelin 1949; Klug, Harshfield et al. 1952; Mc and Portman 1952; Fabre and Truhaut 1956) and its biological role was unknown until 1952 when its essential role was discovered in microorganisms. It is a trace mineral that is essential for humans (Papp, Lu et al. 2007), which was first recognized when when dietary supplementation of selenium prevented liver necrosis in rats efficiently (Mertz and Schwarz 1958; Schwarz, Stesney et al. 1959; Schwarz, Porter et al. 1972). Following these studies, it was found that selenium was essential for glutathione peroxidase (GPX) activity (Flohe, Gunzler et al. 1973; Rotruck, Pope et al. 1973). Sec, now identified as the 21st amino acid was indentified in eubacteria and archaebacteria model systems (Ambrogelly, Palioura et al. 2007; Xu, Carlson et al. 2007). Stadtman and colleagues found that selenium was incorporated into proteins in the form of selenocysteine through labeled *Clostridium sticklandii* with ^{75}Se metabolically (Cone, Del Rio et al. 1976; Axley and Stadtman 1989; Lee, Worland et al. 1989; Leinfelder, Stadtman et al. 1989; Stadtman, Davis et al. 1989). UGA, the stop codon, was discovered for selenocysteine incorporation in murine and GPX1 gene was cloned at the same time (Chambers, Frampton et al. 1986).

Selenium deficiency (Kakturskii, Strochkova et al. 1990), or Keshan disease, is responsible for the cardiomyopathy in children living in parts of China where the soil lacks selenium (Guanqing 1979; Yang and Xia 1995; Yang, Chen et al. 2007). Recently, the gene of Secisbp2 protein, which is related with synthesis of selenoproteins (Copeland and Driscoll 1999; Copeland, Fletcher et al. 2000; Low, Grundner-Culemann et al. 2000; Tujebajeva, Copeland et al. 2000; Berry, Tujebajeva et al. 2001; Copeland and Driscoll 2001; Copeland, Stepanik et al. 2001; Fletcher, Copeland et al. 2001; Copeland and Driscoll 2002; Lescure, Allmang et al. 2002; Lescure, Fagegaltier et al. 2002), has been studied as a possible target for hereditary diseases, an example of which includes human thyroid hormone metabolism disorder (Dumitrescu, Liao et al. 2005). Selenium is also known as an important chemoprevention agent (El-Sayed, Aboul-Fadl et al. 2006; Micke, Schomburg et al. 2009) by inducing apoptosis in cancer cells (Jackson and Combs 2008) and senescence in the early stage of tumorigenesis (Wu, Kang et al. 2010) in a dose-dependent manner.

More than 50 years ago, the ROS (reactive oxygen species) theory of aging was proposed. However, there is still there is a lack of irrefutable evidence in support or opposition of this theory. ROS can be induced by exogenous sources, such as UV or ionizing irradiation (Finkel 2000), which are by-products of mitochondrial respiration and widespread *in vivo* as the form of superoxides, hydroxyl free radicals, and hydrogen peroxides (Harman, 1956). As previous studies have shown, GPX1 contributes much of the H_2O_2-eliminating activity of selenium (Cheng, Ho et al. 1997; Cheng, Ho et al. 1997; Lei, Cheng et al. 2007). Therefore, it is logical to address the ROS theory of aging through investigation of selenium and selenoproteins (Hawkes and Alkan 2010). Some studies have implied that the accumulation of lipid oxidation in mitochondria has the potential to suppress the increased activity of GPX when comparing aged and juvenile rats (Nohl, Hegner et al. 1979). However, there is

no conclusive evidence to identify the biological function of GPX1 in lifespan extension in mouse models (Ladiges, Van Remmen et al. 2009). Interestingly, $GPX4^{-/-}$ mice are embryonic lethal while $GPX4^{+/-}$ mice displays lifespan extension (Ran, Liang et al. 2007).

Most of the selenoproteins that have been identified are enzymes with selenium in their active sites. Through scanning the whole human genome to find SECIS, a total of 25 selenoproteins were discovered (Kryukov, Castellano et al. 2003), which contain five glutathione peroxidases (GPX1-4, GPX6), three thioredoxin reductases (TR1-3) (Hondal and Ruggles 2010), three iodothyronine deiodinases (DI-III), the 15-kDa selenoprotein (Sep 15), selenophosphate synthetase-2 (SPS2), and selenoprotein H, I, K, M, N, O, P, R, S, T, U, V. All selenoproteins only have one selenocysteine residue except for selenoprotein P which contains 10 residues (Burk and Hill 1999). The domains containing selenocysteine residues in selenoprotein are highly related with their enzyme activities (Hill, Zhou et al. 2007). It was noted that cysteine replacing the selenocysteine residue in selenoproteins lead to reduction of catalytic activity (Hazebrouck, Camoin et al. 2000; Lee, Bar-Noy et al. 2000; Korotkov, Novoselov et al. 2002; Kryukov and Gladyshev 2002).

Notably, more than one-third of selenoproteins play a critical role in combating oxidative stress. The GPX family uses glutathione as a reducing equivalent to eliminate hydrogen peroxide, organic hydroperoxides and phospholipid hydroperoxides (Bosch-Morell, Flohe et al. 1999; Flohe, Hecht et al. 1999). The redox status of thioredoxin is under the control of the thioredoxin reductase (TR) family (Bjornstedt, Hamberg et al. 1995; Holmgren and Bjornstedt 1995). Sel P is thought to be not only a selenium carrier, which distributes in body fluids, but capable of eliminating phospholipid hydroperoxides (Saito, Hayashi et al. 1999; Takebe, Yarimizu et al. 2002). It is important to elucidate the mechanism by which selenoperoxidases eliminate ROS in the brain and to identify additional selenoperoxidases and their functions during aging and neuron degeneration.

3. Selenium and selenoproteins in epilepsy

The brain expresses most selenoproteins and is at the apex of selenium retention in the body (Nakayama, Hill et al. 2007). It is proposed that selenium may be necessary for prevention of epilepsy or other degenerative neurological disorders; however, there is no consensus in the field whether selenium plays a direct role or not. Selenium has a role in protection the neurons from excitotoxic insults, such as the continuous stimulation of a nerve cell by glutamate or another neurotransmitter, and thus can decrease the insult burden on the neurons (Savaskan, Brauer et al. 2003).

Although no severe neurological phenotypes have been associated with a selenium deficient diet, this may be due to the brain's preferential sequestration of body selenium. Mice on a selenium deficient diet are, however, more susceptible to neuropathological changes (Hill, Zhou et al. 2004). It is not well known how selenium status in the blood and the brain are correlated (Chen and Berry 2003), but it is known that selenium supplementation can prevent dopamine loss and degeneration of neurons in the substantia nigra, and reduce lipid peroxidation (Aldeeb, Almoutaery et al. 1995; Imam, Newport et al. 1999; Zafar, Siddiqui et al. 2003). Selenium was shown to protect the neurons through selenoproteins (Savaskan, Brauer et al. 2002; Lamarche, Signorini-Allibe et al. 2004; Reeves, Bellinger et al. 2010; Wang, Geng et al. 2010) and other studies that combine selenium treatment with anticonvulsant medication showed a synergistic protective effect against induced seizures (Kutluhan, Naziroglu et al. 2009). Also, in cases of selenium deficiency, neurons are exposed

to increased glutamate-induced excitotoxicity because selenium has an inhibitory effect on the glutamate induced NF-κB and AP-1 activation (Savaskan, Brauer et al. 2002; Santamaria, Vazquez-Roman et al. 2005).

There are a total of 24 selenoproteins in mice, all of which are expressed in the brain, particularly in neurons of the olfactory bulb, hippocampus, cerebral cortex and cerebellar cortex (Zhang, Zhou et al. 2008). In these tissues *Gpx4, Sel K, Sel M, Sel W,* and *Sel 15* mRNAs were expressed the most, but in the choroid plexus over half of the identified selenoproteins were expressed. *Gpx4, Sel P, and Sel W* genes were highly expressed in over 90% of the brain, similar to findings from previous studies that found *Sel P* and *Sel W* were highly expressed in the rodent brain (Dreher, Schmutzler et al. 1997; Steinert, Bachner et al. 1998; Sun, Butler et al. 2001; Hoffmann, Hoge et al. 2007; Nakayama, Hill et al. 2007; Renko, Hofmann et al. 2009).

There are some regions of the rodent brain that express significantly lower levels of selenoproteins, which implies that some structures of the brain are less dependent on selenium availability. Selenoprotein genes are expressed the lowest in the oculomotor nucleus, Edinger-Westphal nucleus, nucleus Raphé pontis, anteroventral periventricular nucleus and dorsal premammillary nucleus; however, these brain regions do express the same selenoproteins that are found to be highly expressed in other regions of the brain, but at a significantly lower level, such as *Sel P, and Sel W* and *Gpx 4* (Zhang, Zhou et al. 2008). In the white matter of the brain, or corpus callosum, selenoprotein expression is considerably less than in other regions of the brain. The corpus collosum consists of mainly myelinated axons and is responsible for carrying the nerve impulses to different regions of gray matter.

Generation of ROS and the resulting oxidative stress are known to be both the cause and consequence of many neurodegenerative conditions (Sander 1993). Post mortem autopsies of people with the neurodegenerative disorders Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis all show that there are increased ROS in the regions of the brains that were affected by the neurodegenerative disorder. The brain has a reduced capacity for regeneration and a high metabolic rate which predisposes it to oxidative damage. The repair mechanisms that the cells have employed to counteract oxidative damage are primarily antioxidant enzymes such as glutathione peroxidases and thioredoxin reductases which are dependent on selenium for their function. Accumulating lines of evidence suggest that selenium-dependent antioxidant enzymes and selenoproteins are integral to epilepsy and have a role in the progression of the disease. It is promising to use selenium as a line of treatment against degenerative free radical diseases such as epilepsy.

3.1 The Se-dependent glutathione peroxidase

The function of the GPX family is to remove hydrogen and other peroxides from the brain and other tissues by coupling its reduction to water and alcohols using glutathione as a reducing equivalent. Glutathione is found in the mitochondria and cytoplasm of brain tissue and has been found to be released into the extracellular space by astrocytes. Interestingly, the first report of selenium status affecting any neurological condition was in infants with intractable epilepsy (Weber, Maertens et al. 1991). Infant brains are particularly susceptible to oxidative damage, which can contribute to abnormal brain electrical signaling, because of the high concentration of unsaturated fatty acids in the infant brain needed to synthesize cholesterol and fatty acids. These nutrients are necessary for the billions of developing nerve connections.

The seizure symptoms of these infants can be reversed with selenium supplementation (Weber, Maertens et al. 1991; Ramaekers, Calomme et al. 1994). Children suffering from intractable epilepsy are deficient in glutathione peroxidase activity. From the four children studied, two had normal blood selenium and high concentrations of GPX in their plasma, but low enzymatic activity. The other children had low intracellular selenium levels and low GPX expression and activity. Selenium supplementation and discontinuation of their anticonvulsant medication decreased seizure activity. The second study found that oral selenium supplements in children (3-5 μg/kg body weight) decreased frequency of seizures, improved electroencephalograph recordings, and restored liver function in the infants after two weeks of treatment (Ramaekers, Calomme et al. 1994). It was thought that supplementation of selenium restored the function of selenium-dependent GPX1 and GPX4 activity. From these results, selenium status is an important triggering factor for the onset of intractable seizures and that selenium-conferred antioxidant protection in the brain can help prevent neuronal damage following frequent seizures.

It has been suggested that treatment with anticonvulsants, such as valproic acid, which induces oxidative stress, depletes total body selenium and decreases GPX activity (Naziroglu 2009). However, others believe that selenium supplementation in congruence with antiepileptic drugs increase the risk of systemic toxicity. The conflicting selenium levels are likely associated with the pharmacology of the particular drug, duration of treatment, age of subject, validity of plasma selenium estimating brain selenium concentrations, and the underlying cause of the patient's epilepsy. It has been found that epileptic patients, without medication, have lower blood and tissue levels of selenium in addition to lower levels of selenoproteins and lower levels of selenium-dependent redox enzymes. In particular, intractable epileptics have been shown to have significantly lower mean serum selenium levels compared to a control group (Ashrafi, Shabanian et al. 2007). Animal models have shown that selenium can prevent the development of iron-induced epileptic symptoms due to selenium's ability to combat peroxidative injury (Rubin and Willmore 1980; Willmore and Rubin 1981).

3.2 Thioredoxin reductases

Thioredoxin reductases (TR) use NADPH for reduction of thioredoxin in various cellular redox pathways (Tamura and Stadtman 2002). TR-3 is expressed in very low levels in the mouse brain (Zhang, Zhou et al. 2008). Although TR-1 is well characterized and expressed in high levels in other organs, it is not highly expressed in the brain. Since TR is important for intracellular redox regulation and antioxidant defense (Schweizer, Brauer et al. 2004), decreased TR expression and activities could lead to enhanced cell loss, thus increasing the risk for epilepsy and other neurodegenerative conditions.

3.3 Selenoprotein P

Selenoprotein P (Sel P) is unique because it contains 10-17 selenocysteine residues, unlike other selenoproteins which contain only one. Sel P is produced mostly in the liver, but all tissues make and secrete Sel P into the plasma (Burk and Hill 2005; Hoffmann, Hoge et al. 2007). In rodents, Sel P is a predominant form of selenium in the plasma. Sel P is responsible for selenium transportation throughout the body, particularly to the brain where selenium is needed for incorporation into other selenoproteins which confer antioxidant benefits (Hill, Zhou et al. 2007). Sel P knockout mice develop seizures and movement disorders when raised on selenium restricted diets.

During seizure activity in epileptics, the neurons in the affected areas of the brain often have redox shifts due to the large influx of calcium through voltage gated channels (Stefani, Spadoni et al. 1997). If cellular redox equilibrium is not quickly restored following seizures, it is probable that the neurons will die and that region of the brain will be unable to function properly (Wirth, Conrad et al. 2010). It is thought that selenium, probably through its function in the GPX or TR families, can help prevent neuronal cell death and neutralize ROS which is generated as a consequence of high intracellular calcium (Xiong, Markesbery et al. 2007). Typically the brain can handle redox shifts and occasional electrolyte imbalances; however, during a seizure these activities happen at a rate and frequency that the cells cannot handle (Howse and Duffy 1975). The neurons eventually will die, and cause an increase in potentially harmful cellular byproducts (Barinaga 1998).

3.4 Selenoprotein W

Selenoprotein W (Sel W) has a redox motif and binds glutathione (Beilstein, Vendeland et al. 1996; Whanger 2009), similar to the well characterized GPX family. The 'W' designation is because of the white muscle disease seen in grazing livestock in areas where the soil is depleted of selenium (Vendeland, Beilstein et al. 1993). Sel W is highly expressed in the four basic brain regions in rodents; the hippocampus, olfactory area, cerebellar cortex, and isocortex (Zhang, Zhou et al. 2008).

The expression of Sel W is induced in the neurons of mesial temporal lobe epilepsy patients more than tenfold and is associated with BCL2 expression, which suggests that the Sel W induction is a defensive response to oxidative stress (Yuzbasioglu, Karatas et al. 2009). Furthermore, the expression of Sel W in cortex, cerebellum, and thalamus remains constant under Se deficiency (Sun et al., 2001b). Thus, Sel W expression is maintained in certain regions of the brain under selenium deficiency, suggesting a critical role of Sel W in the protection against seizure activities that is associated with increased oxidative stress.

3.5 Selenoprotein H

Selenoprotein H (Sel H) is a nucleolar DNA-binding protein (Novoselov, Kryukov et al. 2007) and may function as a transcription factor (Panee, Stoytcheva et al. 2007). It has been found that Sel H mRNA is highly expressed in the hippocampus during development but level falls quickly after birth and is undetectable in the adult brain (Zhang, Zhou et al. 2008); however, in another study the Sel H gene was found to be expressed in the adult mouse brain by using the RT-PCR method (Hoffmann, Hoge et al. 2007).

3.6 Selenoprotein M

Selenoprotein M (Sel M) is a small endoplasmic reticulum protein with unknown function, which is highly expressed in cornu ammonis granule cells of dentate gyrus (Zhang, Zhou et al. 2008). Cornu ammonis is also known as Ammon's horn and is part of the interlocking gyri which makes up the hippocampus. Interestingly, the most common form of neuropathological damage in temporal lobe epilepsy patients is sclerosis (hardening of tissue) of the cornu ammonis, also known as Ammon's horn sclerosis, which is characterized by neuronal cell loss in the hippocampus region of the brain. Common clinical markers of the disease include granule cell dispersion, reactive gliosis, and the segmental loss of pyramidal neurons. Unfortunately, 65% of people with temporal lobe epilepsy suffer from this disease (Blumcke, Thom et al. 2002; de Lanerolle and Lee 2005). Many of the seizures

due to temporal lobe epilepsy are very poorly controlled with anticonvulsant drugs. The role of Sel M is not fully understood and it is unclear whether Ammon's horn sclerosis is the cause of seizures of epileptics, or rather damage from the ongoing seizure activity.

4. Selenium and neurological disorders

Most inherited or congenital forms of epilepsy develop and occur before the age of 2. Older adults who have developed epilepsy have done so mostly because of brain injuries (hematomas and other bleeding vessels) or after a stroke. Generally, the most common cause of epilepsy in the elderly is attributed to cerebrovascular diseases or prescription drug reactions. Older populations are more at risk for drugs causing epileptiform activities because of their increased sensitivity to all drugs due to decreased drug metabolism.
Also other diseases in the elderly, particularly those that cause impaired language function or overall neurological deterioration, have been associated with increased seizure risks. Other factors such as metabolic problems, uraemia, thyroid problems, low blood sugar, electrolyte imbalances and liver damage can all contribute to seizures in the older population. In addition to the metabolic risk factors, epilepsy risk can also increase in patients with neurological disorders such as Alzheimer's (Noebels 2011), down syndrome, and dementia (Ito, Echizenya et al. 2009; Palop and Mucke 2009; Rao, Dove et al. 2009; Scarmeas, Honig et al. 2009; Larner 2010; Westmark, Westmark et al. 2010).
There is a link between neurological disorders and aging (Zhang, Rocourt et al. 2010). Since the body preferentially retains selenium in the brain even during periods of selenium deficiency, which further implies that selenium plays an important role in the brain (Behne, Hilmert et al. 1988; Chen and Berry 2003). Neurological disorders such as Alzheimer's disease, Parkinson's disease, and those disease states resulting from damage caused by environmental toxins, ischemic insults, drug abuse, and brain tumors are exacerbated by ROS (Chen and Berry 2003).
The selenium protection against neurological disorders is primarily conferred through antioxidative selenoproteins (Zhang, Rocourt et al. 2010). Therefore, selenoproteins play an important role in regulating redox reactions in the body, and particularly the brain. For example, the brains in mouse models of Alzheimer's disease show reduced levels of Sel M, which could mean that this selenoprotein plays a protective role in the development of Alzheimer's disease (Hwang, Cho et al. 2005).
Sel P knockout mice have movement disorders and seizures when given selenium-restricted diets from their birth (Hill, Zhou et al. 2003; Schomburg, Schweizer et al. 2003; Kutluhan, Naziroglu et al. 2009). Rats on selenium deficient diets have an increased risk of seizures and neuronal loss, further providing evidence that selenium can help prevent seizures. Because epilepsy is characterized by neuronal loss resulting from pro-apoptotic factors in association with oxidative stress (Savaskan, Brauer et al. 2003), selenium might help alleviate the underlying causes of brain injury that precipitate seizures.

5. Nutrigenomics perspectives

There is much evidence that speculates on the genetic aspect of epilepsy (Rees 2010; Sisodiya and Mefford 2011). Nonetheless, since epilepsy is a syndrome and not a disease, the cause of seizures in epileptics varies widely. Furthermore, even if epilepsy could be divided into groups based on the respective, underlying cause, it would still be difficult to determine the

genetic influence because of changes across clinical subgroups and amongst families. However, some types of epilepsy have been shown to have strong genetic components (Greenberg and Subaran 2011): 1) partial epilepsy has been linked to chromosome 10 q; 2) benign familial neonatal convulsions have been mapped to chromosome 20 q and another locus found on 8q; 3) a progressive form of myoclonus epilepsy has been localized to chromosome 21 q; 4) juvenile myoclonic epilepsy has been associated with regions of chromosome 6. Interestingly, the types of epilepsy syndromes that have genetic linkage evidence are a very small proportion of all epilepsy cases and no genetic basis has been found for most types of epilepsy.

Nutrigenomics is the study of how nutrition impacts the variation of gene expression across individuals. Seeing as how genetically diverse individuals are, it would stand to reason that individualized diets based on a person's genetic background can contribute to better overall health. Nutrigenomic approaches will become increasingly necessary in treating chronic diseases, including epilepsy. In the future it is expected that we will uncover genetic evidence that explains why certain people have different responses to similar diet, and how such variations help alleviate neuronal disorders.

6. References

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Part 2

Molecular Genetics of Epilepsy

5

The Molecular Genetics of the Benign Epilepsies of Infancy

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1. Introduction

Three benign epilepsy syndromes with autosomal dominant inheritance are recognised in infancy. These are Benign Familial Neonatal Epilepsy (BFNE), Benign Familial Neonatal-Infantile Epilepsy (BFNIE) and Benign Familial Infantile Epilepsy (BFIE). These disorders were previously known as Benign Familial Neonatal Seizures, Benign Familial Neonatal-Infantile Seizures and Benign Familial Infantile Seizures, respectively (Berg et al., 2010). They were recently renamed by the International League Against Epilepsy Classification Committee. The three disorders differ in their average ages of onset but age of onset distributions overlap between the syndromes. Ages of onset vary within syndromes and within families affected with any of the syndromes.

BFNE patients have lateralised motor seizures with a mean age of onset of around 3 days of age and a mean age of seizure offset of 3 months of age. Subsequent neurological development usually proceeds normally. However, about 15% of patients have later seizures or epilepsy and occasional cases deteriorate into severe epileptic encephalopathies with intellectual disability (Dedek et al., 2003; Borgatti et al., 2004; Steinlein et al., 2007). The benign form of the disorder was originally described in an Austrian family by Rett and Teubel (1964). Since then numerous BFNE families have been described, many with potassium channel subunit mutations, to provide a well established range of presentations.

BFIE was originally described by Watanabe and colleagues in 1987, and is sometimes referred to as Watanabe syndrome. Watanabe observed that a subgroup of infants with generalised motor seizures had subsequent normal development and became seizure-free by 3 years of age. Similar families were subsequently described by Vigevano and colleagues (1992). Onset of seizures in BFIE occurs between 4 and 6 months of age with seizure offset generally occurring by 12 months of age. Seizures are generally not seen in later life. However, some cases develop paroxysmal dyskinesia or paroxysmal kinesigenic choreoathetosis in adolescence. This is known as infantile convulsions and choreoathetosis (ICCA) syndrome.

BFNIE patients have similar seizures to those seen in BFNE and BFIE, with the average age of onset intermediate between the other two. Although BFNIE differs from these disorders in the average age of seizure onset, the age of onset distribution which can range within a single family from 3 days up to 13 months of age (Herlenius et al., 2007), significantly

overlaps with BFNE and BFIE. Seizure onset in BFNIE most commonly occurs at between 2 and 4 months of age. Clinically the condition was first described by Kaplan and Lacey (1983) and molecularly delineated two decades later (Heron et al., 2002).
The aim of this chapter is to describe the current state of knowledge of the molecular genetics underlying these disorders and the significance that this has for patient care.

2. The molecular genetics of benign familial neonatal epilepsy

Benign familial neonatal epilepsy is most commonly caused by mutations in two related voltage-gated potassium channel subunit genes, *KCNQ2* and *KCNQ3*. Together, these form the pore of the neuronal M-channel, which plays an important role in neuronal inhibition by acting as a "brake" on repetitive action potential discharges (Delmas & Brown, 2005). Mutations in *KCNQ2* and *KCNQ3* were originally described in 1998 (Biervert et al., 1998; Charlier et al., 1998; Singh et al., 1998). There are now more than 80 known mutations in *KCNQ2*; but only four described for *KCNQ3*. There may be other, rare, families with mutations in other genes.

2.1 BFNE caused by mutations in the potassium channel subunit *KCNQ2*

KCNQ2 (OMIM 602235) encodes one of the pore-forming subunits of the M-channel. The M-channel is a heteromultimer consisting of two KCNQ2 and two KCNQ3 proteins, as well as accessory subunits. The gene is located close to the q-arm telomere of chromosome 20, at 20q13.3. It consists of 17 exons embedded within 87kb of genomic DNA. The gene codes for an 872 amino acid protein consisting of three domains: a short N-terminal domain; a transmembrane domain; and a large C-terminal domain, which contains regions that interact with other channel subunits. The gene has five splice variants, which produce proteins that differ in the C-terminal domains. The transmembrane domain consists of six transmembrane segments (S1-S6) and the pore-loop between segments S5 and S6, which forms the lining of the ion pore. The S4 domain is the voltage sensor and contains a number of positively charged arginine or lysine residues, which are required for normal channel function. The S4 segments move within the membrane in response to a change in voltage, opening the channel.
BFNE was originally mapped to the *KCNQ2* locus in 1989 (Leppert et al.) by linkage analysis of a large, four-generation pedigree. Further families were reported that confirmed the chromosome 20q localisation (Ryan et al., 1991; Malafosse et al., 1992). *KCNQ2* was identified as the BFNE gene at this locus when a submicroscopic deletion containing *KCNQ2* was detected in a single BFNE family. Screening of additional BFNE families revealed missense, frameshift and splice-site mutations in *KCNQ2* (Singh et al., 1998). A frameshift mutation was simultaneously identified in *KCNQ2* when it was screened as a candidate gene in another family mapped to the 20q locus (Biervert et al., 1998).
In total, 85 mutations in *KCNQ2* have been described in the scientific literature (Heron et al., 2007; Soldovieri et al., 2007; Wuttke et al., 2007; Yalçin et al., 2007; Neubauer et al. 2008; Sadewa et al., 2008; Goldberg-Stern et al., 2009; Ishii et al., 2009; Kurahashi et al. 2009; Lee et al., 2009; Miceli et al., 2009; Volkers et al. 2009; Yum et al., 2010). These include missense, nonsense, splice site and frameshift mutations as well as deletions or duplications affecting multiple exons. The latter type of mutation accounts for over 10% of the published *KCNQ2* mutations and for around 20% of the *KCNQ2* mutations in one BFNE cohort

comprehensively analysed for all classes of mutation (Heron et al., 2007). Several deletions have been described which also affect the genes surrounding *KCNQ2* (Kurahashi et al., 2009). Patients carrying these deletions do not exhibit different phenotypes to BFNE patients with *KCNQ2* missense mutations, indicating that the loss of one copy of these contiguous genes is no more pathogenic than the loss of one copy of *KCNQ2* alone. The deletions and duplications affecting *KCNQ2* are shown in Figure 1.

Approximately one-third of the mutations in *KCNQ2* are missense mutations. Unlike the truncating mutations, which are spread throughout the gene, most of the missense changes occur in regions of functional importance. A cluster of missense mutations is seen in the transmembrane domain, with mutations particularly frequent in the S4 segment and the pore-loop. Functional studies of some of these mutations showed that they reduce channel activity and thus reduce neuronal inhibition (Soldovieri et al., 2007). Clusters of missense mutations are also seen in two regions of the C-terminal domain involved in binding to calmodulin. This interaction is known to be required for optimal channel function and mutations affecting it can disrupt the interaction and thus the normal function of the M-channel (Wen and Levitan, 2002; Yus-Najera et al., 2002; Richards et al., 2004).

The published mutations in *KCNQ2* are summarised in Table 1. More than half of the mutations in *KCNQ2* are either truncating mutations or large deletions or duplications that disrupt the structure of the gene. Both of these types of mutation extinguish protein expression from the mutated allele. Haploinsufficiency is the most significant mutational mechanism affecting *KCNQ2* that manifests as BFNE.

Mutation Type	Number Reported
Start Codon	2
Missense	28
Nonsense	9
Frameshift	21
In-frame deletion	3
Splice site	11
Large deletion or duplication	10
Other	1
Total	**85**

Table 1. Summary of mutations reported in *KCNQ2*

2.2 BFNE caused by mutations in the potassium channel subunit *KCNQ3*

KCNQ3 (OMIM 602232) encodes the second pore-forming subunit of the M-channel. The *KCNQ3* gene is located on the long arm of chromosome 8, at 8q24, and consists of 15 exons spread across 360kb of genomic DNA. The 849 amino acid KCNQ3 protein has a structure very similar to that of the KCNQ2 protein described above. The two proteins have highly homologous sequences in the transmembrane domains, but the sequences of the N- and C-terminal domains differ markedly (GenBank accession numbers NP_742105 and NP_004510).

Following the linkage of BFNE to the *KCNQ2* locus at 20q13.3, families were described that did not link to the region, indicating the presence of a second locus for the disorder. Some of these families were found to have evidence of linkage to a locus on chromosome 8q (Lewis et al., 1993; Steinlein et al., 1995) where the *KCNQ3* gene, with similarities to the *KCNQ2*

gene, was known to map. A mutation in *KCNQ3* was described at the same time as the first mutations in *KCNQ2* (Charlier et al., 1998).
Only four mutations in *KCNQ3* associated with BFNE have been reported in the literature. All of these affect amino acid residues located in or near the pore-loop (Charlier et al., 1998; Hirose et al., 2000; Singh et al., 2003; Li et al., 2008a). These mutations have not been functionally investigated, so their precise effect on channel function is unknown. The transient occurrence of seizures in BFNE may be due to changes in the expression of *KCNQ2* and *KCNQ3* that occur during development (Weber et al., 2006; Geiger et al., 2006; Kanaumi et al., 2008).

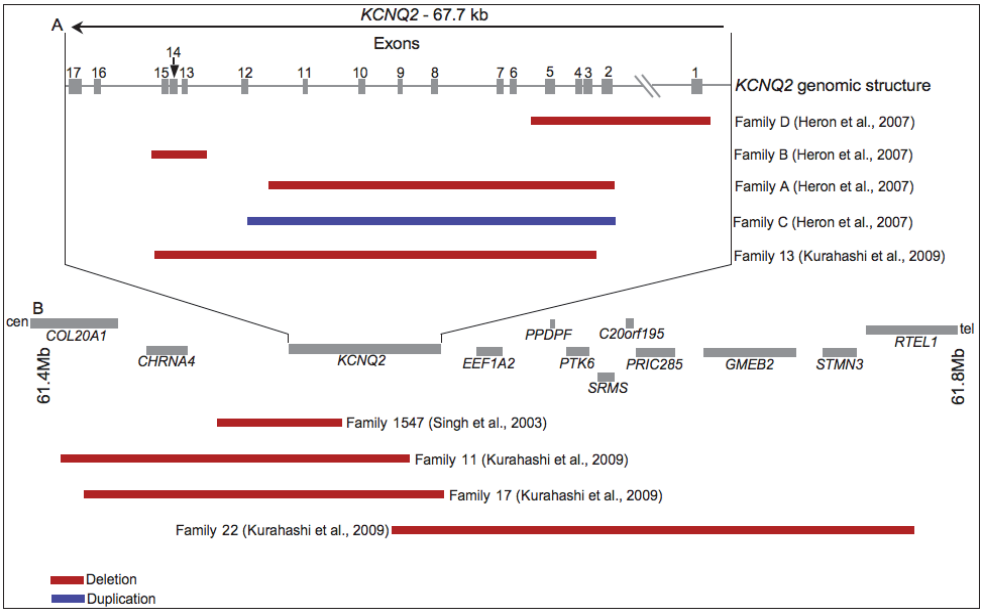

Fig. 1. Nine microchromosomal rearrangements described in *KCNQ2*. A: Intragenic deletions (red) and duplication (blue). B: Deletions extending beyond *KCNQ2*. A tenth deletion has been reported (Steinlein et al., 2007) but its breakpoints were not described.

2.3 BFNE with other causes

Occasional cases of BFNE have been reported with causes other than mutations in *KCNQ2* or *KCNQ3*. A small family with a pericentromeric inversion on chromosome 5 was reported in 2002 by Concolino and colleagues. The breakpoints for this inversion were mapped to 5p15.1 and 5q11.2. This inversion is presumed to disrupt a gene at one of these breakpoints, but this gene has not been identified. However, it is possible that further BFNE families have missense or other mutations affecting this gene on chromosome 5. It is also possible that the inversion disrupts a regulatory sequence for a gene located at some distance from the breakpoint.
Mutations have also been reported that cause BFNE with co-morbidities (Dedek et al., 2001; Dedek et al., 2003; Borgatti et al. 2004; Schmitt et al., 2005; Stenlein et al., 2007). Recently a

family with familial neonatal seizures and intellectual disability caused by a microduplication at chromosome 2q24.3 has been reported (Heron et al., 2010). The four affected members of this family all had neonatal seizures and some degree of intellectual disability ranging from borderline intelligence to an IQ score less than 40. A second patient with a similar microduplication has been described with seizures starting at 2-3 weeks of age and developmental delay (Raymond et al., 2011). This suggests that microduplications at chromosome 2q24.3, which contains a cluster of five voltage-gated sodium channel genes, could be a recurrent cause of neonatal seizures accompanied by intellectual disability. Such mutations can be easily detected and characterised using comparative genome hybridisation (CGH), therefore patients with neonatal seizures and intellectual disability should be tested for microduplications using this method. The pathogenic mechanism of these microduplications is hypothesised to be an increase in the expression of the duplicated sodium channel genes, in contrast to the haploinsufficiency cause by truncating or deletion mutations or the changes in function caused by missense mutations in *KCNQ2*. The two duplications described so far have slightly different breakpoints (Raymond et al., 2011), but are probably both the result of non-homologous recombination between the five sodium channel genes in the region.

These reports suggest that not all cases of BFNE are caused by *KCNQ2* and *KCNQ3* mutations. However, these exceptions are not common as mutations in these two genes account for around 90% of cases of BFNE.

3. The molecular genetics of benign familial neonatal-infantile epilepsy

Patients with benign familial neonatal-infantile epilepsy (BFNIE) have similar seizures to those seen in BFNE and BFIE. BFNIE differs from these disorders in the age of onset, which can range within a single family from 3 days up to 13 months of age (Herlenius et al., 2007), although seizure onset in BFNIE most commonly occurs at between 2 and 4 months. BFNIE was first described as a distinct clinical entity by Kaplan and Lacey in 1983. They described an American family with 12 affected individuals who had seizures with onset ranging from 3 days to 3½ months. All these individuals had subsequent normal development. Another family was subsequently described with seizure onset also occurring between 3 days and 3½ months of age (Lewis et al., 1996). Linkage to *KCNQ2* and *KCNQ3* was excluded for this family, indicating the existence of an additional locus for seizures occurring in early infancy.

3.1 Identification of mutations in the sodium channel subunit gene *SCN2A*

SCN2A (OMIM 182390) is located at chromosome 2q24.3 and contains 26 exons spread across 150kb of genomic DNA. The gene codes for the voltage-gated sodium channel alpha subunit $Na_v1.2$, a large protein of 2005 amino acids. Voltage-gated sodium channels consist of the large alpha subunit and two small accessory beta subunits. The alpha subunits consist of a small N-terminal domain, four transmembrane domains (DI-DIV) linked by intracellular loops, and a large C-terminal domain. The transmembrane domains all comprise six transmembrane segments (S1-S6) with a pore-loop between segments S5 and S6. The S4 domains act as voltage sensors. Overall, the structure of one of these transmembrane domains is very similar to the structure of the transmembrane domains of the voltage-gated potassium channels associated with BFNE described above. Indeed, it is thought that the voltage-gated sodium channels evolved from the assembly of four voltage-gated potassium channels (Marban et al., 1998).

SCN2A was originally suggested as a candidate gene for BFIE by Malacarne and colleagues (2001) when they mapped four BFIE families to the chromosome 2q24.3 locus. Mutations in *SCN2A* were then described in two families: the family described by Lewis and colleagues (1996) and a newly described family (Heron et al., 2002). Mutations have been subsequently described in another ten families and one sporadic case diagnosed clinically with neonatal seizures, who had a *de novo* mutation (Berkovic et al., 2004; Striano et al., 2006a; Herlenius et al., 2007; Liao et al., 2010). These families included the one originally described by Kaplan and Lacey (1983) and one of the families mapped to the *SCN2A* region by Malacarne and colleagues (2001). Interestingly, two of these families were originally described by clinical criteria as having BFIE (Malacarne et al., 2001; Striano et al., 2006a), highlighting the clinical overlap between BFNIE and BFIE.

The mutations in *SCN2A* associated with BFNIE are distributed throughout the SCN2A protein. Most of them occur either in the transmembrane segments or in intracellular loops, as shown in Figure 2. The mutations were originally predicted to increase sodium current, as increased sodium current leads to hyperexcitability (Heron et al., 2002). Functional studies done for six of the mutations supported this prediction (Scalmani et al., 2006; Xu et al., 2007; Liao et al., 2010). There have been two possible mechanisms suggested as the basis for the transient occurrence of the seizures in BFNIE. The first of these is age-dependent alternative splicing of *SCN2A*. The second is developmental changes in the expression of the gene. Studies showing a decrease in the expression of the *SCN2A* gene during development and a change in the intracellular distribution of the $Na_v1.2$ protein suggest that the latter mechanism is the more plausible of the two (Liao et al., 2010).

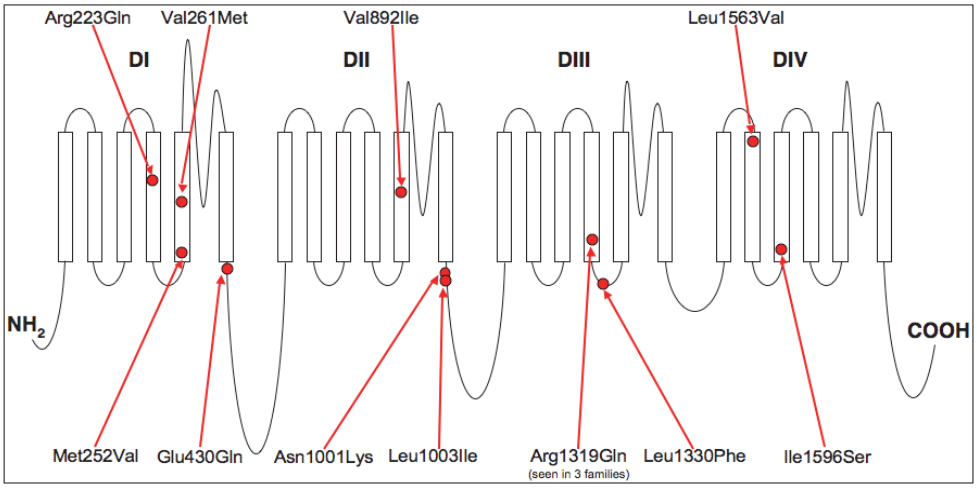

Fig. 2. Diagram showing the structure of the SCN2A protein and the locations of published mutations associated with BFNIE (red circles). The four transmembrane domains are indicated in bold.

3.2 Clinical overlap between BFNE and BFNIE

There is considerable similarity in the phenotypes of BFNE caused by potassium channel mutations and BFNIE caused by *SCN2A* mutations. The epileptic seizures in BFNE and

BFNIE are similar except for a difference in average age of onset and the two disorders cannot always be distinguished on the basis of age of onset (Mulley et al., 2011). As discussed above, the age of onset in BFNIE ranges from a few days up to one year of age. While most BFNE patients have onset in the neonatal period, occasional patients are reported with later ages of onset up to around 4 months of age, which is the typical age of onset for BFNIE (Singh et al., 2003; de Haan et al., 2006; Heron et al., 2007). A Chinese family with a *KCNQ2* mutation has been reported in which the entire family had seizure onset at between 2 and 4 months of age (Zhou et al., 2006). This variation in the age of seizure onset means that, especially in small families or those with limited clinical information, it is difficult to distinguish between BFNE and BFNIE in both familial and sporadic cases.
This clinical overlap means that determining the genetic defect is the only certain method to distinguish between BFNE and BFNIE. Making the distinction accurately is of clinical significance as 15% of patients with *KCNQ2* mutations have seizures in later life, while this is rare in patients with *SCN2A* mutations. Seizures in later life have also not been reported in patients with *KCNQ3* mutations, although the total number of patients described with these mutations is small (Hirose et al., 2000; Singh et al., 2003; Li et al., 2008a). Genetic diagnosis therefore has prognostic value in these disorders in the context of predicting the likelihood of later seizures. Severe epileptic encephalopathies have also been reported in families with *KCNQ2* mutations and this is another consideration when counselling families with *KCNQ2* mutations as opposed to those with *SCN2A* or *KCNQ3* mutations (Dedek et al., 2003; Borgatti et al., 2004; Schmitt et al., 2005; Stenlein et al., 2007). Extension of genetic diagnosis to BFIE awaits the discovery of the BFIE genes.

4. The molecular genetics of benign familial infantile epilepsy

Benign familial infantile epilepsy (BFIE) has a later average age of onset than BFNE and BFNIE (Berkovic et al., 2004). The disorder was originally described by Watanabe and colleagues (1987), who observed that a subgroup of infants with generalised motor seizures had subsequent normal development and later became seizure-free. Additional families were described by Vigevano and colleagues (1992) several years later. The disorder was demonstrated to be genetically distinct from BFNE in 1994 when Malafosse and colleagues excluded BFIE families from linkage to the BFNE region containing *KCNQ2*.
A subset of BFIE patients also have paroxysmal movement disorders with onset in adolescence: either paroxysmal kinesogenic choreoathetosis or paroxysmal dyskinesia. The co-occurrence of these disorders is referred to as infantile convulsions and choreoathetosis (ICCA) syndrome. None of the genes associated with BFIE have been identified. The disorder has been mapped to loci on chromosomes 1, 16 and 19 (Guipponi et al., 1997; Szepetowski et al., 1997; Li et al., 2008b). A single family has been described with partial co-segregation of BFIE and familial hemiplegic migraine (FHM) and a mutation in *ATP1A2* (Vanmolkot et al., 2003).

4.1 Rare BFIE loci

The first locus for BFIE was described in 1997. Five Italian families were mapped to a locus on chromosome 19q. The five families shared partial marker haplotypes for the region, possibly suggesting a founder effect – that is, that the five families were descended from a common ancestor and had the same mutation. No further families have been described which map to this locus and the mutation in the region has not been identified. Study of a further seven

Italian BFIE families showed that they were not linked to the locus (Gennaro et al., 1999). This suggests that the putative mutation at the chromosome 19 locus is a rare cause of BFIE restricted to a localised geographic region. Indeed, given the possible founder effect in the five families originally linked to the locus, it may harbour a single BFIE founder mutation.
Another single BFIE family has been mapped to a locus on chromosome 1. This was a large Chinese family containing eight affected individuals. The family was mapped to a 12.4 centiMorgan region on chromosome 1p36.12-p35.1 (Li et al., 2008b) with a maximum LOD score of 3.14. Analysis of 45 candidate genes selected from the 315 genes in the region on the basis of function or neuronal expression did not identify any causative mutations in the family (Li et al., 2010).

4.2 BFIE associated with *ATP1A2*

A single family has been described in which BFIE partially co-segregates with familial hemiplegic migraine (FHM) (Vanmolkot et al., 2003). FHM has been associated with several genes, including *ATP1A2*, the gene mutated in the family with BFIE. No further families have been described with BFIE and mutations in *ATP1A2*. However, several families have been described with mutations in the gene and other epileptic phenotypes (Haan et al., 2007). It appears that mutations in *ATP1A2* can cause a predisposition to seizures, without being associated with any particular seizure type. Given that there was only partial co-segregation of BFIE and the *ATP1A2* mutation in the family described by Vanmolkot and colleagues (2003), it is possible that additional mechanisms are contributing to the seizures in the family.

4.3 The chromosome 16p11.2-q12.1 BFIE locus

By far the most common locus for BFIE is located in the pericentromeric region of chromosome 16, between 16p11.2 and 16q12.1. This locus was originally described by Szepetowski and colleagues (1997), who mapped four French families with co-occurrence of BFIE and paroxysmal kinesigenic choreoathetosis (PKC) to the region. Numerous other families have subsequently been mapped to the region for similar conditions. Numerous additional smaller families are consistent with mapping to the region, showing no recombination, although they are of insufficient size to demonstrate linkage. Families mapping to the region include those with BFIE or PKC and PKD alone as well as those with both disorders (ICCA). The intervals for ICCA, BFIE and PKC/PKD from 13 published studies are shown in Figure 3. Two of these localisations (Valente et al., 2000; Callenbach et al., 2005) are small and do not overlap, suggesting that there may be more than one causative gene in the region. However, most of the localisations are large and span the centromere, a region of greatly reduced recombination on chromosome 16, especially in female meioses.
Despite the large number of families that have been mapped to the locus (approximately 50 have been described in the literature), no causative mutation has been identified at the chromosome 16 BFIE locus. A recent analysis of copy number variants in the region did not reveal any pathogenic copy number changes in the region, although low copy numbers of a particular variant were observed with increased frequency in a small cohort of BFIE patients (Roll et al., 2010). This suggests that the BFIE mutation on chromosome 16 has an unusual mechanism or is in an unannotated gene. Such unusual mutational mechanisms include: mutations in regulatory regions; balanced inversions; small copy number changes, which would not be detected by standard CGH arrays; and repeat expansions or contractions as in unstable triplet repeats.

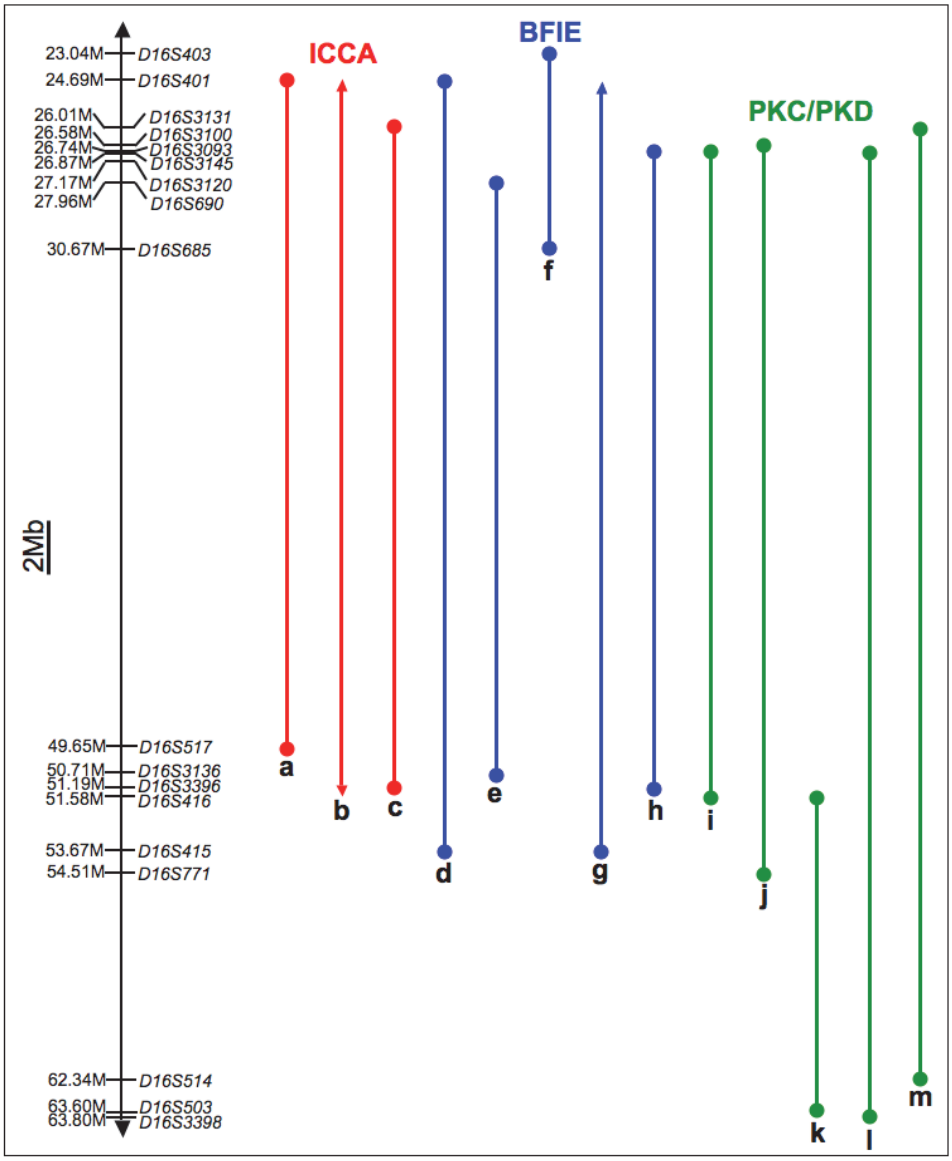

Fig. 3. Physical map showing the positions of microsatellite markers in the pericentromeric region of chromosome 16 and the linkage intervals for ICCA (red), BFIE (blue) and PKC/PKD (green) determined by analysis using the markers. These intervals are derived from the following 13 publications: [a]Szepetowski et al., 1997; [b]Lee et al., 1998; [c]Swoboda et al., 2000; [d]Caraballo et al., 2001; [e]Weber et al., 2004; [f]Callenbach et al., 2005; [g]Striano et al., 2006b; [h]Weber et al., 2008; [i]Tomita et al., 1999; [j]Bennet et al., 2000; [k]Valente et al., 2000; [l]Cuenca-Leon et al., 2002; [m]Kikuchi et al., 2007.

The identification of the mutation in the chromosome 16 BFIE locus will potentially require the application of techniques for the detection of alterations in gene expression, analysis of the non-coding sequence or the identification of small copy number changes. These techniques have not traditionally been applied for mutation detection. However, they may be required for the identification of the mutation or mutations causing BFIE linked to the pericentromeric region of chromosome 16.

5. Conclusions

The molecular genetic basis of the benign epilepsies of infancy has not been fully determined. The molecular basis for BFIE remains elusive. However, striking progress has been made in determining the mutations underlying BFNE, of which around 90% are in the potassium channel subunit gene *KCNQ2*. A significant proportion of these are exonic deletions or duplications, so in addition to DNA sequencing comprehensive screening for mutations in this gene requires the application of a technique such as multiplex ligation dependent probe amplification (MLPA) for detecting copy number mutations. A small number of BFNE mutations have also been found in the *KCNQ3* subunit, which appears to be far less mutable than *KCNQ2*. It is apparent from the report of a BFNE family with a pericentromeric inversion of chromosome 5 that other, rare, BFNE loci exist.

Some cases of BFNE associated with developmental delay or intellectual disability are caused by duplications of chromosome 2q24.3. This region contains a cluster of voltage-gated sodium channels and the overexpression of these and perhaps other contiguous genes within the microduplication is hypothesised to cause the seizures and other phenotypes associated with the duplications. Only two cases with these duplications have been described so far, but it is likely that more will emerge with the increased routine application of array comparative genome hybridisation in cases that do not fit within conventional syndrome parameters.

Mutations in the voltage-gated sodium channel *SCN2A* have been identified as a cause of BFNIE. There is considerable phenotypic overlap between BFNE and BFNIE, which in some cases can only be resolved by identifying a genetic defect in either *SCN2A* or *KCNQ2*/*KCNQ3*. This is of clinical significance as patients with neonatal or infantile seizures caused by *KCNQ2* mutations have a significantly higher risk of later seizures than patients with *SCN2A* or *KCNQ3* mutations, based on current observations of families that have been studied in detail.

Several loci have been mapped for BFIE, but no genes have been identified at any of these loci. The most important locus located in the pericentromeric region of chromosome 16 has been the focus of considerable research, but no causative gene has been identified as yet. The identification of the BFIE gene on chromosome 16 remains is a major challenge for completing the molecular picture for the benign epilepsies of infancy.

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Part 3

Animal Models

6

Audiogenic Seizures - Biological Phenomenon and Experimental Model of Human Epilepsies

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1. Introduction

Animal models that recapitulate human epilepsies with more or less details are believed to be of importance for clinics – for studies of anticonvulsants (Reigel et al., 1986, Dailey et al., 1996, Fedotova et al., 1996, Kosacheva et al., 1998, Ross, Coleman, 2000 et al.), for dissection of molecular and biochemical pathogenesis of epilepsy, and for the search of epilepsy susceptibility genes. The use of audiogenic epilepsy in rodents as the model for anticovulsant activity of different drugs is popular, the PubMed search for the key words combination "anticonvulsant and audiogenic and model" resulted in about 100 citations. Thus the detailed investigation of this type of seizures has great value for iur knowledge concerning the genesis of seizure (and epileptogenesis) in general and their genetic basis in particular.

Apart of its practical importance, the rodent audiogenic epilepsy is the enigmatic biological and genetic phenomenon. Actually the biological significance of high sound sensitivity for rodent survival was never questioned but at the same time its connection with audiogenic epilepsy (AE) was not analyzed from this point of view either. This chapter aims first to introduce several data items concerning investigations of Russian audiogenic prone rat strain (Krushinsky-Molodkina, KM) as they were not fully represented in English literature and second – to discuss the general biological mechanisms of audiogenic seizure. It is well known that the good theory is the best friend of practice. There is some hope that elucidating the origin of animal audiogenic epilepsies will bring us closer to still unsolved problems of seizure states in general. The methods which were used in studies of audiogenic epilepsies marched along with neurobiology methods starting from the middle of XX century. The same is now – new technologies are going to be applied and will be mobilized in future as these valuable models (seizure states developing in response to loud sound) are of great use for medical practice and clinic in particular.

2. General features of audiogenic epilepsy in KM rat strain and rodents of other genotypes

The data on increased sound sensitivity in audiogenic rats and mice are known for rather long time (Ross, Coleman, 2000, Semiokhina et al., 2006). Although the anomalies in "sensory part" of acoustic impulses in the brain pathway, promoting the acoustic seizure fit is not the single prerequisite for AE development. The abnormal biochemical and

physiological status of central auditory (and other) structures are also the important issues (Garcia-Cairasco, 2002). As several audiogenic rat strains exist, the phenomenon rat AE had been explored in different laboratories.

The audiogenic epileptic fits in rats were noted in Wistar Institute at the beginning of rat breeding program (Ross, Coleman, 2000), while the first observation of mouse audiogenic feature was made by N. Studentsov in I.P. Pavlov laboratory. The instrumental conditioning in mice using loud sound as CS unexpectedly resulted in seizure reaction of animals. In mid-1940-s the AE was noted in DBA/2J strain (Hall, 1947) and was also described in different laboratory mouse strains both inbred (Fuller, Smith, 1953) and outbred. Frings and Frings (1953) selected the albino mouse strain (Frings strains) which further was used later to identify the *mass-1* gene (Skradski et al., 2001). The selection experiment was also performed in mice later (Chen, Fuller, 1976) in order to investigate the relationship between "inborn" AE fits and fits induced by priming. In 1948 L. Krushinsky, L. Molodkina and D. Fless from Moscow State University started the selection of rats for high susceptibility to"sound seizures". The data, describing the seizure pattern in rats, selected for audiogenic epilepsy, were first published in Russia in 1949 in the "Advances of Contemporary Biology" (v 28, p. 108-133). In English they became available in 1962, as the translation of L. Krushinsky monograph had been issued (Krushinsky, 1964), and in Krushinsky et al. (1970) paper. Starting from the mid-50-s this new KM (for Krushinsky-Molodkina) strain had been extensively used as epilepsy model and model of catatonic state in pharmacological studies (Semiokhina et al., 2006). At that time authors avoided to describe the genetic aspect of the problem because scientific genetic investigation had been formally suppressed in Russia up to mid 60-s. Later the genetic study of AE inheritance had been performed (Romanova et al., 1993). In 1986-87 the inbreeding of KM strain was initiated, and up to the present the KM rat strain is maintained as inbred (more than 45 generations of inbreeding).

The marked phenotypic similarity is characteristic for audiogenic seizure fit and concomitant phenomena in rat strains selected in Russia, USA, France and Brazil. This includes the specific pattern of seizure stages, audiogenic kindling and postictal catalepsy, brain metabolite levels and their changes after the seizure fit. The phenomenology of audiogenic fit as well as numerous data on so called "priming" procedures in large number of mouse and rat genotypes were extensively presented in the review of Ross and Coleman (2000, see also below).

The brief description of rat audiogenic seizure fit will follow. It should be noted that the audiogenic seizures in mice have in general the pattern similar to that of rats with relatively larger proportion of animal deaths as the result of breath arrest (Tupal, Faingold, 2006). Mice of DBA/2J strain is most well known as demonstrating the AE phenotype with the peak intensity in the third decade of the first postnatal month and the decline of the trait expressivity at the age 40-45 days. Frings mice which were selected at the early 1950-s demonstrate the adult age AE (Klein et al., 2004). Later the audiogenic seizure proneness was discovered in mice of 101/HY strain carrying the mutant locus *mut-1* which increase their chromosomes sensitivity to the chemical mutagens (Poletaeva et al., 1996).

2.1 The audiogenic epilepsy phenotype

The seizure fit develops according to standard pattern in almost 100% of KM rats with wild run onset in 2-3 s and the clonic-tonic seizures developing in 7-9 s after the sound onset. Audiogenic seizure starts as the wild run stage, consisting of intense running mixed with high jumps of an animal. Wild run stage could stop and the normal waking state of an

animal gradually restores. If an animal display the single wild run bout and stopped, thus displaying the low intensity fit score (KM arbitrary unit "1"). Sound-evoked wild running induced the pattern of c-Fos similar to that of full AS in naive rats, this fact confirming the epileptic nature of this early audiogenic fit component (Simler et al., 1994). The "epileptic" nature of this stage was accepted as the fact by Fehr et al. (2004), who named it "clonic run". At the same time two earlier studies demonstrated that the wild-run stage is of "mixed" nature, which include the intense movements and jumps characteristic for avoidance reaction (fleeing from the fearful stimulus) and the involuntary seizure-like movements. The "avoidance" component in wild run stage was demonstrated when rats were provided with the capacity to escape the box in which the sound was presented, although such escape occurred only in the part of animals (Plotnikov, 1963, Fless, Salimov, 1974). The recent data confirm the relationship between these two behavior manifestations. N. Garcia-Cairasco (2002) made the detailed analysis of this problem basing on chemical stimulation data (injections of drugs into the restricted brain regions and analyzing the seizure pattern induced). He claims, in particular, that audiogenic-like seizures induced by chemical manipulations of specific subnuclei of inferior colliculi suggest overlapping of convulsive and aversive responses. In the review of Garcia-Cairasco the comparison was presented of audiogenic seizure pattern between the animals of selected strain WAR and resistant rats with audiogenic fit induced by chemical injections into inferior colliculli and other midbrain structures. This comparison demonstrated both common fit features and the peculiarities inherent for rats of selected strain (Garcia-Cairasco, 2002). As this author notes: "Obvious discrepancies could be derived from the use of genetic strains versus normal animals with acquired audiogenic responses" (Garcia-Cairasco, 2002).
At the same time all AE susceptible rodent strains demonstrated the phenomenon of the two phases of wild run (or "two waves of motor excitation" in Krushinsky terms). In such cases the wildly running rat could stop, rat remained quiet for several seconds and then the second run phase would resume. Although in many cases the AE fit proceeds and wild run is followed first by clonic and then by clonic-tonic and tonic "full" seizure with extension of extremities. After convulsions developed the normal state gradually returns. The postictal state of an animal could be represented by either the cataleptic state development (see below) or by the prolonged motor excitation, which is locomotion bout, different from wild run and which at least in KM strain was poorly analyzed. This type of motor "after-excitation" could be regarded as the phenotypical equivalent of prolonged abnormally high motor excitability.

2.2 Audiogenic epilepsy prone strains

The marked phenotypic similarity is characteristic for audiogenic seizure fit and concomitant phenomena in rat strains selected in Russia, USA, France and Brazil. This includes the specific pattern of seizure stages, audiogenic kindling and postictal catalepsy, brain metabolite levels and their changes after the seizure fit. The phenomenology of audiogenic fit as well as numerous data on so called "priming" procedures in large number of mouse and rat genotypes were extensively presented in the review of Ross and Coleman (2000). The brief historical notes concerning other AE susceptible rat strains will follow. At late 1940-s-begin of 1950s several papers were published on audiogenic rats sensitivity presumably using Wistar rats. Recent evaluations show that about 20-25 % of Wistar rat population develop the audiogenic fit of low intensity (demonstrating mainly wild run stage). The strains GEPR-3 and GEPR-9 (Genetic audiogenic prone rats) were created by

Albert Picchioni and Lincoln Chin in University of Arizona, USA at late 50-s (see Consroe et al., 1979, Reigel et al., 1988, Jobe et al., 1995). There are also WAS-GAERS rats (Wistar audiogenic epilepsy susceptible rats), selected in Strasbourg (Depaulis et al., 1990) and WAR (Wistar aufiogenic rats), which were bred in San-Paolo, Brazil (Doretto et al., 1996, Garcia-Cairasco 2002 et al.).

The genetic variability in AE propensity was the basis for the successful selection to the high AE seizure intensity and made it possible to create the respective AE susceptible rat strains. These strains were founded using at least three laboratory rat populations (outbred strains). In Wistar rats the successful selection for audogenic epilepsy was performed at least three times. These strains were: KM strain (already mentioned above), Wistar audiogenic sensitive rats (the detailed history not described, Simler et al., 1994) and the WAR strain which was extensively investigated by N. Garcia-Cairasco and his colleagues (Doretto et al., 1994, 2003, 2009, Garsia-Cairasco, 2002). Long-Evans rats served as the basic population for the strain of AE prone rats with about 100% penetrance and high expressivity. This strain had been bred by T. Kalinina and A. Volkova from Moscow Institute of Pharmacology (Russian Academy of Medical Sciences). Unfortunately the strain was lost due to breeding problems and only one paper which describes the experimental data using this strain is available (Surina et al., 2011).. The possibility to breed AE prone rats from Long-Evans population was very informative as this strain previously was reported as being very audiogenic-seizure resistant. The sound-induced seizures in these rats were possible to induce only by means of priming procedure (Ross, Colemann, 1999). The GEPR strains were selected using Sprague-Dawley population (Jobe et al., 1973, Consroe et al., 1979). Rather rapid selection response for this trait was characteristic for each of these experiments. In each case it resulted in creating the viable and long-lasting laboratory population of AE-susceptible rats with high penetrance and expressivity of this trait. In general, it means that the alleles of loci involved in audiogenic seizure propensity are not deleterious to the general neurological and/or somatic state of the animals. Although the oldest strain – KM (which has now more than 45 inbreeding generations and about 150 generations of selection breeding from 1947) demonstrates rather low fertility at present with the delayed start of reproductive age (3.5-4 months).

Audiogenic seizures were also described in the WAG/Rij rat strain which was initially studied as the laboratory model for the "absence" epilepsy (Kuznetzova et al., 1996). Midzyanovskaya et al. (2004) noted that about one third of WAG/Rij rats develop audiogenic seizure fit in response to loud sound stimulation, although these seizures were of low intensity –the tonic seizure stage was demonstrated rarely. GAERS rats also represent the model for "absence" epilepsy although the degree of relatedness between this strain and audiogenic prone strain of Wistar origin in Strasbourg laboratory (Simler et al., 1994) had not been clarified.

Brain structures involved in the development of audiogenic seizures

Audiogenic seizure fit initiates as the excitation arrives into the brain stem structures, namely in inferior colliculli. This fact has been extensively demonstrated in GEPR strains (Millan et al., 1988, N'Gouemo & Faingold, 1998, 1999, Faingold, 1999), as well as in WAR and WAS strains and mice (Willott & Lu, 1980, Garcia-Cairasco, 2002, Garcia-Cairasco & Sabbatini, 1991, Simler et al., 1994). Bilateral lesions of inferior collicculi, lateral lemniscus and the connections between these structures blocked audiogenic seizures expression in rats and mice (Garcia-Cairasco, 2002, Browning et al., 1999 Garcia-Cairasco, Sabbatini, 1991, Kesner, 1966, cited by Garcia-Cairasco, 2002).

The epileptic EEG activity concomitant with AE fit was demonstrated in medulla (Krushinsky et al., 1963, Krushinsky et al., 1970), midbrain, namely PAG and substantia nigra (N'Gouemo & Faingold, 1998, 1999, Doretto et al., 1994), lateral geniculate bodies (Krushinsky, 1963, Ribak et al., 1994), but not in hippocampus or neocortex (Merill et al., 2005, Moraes et al., 2005, Semiokhina et al., 2006). The latter fact was demonstrated both by lack of EEG epileptic pattern during clonic and tonic seizures and by audiogenic fit being unaffected after cortex ablation and during neocortex inactivation in the course of cortical spreading depression (Semiokhina, 1969). Detailed pattern of AE fit development was described in details for GEPR-3 and GEPR-9 by data on EEG and c-fos expression as well by data on brainstem sections and structure ablations (Ribak et al., 1994, Doretto et al., 1994, 2009). The main structures involved in audiogenic fit development are shown on the scheme (Fig.1).

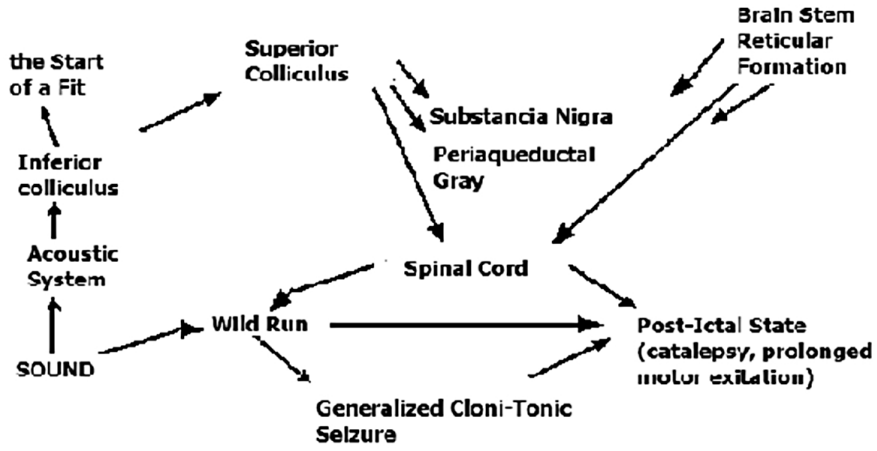

Fig. 1. The schematic representation of audiogenic seizure discharge development involving brain stem structures (see the text)

In several studies of AE, performed on DBA/2J model, the cortical EEG demonstrated the epileptic activity focus (Takao et al., 2006). Although it could be possible that high voltage epileptic potentials could result from electrotonic spread of potentials in the volume conductor, although the active participation of cortical neurons in AE fit development was demonstrated in mice using markers of glia cells activation (Guillaume et al., 1991), demonstrating the cortical catecholamine system activation and suggesting the protective role of neocortex in this process.

Another example of deviations from the commonly accepted view about the lack of EEG activity in the cortex during tonic AE fit was obtained by Zivanovic et al. (1997). The audiogenic seizures induced in non-epileptic Wistar rats by means of metaphit injections (competitive antagonist of NMDA-receptor) manifested the typical epileptic pattern in cortical EEG recordings. Thus the chemically induced AE fits are different by the pattern of pathological discharge propagation from that of "naturally" occurring AE fit in the genetically susceptible rats.

The participation and the key role of inferior colliculi in initiation and development of wild run and motor seizures had been investigated by all techniques available – EEG, activity of

single neurons (Faingold, 1999), 2-deoxiglucose uptake (Clemmesen et al., 1988) and the investigation the c-Fos expression pattern both in mice (Klein et al., 2004) and rat (Eells et al., 2004). The arrival of excitation to the superior colliculus is the first step in the seizure generalization (Ribak et al., 1997). Ribak & Morin (1995) showed (using immunocytochemistry and in situ hybridization) the significant increase in GABA level and the larger number of GABAergic neurons in the central nucleus of the inferior colliculus in GEPR-9 strain in comparison to Sprague-Dawley non-epileptic rats. The number of small cells with diameters less than 15 microns showed the greatest increase. The causal connection of this anatomical trait with AE was proved by the fact that in KM rats (the independent selection experiment from different initial population) the similar increase was recently shown in the number of neurons expressing proteins co-localized with GAD in rat brain of 2, 4 and 6 weeks of age (unpublished data). Ribak & Morin (1995) using the knife cuts through the midbrain indicated that in sound-induced seizure propagation the important stage was the activation of the inferior colliculus external nucleus via projections from the central nucleus.

It was known for rather long time that audiogenic-like seizures could be evoked by microinjections of bicuculline (GABA-A receptors antagonist) into the inferior colliculi, namely into the central nucleus of this structure (Bagri et al., 1989, Terra & Garcia-Cairasco, 1992). The more detailed analysis of morphological substrate of wild run stage in rat by means of stimulation as well as by self-stimulation of collicular structures gave similar results. The "wild run" seizures which follow biccuculine microinjections into inferior colliculus and resemble AE fit were never followed by clonic-tonic stage. The infusion of NMDA in this structure had the similar results. Animals displayed more severe spontaneous audiogenic-like seizures after NMDA injection into central ventral or cortical dorsal inferior colliculus nuclei. which were potentiated by the acoustic stimulus. In general these responses resembled the flight reaction. This reaction could be interrupted by an animal only in cases when electrodes were placed into ventral part of central nucleus (of the inferior colliculus), but not in the cortical region of this structure (Garcia-Cairasco, 2002). Most part of these seizure responses developed prior to sound stimulation and they never ended in tonic-clonic seizures. These audiogenic seizures were blocked by microinjections of 2-amino-7-phosphono-heptanoate (AP7) applied just before NMDA microinjections into central or cortical nuclei (Terra & Garcia-Cairasco, 1994).

The activation of connections between interior and superior colliculli are obligatory for the "sensory-motor transduction in the midbrain", which means that this connection wais activated prior to audiogenic seizure appearance (Garcia-Cairasco, 2002). Thus, the integrity of connections between inferior and superior colliculi projections looks to be the crucial, or "key" point not only for adaptive responses to loud sound (startle, flight) but also for audiogenic seizures (Dutra Moraes et al., 2000). The non-acoustic structures – periaqueductal grey, PAG, and substantia nigra, pars lateralis are also critically involved in the network, activated during audiogenic seizure development. The evoked audiogenic-like seizures (by bicuculline application) could not be modified by lesions in the PAG, which suggest that this structure is not necessary for the senso-motor processing of these seizures (Bagri et al., 1991). In contrast, the ventrolateral portion of this structure is involved in the modulation of audiogenic seizures in GEPRs via a complex mechanism mediated by NMDA, GABA and opiates (N'Gouemo & Faingold, 1998, 1999, Faingold, 1999). In WAR the bilateral microinjections of clobazam into substantia nigra pars reticulata completely blocked mentioned above audiogenic like seizures evoked by unilateral microinjection of

bicuculline into inferior colliculus (Terra, Garcia-Cairasco, 1992). It made possible to suggest that WARs might be genetically deficient in GABA release or GABA receptors number and/or specificity. In another AE model the block of audiogenic seizures in similar situation was not possible, and the non-involvement of nigro-tectal connections in AE had been suggested (Depaulis et al., 1990).
The study of EEG peculiarities (wavelet analysis) in the *striatum – substantia nigra pars reticulate – superior* colliculus in WAR strain demonstrated the active participation of this system in audiogenic fit development. It was suggested that this circuitry could probably participate in active seizure fit termination as well as the participation of cerebellum in such process was not investigated in this model. Although the authors claim that methodology employed cannot answer the question – whether the increase of EEG frequency oscillations means the "desinhibition" of a nucleus, or the decrease of EEG frequency means an "inhibition". These data are of rather big importance as the active nature of seizure cessation is still poorly understood (Doretto et al., 1994, Kryzhanovsky, 2002).
The fact that brain stem nuclei are involved in the audiogenic seizure propagation does not necessarily mean that the wild run and tonic convulsions are mediated by the identical neuronal circuitries. For instance two different kinds of glutamate receptors (NMDA and AMPA) were involved in these two stages of the AE fit (Yasuda et al., 1998). The pattern of EEG and init activity in GEPRs permitted to suggest the way by which the excitation spreads in the brain during seizure fit (Dailey et al., 1996, Deransart et al., 2001). Muscimol intranigral inactivating injections were capable to suppress clonic and absence-like states in audiogenic seizure prone Wistars but were not capable to stop the tonic seizure. The inferior collululi seem to be the critical structure as the increase of neuronal firing there precedes the start of the fit. The excitation spread to the deep structures of the superior colluculus plays the similar role for wild run stage as neuronal firing increase also precedes the start of audiogenic fit. When the excitation reaches the pontine reticular nucleus and PAG the clonic-tonic stage of the fit starts (Molnar et al., 2000). After the end of the seizure fit the electric activity in these structures is depressed (dorsal cortex of inferior colliculus being the exception). The existing evidence in GEPR-9 claims that abnormal epileptic activity is determined by the increase of glutamatergic and decrease of GABAergic brain stem systems (Chakravarty & Faingold, 1999).

2.3 Priming procedure, hearing defects and audiogenic seizure fit

Priming procedures, as well as several other treatments (e.g. methaphit injections, Stanojlović et al., 2000) could induce AE in non-susceptible rats and mice, this phenomenon in mice being first analyzed by K. Henry (1967).
Innate susceptibility to audiogenic seizures appears and declines with age at varying rates, depending upon genotype and environmental conditions as well (Henry, 1985). Auditory dysfunctions could be experimentally produced which induce susceptibility in otherwise non-susceptible mice or rats. In order to study the correlation between the cochlear functions and audiogenic seizures in genetically susceptible mice, both measures were obtained from LP/J mice, at ages ranging from 8 to 120 days (Henry, 1985). Susceptibility to sound-produced convulsions was first noted at the age of 12 days, was maximal in the period from 18 to 32 days, and declined rapidly by 40 days, disappearing totally by 120 days of age. Cochlear nerve-evoked potential thresholds were very high at 12 days, were lowest between 18 and 32 days and increased thereafter. The correlation between susceptibility and cochlear thresholds was greatest for high frequencies ($r = -.93$), intermediate for

midfrequency (r = -.77), and poorest for low frequencies (r = -.56). It was concluded that either genetic or environmental factors which produce an intermediate level of cochlear damage (for high frequency perception) in the young mouse will produce susceptibility to audiogenic seizures (Henry, 1985).

The detailed analysis of "acoustic" behavior and cochlear functions in albino Frings mice, namely in inbred descendants of them was also performed (Henry, Buzzone, 1986). The cochlear action potential thresholds of the susceptible RB/1bg mice were abnormally high, while the resistant inbred RB/3bg mice had normal audiograms of evoked potentials. The F1 hybrid showed heterosis for cochlear function. This RB/1bg line showed little age-related cochlear loss, which probably accounts for its robust sensitivity to audiogenic seizures over most of its lifespan. Earlier studies had demonstrated that the susceptible RB line had a robust evoked potentials, but little or no cochlear microphonic. The susceptible RB/1bg mice had well-defined potentials and cochlear microphonic (Henry, Buzzone, 1986).

GEPRs cochleas hair cell and electrocochleographic alterations, particularly, were investigated (Faingold et al., 1990) being in accordance with mouse data. Furthermore, several neonatal manipulations such as acoustic trauma (Pierson & Snyder-Keller, 1994) or kanamycin (Pierson & Swann, 1991), perinatal antithyroid treatment (Middlesworth & Norris, 1980) induced audiogenic seizure susceptibility in normal rats.

The detailed reviews of AE after the priming procedure were presented by Ross & Coleman (2000) and Garcia-Cairasco, (2002). In general, priming is thought to be a disruption in the normal development of activity dependent auditory pathways.

When topography of pure-tone responses in Wistar rat inferior colliculus was mapped (using Fos expression), it was demonstrated, that auditory deprivation, starting at the age of 14 days, as well as neonatal exposure to potentially deafening noise (which resulted in hearing losses) change drastically the topographic frequency representation in inferior colliculus (Pierson & Snyder-Keller, 1994). As these treatments are known to result in inducing the audiogenic seizure susceptibility, it was believed that this susceptibility might depend on derangements of hearing due to neonatal auditory deprivation. Pierson & Snyder-Kelly (1994) suggested that the deteriorations in tonotopic organization of inferior colliculi in these cases could be the probable basis of AE. This idea was supported by the fact that the ontogenetic differentiation processes in cochlea and inferior colliculi developed in the similar order – from low to high frequencies. This tonotopic organization was the result of activity-dependent process, and thus the different "priming" treatments which interfere into integrity of normal functional development of inferior colliculus and hearing were effective in AE production.

The hypersensitivity of the inferior colliculus neurons to a high-intensity auditory stimulation and hyperexcitability of these neurons in a stimulation experiment have been reported after priming procedures (Urban & Willott, 1979; Willott & Lu, 1980, Sakamoto & Niki, 2001, cited by Ross, Colemann, 2000). Thus it was more or less established that in majority of cases the AE phenotype could be revealed when inner ear severe damage of different nature took place. Black Swiss mice were audiogenic seizure prone with typical audiogenic fit which peaked in 21 days old animals and declined further (monogenic autocomal-recessive, chromosome 10). The hereditary hearing thresholds increase was found in these mice which were fully developed to the age of 4-5 month. It is interesting to note that this hearing defect was shown not to co-segregate with AE proneness, these two traits being thus independent (Misawa et al., 2002).

The functional and morphological integrity of the peripheral acoustic organ seems to be very important for development of AE fits. The structure of mammal cochlea provides the high auditory sensitivity in the broad frequency range (Dallos, 1992).
The mammalian cochlea contains two types of hair cells – inner hair cells and outer hair cells, embedded in a sensory epithelium, which is organ of Corti. Detailed morphology and function of mammalian cochlea was presented in the instructive paper of Dallos (1992). The organ of Corti is placed on the basilar membrane, which is situated along the whole cochlea length being different in firmness from its base to the apex. Basilar membrane moves as the sound vibrations enter the inner ear. Sound signals in vivo are thought to be enhanced by active mechanisms in outer hair cells – they amplify the sound-induced displacements of the basilar membrane (Dallos, 1992). Thus outer hair cells of the mammalian cochlea besides serving as sensory receptors also generate force to enhance auditory sensitivity and frequency selectivity (Mahendrasingam et al., 2010). Inner hair cells function is to relay auditory signals to acoustic nerve endings (which then travel to the brain). Reciprocally, efferent axonal fibers from the medial olivocochlear system innervate sensory cells (Ryan et al., 1990). The function of outer hair cells is based on the voltage-dependent contractility of the outer hair cells, which, in turn, depends on pristine, motile protein specified not long ago (Frolenkov, 2006).Prestin is located in the basolateral wall of outer hair cells, and is thought to alter its conformation in response to changes in membrane potential (Mahendrasingam et al., 2010). Prestin is a member of a gene family, *solute carrie*r (SLC) family 26, which encodes anion transporters and related proteins. In humans three genes of this family (SLC26A2, SLC26A3 and SLC26A4) are associated with different human hereditary diseases (Liu et al., 2003). The details of signal transduction and role of the feedback mechanism in the sensitivity of the peripheral acoustic organ could be very important being the part of presumably pathological mechanism of the AE. The medial olivocochlear efferent system is an important component of an active mechanical outer hair cells system in mammals. It seems that no data on this system functional and/or morphological characteristic in audiogenic prone rats or mice exist at the beginning of 2011, although the protective role of medial olive to cochlea in mammalian ear was well described (Christopher & Smith, 2003).
Morphological and functional changes in auditory path periphery are related to AE in rodents, although this relationship is not simple (Ross & Coleman, 2000). It could be mentioned, for instance, that igf-1 (insulin-like growth factor-1 or somatomedin C) plays the important role in cohlea intrauterine and early post-natal development, although there were no indications that the defects of cochlea development in igf-1 knockouts are accompanied by audiogenic seizures (Camarero et al., 2001). In mouse mutants – Bronx waltzer (bv/bv) – the 75 % loss of inner hair cells cause only moderate elevation of hearing threshold (Schrott et al., 1989). These facts could signify that specific gene-knockout procedure acts selectively inside cochlear cell sparing the neural elements and thus the knockout consequences does not interfere the outer hair cells feedback connections from olive nucleus. At the same time, priming procedures which induced unspecific cochlear damages presumably involving the innervation impairments too, were accompanied by AE. The AE development after priming procedures (early exposure to the loud sound, deafening or toxic kanamicin injections in early postnatal age) does not induce the cochlear damage. So for priming procedure it is necessary and sufficient to change activity in the acoustic neuronal network (e.g. Pierson & Li, 1996).

It should also be mentioned that mapping of fos-like immunoreactive neurons in brain nuclei demonstrated the unexpected decrease of fos expression in the cochlear nuclei and the central nucleus of the inferior colliculi in animals having AE seizures in comparison to controls (Clough et al., 1997)

One of the conclusions which could be drawn on the basis of our present knowledge is that AE develops when acoustic sensitization takes place. Such sensitization could be the result of hereditary inner ear structural (or functional) anomalies of innervation pattern in combination with brain anomalies.

One more source of variability in the function of audiogenic seizure substrate is the level of thyroid hormones, which was reported to influence the AE proneness, although the information was partly controversial. In general the early postnatal onset of hypothyroidism produces audiogenic seizure susceptibility in rodents (Yasuda et al., 1998). These data were supported by developing the audiogenic seizure phenotype in mouse KO mice lacking specifically TR beta (Thrb (tm1/tm1)) (Ng et al., 2001).

In early 1950s Krushinsky and his colleagues found that the artificial increase of thyroxin levels in KM rats increased the audiogenic fit intensity (Semiokhina et al., 2006). The authors suggested that the elevation of thyroid hormone level increased the general level of CNS excitability thus inducing the fit severity.

The opposite data were reported by Mills & Savage (1988), who demonstrated, that GEPRs-9 was hypothyroid from the second week of life up to at least 1 year of age.

Using the battery of seven C57 X DBA (BXD) recombinant inbred mouse strains and the D2.B6-Iasb congenic strain, Seyfried et al. (1984) demonstrated in details that there was no genetic correlation of audiogenic seizure susceptibility and the serum thyroxin levels, although in earlier papers the existence of this relationship was more or less obvious as more simple genetic approach was used (Henry et al., 1981).

2.4 Genetics

Genetic researches of audiogenic seizures in mice and rats have the separate histories. The identification of Asp loci in DBA/2J mouse strain had been possible on the basis of AE susceptibility scores in F2 hybrids of C57BL.6J (resistant) and DBA/2J susceptible strains (Neumann & Collins, 1991). The chromosomal location of three Asp (audiogenic seizure proneness) loci was determined by means of classical genetics approach (Fuller et al., 1950). Later J.Fuller performed the new selection experiment creating 4 strains contrasting by initial AE susceptibility and audiogenic seizures induced by priming. The results of new four lines AE testing permitted to conclude that both traits have the independent genetic determination (Fuller, 1975, Chen & Fuller, 1976).

T. Seyfreid at al. (1980) investigated AE in mice using two parental strains C57BL and DBA mice, their F1 hybrids, backcrosses to both parental strains as well as the battery of 21 recombinant inbred strains. The authors' conclusion was that this physiological trait possessed the hereditary pattern which was characteristic for so called threshold traits. This means that genetic and environmental factors influencing trait in question influence its expressivity but the trait appears as the definite phenotype if the summarized influence of factors exceeds certain "threshold" (Seyfried et al., 1980).

Genetic researches of audiogenic seizures historically bases mainly on data for mouse AE. Three genes were identified on the basis of DBAxC57BL crosses. The first was *asp-1* (a major gene, chromosome 12**,** between Ah and D12 Nyul), the second - *asp-2* (with smaller effect, chromosome 4), and the third one – *asp-3*, which is located in the proximal region of

chromosome 7 (Neumann & Seyfried, 1990., Neumann & Collins, 1992). The authors ascribe the significant role in trait determination to the genomic imprinting. The model, representing these processes, is presented in which the maternal Asp3 allele is repressed, providing an influence largely from the paternal allele (Banko et al., 1997).
The investigations showed the genetic associations of the *asp-1* locus with the brain stem $Ca^{2(+)}$-ATPase activity level (Neumann & Seyfried, 1991).
The general seizure susceptibility (maximal electroshock seizure threshold) genes were also investigated by QTL technology using C57BL/6 (B6, seizure resistant) and DBA/2 (D2, seizure susceptible) mice. The data obtained demonstrated a significant effect originating from middle region of chromosome 5. Reciprocal congenic strains between B6 and D2 mice were created by a DNA marker-assisted backcross breeding strategy. Comparison of seizure thresholds between congenic strain and that one which contain the parental genetic background genes indicated that mice from strains having chromosome 5 alleles from D2 and B6 genetic background exhibited significantly lower thresholds, than control littermates, and v.v. - congenic mice harboring B6 chromosome 5 alleles on a D2 genetic background exhibit significantly higher thresholds (Ferraro et al., 2007). Thus that was another chromosome locus which could be also responsible for AE proneness differences between DBA/2J and C57BL/6J mice. Similar data were got using chemically induced seizures, demonstrating the plausible connection between audiogenic seizures and seizures induced by the benzodiazepine receptor inverse agonist methyl-beta-carboline-3-carboxylate (beta-CCM). As the injections of this drug induce seizures and the susceptibility for it had the genetic component, both traits were analyzed using recombinant congenic strains of mice bred from B10.D2 and DBA/2J. Although both types of seizures have similar behavioral patterns and might involve GABAergic mechanisms, no correlation was observed between the occurrences of the two types of seizures across the strains, suggesting that these two types of seizures depend on different genetic mechanisms (Martin et al., 1992). These data could be in contrast to the generally increased seizure susceptibility in audiogenic rats (see below).
Another gene had been identified in Black Swiss mice **(**Misawa et al., 2002), which develop the audiogenic seizure with the pick intensity at 21 postnatal day. Genetic mapping and linkage analysis of hybrid mice localized the gene, *jams1* (juvenile audiogenic monogenic seizures), to the region of chromosome 10, delimited by the gene *basigin* (*Bsg*) and marker *D10Mit140*. It is worth to note, that the majority of this critical region is syntenic to a human chromosome 19p13.3 region, which is implicated in a familial form of juvenile febrile convulsions (Klein et al., 2005).
In Frings mice the single gene responsible for AE was mapped on mouse chromosome 13 (Skradski et al., 2001). This locus was named monogenic audiogenic seizure-susceptible (*mass1*). The protein coded by *mass1* (now referred to as Mgr1) is unique in that it is one of only two identified seizure loci that are not associated with an ion channel mutation. This gene codes for *very large G-protein-coupled receptor-1* (vlgr1) which contains about 6300 amino acids, and which is the largest known cell surface protein. It is expressed at high levels within the embryonic nervous system, especially in the ventricular zone. A naturally occurring nonsense mutation in this gene - V2250X, was shown to be linked to susceptibility to audiogenic seizures in mice (McMillan & White, 2004). Vlgr1d and Vlgr1e - alternatively-spliced variants of Vlgr1b/MGR1 - were shown to be transcripts from a locus *mass1*. Experiments performed suggested that Vlgr1d and Vlgr1e are secretory molecules, while Vlgr1b is a receptor. Knockout mice lacking exons 2-4 of Vlgr1 were susceptible to

audiogenic seizures without priming with any apparent histological abnormalities in their brains (Yagi et al., 2005). Mice of other genotypes possessing the Frings Mgr1 allele exhibited a mild to moderate level of hearing impairment which was already present during the days following hearing onset in ontogeny (Klein et al., 2005).

The heritability of audiogenic seizure in rats was no less simple issue. In KM rats the diallelic cross showed that AE heritability is polygenic with additive effects, and alleles determining the resistance to sound induced seizures were dominant (Romanova et al., 1993).

The data on the genetic experiments in other AE rat strains are the following. Audiogenic seizure predisposition in GEPRs was inherited as dominant polygenic autosomal trait (Kurtz et al., 2001). The penetrance and expressivity of the trait was evaluated in 20,373 animals of GEPR strains. The GEPR-3s and GEPR-9s animals both showed incomplete penetrance and variable expressivity of the underlying genetic predisposition to AE. The GEPR-9 strain had more animals with the variable levels of seizure predisposition (as measured by a scoring system that denotes the severity of generalized tonic/clonic seizures) and a greater percentage of animals that exhibited no susceptibility to such seizures induced by sound. Both strains had a number of animals that were not susceptible to sound-induced seizures and that exhibit some variability in seizure severity. The GEPR-9 males show greater differences in expressivity and penetrance compared to GEPR-9 females. The GEPR-3 animals also show sex-associated variable penetrance and expressivity of the epileptic phenotype, although the differences are much smaller. It should be noted that in contrast to GEPRs the trait penetrance and expressivity in rats of KM strain were very high – reaching the level of about 99% (Semiokhina et al.., 2006), probably the different genetic background (Wistar versus Sprague-Dowley) could be responsible. It should be remembered that the KM strain is inbred and display breeding difficulties as well.

Practically no published data could be found concerning the inbreeding used in GEPR strains, while in WAR strain the inbreeding started in parallel with the selection for high AE fit intensity (Garcia-Cairasco, 2002). The selection for high AE predisposition in WAR strain was rather quick (from 3 to 17th generations) the intensity of the fit increasing twice in parallel with fit latencies significant reduction (Doretto et al., 2003). This indicates the additive type of gene action and relative low effect of non-allelic interactions, which resemble the type of AE inheritance in KM strain.

The new experiment is now in progress in which the rats were selected for the lack of audiogenic seizures and the basic population was the F2 and backcross hybrids KM x Wistar. The "0" sensitivity rats which are under selection in this strain share the portion of KM genotype (which is presumably different from the Wistars after more than 100 generations of separate breeding).

The selection success in first ten selection generations of this selection experiment is demonstrated in fig 2. The percentage of rats, demonstrating "0" reaction (which was verified by sound exposure for 2-3 times with no less than one week intervals) was never very high, being about 40% in average. The more thorough analysis performed at the initial stages of selection demonstrated that the distribution of audiogenic sensitivity in F2 and backcrosses corresponds significantly to the two-gene-incomplete penetrance model (Fedotova et al., 2005).

The spontaneous mutation in the rat Wwox gene (Ide/Ide rats) induced dwarfism, postnatal lethality, male hypogonadism, as well as high incidence of epilepsy, typical audiogenic seizures as well, and many structural cell anomalies in the hippocampus and amygdale (Suzuki et al., 2009).

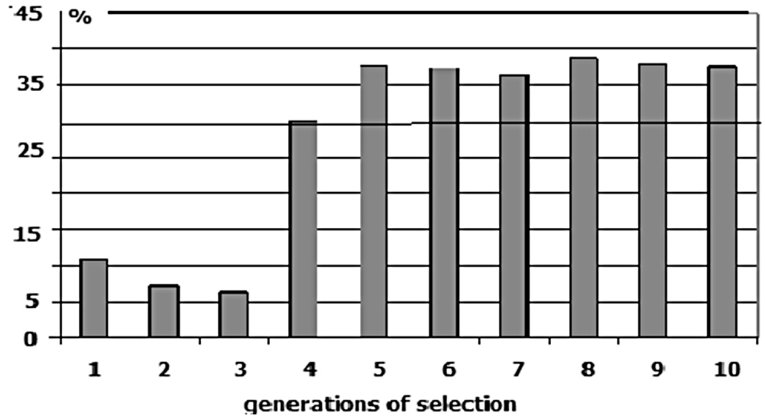

Fig. 2. The percentage of rats, selected for "0" audiogenic seizure susceptibility, starting from hydrids between Wistar rats with "0" susceptibility and KM rats with maximal seizure intensity. Ordinate – the proportion of "0" rats in respectiove generations.

The new data in the field had been brought by new technologies used. KO of several mouse genes resulted in the audiogenic seizure phenotype - fmr-1, interleikin-6, 5-HT2c receptor (Chen, Toth, 2001, Pacey et al., 2009, Brennan et al., 1997, De Luca et al., 2004). Probably these data are only the start point of new discoveries in this area using gene engineering techniques.

2.5 Channelopathies

Seizure states including epilepsy are determined by brain cells pathologies which induce functional changes in neurons. It was demonstrated that in the majority of cases both epileptic neuronal discharges and motor seizures are determined by the deteriorations in ion channels structure and/or function, which were named channelopathies (Mulley et al., 2003, Heron et al., 2007). The changes in the expression of the gene coding for one of the subunits of calcium channel Cav3.2 (as the response to pilocarpin) provoke the anomalous increase of neuronal discharges (Becker et al., 2008). It was demonstrated that in GERPs the dysfunction of voltage-gated Ca^{2+} channels are of primary importance in the development of audiogenic seizures (N'Gouemo & Morad, 2003, N'Gouemo et al., 2009, 2010). In the GEPR strains Ca^{2+} channel blockers (L-type) suppressed the audiogenic fits. The authors demonstrated this dysfunction in neurons of inferior colliculi in GEPRs, while it was unclear whether this "channelopathy" is also characteristic for other brain regions involved in AE. It stayed still unclear whether these Ca^{2+} current properties are inherent to other AE genotypes, although the general similarities between different AE models suggests that this could be the case. Ca^{2+} currents are also known to activate K^+ current that initiate the repolarization of action potential and generate after-hyperpolarization potentials. Such Ca^{2+}-activated K^+ channel may represent an intrinsic inhibitory mechanism that would restore the resting state and maintain normal physiological excitability. The anomalies in this "membrane" trait could be the cause of increased AE susceptibility in rodents. It was demonstrated, that the molecular basis for the enhanced current density of L- and R-type of HVA Ca^{2+}channels in the GEPR inferior colliculli neurons was the upregulation in the expression of Ca^{2+}channel α1D and α1E subunits, and it could contribute to the genetic basis of GEPR enhanced seizure susceptibility. The up-regulation of Ca^{2+}channel α1A subunits

induced by seizures which was demonstrated, could contribute to the increased neuronal excitability in inferior collicullus. This trait developed after repetitive seizures in the GEPR (N'Gouemo, Morad , 2003, N'Guemo et al., 2010).

2.6 Myoclonic seizures induced by repetitive audiogenic seizures

The general notion of brain "kindling" describes the gradual increase of electric responses in limbic system (e.g. hippocampus or amygdala) to the initially subthreshold electrical (or chemical) stimuli (see Galvis-Alonso et al., 2004). Audiogenic kindling (myoclonic seizures, which develop after numerous daily sound exposures) is the result of the spread of seizure discharge to forebrain structures (hippocampus, amygdale, neocortex) after such repetitive stimulation and induces seizure fits of another type. The phenomenology of audiogenic kindling in KM strain was first described by A. Semiokhina in Russian paper (1958, cited by Krushinski, 1963). These seizures are represented by the rhythmic convulsive jerks which involve facial muscles, neck muscles and, as their highest intensity, the musculature of the whole body. In KM rats these seizures appear in response to sound exposure after 10-15 daily sound exposures. These type of seizure (audiogenic kindling) was described and studied extensively in GEPR, WAR and WAS rats (Doretto et al., 2009, Garcia-Cairasco, 2002, N'Gouemo & Faingold, 1996, Dutra Moraes et al., 2000, Ross & Coleman, 2000, Simler et al., 1994). Myoclonic seizure fits severity increases in both GEPR substrains and resulted in prominent cortical epileptiform EEG activity (Feng et al., 2001). The same feature was described in KM rats (Semiokhina et al., 2006).

The audiogenic kindling phenotype in GEPRs seems to be different from that observed in KM strain (N'Gouemo & Faingold, 1996), although War and KM kindling patterns seem to resemble one another (Dutra Moraes et al., 2000). These facts could be ascribed to genetic background similarity/dissimilarity in KMs and Wars from one side and GEPRS – from another.

It appears evident thus, that two factors - the audiogenic seizure proneness and the audiogenic fit "experience" after repetitive sound exposures-form the basis for audiogenic kindling development. The involvement of forebrain structures in the kindling development was demonstrated by EEG techniques as well as by means c-fos expression mapping (Doretto et al., 2009, Garcia-Cairasco, 2002, Eells et al., 2004, Ross & Coleman, 2000, Semiokhina et al., 2006,). The comparison of brain c-fos expression patterns which was characteristic for audiogenic seizure fit and for audiogenic kindling (mioclonic seizures) demonstrated that during kindling procedure the new process takes place - the "transformation" of midbrain seizure fit into forebrain seizure state. The latter involve the structures which initially were not connected directly with overt motor function (Eells et al., 2004). This process was determined by changes in the neuronal excitability in the genetically abnormal brain of AE susceptible rats.

The special interest was paid to the amygdala participation in audiogenic kindling phenomenon. Bilateral microinjection of a GABA(A) agonist, muscimol into amygdale (0.3 nmol/side) significantly reduced the duration of kindling clonic seizures, and they were suppressed by histamine injections. At the same time wild running and tonic components of AE fit were never affected by microinjection of these agents into the amygdala. Thus amygdala becomes critical for seizure development after the expansion of the seizure network (audiogenic kindling), and this type of seizures were negatively modulated by increased GABA function in this structure (Feng et al., 2001). It was also noted that severe brainstem seizures prevent the behavioral expression of forebrain (kindled) seizures in

GEPR-9s, although the EEG spike-wave discharges in the forebrain were present. The authors suggested that it was the high intensity of brainstem seizures which did not allow the forebrain seizure to be manifested (Merrill et al., 2005).
In an effort to identify genes involved in molecular mechanisms underlying acute and kindled audiogenic seizures, the suppression-subtractive hybridization was used in order to construct normalized cDNA library enriched for transcripts expressed in the hippocampus of WARs (Gitai et al., 2010). The most represented gene among the 133 clones sequenced was the ionotropic glutamate receptor subunit II (GluR2), a member of the alpha-amino-3-hydroxy-5-methyl-4-isoxazoleopropionic acid (AMPA) receptor. The hippocampal levels of the GluR2 subunits do not differ between naïve WARs and their Wistar controls, while the transcript encoding the splice-variant GluR2-flip expression was increased in the hippocampus (namely in CA1 region) of WARs submitted to both acute and kindled audiogenic seizures (Gitai et al., 2010).
Audiogenic kindling is the phenomenon inherent to rats with elevated AE predisposition, and for those which are predisposed genetically. Although the special analysis of the genetic basis of audiogenic kindling in KM rats was performed using the genetic selection. L. Romanova selected KM rats for quick and slow audiogenic kindling development. This selection was successful and the maximum selection effect in both new substrains was achieved in 4-5 generations. This made it possible to suggest that the predisposition for audiogenic kindling development is the oligogenic trait (Romanova et al., 1993).
In mouse no data exist about the possibility to induce the audiogenic kindling. Although in C3H male mouse brain, which was kindled by electric stimulation of hippocampus differentially expressed genes were screened in various time intervals after kindling. 50 from about 30,000 bands obtained were displayed differentially. Differential expression of genes identified was demonstrated in hippocampus and forebrain, but not in brainstem or cerebellum (Liang & Seyfried, 2001).

2.7 Audiogenic postictal catalepsy

In KM rats as well as in WARs (Garcia-Vairasco, personal communication) the audiogenic seizure fit is followed by the cataleptic state (Semioknina et al., 2006), which was named audiogenic postictal catalepsy. Catalepsy is characterized by the areflexia and drastic changes in the pattern of muscle tone – the so-called waxy flexibility. The duration of postictal audiogenic catalepsy correlated positively with seizure fit intensity (Fedotova et al., 2008). The pharmacological study as well as comparison of catalepsy intensity in rats of different genotypes permitted to conclude that the dopaminergic system was the key structure for this anomaly while glytamate- and GABA-ergic systems participate in its genesis as well playing the roles of modulators. It was possible to "disentangle" audiogenic fit and catalepsy correlation when glutamatergic agents (D-serine and MK-801) were injected (unpublished data). As catatonic syndrome in humans share several features with rat catalepsy we suggest that postictal audiogenic catalepsy could serve as valid laboratory model of this pathology, which would permit to make the preclinical anti-cataleptic drug testing (Fedotova et al., 2008).

2.8 Brain vascular anomalies

Early experiments in L.V. Krushinsky laboratory demonstrated that in cases when the rat was exposed to the sound stimulation for the longer period (e.g. ten minutes or more with special pattern of sound "offs" and "ons") the brain vascular disturbances could be noted in

many cases sometimes with lethal outcome (Krushinsky, 1962, 1963, Krushinsky et al., 1970). The causes of these deaths were the acute brain hemorrhages both epidural and ventricular, which were provoked by drastic changes in the permeability of brain capillary and erythrocyte diapedesis.

In rats which survived the prolonged sound exposure the less serious brain hemorrhages develop as well, which were verified morphologically. These hemorrhages manifested in vivo as hind limbs paresis after sound exposure which restored in several minutes.

The increase in arterial blood pressure was described as the initial phase of audiogenic fit which was followed by its' decrease (up to 87-90 mm Hg) (Krushinsky, 1963). These AP changes are in parallel with capillary permeability anomalies mentioned above. Changes in systemic and regional hemodynamic during sound-induced convulsions were measured in KM rats with microsphere technique. Blood pressure increased from 103 till 178 mm Hg and cardiac index rose from 27.3 to 49.3 ml/min/100 g b. w. during convulsion stage. Blood flow was increased in the brain and in the heart by 140-700%, whereas in most of internal organs it was decreased by 40-94% (Ivashev et al., 1991)...

The venous tone was estimated by means of circulatory filling pressure during a short arrest of circulation by inflating a balloon in the right atrium. It was confirmed that, during audiogenic seizure, AP raised from 104 to 156 mm Hg on the average, while mean circulatory filling pressure decreased from 8.9 to 7.4 mm Hg. The intensity of subdural and subarachnoidal hemorrhages correlated with the raise of AP during seizure. The hemorrhagic area spread over 75 mm^2 when the AP elevated above the 200 mm Hg level, while in lower increment it was only 2.56 mm^2 (Vlasov et al., 1991).

The rates of local cerebral blood flow during audiogenic seizures in Wistar audogenic seizure rats belonging to a genetic strain selected for AE at the Centre de Neurochimie (Strasbourg, France) were analyzed. During single audiogenic seizures, rates of local cerebral blood flow increased over control values in almost all brain areas, the highest increases being in brain stem (inferior and superior colliculus, reticular formation, and many thalamic and hypothalamic regions). The lowest increases were in forebrain limbic regions and cortical areas. In kindled rats the increase of cerebral blood flow was of lower amplitude, than in controls. Thus the good correlation between the involvement of structures in the AE progress and the increase of blood flow in them was discovered. The circulatory changes in kindled rats consisted in the decrease of blood flow, this fact reflecting the differences in respective mechanisms of brain stem AE seizures and audiogenic kindling (Nehlig et al., 1995).

In KM rats the inhibitor of NO-synthase (L-NNA) increased the severity of seizures, mortality rate and intracranial hemorrhages. In contrast, L-arginine (which is the substrate for NO synthesis) elevated the resistance of animals to acoustic stress. Preliminary exposure of rats to hypoxic conditions (1 hour in hypobaric chamber, simulated altitude of 5000 m above the sea level) decreased the development of dysfunctions induced by acoustic stress. At the same time the protective effect of hypoxic "training" disappeared after the administration of NO-synthase inhibitor (L-NNA). The study demonstrated that nitric oxide (NO) participates in adaptive reactions of cerebral hemodynamics linked with the significant increase of cerebral blood flow (Fadiukova et al., 2005).

2.9 Neurochemical correlates of audiogenic seizure susceptibility and fit development

Brainstem and forebrain neurochemical systems in GERPs and KM rats (to lesser extent) have been characterized. In brief, audiogenic seizure proneness in rats and mice is

accompanied by the changes in the background neurotransmitters levels, including both the background level and seizure aftereffects. The neurotransmitter systems involved in the generation of AE fits include noradrenergic, dopaminergic, serotoninergic systems, as well as glutamatergic and GABA-ergic neurons and neuronal networks (Laird et al., 1984, Jobe et al., 1986, Ribak et al., 1988, Lasley, 1991, Fedotova et al., 1996, Kosacheva et al., 1998). Fig 3 demonstrates the neurotransmitter aminoacid levels in brain regions of KM strain.

Jobe et al. (1986) found the "reciprocal relationship" between both noradrenergic and serotonergic transmissions and the severity of audiogenic seizures, thus indicating the deficit of brain monoamines as one of the concomitant neurochemical features of AE. The serotoninergic brain system participates in AE of DBA/2J mice. Interesting enough was the fact that the number of 5-HT2 binding sites was 20% higher in the cerebral cortex of DBA/2J in comparison to C57 BL/6 mice at the age of "audiogenic" susceptibility of DBA/2J mice but did not differ at other ages. There were no differences in these parameters between the two strains in forebrain, mid-brain, hippocampus and pons-medulla (Jazrawi et al., 1989).

The failure to replicate previously reported significant differences between the susceptible (DBA/2J) and nonsusceptible (C57BL/6J) mice in the brain monoamine levels at the age of peak seizure susceptibility (in DBAs) demonstrated the difficulties of the whole issue, which was probably due to different techniques used (Lints et al., 1980). The cordotomy and brain monoamine levels approach proved, as authors claimed (Willott et al., 1979), that NE and 5-HT are not responsible for attenuation of audiogenic seizures. These data demonstrate that inter species differences (mouse genotypes compared) could be responsible for differences in neurochemical changes during AE fit.

Compared to controls, GEPR-3s and GEPR-9s had a modest and larger increase respectively in Bmax for both high and low affinity GABA sites, with no change in Kd. Chloride-dependent, barbiturate-enhanced GABA binding (increased Bmax) was observed for all conditions and groups. Likewise benzodiazepine binding (Bmax) increased slightly in GEPR-9 animals. There were no observed changes in binding sites for a survey of biogenic amines. Seizure-prone animals appeared to have compensatory denervation-like supersensitivity for their most prominent inhibitory receptor, which may or may not be linked to the seizure event (Booker et al., 1986). This fact was also demonstrated in the direct experiments by chronic depletion of brain 5-HT by i.c.v. administration of 5,7-dihydroxytryptamine. This procedure induced the significant increase in seizure severity, which could be noted in 2, 3 and 4 weeks after drug injections (as compared to vehicle-injected controls) (Statnick et al., 1996).

The thorough comparison of serotoninergic system was performed by Bakhit et al. (1982). Tryptophan hydroxylase activity (the rate limiting enzyme of serotonin synthesis) was significantly lower in brains of Frings mice compared to CF1 control mice probably due to altered kinetic characteristics of this enzyme. However, brain levels of serotonin were similar in both strains, as well as the uptake of tryptophan and accumulation of 5-HT (following MAO-inhibitor pargyline infusion). These data represent the information suggesting that shifts in brain monoamine levels are not the direct cause of AE seizures (Bakhit et al., 1982).

In KM rats the diazepam binding in synaptic membrane of different brain regions was significantly lower than in control non-epileptic Wistars (with highest differences being in cerebellum (Joulin & Pleskacheva, 1991). GABA binding in the cerebellum (but not in the brain stem) was also lower in KM. Presumably the receptor numbers differences but not receptor affinity were the cause of data obtained. Low scores of cerebellum synaptic

membranes binding should be analyzed in the framework of brain anticonvulsive system concept (Kryzhanovskiĭ, 2002), as this structure is suggested to be central in the process of seizure suppression.

Data of the opposite "sign" were obtained in mice by Robertson (1980). The specific binding of 3H-flunitrazepam was higher in the seizure-susceptible DBA/2J strain (at about 22 days of age) when compared to age-matched, seizure-resistant C57BL/6J mice. The DBA/2J strain had higher benzodiazepine binding both in normal state and in seizures. This higher binding in DBA/2J was due to a higher benzodiazepine receptor density (Bmax) in this strain.

The neurochemical studies demonstrated that brain monoamine deficits could be detected in the projection areas of both NA-containing neuronal groups - locus ceruleus and the lateral tegmental area. These deficits existed in GEPRs without seizure experience and were more pronounced in the GEPR-9s as compared to GEPR-3s. GABA and taurine levels were also abnormal (Laird et al., 1984). It was proposed that the NA and 5-HT deficits were the causes of AE proneness, while GABA-taurine anomalies were "the inadequate attempts of the central nervous system to compensate for the seizure-prone state". This statement was proved not to be the case later (see Lasley, 1991).

GABA concentrations were lower in GEPRs compared to non-epileptic controls in several brain regions. Aspartate and taurine content was also elevated although not in all brain areas investigated (Lasley, 1991). Seizure experience induces the changes in this pattern – the increases in aspartate, glutamate and glycine levels took place (as compared to seizure-naive rats) in inferior colliculus and in sensomotor and frontal cortices. Author suggested that the high levels of taurine in GEPR-3s and the elevated content of aspartate in GEPR-9s play certain roles as determinants of seizure severity, as well as low concentrations of GABA did. The seizure-induced changes in brain aspartate and glutamate supported the concept that these excitatory amino acids mediate changes in seizure predisposition (Lasley, 1991, Molnar et al., 2000). In estimating the deviations of brain neurotransmitter levels the main attention was paid to their regional concentrations, inferior colliculi, PAG and substantia nigra being of prime interest. The incomplete seizure suppressant effect of naloxone was moderate, while blockade of NMDA receptors by AP7 or activation of GABA (A) receptors in the PAG really suppressed AE susceptibility. Thus, PAG was viewed as the important link in the chain of structures, necessary for AE fits with GABA (A), opioid peptide and NMDA receptors participating in fit severity regulation (N'Gouemo & Faingold, 1999). The 1,9-fold increase of brain glutamate, 2,3-fold increase in GABA, 2,4-fold increase of taurine in comparison to non-epileptic rats was found in inferior colliculi by Ribak et al. (1988). In the GEPRs bilateral microinjections of NMDA receptor antagonists in substantia nigra blocked or reduced the seizure severity. The technique of brain micro dialysis permitted to measure the reactivity of respective neurotransmitter systems in GEPRs and non-epileptic rats. It was demonstrated that the increases (relative to basal levels) for non-epileptic controls were 35%, 74%, 68%, 847% and 283% for aspartate, glutamate, glycin, taurine and GABA respectively. Corresponding increases for GEPR-9s were less intense - 14%, 10%, 41%, 505% and 123% (Doretto et al., 1994).

Akbar et al. (1998) demonstrated differences in the expression levels of mRNA for three amino acid transporters - glial and neuronal glutamate transporters (GLT-1 and EAAC-1), and the neuronal GABA transporter (GAT-1). Reductions in GAT-1 mRNA were found in genetically epileptic-prone rats in all brain regions examined. Similar reductions in GLT-1 mRNA expression levels were seen in cortex, striatum, and hippocampal CA1 of genetically

epileptic-prone rats; the largest reduction being in the inferior colliculus. Differences in messenger RNA levels for GLT-1 and EAAC-1 were not reflected or were reflected only partially in the expression of the corresponding proteins (Akbar et al., 1998).
The data obtained for KM strain were more or less similar (Raevski et al., 1995, Kosacheva et al., 1998). The baseline content of serotonin and its metabolite (5-oxyindoleacetic acid) in the temporal cortex, hippocampus and medulla as well as the striatal content of dopamine and 3, 4-dihydroxyphenilacetic acid were higher in KMs than in non-epileptic Wistars. The noradrenalin level in striatum was reduced. Audiogenic seizure fit development in KM rats induced the decrease of GABA level in: striatum, hippocampus and temporal cortex, as well as the reduction of striatal and hippocampal glutamate levels (fig.3). In KM rats the histamine levels in the striatum, hippocampus, amygdala, midbrain, thalamus and hypothalamus were significantly lower than in the epilepsy-resistant Wistar rats (Onodera et al., 1992). At the early stage of KM rats selection the caffeine infusions were the experimental technique to increase the audiogenic fit severity (Krushinsky, 1964). Thus the purinergic system could be considered as being involved into generation of the AE fit.

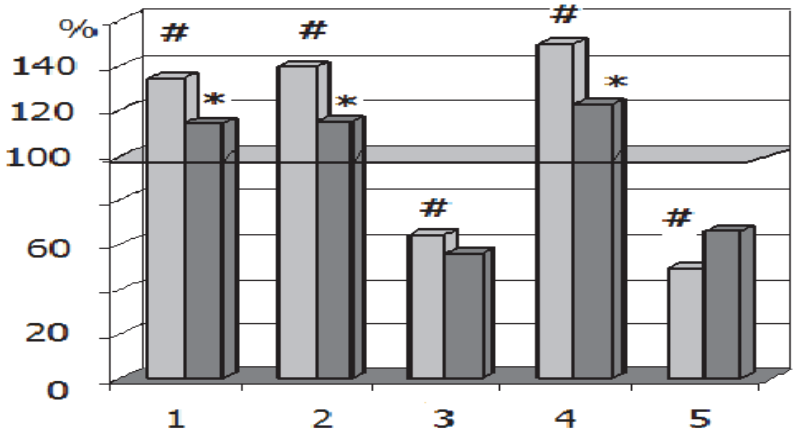

Fig. 3. Medulla levels of excitatory aminoacids (KM rat strain) without seizures (light gray columns) and at the maximum of tonic convulsions in response to sound (dark grey columns) (Raevski et al., 1995). The data presented as the percentage from intact Wistar rats respective level (100%). 1- aspartate, 2-glutamate, 3-GABA, 4-glycine, 5 – taurine. # - significantly different from Wistar rats, $p<0.05$, * - significantly different from the pre-seizure level, $p<0.05$

Brain dopamine system also participates in the complicated pattern of monoamine "audiogenic" pattern (Yu et al., 2000, Sorokin et al., 2004). The combined action of dopaminergic and serotonergic agonists suppressed audiogenic seizures, with local increase in dopamine and reduction in serotonin in the striatum (in rats not selected for AE). The audiogenic seizures which were provoked in rats by the withdrawal from chronic ethanol consumption were associated with increase in striatal dopamine and a reduction in striatal serotonin (Yu et al., 2000). In KM rats and the basal striatal dopamine level measured by in vivo microdialysis was 25% higher that in non-epileptic Wistars. A single amphetamine injection (1 mg/kg body weight, intraperitoneously) caused a significant increase in the

dopamine basal level up to 250-260% in animals of both genotypes. However, in Wistar rats, the dopamine level reached maximum as soon as 20 min after drug infusion, whereas in KM rats, this happened after 120 min. The increase in the dopamine level after a single injection of raclopride (antagonist of D2 and D3 receptors) was also similar in amplitude in rats of both genotypes (up to about 210%); however, this occurred 20-30 and 100 min after raclopride administration in Wistar and KM rats, respectively. This evidence confirmed that the genetic "defect" in audiogenic seizure prone KM rats is connected with brain monoamines anomalies. At the same time the peculiar scheduling of changes in dopamine increase could be connected with regulatory gene(s) dysfunction (Sorokin et al., 2004).

It is now recognized that neuronal hyperexcitability and excessive production of free radicals have been implicated in the pathogenesis of epilepsy. The high rate of oxidative metabolism, coupled with the low antioxidant defenses and the richness in polyunsaturated fatty acids, makes the brain highly vulnerable to free radical damage (Devi et al., 2008). Prolonged seizures may result in the mitochondrial dysfunction and increased production of reactive oxygen species and nitric oxide, which precede neuronal cell death and cause subsequent epileptogenesis. Emerging evidences also showed that intrinsic mitochondrial apoptotic pathway may contribute to the neuropathology of human epilepsy (Chuang, 2010).

In KM rats the background levels of the some membrane lipid fractions (containing endogenous antioxidants) were higher in comparison to non-epileptic Wistars. The development of audiogenic seizure fit was accompanied by the significant increase in free radicals levels and lipid peroxides while the lipid antioxidative activity decreased. These changes were found in medulla, thalamus and hippocampus but not in the neocortex (Fedotova et al., 1988). The decrease of brain antioxidant levels developed in parallel with the audiogenic fit stages. Thus it was concluded that these oxidative stress mechanism which participated in these processes also play the important role in the brain reaction to sound induced seizures.

The *in vitro* studies (incubation of brain synaptosomes and mitochondria of KM rats with lipid peroxidation inducers) demonstrated the decrease of deamination of serotonin (which is the substrate of MAO-A) in mitochondria, but not in synaptosomes with simultaneous stimulation of GABA deamination process. The latter was apparently due to modification of catalytic properties of brain membrane-bound MAO (Medvedev et al., 1992). The similar parameters of KM brain taken at the height of AE seizures demonstrated the stimulation in brain synaptosomes and mitochondria of lipid peroxidation. This was accompanied by a marked decrease in serotonin deamination, with a simultaneous increase in GABA deamination in both fractions. The data obtained suggested that appearance of GABA-deaminating activity might be the important component in the development of epileptic seizures and it was shown that this deamination was determined by the modification of catalytic properties of MAO (Medvedev et al., 1992).

In DBA/2J mice the combined inhibition of serotonin uptake and oxidative deamination attenuated audiogenic seizures. The administration of MAO-A inhibitor (clorgyline) suppressed these AE seizures and the inhibition of seizures by tryptophan was potentiated by combination with either of the mixed MAO inhibitors (Sparks, Buckholtz, 1985).

Brain cytokine system (interleukins -1, -2, -6) also participate the seizure development and demonstrate differences in mice of different genotypes. In Frings and DBA/2J mice the expression of interleukine-1-alpha transcripts in different brain regions after the AE seizures was elevated in hypothalamus but not in hippocampus presumably by dexamethasone-sensitive pathway (Gahring et al., 1997). Intracerebroventricular administration of

interleukin-2 (both recombinant human and mouse varieties) in DBA/2 mice, increased the incidence of seizures. Since interleukin-2 proconvulsant properties were antagonized by specific monoclonal antibodies, it was suggested that some epileptic phenomena could be linked to stimulation of IL-2 receptors (De Saro et al., 1994). Deficiency in interleukin-6 (IL-6 -/- KO) induce numerous changes in brain metabolism, namely in glutamate, aspartate, GABA, glycine and taurine in different brain structures and these mice developed the audiogenic seizure fits, although no differences between IL-6 WT and KO mice in the susceptibility to maximal electroshock were noted (De Luca et al., 2004).
As mentioned above, brain monoamines levels and metabolism were consistently reported to be different from non-epileptic animals in cases of rodent audiogenic seizures. The biological degradation of monoamines is catalyzed by monoamine oxidase. Tribulin is a fraction of endogenous MAO inhibitors which are detected in human and animal tissues and biological fluids. An investigation of the biological properties of tribulin revealed its heterogeneity and some chemical components were identified. The development of several brain pathologies (epilepsy among them) are accompanied by qualitative changes in catalytic ability of the membrane-bound MAO-A and MAO-B (Medvedev, 1999). Brain tribulin activity in KM rats was studied after audiogenic seizures of different intensity. Moderate and especially maximal audiogenic seizures were accompanied by the increase of both MAO A inhibitory activity (up to 2.5-fold in case of tonic seizures), this increase in sound exposed non-epileptic Wistars was significantly less intense (Medvedev et al., 1991).
Investigation of phosphorylation in homogenates of neocortex and hippocampus, aiming to discover AE proneness influence on Ca(2+)/calmodulin- and cAMP-dependent systems was performed. Non-epilectic Wistar rats, not-pure bred KM audiogenic seizure prone rats and the same rats after being exposed to audiogenic kindling were the subjects of this study. The significant differences in phosphorylation of 270, 58, 54 and 42 kDa proteins in neocortex and hippocampus were found. The activity of PKA was higher both in neocortex and hippocampus in audiogenic sensitive rats. Daily repeated audiogenic seizures induced the decrease of Ca(2+)-independent CAMKII activity in hippocampus and the increase of PKA activity in non-epileptic rat neocortex in comparison to audiogenic rats (Yechikhov et al., 2001).
Thus the neurochemical studies demonstrated both the similarity and differences in the shifts of main neurotransmission systems in rats and mice, susceptible to AE. The current problem seems to be to detect which brain chemistry and/or gene expression peculiarities are the primary ones (more close to initial genetic defect) and which – develop as the consequences of primary defects. The high complexity of neuronal signaling pathways permit such suggestion, and could tune researchers to the pessimistic mode. Although the detailed knowledge of gene expression during brain development which is extensively investigated at present could bring the quick and may be unexpected data in this respect.

2.10 Krushinsky's views on AE phenotype: Pure historical interest or the insight with present scientific significance

At the start of audiogenic epilepsy studies (at the beginning of 1950s) L.V. Krushinsky created the hypothesis that this pathology could be explained by the misbalance between brain excitation and inhibition processes. We should remember that Moruzzi and Magoun first published their data on reticular activation system only in 1949, and there were no ways to discuss in the USSR the possible impact of genotype in AE development, although KM data were obtained via the genetic selection. According to L. Krushinsky's hypothesis the following sequence of events took place in AE rat CNS after the sound onset. Strong

sound induces the excitation which "irradiates" along the brain stem structures and wild run stage starts. In response to this start the inhibitory process initiates which could be regarded as the reaction against anomalous excitation. As the result the wild run stage could stop. Then there could be two options of events development: i) the fit stops and no further abnormal reactions take place (that will be the audiogenic fit of low intensity, Krushinsky's arbitrary unit "1"), or ii) there occurs only the "inhibitory pause" in the wild run behavior lasting several seconds. As this pause ends the wild run renews and in majority of cases the motor seizure occurs (arbitrary units 2-4). According to Krushinsky's view the prolongation of sound action during the inhibitory pause leads to the "exhaustion" of inhibition as it is too strong. He hypothesized that the inhibitory pause which inserts into the wild run stage (creating "two waves" of wild locomotion) is the "active" inhibitory process. The finish of motor seizures after the "second wave" of the wild run and clonic-tonic convulsions is the result of "over-limit" inhibition (that is of inhibition which develops as the response to very strong stimulation). Bromide infusions accentuated the "inhibitory pause" in the audiogenic fit sequence, and induce such pause in rats which previously showed the "single wave" pattern of wild run. Caffeine's effect was the reverse – the "inhibitory pause" of wild run dissappeared and the fit intensity increased.

At present some arguments in the favor of that old theoretical consideration could be found, that is arguments for benefit of the postulated brain macro-processes and their interactions. One of them is the well known fact concerning the lack of epileptic EEG discharges in the neocortex and hippocampus during the AE fit. It could be partly ascribed to the "inhibited state" of these structures. Now it was demonstrated that audiogenic fit development is accompanied by the neocortical spreading depression (Vinogradova et al., 2005), which also inhibit both EEG and single unit activity in this structure. The anomalies in the balance of excitatory and inhibitory aminoacid transmitters which are inherent to AE and which are accentuated during and after the AE fit also could be the manifestation of these two processes interactions. The data also exist which concern the induction of c-Fos expression in neocortex as the result of audiogenic seizure fit, which means that the excitation of GABA-ergic interneurons could explain this finding (e.g. Batuev et al., 1997).

3. Conclusion

Many data obtained evidence that the AE susceptibility is associated with the decreased thresholds for evoking seizures of other types (e.g. Scarlatelli-Lima et al., 2003). In WARs the general seizure susceptibility was elevated in comparison to non-epileptic Wistar rats in experiments using other pro-convulsive stimuli (apart from loud sound) - transauricular electroshock, pentylenetetrazole and pilocarpine).

The similar patterns of relationships were found in mouse studies. Audiogenic seizures in mice selectively bred for susceptibility and resistance to ethanol withdrawal (induction of seizures during handling) were investigated. Resistant mice exhibited no AE response at any age, whereas "seizure withdrawal prone" mice were sensitive on several age (days 17, 22, and 28). These data suggested that susceptibility to AE and handling-induced convulsions during EtOH withdrawal may share some common genetic determinants and presumably some common neurochemical systems. This phenomenon could be regarded as the AE susceptibility induced by changes in brain neurochemistry induced by ethanol consumption. Authors suggest that these two types of seizures may share some common genetic determinants and presumably some common neurochemical systems (Feller et al., 1994).

The AE fit pattern in specialized genetic strains of rats and mice is more or less similar and this fact gives the possibility to suggest the existence of the specific "audiogenic epilepsy endophenotype". Gould, Gottesman (2006) introduced the endophenotype notion and described the endophenotypes as "quantifiable components in the genes-to-behaviors pathways". The endophenotype concept has emerged as a strategic tool in neuropsychiatric research, but it has more general applicability. Endophenotypes, as Gould & Gottesman (2006) stated, can be neurophysiological, biochemical, endocrine, neuroanatomical, cognitive or neuropsychological. Such pattern of specific features could be regarded as complex but rather specific trait of given phenomenon. It is evident that the complex relationships between genes and behavior could not be exactly reproduced in different experiments with different objects (namely in different AE models). Although the analysis of whole plethora of AE data obtained from this point of view - that there is hypothetical "common pathway" by which this pathology could be realized in the brain - could help in understanding of this phenomenon. The endophenotype for audiogenic epilepsy could be the specific seizure-provoking constellation of genetic and neurochemical events, which act both at the level of peripheral hearing organ and at the level of brain structures. The development of regional specific channellopathies and/or of the misbalance in GABA and glutamate systems (both central and cochlear) could be the possible links which help in the search of the endogphenotype of interest. The key components of any useful endophenotype are heritability and stability (state independence). Endophenotype approach is useful as "it reduces the complexity of symptoms and multifaceted behaviors" (Gould & Gottesman, 2006) which result in identifying units of analysis that could be explored in animal models.

The data reviewed above demonstrated that apart from phenotypical similarity of AE seizures there are not many traits common for all models explored – these are the disturbances of hearing system and the misbalance of brain glutamate and GABA-ergic system. The malfunction of other traits - brain monoamine, purinergic, histaminergic, cytokine systems dysfunction were also described, although the pattern of changes in cases of AE were not always consistent in different models and vary with different techniques used, animal models and species chosen (rat-mouse).

It looks more or less plausible that the disturbances in gene expression patterns rather early in development could underlie the AE, although these disturbances (mutations) should be still compatible with the fetus survival and future animal viability (although probably reduced). Such mutations are still to be identified, although the examples of genetic elements which could belong to such category are now available.

The simultaneous genetic inactivation of three transcriptional factors connected with circadian rhythmicity (albumin D-site-binding protein proline, hepatic leukemia factor and thyrotrophic embryonic factor) induce the audiogenic epilepsy in young mice (Gachon et al., 2004). The target gene of this transcription factors family is the pyridoxal kinase (*Pdxk*), which catalyses the conversion of vitamin B12 derivatives into pyridoxal-phosphate. The latter is the coenzyme of many enzymes participating in the neurotransmitter metabolism. It was demonstrated that in the triple mouse KOs the levels of brain pyridoxal-phosphate, serotonin and dopamine were decreased (Gachon et al., 2004).

Mutations of other genes which are identified as being involved in the early stages of CNS development could also be the possible candidates for causes of AE development. One example of such mutation could be the *disheveled* genes. The proteins coded by this gene family are the important signaling components of beta-catenin/Wnt pathway, which controls cell proliferation and patterning, and the planar cell polarity pathway. Dvl3 (-/-)

mice died perinatally with several developmental defects, the misorientated stereocilia in the organ of Corti being among them (Etheridge et al., 2008).
The specific pattern of the hypothesized abnormal "developmental" genes expression could be the cause of the CNS disturbances and of the AE in particular. These anomalies presumably could induce the region specific channelopathies in the structures involved in sound-seizure production. As different channellopathies (not yet well understood) are the prominent trait of many human types of epilepsy, the study of genetic/physiological basis of their regional specificity (in AE models) could be of certain clinical value. Although another hypothesis could be tested as well, that such region-specific channelopaties arise in rats and mice as the result of the abnormal pattern of CNS development per se, the rodent brain being especially vulnerable for loud sound as these animals rely on sound sensitivity in avoiding danger in natural habitats.

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Part 4

Ion and Channels

7

Ionic Imbalance

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1. Introduction

Seizures are accompanied by significant alterations in both intra- and extracellular ion concentrations (Hotson, 1973; Kager et al., 2000). These concentration gradients, in concert with the conductances of a variety of ion channels, determine the resting membrane potential, the degree of membrane excitability, and the strength of excitatory and inhibitory synaptic responses. Changes in these concentration gradients have profound effects on the signaling and excitability of cells and can therefore play an important role in seizure generation, maintenance and cessation (Kager et al., 2000).

In this chapter, we describe the network of pumps, channels, and exchangers that make up the ionic regulatory system (IRS). This system runs in a nearly steady state while the neuron is at rest and responds to increased neuronal signaling by reestablishing normal ion distributions. We give a brief description of the basic elements of the IRS and their role in ionic maintenance. The IRS has the ability to redistribute intra- and extracellular ions rapidly, but this system can be overwhelmed by excessive activity, allowing ion concentration to drift towards equilibrium permitting their effects to dominate neuronal dynamics. (Ullah et al., 2009; Ransom et al., 2004) In addition to excessive neuronal firing, the IRS can be compromised through a broad range of alterations to its constituent mechanisms. We give a broad overview of how malfunctions in critical elements of the IRS may influence seizure onset or duration and identify several motifs that may play a role in a wide range of seizure disorders.

We will present a brief review of the known etiologies for a small number of seizure disorders. We discuss the ways in which idiopathic causes of epilepsy such as channelopathies, as well as symptomatic origins such as structural malformations and scarring, interact with the IRS to modulate or even generate seizure activity. In the second part of this section we outline the role of the IRS in experimental seizure models, both acute and chronic. We conclude this section by evaluating experimental seizure models and mathematical models based on the mechanisms that comprise the IRS.

In the final section we discuss the action of common anti epileptic drugs and note whenever possible their proposed mechanism in seizure prevention or cessation. We discuss direct and indirect interactions of these drugs upon the IRS and evaluate potential consequences of these interactions.

2. The ionic regulatory system

2.1 Components of the ionic regulatory system

The neuron at rest is subject to continuous leakage of ions across the plasma membrane. It therefore requires a steady flow of energy to maintain ionic concentrations necessary for normal neuronal firing. Ions leak down their electrochemical gradients via a combination of diffusion through the membrane as well as channels whose conductances are relatively insensitive to voltage but highly selective to ionic species. These leak currents determine the resting membrane potential and serve to reestablish the resting membrane potential after perturbations, wiping out the effects of inputs received by the neuron in the distant past. It may even be said that leak currents allow the neuron to "forget" remote inputs. The conductance through other channels depends upon factors such as transmembrane potential, ionic concentrations, chemical ligands, and more sophisticated cellular processes. These channels are involved in neurotransmitter signaling and action potential generation.

The electrical state of the neuron is determined by the flow of ions across the plasma membrane. The passage of ions through these channels is due to a competition between their charge flow down the electrical gradient and chemical flow down their concentration gradient. The potential at which these mechanisms are balanced is described by the logarithm of the ionic concentrations for a given ion outside and inside the cell and is known as the Nernst potential.

$$V = 26.6 \ln\left[C_o / C_i\right] \tag{1}$$

The further the actual membrane potential is from this Nernst potential, the greater the electrochemical gradient for that species of ion. The amount of charge that flows down this gradient can be approximated by Ohm's law, where resistance equals one over the membrane conductivity, σ, for the cell and the voltage is the difference between the membrane potential and the reversal potential for the ion. In reality, the conductivity is dependent on the ionic concentrations and is better modeled using the Goldman-Hodgkin-Katz equation. The approach we outline is a good approximation to the GHK analysis and is far more transparent.

$$I_i = \sigma_i (V_m - V_i) \tag{2}$$

At rest, the electric current produced by the leak of sodium, potassium, and chloride are balanced, and the resting potential, aside from osmotic effects and immobile ions, is determined by a conductance-weighted average of their reversal potentials. The net current through the ion channels is described as:

$$I = \sigma (V_m - V_{rev}) = \sum_i \sigma_i (V_m - V_i) \tag{3}$$

We can therefore express the total reversal as:

$$V_{rev} = \frac{\sum_i \sigma_i V_m}{\sum_i \sigma_i} \tag{4}$$

Similarly, the reversal potential for channels that are permeable to multiple ions, such as the NMDA, AMPA, and GABA channels, is roughly a conductance-weighted average of the reversal potentials for the permeable ions. In any case the flow of ions through a pore is proportional to the difference between the membrane potential and the reversal potential for the ion. This difference is the electrochemical gradient experienced by these ions.

Individual ions are not regulated independently, but rather the pumps, channels, and cotransporters providing steady state concentration gradients often exchange precise ratios of different ionic species therefore connecting their individual dynamics. Therefore, the IRS forms a driven-coupled-dynamic system that can display complex behaviors when forced from normal steady state conditions (Cressman, 2009). These emergent behaviors may play a role in seizure dynamics.

Ultimately the driving force behind all ionic gradients is a set of ATPases embedded in the cellular membrane that use the energy stored in ATP to move chemical species and charges against their electrochemical gradients. There are numerous electrogenic exchangers in the human brain, but a few of these play a disproportionately large role in establishing ionic gradients. The primary source of electrochemical gradients across the plasma membrane of neurons is the 3/2 sodium potassium ATPase. This pump extrudes three sodium ions into the extracellular space in exchange for the uptake of two potassium ions into the intracellular space, moving both elements up their chemical gradient as well as +e charge up the electrical gradient. The rate of exchange increases with increasing intracellular sodium and extracellular potassium concentrations, providing a mechanism that compensates for increased ionic drain produced by electrical signaling (Lennie, 2003; Attwell, 2001). It is also responsive to the membrane potential, magnesium concentration, and ATP availability. Pumping rates and ATP production are limited by glucose, oxygen and mitochondrial function, therefore there are multiple constraints upon the compensatory action of the pump.

Gradients are further tuned by the action of electroneutral cotransporters that move ions up their concentration gradient at the expense of a second, electrochemically favorable ionic gradient. For instance, chloride gradients are largely established through the cotransport of potassium and chloride ions through the KCl cotransporters (Payne, 1997; Stein, 2003). The action of this cotransporter allows potassium to exit the cell down its electrochemical gradient while forcing chloride up its electrochemical gradient. Therefore the steady state chloride gradients are directly influenced by the potassium gradients establishing more linkage between ion species.

The neuronal environment is also highly regulated by the activity of glial cells surrounding the neurons. Glial cells help maintain a normal neuronal environment through a range of actions, including reuptake of extracellular glutamate and buffering of extracellular potassium concentrations. Astrocytes posses a high density of inward rectifying potassium channels (Kir). The phenomenon of rectification is produced by polyamines or magnesium ions that block the channel at positive potentials, preventing potassium from flowing down its electrochemical gradient into the extracellular space. As potassium is dumped into the extracellular space, its reversal potential increases towards zero and becomes greater than that of the glial membrane potential, thus leading to an inward flow of potassium (Butt, 2006). Once siphoned into the glial cell, potassium diffuses through the network of glial cells via gap junctions that allow networks of glia to form extensive functional groups. These glial networks can move potassium from areas of high to low extracellular potassium concentration. They may also serve to move ions into the vasculature by interacting with

specialized endothelial cells of the blood brain barrier (BBB). This buffering may mitigate the local excitatory effects of increased extracellular potassium, but it may also facilitate the spread of potassium over relatively large spatial networks (Somjen, 2004).

In addition to glial buffering, simple ionic diffusion and osmosis will blur ionic gradients in the extracellular space. On the scales of the extracellular space the diffusion constant for water can be assumed constant, however the effective diffusion is limited by the path the ions can take. This effect, or tortuosity, can be greatly influenced by the geometry of the extracellular space. Increased extracellular potassium concentrations leads to potassium uptake by glial cells and the subsequent absorption of water through osmotic action. This influx of water leads to cell swelling that in turn can cause the extracellular space to become more confined resulting in an increased tortuosity (Somjen, 2004; Sykova, 2005).

Finally, the brain needs a continual supply of energy, oxygen, and other building blocks as well as the removal of carbon dioxide and other waste. As in the rest of the body this role is provided by the vasculature, however endothelial cells of the central nervous system and gonads are far less permeable than the in the rest of the body. This increased regulation is provided by the BBB and the cerebral spinal fluid barrier (CSFB). Endothelial cells with tight junctions line the capillaries in grey matter to make the BBB, and similar cells line the blood vessels of the choroids plexus and pial and arachnoid spaces as well as the outer layer of cells of the arachnoid (Somjen, 2004; Zheng et al. 2005). These cells actively move ions, amino acids, glucose and more in and out of the vasculature and allow the passive diffusion of oxygen and carbon dioxide. The endothelial cells of the BBB and their tight junctions are influenced by the endfeet processes of the glial syncytium, interactions that are believed to play an important role in the glial buffering of potassium, as discussed above.

One should not think of the IRS as exerting strict control over ionic composition; rather this set of mechanisms supports certain fixed states towards which the system will evolve. However this system can potentially support multiple fixed points, and limit cycles as well as a range of long lived transient states. This lack of strict control could be viewed as a shortcoming of neuronal systems, but in reality it affords them a great degree of dynamics that can be used for changing neuronal function. For instance, calcium entering the buton after the arrival of an action potential is not immediately expelled, and this makes subsequent action potentials more effective at producing vesicle release (Eccles, 1941, Feng, 1941). This common mechanism of synaptic potentiation is utilized by some cells and has been proposed as the basis for information encoding. Other transient neuronal states, such as cortical up-states, may also make use of transient deviations from ionic balance(Lennie, 2003; Haider, 2006; Ullah et al., 2009).

2.2 Breakdowns of the IRS

Seizure disorders may be caused by a number of distinct etiologies. Causes are generally classified either as idiopathic, that is, of unknown but likely genetic origin, or symptomatic, that is, due to a lesion or structural malformation (Shimizu-Okabe et al., 2007). A number of genetic abnormalities have been identified as causing idiopathic epilepsy disorders, and for many of these abnormal protein structure and function have been identified. The genetic culprits tend to encode ion channels and receptors for neurotransmitters (Lu & Wang, 2009). For epilepsy due to structural changes or defects, the cause of the seizure is due to malformations or scarring that cause far less subtle changes in neuronal function. We will proceed by revisiting the main components of the IRS, discuss which components would

make the system vulnerable, and present examples of epilepsies that are influenced by these breakdowns. We will finish this section by discussing experimental and computational models of epileptic seizures and discuss how these are predicated on disruptions to the IRS. We will begin by describing some general effects of disturbances to the ionic environment on seizure generation and propagation. We will refer back to these motifs in each subsequent section. They are segregated into discussions of resting membrane potential, excitability and refractory periods, edema, acidosis, and synaptic release and response.

2.2.1 Resting potential

The magnitude of the resting membrane potential is approximated as the weighted average of the reversal potentials for the ions conducted at rest as described in the previous section. Reversal potentials decrease in magnitude with decreasing concentration gradients, and so decreases in potassium and/or chloride gradients will result in an upward shift of the resting membrane potential and can lead to increased excitability and spontaneous spike generation. On the other hand, decreases in sodium gradients have the reverse effect, making the cell less excitable. The membrane potential is more sensitive to absolute changes in potassium concentrations than absolute sodium concentrations, since potassium is more conductive than sodium. Therefore potassium is the most important ion for establishing the resting membrane potential (Kager et al., 2000).

The potassium reversal potential is also more sensitive to changes in potassium concentrations than are reversal potentials for sodium and chloride. Since reversal potentials depend on the ratio of the extra- and intracellular concentrations an absolute change to the smaller of the two concentration has a greater effect on the reversal potential than would the same change in the larger concentration. Ionic flow across the membrane also produces a larger concentration change in the exterior than in the substantially larger interior space. Since potassium concentrations are lowest in the extracellular space and sodium and chloride concentrations are lowest in the interior, the external potassium concentration is most easily altered. The physiological concentration of potassium (4mM) is low as compared to sodium (18mM) and chloride (6mM), making it more susceptible to large percent changes in concentration and therefore large changes in the its reversal potential.

2.2.2 Excitability and refractory periods

Neuronal excitability is a function of both the resting membrane potential as well as the threshold for firing. This threshold is determined by the voltage gated response of sodium channels as well as the electrochemical gradient for sodium. If sodium gradients are diminished they will cause the threshold to rise to higher voltages making the cell less excitable, providing some amount of negative feedback on continual firing. This mechanism is widely accepted as the means by which some bursting neurons intermittently cease their activity (Catterall, 2000; Hodgkin & Huxley, 1952).

After spike initiation, sodium influx, and depolarization, voltage gaited potassium channels open and release potassium from the cell, thus repolarizing the membrane. The rate of this repolarization is determined by the electrochemical potassium gradients and potassium conductance. If either of these mechanisms are impaired the duration of the depolarization is lengthened, resulting in increasing transmitter release. The magnitude of the hyperpolarization can also become diminished and cause a reduced or eliminated relative

refractory period. On the other hand, in neurons with inactivating sodium channels, the loss of hyperpolarzation may lead to a prolonged absolute refractory period and therefore reduced potential for fast action potential generation (Catterall, 2000).

2.2.3 Synaptic release and response

Synaptic release is initiated when an action potential enters a buton and depolarizes its membrane. This depolarization activates voltage-gated calcium channels and allows calcium to enter the buton, producing a fusion of vesicles with the plasma membrane and the release of transmitter into the synaptic cleft. The amount of calcium entering the buton depends on the duration of depolarization by the action potential as well as the electrochemical drive forcing calcium into the cell. Therefore low extracellular levels of calcium work to inhibit synaptic release.

As stated in the previous section the reversal potentials for all mixed ion channels are approximated by the weighted average of the ions they conduct. This treatment can be applied to any ion channel. Here we will discuss the effects on the AMPA and GABAa receptors. The AMPA channel is in general excitatory, and it conducts calcium, potassium, and sodium. Decreased potassium gradients will make these channels more excitatory while decreased calcium and sodium gradients will lead to decreased excitation (Kager et al., 2000).

The GABAa receptor is permeable to both chloride and bicarbonate. The reversal is generally very low due to the chloride reversal potential. Maintenance of the chloride gradient is essential to produce strong inhibitory responses. If the breakdown in this gradient is significant, chloride can flow out of the cell upon receptor activation leading to excitation. As mentioned above the chloride gradient is intimately linked to the potassium gradients. Therefore, losses in potassium gradients are once more implicated in making neural networks more excitable.

2.2.4 Edema

Ionic imbalances can lead to transient osmotic gradients between neurons and glia and the extracellular space. These gradients are diminished by the flow of water towards regions of high concentration. When concentrations are higher in cells, as is the case during metabolic malfunction, or when glia absorb extracellular potassium, water movement into the tissue results in edema. This swelling can have detrimental effects on further potassium clearance through diffusion (Somjen 2004; Ragaišis, 2002; Park et al., 2008).

2.2.5 Acidosis and alkalosis

Acidosis (a lower than normal pH) and alkalosis (a greater than normal pH) can affect neuronal excitability. In general, acidosis lowers excitability, and alkalosis increases excitability. This is because H^+ ions directly modulate ion channels, both voltage gated and ligand gated.

H^+ ions inhibit all voltage gated ion channels, but not equally. If all channels were equally inhibited, there would be no impact on excitability, because inward currents would still balance outward currents. However, calcium channels (the most inhibited) and sodium channels are inhibited much more strongly than potassium channels. In fact, in pH ranges that are consistent with life, potassium is relatively unaffected by H^+ ions. This means that

an increased concentration of H^+ ions (acidosis) inhibits inward currents (such as Na^+ and Ca^{2+}) while leaving outward currents unaffected. The overall net result is more outward current relative to inward current, and a hyperpolarized neuron. This is consistent with lower excitability. On the other hand, an absence of H^+ ions (alkalosis) would boost inward currents, and somewhat depolarize the cell, leading to excitability. In addition, at the synapse, an increased flow of inward Ca^{2+} results in an increased release of neurotransmitters, which also helps explain why alkalosis causes excitability(Somjen, 2004).
H^+ ions also impact ligand-gated ion channels. Glutamate modulated ion channels are depressed by H^+, resulting in inhibition. In contrast, GABA modulated ion channels are enhanced by H^+, also resulting in inhibition (Somjen, 2004).

3. Epilepsies

3.1 Idiopathic

Out of the vast array of proteins expressed in the brain, only a small number are responsible for epilepsies. The complexity of the brain and its behavior may lead one to expect a greater variety of disorders. However, insensitivity to most parameters, redundancy, and the fact that phenotypes can only survive as long as their seizures remain transient, all limit the range of genetic variation that can lead to seizure disorders.
Familial hemiplegic migraines are associated with mutations in the genes encoding sodium/potassium ATPase and in rare cases patients exhibit epileptic seizures (Ashmore et al., 2009; Gallanti et al, 2008). With decreased pumping, normal neuronal activity results in the general loss of all ionic gradients. Complete loss of gradients will however more likely lead to spreading depression and perhaps migraine rather than seizures. In order to produce sustained seizure activity the pumps must remain at least partially functional.
In a similar fashion, without glucose, oxygen, and an intact metabolic system to produce ATP, sodium/potassium pumps are incapable of maintaining the resting potential and cells quickly depolarize once they drift above threshold and begin firing action potentials. If ATP is not produced in a relatively short time, loss of mitochondrial gradients as well as elevated calcium concentrations will initiate apoptotic pathways. There is then the potential for the creation of lesions whose spatial extent depends on the scale, duration, and severity of the metabolic deprivation.
Even though there are a large number of ion channels, only a small number have been found to be directly responsible for epileptic syndromes. Generalized epilepsy with febrile seizures plus (GEFS+) encompasses a number of disorders including severe myoclonic epilepsy of infancy (SMEI), border-line SMEI (SMEB), and intractable epilepsy of childhood (IEC) (Kapur 2002; Lossin et al. 2002). In total, there are four types of GEFS+, each defined by its causative mutation (Lossin et al., 2002). Three types of GEFS+ include mutations to either the alpha or beta subunit of the NAv1.1 or Nav 2.1 sodium channels (Fujiwara-Tsukamoto et al., 2004; Rosenberg et al., 1997). There are a number of different direct effects on sodium channel conductance, including changes in inactivation and frequency-dependent rundown. The loss of inactivation removes a strong governing effect on neuronal firing, increasing afferent signaling as well as ionic accumulation around the effective cells. The former obviously leads to greater excitation, but would also lead to greater inhibitory output. Ionic accumulation not only increases due to the increased activity, but also because the loss of inactivation allows higher sodium currents to keep the cell from repolarizing. This windowing effect will be discussed in greater detail below.

Epilepsies can arise from disfunction to the inhibitory mechanisms. Mutations to $GABA_A$ receptors are implicated in GFES+ type 3. In neuronal networks with these mutations, inhibitory currents are one-tenth of their normal strength. The direct loss of inhibition should be weakly compensated for by the reduction of potassium flowing out of the KCl cotransporters, as decreased potassium flow should reduce the potassium accumulation outlined above. This effect is most likely insignificant in comparison to the loss of inhibition and potential for uncontrolled excitatory discharge.

As previously mentioned, cotransporters are important for establishing chloride gradients. Unlike sodium, calcium, and potassium, chloride has no other means to produce a gradient than by utilizing cotransporters. There is evidence that reexpression of juvenile sodium-potassium-chloride cotransporters occurs after trauma (Shimizu-Okabe et al., 2007; Payne, 1997), and upregulation of the gene for the sodium-potassium-chloride cotransporter has been demonstrated in epileptic tissue (Shimizu-Okabe et al., 2007). In the adult form these cotransporters moves both potassium and chloride out of the cell functionally increasing the strength of post synaptic inhibitory currents. The juvenile form moves potassium sodium, and chloride into the cell. This juvenile form diminishes the strength of GABA and its reversal potential can even lie above the resting potential resulting in GABAergic excitation. On the other hand decreasing the potassium and sodium reversal potentials causes a relative inhibitory effect on the neurons.

It is interesting to note that there is no significant evidence that mutations to potassium channels leads to seizures disorders. It is easy to imagine that any source of significant change in potassium conductances would have a severe effect on both the resting potential, excitability, and synaptic strength. Perhaps it is because of this sensitivity that phenotypes with potassium channel dysfunction are not viable.

3.2 Symptomatic

Symptomatic epilepsies are caused by lesions, malformations, and tumors. Although these seizures are due to identifiable structures, the specific cellular alterations and therefore etiologies are quite different. These alterations can include structural and functional changes due to direct mechanical insult or genetic regulation due to more complex processes such as immunological responses.

Brain lesions can either be highly localized as in the case of traumatic injury or ischemia, spread across elements of extensive neuronal circuits like the limbic system, or present diffusely as in a systemic metabolic crisis. The tissue within and surrounding a lesion is different from normal brain. In certain severe lesions complete neuronal death occurs, leaving microglia and astrocytes to occupy the interior of the lesion. In other cases a reduced number of neurons remain within the lesion. In these cases, the behavior and morphology of these neurons can change. In the next section we will discuss experimental models aimed at investigate these post trauma alterations. They suggest that seizures may more easily arise and persist due to reduced potassium buffering by glial cells affected by the trauma, perforation of the BBB, and subsequent immune response.

Tumors, malignant and benign, can induce epileptic seizures. In many cases, these seizures appear to originate from the borders of the tumor (de Groot et al., 2010) and may not even present until the tumor is removed. In other cases the cells within the tumor are functionally active and may play a role in seizure generation themselves (de Groot et al., 2010). There are numerous types of tumors that can result in epileptogenesis. Some gliomas expand into the

surrounding parenchyma by releasing large quantities of glutamate. This glutamate excites local neurons, depolarizes them, inducing high levels of activity that eventually leads to neuronal death. As the neurons die off the cancer can expand into the vacated space. Prior to cell death the excessive excitation, in tandem with other modifications to the tissue can lead to seizure generation.

Seizures caused by malformations, such as cortical dysplasia, are associated with the presence of abnormal cells in the region of the malformation. The effects of these morphological and structural abnormalities on seizure generation is not well understood. However as has been discussed above in the context of edema, relatively large cellular volume can lead to reduced potassium buffering by the glial network. Similarly, structural changes associated with other diseases such as Sturge Weber syndrome can cause seizures. In this case vasculature malformations exerts stresses on the local neuronal and glial tissue.

As is often the case, it is straightforward to identify qualitative mechanisms whose actions could lead to epileptic behavior. However we are often presented with opposing mechanisms whose combined effects cannot be easily ascertained. As these systems are complex it is often impossible to analytically determine which effects will dominate and further investigation via computational modeling or experiments are necessary.

4. Models

As already mentioned, the IRS and neuronal dynamics in general can produce complicated dynamics. Therefore altered function of a specific mechanism cannot always be extrapolated to overall behavior. To this end it is often necessary to perform experiments or computational simulations.

4.1 Experimental models

4.1.1 In vivo

Most experiments aimed at understanding the electrophysiology of epilepsy are performed in rodent models. Most in vivo models involve producing damaged, or lesioned, neuronal circuits. Whether it be through electrical, chemical, or mechanical insult the resulting brain will show significant and stereotypical damage to susceptible regions and cell types. As in human epilepsy, the development of chronic seizures in these animal models is not immediate. Epileptogensis is due to changes to the neuronal circuits that involve apoptotic and necrotic cell death, astrocyte and microglia migrations, and complex alterations to neuronal and astrocytic function. A complicated set of observable changes is well documented in the literature and we will only discuss a small number of them here.

It has recently been proposed that epileptogenesis occurs at least in part due to a breakdown in the BBB trauma, infection, or ischemia (Friedman et al., 2009). Animal models demonstrate that neuronal tissue undergoes a set of stereotyped alterations in response to albumin entering the extracellular space through the breeched BBB. First, there is a reduction in the expression of Kir4.1 channels in glial endfeet. These channels as discussed above are one of the essential features of glia that allow them to buffer potassium from the extracellular space. Secondly, there is a reduction in the expression of gap junctions in glial cells. These gap junctions link together glial cells forming a syncytium that facilitates potassium buffering as discussed previously. As with a reduction in Kir channels the impaired syncytium limits the ability of the glia to remove potassium from the extracellular

space. Experiments on genetically modified animals confirm that these effects facilitate the generation of seizures (Binder & Steinhäuser, 2006; Djukic et al., 2007; Wallraff et al., 2006).
Dysfunctional glutamate metabolism in glial cells is also implicated in epileptogenesis following trauma. As discussed above one of the primary functions of glial cells is to recycle glutamate. In addition glial cells can also release glutamate on their own and may play a more central role in seizure generation than previously thought (Tian et al., 2005). Of course tonic, or large releases of glutamate will lead to increased activity and therefore strain the IRS. Finally, transformed astrocytes release more inflammatory mediators than normal glia. The release of inflammatory mediators has been implicated in epiletogenesis (Hinterkeuser et al., 2000).
All in all these modifications to the glial tissue result in a compromised potassium buffering system, with the potential for greater excitation through poorly regulated glutamate trafficking and increased inflammatory interference.

4.1.2 In vitro

There is a large array of experimental models for seizures, that are in large part based on what is observed in epileptic seizures. Most experiments are carried out in brain slice preparations, usually dissected from rats or mice. Experimental models are designed to subject the neuronal tissue to conditions with combinations of ionic imbalance, excess excitation, reduced inhibition, or hyperactivity. For brevity, we will only discuss a few of these models and the role ionic imbalance plays in the behaviors seen.
Several seizure models are based on asserting ionic imbalances. Increasing extracellular potassium leads to the effects outlined above and is in almost all ways excitatory (Jensen & Yaari, 1997). Substantial increases in potassium can directly lead to seizure-like activity, and slightly elevated potassium is often used to promote activity in other models. Seizures can also be induced by reducing the extracellular calcium concentrations(Bikson et al., 2003). Both of these models are expected to exhibit reduced synaptic transmission, suggesting that these seizures are largely mediated by concentration dynamics.
Seizures are generally thought to be at least partially due to an imbalance between excitatory mechanisms and inhibitory mechanisms. A number of models attempt to produce these conditions by pharmacologically reducing inhibition with such agents as bicuculine or picrotoxin, or increasing excitatory mechanisms with agents such as carbachol. Not surprisingly these models produce uncontrolled excitatory output and seizure like events. Though the primary reason for these uncontrolled events is a tilting towards excitation, the dynamics of the seizures are undoubtedly influenced by the ionic imbalances produced by the hyperactivity.
There is some debate as to what degree inhibitory mechanisms are compromised in epileptic tissue. Some experimental models, such as 4-aminopyridine, are based on the assumption that it is not inherent loss of inhibition that leads to seizures but temporary loss due to the interactions between the networks of cells (Ziburkus et al., 2006). There it was demonstrated that prolonged seizures-like events can occur with intact inhibition. Excitatory output did not peak due to lack of activity in the interneuron network, but rather was accompanied by the interneurons being forced into a depolarized state through excess activity. This state, known as depolarization block, sets a threshold on cellular, especially inhibitory, output. Depolarization block, as will be discussed in the next section is influenced by, or even caused by, ionic imbalance. In addition to influencing the membrane potential, ionic imbalance will also shift the reversal potentials for synaptic transmission. Understanding

these effects may greatly enhance our understanding of the balance between excitation and inhibition during pathological states.

4.2 Mathematical models

Computational models are an efficient and relatively transparent method to investigate neuronal dynamics. For the sake of simplicity most computational models of neurons do not incorporate changes to ionic concentrations, even though one could argue that even small changes in the ionic distributions can influence network activity, and may in fact be used by the healthy brain to prime or suppress the responsiveness of the network (Frohlich et al., 2010). Either way there is no question that one must include concentration dynamics in order to understand the dynamics of seizures. This has been done on several different levels of complexity, from modeling individual neurons to networks of neurons (Somjen, 2004; Bazhenov et al., 2004; Cressman, 2009; Barreto & Cressman, 2011). Models are always approximations to physical systems, therefore results and predictions must be interpreted within the narrow scope of the models focus.

Although it is often possible to ascertain the general mechanisms involved in an epileptic disorder, the non-linearity of these mechanisms as well as coupling between different mechanisms often rules out a simple linear analysis. In these cases it is most often necessary to mathematically formulate these mechanisms and computationally evolve their dynamics in order to determine their effects.

4.2.1 Single cell models

Single cell models can either be optimized for the most transparent analysis, or be extremely complex in order to understand the detailed interactions of pumps, channels and transporters. Both of these approaches give their own insights into seizure dynamics.

The most detailed neuronal models include multiple compartments for the soma, dendrite, extracellular space, and glia (Kager et al., 2007). These models include numerous ionic channels, pumps, and cotransporters for potassium, sodium, chloride, and calcium, inserted at different densities for different compartments. This sophisticated model allows a detailed inspection of the mechanisms responsible for sustained after discharges.

One of the main results of (Kager et al., 2007) relates to the inactivation of sodium channels. They showed a strong relationship between seizure susceptibility and the permeability of the persistent sodium current. In their model, prolonged self sustained after discharges are maintained by a nearly balanced outward flow of potassium and inward flow of sodium. If sodium channels inactivate, this balance cannot be maintained and potassium flow will outstrip the sodium, and repolarize the cell. However with persistent sodium currents or incomplete inactivation of transient sodium this near balance can sustain long-term depolarized states. This incomplete inactivation is called windowing, due to the overlap between activation and inactivation curves for the transient sodium channels. Seizures finally subside as pump rates increase and currents decrease as ions approach equilibrium. They also show that the ionic imbalance produced leads to sustained depolarization and if severe enough, depolarization block.

On the other hand extremely simple models of single neurons have been employed to investigate the slow modulating effects of ionic changes with less attention paid to the individual currents responsible for the membrane potential and concentrations (Cressman et

al., 2009). In this study the authors investigated how seizures can arise from alterations to the IRS. The model was designed to replicate high potassium in vitro experiments described above. As the bath concentration in the model is increased to values used to produce seizures in experiments this model supports stable oscillatory behavior that could be the basis for seizures seen in experimental models. This model has also been utilized to investigate the role of depolarization block in seizure dynamics (Barreto & Cressman, 2011). Here the model was used to determine the parameters for glial function and potassium diffusion that produces the different patterns of neuronal firing and depolarization block seen in experimental models.

4.2.2 Network models

Of course seizures are the manifestation of activity that emerges from networks of interacting cells. There are several mechanisms that couple the activity of cells, namely synaptic transmission and extrasyaptic neurotransmitter release, gap junctions, and ionic flow through diffusion in the extracellular space as well as through the glia syncytium. A number of models incorporate these mechanisms (Frohlich et al., 2010; Park et al., 2008; Ullah et al., 2009).

In all of these models extracellular potassium concentrations play a role in coupling the activity of neighboring cells. This effect is investigated to varying degrees in a number of computational models(Park et al., 2008, Frohlich et al., 2010; Ullah et al., 2009). Durand's work focuses on the effect of extracellular coupling to synchronize bursting activity in networks of synaptically uncoupled neurons. Their main results suggest that larger networks require greater extracellular coupling to synchronize. Their work suggests that small networks with limited synaptic connectivity are more susceptible to synchronization.

The works of Frohlich et al. and Ullah et al. focuses on the effect of potassium on the excitability and stability of activity within neuronal networks. Both of these models investigate one of the most difficult aspects to understanding seizures. Namely, how do networks only occasionally support seizure like states? In Ullah et al., 2009 the authors demonstrate that finite sustained activity, such as that implicated in short term memory (Gutkin et al., 2001), can be stabilized or destabilized by changes in glial function.

Frohlich et al. present a different form of multistability in networks of neurons with ionic and synaptic coupling. They demonstrate that networks can be forced into one of two dynamic states by varying the degree of perturbation. The two stable dynamic states in their model represent normal and pathological function. They demonstrate that the transition between these two states can be tuned through modifications to their ionic regulatory system. Both of these models implicate glial function and ionic balance in general as powerful modulators of network function as well as possible culprits in establishing pathological conditions.

5. Anti epileptic drugs

There are over 50 million diagnosed cases of epilepsy throughout the world, and many people are able to live without seizures thanks to the availability of a wide array of antiepileptic drugs. The first rudimentary antiepileptic drug was introduced in the mid-1800s, and numerous more have been developed since, each acting via a slightly different

set of mechanisms that lend them their anti-convulsant capacity. However, despite the advancement in antiepileptic drugs, as many as 30% of patients suffer from refractory epilepsy, meaning that no combination of drugs render them completely seizure free. This fact, along with an array of serious negative side-effects conferred by some drugs, provides incentive for continued research in AED development.

The basic goal of AEDs has been to dampen excitatory networks (or amplify inhibitory networks) in seizures without impacting day-to-day healthy behaviors. Broadly speaking, there are four major categories of anticonvulsant mechanisms. Most of these mechanisms are firmly rooted in basic ionic processes. Unfortunately for the scientists studying AEDs, in many cases these drugs do not have a single mechanism, but rather rely on multiple mechanisms that could be used to explain the anticonvulsant behavior. The majority of AEDs modulate sodium ion channel activity. Another group of AEDs modulate calcium ion channel activity. Both these mechanisms are effective in part because they limit Sustained High-Frequency Repetitive Firing (SRF). A third group comprises those AEDs that modulate the GABAergic system, particularly through the GABAr receptor. Finally, the fourth category of AEDs limit the effectiveness of the excitatory neurotransmitter glutamate. There are a variety of additional minor mechanisms of AEDs, including modulation of potassium channels, inhibition of carbonic anhydrase, modulation of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, and even modulation of Na+/K+ ATPase pumps.

5.1 Inward cations

5.1.1 Voltage-gated Na+ channels

Many AEDs selectively decrease current through voltage-gated sodium channels. These AEDs include carbamazepine, lamotrigine, oxcarbazepine, phenytoin, topiramate, valproic acid, and zonisamide, and possibly a new drug, Carisbamate (Liu, 2008). Evidence strongly supports the idea that these AEDs bind to voltage-gated sodium channels when they are in an inactivated state brought on by an action potential (AP). Because these AEDs bind voltage-gated sodium channels only in the inactivated state, they become more bound (and thus more effective) during repetitive APs. This use-dependent effect is critical to these AEDs having increased activity during rapid firing, as in seizure activity, but little or no effect during non-pathologic neuronal firing.

Once bound, these AEDs delay the sodium channel recovery from an inactivated state to a resting state and shift gating to a more negative voltage. For example, a normal voltage-gated sodium channel may recover from the inactivated state in an average of 2ms, but in the presence of phenytoin this recovery make take as long as 90ms (Delorenzo & Sun, 2002). With the sodium channel in an inactivated state, the neuron becomes refractory to subsequent APs until the AED becomes unbound. In this way, AEDs that modulate sodium channels significantly reduce SRF, which is thought to be a major component of seizure formation and spread. Hyperpolarization of the membrane removes all bound AEDs and allows the sodium channel to fully recover to a resting state. While most drugs that modulate voltage-gated sodium channels utilize this mechanism, there are slight variations from drug to drug. For example, while Topiramate modulates voltage-gated sodium channels in a voltage and use-dependent way, it does not fully block SRF (White, 2002). It is also believed that each drug acts at a different binding site on the sodium channel protein.

These AEDs act in a similar manner as the frequency dependent rundown mechanism compromised in GEFS+ type I. The role of sodium inactivation as a control parameter for

seizure susceptibility is also highlighted by the models of Somjen et al. There they suggest that the seizure length is very sensitive to the persistent sodium channel conductivity. Their and other models show that it is the persistent or windowed transient sodium channels that enable the self regenerating discharges. Therefore the main action of these drugs is most likely not the direct reduction on excitatory output, rather it is through limiting the self regenerative period for the active neuron itself.

5.1.2 Ca++ channels

Many AEDs modulate voltage-gated calcium channels, all having the effect of inhibiting calcium current. However, different AEDs target different calcium channels. For example, lamotrigine inhibits high-voltage gated N/P type calcium channels, which are associated with neurotransmitter release at the synapse. By inhibiting these channels, lamotrigine is probably diminishing neurotransmitter release. Similarly, levitaracetam inhibits N-type Ca2+ channels. However, by contrast, Succinimides (such as thosuximide), valproic acid, phenytoin, and zonisamide have been shown to inhibit T-type calcium channels, and are widely used for their anti-absence affects. T-type calcium channels are implicated in the generation and spread of bursts, particularly in absence seizures (Kito et al., 1996).

In addition to inhibiting T-type calcium channels, phenytoin is also thought to inhibit calcium uptake into mitochondria, which serve as a buffering system for intracellular free calcium. This inhibition can increase the intrasynaptosomal Ca^{2+} and cause hyperexcitability through an increase in miniature end-plate potentials. In fact, phenytoin is known to cause hyperexcitability at high concentrations, a reversal from its normally anticonvulsant behavior. This buildup of free calcium in the intrasynaptosomal space may explain this aspect of the drug (Delorenzo & Sun, 2002).

Studies suggest that topiramate targets L-type high-voltage calcium channels and decreases the peak current through the channel. In addition, it has been suggested that gabapentin inhibits high-voltage calcium channels with a particular specificity to the a2d subunit. This subunit specificity allows gabapentin to modulate some neuronal types more than others (Rogawski, 2002).

5.2 Synaptic modulation

5.2.1 GABAergic inhibition

As GABA is the primary inhibitory neurotransmitter in the brain, it is a common target for AED drugs. There are two primary ways that AEDs can modulate GABAergic transmission: 1) By modulating either $GABA_A$ or $GABA_B$ receptors, or 2) By increasing the amount of GABA in the synapse.

Most drugs that modulate GABA receptors do so via the $GABA_A$ receptor. Drugs such as benzodiazepines, topiramate, and barbituates such as phenobarbital allosterically enhance the $GABA_A$ receptor. Since the $GABA_A$ receptor is linked to chloride ion channels, enhancing these $GABA_A$ receptors enhances the chloride ion current, hyperpolarizing the neuron. These chloride channels open in bursts that have a short, medium, or long duration (corresponding to 0.5ms, 2.5ms, and 7.3ms) (MacDonald, 2002). Phenobarbital will increase burst time (favoring longer bursts) without impacting burst frequency (Olsen, 2002). Conversely, benzodiazepines will increase burst frequency without impacting burst time (MacDonald, 2002). Despite subtle differences in mechanism, the end result is the same: an increase in

chloride ion current. Barbituates are not widely prescribed as anti-convulsants because of sedative side effects. Benzodiazepines are largely free of these side effects, probably because they target a specific sub-type of $GABA_A$ receptors, limiting their effect throughout the brain. Barbituates are non-selective, and thus widely effective in all areas of the brain. This probably explains the notable sedative side effects compared to benzodiazepines (Olsen, 2002).
Presynaptic $GABA_B$ receptors inhibit calcium channels (which prevents neurotransmitter release, while postsynaptic $GABA_B$ receptors open potassium channels (which hyperpolarize the neuron). Baclofen is a non-selective $GABA_B$ agonist, and so increases activity at both presynaptic and postsynaptic receptors. This can result in both anti-convulsant, and pro-convulsant behaviors. The pro-convulsant behaviors are thought to be caused by the presynaptic $GABA_B$ receptors, which inhibit neurotransmitter release. While baclofen results in a reduction of excitatory neurotransmitter release, it can also reduce GABA release from inhibitory neurons, which play an important role in seizure prevention. There is some evidence that gabapentin modulates postsynaptic $GABA_B$ receptors, however this is still debated. By activating only postsynaptic receptors, the pro-convulsant effects of baclofen are eliminated (Meldrum & Rogawski, 2007).
In addition to modulating GABA receptors, some AEDs also modulate the amount of GABA in the synapse. Tiagabine blocks the uptake of GABA back into the neuron by blocking the GABA transporter, GAT-1 (Giardina, 2002). This has the effect of leaving GABA in the synapse longer, prolonging inhibitory postsynaptic potentials. Valproic acid increases GAD activity, an important synthesizing enzyme for GABA. Increased GABA synthesis results in increased GABA in the brain (Löscher, 2002). Finally, vigabatrin is an irreversible GABA-T inhibitor. GABA-T catalyzes the conversion of GABA to succinic semialdehyde, and so reduces GABA levels in the brain. By inhibiting GABA-T, GABA is increased in the brain (Ben-Menachem, 2002).

5.2.2 Glutamatergic transmission

Glutamate is the most abundant excitatory neurotransmitter, and acts by binding to NMDA, AMPA, and kainate receptors at the synapse. Some AEDs target these receptors as a means to limit excitatory transmission. For example, felbamate and valproic acid act as antagonists of the NMDA receptor, thus inhibiting glutamatergic transmission. This is indirectly an ionic effect, because an activated NMDA receptor opens a nonspecific cation pore, which allows K^+ to flow out of the cell along its gradient, and Na^+ and Ca^{2+} to flow in. This has the effect of depolarizing the cell, contributing to excitability. Many traditional AEDs that inhibit glutamatergic transmission have intolerable side effects, and so are not used. However, felbamate is a drug that is well tolerated, despite acting on NMDA receptors. It has been shown that felbamate is a much more successful antagonist of NMDA receptors containing NR2B subunits, as opposed to NR2A. NR2B subunits are mainly found in forebrain areas, while NR2A subunits are found throughout the brain. Felbamate's tolerability may be owed to it is focused antagonism of NMDA in the forebrain areas of the brain, which play an important role in seizure generation, while other areas are left relatively unaffected (Rogawski, 2002). Kainate and AMPA receptors are also targets for AEDs. For example, topiramate is thought to inhibit Kainate receptors.

5.3 K+ channels

While voltage-gated sodium and calcium ion channels have long been targets of AEDs, targeting voltage-gated potassium channels is relatively new. A new AED, Ritagabine, has

been shown to modulate voltage-gated potassium channels. Research also indicates that lamotrigine gets some anti-convulsant properties by modulating potassium channels (Czuczwar et al, 2010).

Ritagabine increases current through the Kv7.2-7.3 subunits of the voltage-gated M-type K+ channel. This is done by lowering the activating potential required to open these ion channels down to levels close to the resting membrane voltage. This is either done by stabilizing the M-type channels in their open state, or destabilizing them in their closed state. Evidence suggests that the binding site is exposed when the channel is in the open state, and thus the channels are stabilized in their open state (Czuczwar et al, 2010; Schener et al., 2005). Binding of Ritagabine probably interferes with the closing of the channel until lower voltages.

Opening these potassium channels allows potassium to flow out of the cell due to its gradient. This has the effect of lowering the membrane potential back towards resting or even hyperpolarizing levels, thus reducing excitability. Ritagabine has been shown to hyperpolarize neurons, which supports this explanation for some of its anti-convulsant properties.

5.4 Carbonic anhydrase and acidosis

Research suggests that some AEDs, such as topiramate and zonisamide derive their anti-convulsant properties in part from their ability to modulate the pH in the brain. This is done by inhibiting Carbonic Anhydrase (CA), the enzyme responsible for interconverting CO_2 and H_2O with protons and bicarbonate. The inhibition of CA results in the buildup of H^+ ions from the metabolic process, and mild acidosis results. This is significant because the presence of an excess of H^+ ions modulates ion channel activity, and causes inhibition, as explained previously (Mizra et al., 2009).

5.5 HCN channels

There is recent evidence that gabapentin and lamotrigine increase the inward I_H current in some neurons. The I_H current is an important part of the ability of cardiac cells and neurons to control rhythmicity. Hyperpolarization-Activated, Cyclic Nucleotide-Gated K^+ (HCN) ion channels are responsible for the I_H current. As the name would suggest, these ion channels are hyperpolarization activated, and allow Na^+ and K^+ to flow into and out of the cell respectively (K^+ is four times more permeant than Na^+) (Clapham, 1998). While the role of HCN channels in cardiac tissue is more clearly understood, the distribution and effect on neurons in the brain is still a matter of research and debate. However, it is clear that I_H plays a role in both pyramidal cells and inhibitory interneurons in the hippocampus, and may help to control the synchronized firing that is important for information processing. Studies show that HCN channels are active at the resting membrane potential for many hippocampal neurons. This net inward flow has the effect of depolarizing the neuron slightly, thus increasing excitability. At first glance, it would seem that I_H-induced excitability of inhibitory interneurons would cause overall inhibition, while I_H-induced excitability in pyramidal cells would cause excitation. However, research shows that the overall effect of increased I_H currents is inhibition.

The reason for this is more obvious in interneurons. An increase in I_H currents in inhibitory interneurons has a slight depolarizing effect by about 5-10mV (George et al., 2009), making it easier for these cells to fire. In fact, research indicates that this is one way gabapentin and

lamotrigine get their anti-convulsant properties (Peng et al., 2011). Despite having a similar effect, lamotrigine and gabapentin modulate I_H currents differently. Lamotrigine changes the activation potential of HCN channels (Poolos et al., 2002), while gabapentin increases the conductance while leaving the voltage gating unchanged (Surges et al., 2003).
The impact on pyramidal CA1 cells is perhaps less obvious. Interestingly, increasing I_H current in CA1 pyramidal cells has a nonlinear effect. Increasing I_H currents in conjunction with weak, subthreshold Excitatory Post-Synaptic Potentials (EPSPs) results in an overall excitatory effect. The peak voltage achieved by the EPSP is increased by the I_H currents, though still not enough to cause an action potential. However, strong subthreshold EPSPs have an overall inhibitory effect. The peak voltage achieved by these stronger EPSPs is actually lessened by I_H currents. If increased I_H currents have a depolarizing effect on neurons, how does this lead to inhibition? The answer rests with M-type voltage-gated K+ channels. These channels are activated by modest depolarizations of the neuron, and allow K+ to flow out the neuron along its concentration gradient. M-type K+ channels thus have a hyperpolarizing (inhibitory) effect on neurons. Weak EPSPs are not enough to fully activate M-type K+ channels, and excitation results. However, as the EPSPs get stronger, K+ channels are activated, and inhibition results. Thus, the overall impact of gabapentin and lamotrigine is to make it more difficult for EPSPs to reach the action potential threshold, and thus is inhibitory (George et al., 2009).

5.6 Na+/K+ ATPase pumps

Some research indicates that AEDs, such as phenytoin, can increase the activity of the sodium potassium pump. Increasing the sodium potassium pump draws more potassium into the cell, and forces more sodium out, hyperpolarizing the cell. (Guillaume et al., 1986) There is also some evidence that this effect is dependent on the K+/Na+ ratio in the extracellular space. When there is a higher ratio of K+/Na+, phenytoin is more effective at increasing Na+/K+-ATPase activity. Because extracellular K+ levels can often rise during seizures, this provides a mechanism where phenytoin can modulate seizure behavior without influencing healthy behaviors (Delorenzo & Sun, 2002). This is one of the few targets which is aimed at interacting with the IRS directly.

6. Summary

Although the importance of ion concentrations in seizure dynamics has long been established (Green & Petsche, 1961), the effects of ionic imbalances are still not well understood. Mounting experimental and computational results are beginning to untangle the role of the IRS in seizure dynamics. These findings suggest that seizure initiation, evolution, and termination are all highly influenced by changes in the ionic balance. The knowledge gleaned from this body of work should be used to better understand current pharmacological agents as well as used to inform new pharmacological targets, and other forms of treatment such as electrical or magnetic stimulation.
We have presented the basic mechanisms responsible for ionic regulation as well as their role in maintaining normal ion concentrations. We also described a number of common interactions between neuronal activity and ionic concentrations that directly influence neuron behavior and signaling. By influencing a wide array of neuronal properties, including, membrane excitability and synaptic potency, ionic imbalance can play a large role in establishing the behavior of networks of neurons.

In order to further establish the relevance of ionic imbalance in epilepsy we looked at the role of these mechanism in known etiologies. For known idiopathic epilepsies the function of the implicated proteins will often suggest a mechanism for seizure susceptibility. For instance, the loss of sodium channel inactivation may lead to prolonged depolarization and a greater increase in ionic imbalance. Even in diseases where the obvious culprit is inhibitory malfunction the effects of ionic imbalance are still highly relevant to the dynamics of these seizures.

In symptomatic seizures the alterations to neuronal and glial cells in and around lesions lead to a variety of abnormal behavior. Glial cells are highly implicated in many symptomatic seizures. Seizure susceptibility in some of these cases could be due to the diminished ability of glial cells to maintain ionic balance.

We concluded by looking at the major classes of antiepileptic drugs and their effects on the IRS. Though all of these pharmacological agents effect, to some degree, ionic balance, only a few directly act on it.

Experimental and mathematical models strongly suggest that glial malfunction is implicated in seizure dynamics. To this point, there are no drugs targeted at glial function. This could be a promising avenue for new antieleptic agents. Since the IRS composes a coupled system it important to realize that no target is entirely specific. Therefore one cannot expect to solely interact with any one ionic gradient. It is therefore essential to make use of experimental and theoretical models to evaluate the effectiveness of pharmacological manipulations.

7. Conclusions

It was our goal to inform the reader of the importance of ionic imbalance in seizures, both to better understand the nature of seizures and their manifestations, as well as to think of better ways to directly interact with the Ionic Regulatory System to maintain normal neuronal states.

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Part 5

New Treatments

8

Antiepileptic Medicinal Plants Used in Traditional Medicine to Treat Epilepsy

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1. Introduction

Epilepsy is a disease that affects about 40 million people worldwide (Njamshi et al., 2010). In 1968, the prevalence of epilepsy in Africa was about 4.8 to 40 ‰. In 1996, Diop and collaborators reported in Senegal a prevalence of epilepsy of 21 ‰ (Diop et al., 1996). In 2006, Ngoungou and collaborators estimated the prevalence in sub-Saharan Africa to be two or three time highest than the rate in developed world (Ngoungou et al., 2007). In Cameroon, some epidemiological studies on epilepsy have shown that, the prevalence of epilepsy is estimated to vary from 5-136/1000. The highest ones are reported in some villages of the Cameroon Central Province located in the Sanaga and Mbam River Valley (Nchoji Nkwi & Tioko Ndonko, 1989; Dongmo et al., 2000; Preux et al., 2000; Boussinesq et al., 2002; Kamgno et al., 2003; Dongmo et al., 2004; Prischich et al., 2008). Cameroon is one of the countries most affected by epilepsy in Africa and in the world. Thus, epilepsy is among the major public health problems in Cameroon. In Africa and in Cameroon particularly, phytotherapy in traditional medicine still plays an important role in the management of diseases, mainly among populations with very low income (Geoffrey & Kirby, 1996). And phytotherapy relies on the use of a wide variety of plant species. *Annona muricata* Linn (Annonaceae), *Annona senegalensis* Pers (Annonaceae), *Bidens pilosa* Linn (Asteraceae), *Bryophyllum pinnatum* (Lam) Oken (Crassulaceae), *Citrus sinenis* (Linn) Osbeck (Rutaceae), *Clerodendron thomsoniae* Balf (Verbenaceae), *Daniellia oliveri* (Rolfe) Hutch and Dalz (Caesalpiniaceae), *Datura stramonium* Linn (Solanaceae), *Detarium microcarpum* Guil et Perr (Caesalpiniaceae), *Euphorbia hirta* Linn (Euphorbiaceae), *Flacourtia indica* Willd (Flacourtiaceae), *Hymenocardia acida* Tul (Hymenocardiaceae), *Jatropha gossypiifolia* Linn (Euphorbiaceae), *Khaya senegalensis* A Juss (Desrousseaux) (Meliaceae), *Mentha cordifolia* Auct (Lamiaceae), *Prosopis Africana* Guill and Perr (Taub) (Mimosaceae), *Ricinus communis* Linn (Euphorbiaceae), *Securidaca longepedunculata* Fres (Polygalaceae), *Senna singueana* (Delile) Lock 1988 (Caesalpiniaceae), *Terminalia glaucescens* Planch. ex Benth (Combretaceae), *Terminalia mollis* Laws (Combretaceae), *Tetrapleura tétraptera* Taub (Schum Thonn)

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(Mimosaceae), *Trichilia emetica* Vahl (Meliaceae) and *Vitellaria paradoxa* C F Gaertn (Sapotaceae) are plants that are being used empirically in traditional medicine in Cameroon to treat epilepsy and diseases related to the brain like agitations, anxiety, convulsions, dizziness, headaches, insomnia, migraines, pains and schizophrenia according to our traditional Healers and the literature (Abbiw, 1990; Adjanohoun et al., 1984, 1996; Arbonnier, 2000; Berhaut, 1975; Biholong, 1986; Bouquet, 1969; Brenan, 1959; Dalziel, 1937; Hutchinson & Dalziel, 1958; Iwu, 1993; Joyner, 2004; Malgras, 1992; Mutasa et al., 1990; Nwaiwu & Akah, 1986; Pousset, 1989; Raponda-Walker & Silans, 1961; Saulnier, 1998) (Table 1). Though the literature showed a lot of pharmacological studies done

Name of the plant	Part of the plant used	Form of the medicine	Diseases	Chemical characterization	Pharmaco-logical properties	Country
Annona muricata	Leaves	Infusion Decoction	Insomnia, diabetes Spasms, Fever	Steroid, cardiac glycosides	Antimicrobial	Cameroon, Forest areas
Annona senegalensis	Leaves Roots	Infusion	Convulsions, Epilepsy Sterility, diarrhoea, dysentery		anticonvulsant	Cameroon, Central Africa West Africa, South Africa
Bidens pilosa	Leaves	Decoction	Dizziness, migraines, headaches, rheumatism		Anti hypertensive	Cameroon, Central America
Bryophyllum pinnatum	Leaves	Applica-tion on head Decoction	Convulsions, rheumatism Arthritis	Flavonoids, antraquinones	Antinociceptive, anti-inflammatory antidiabetic	Central Africa
Citrus sinenis	Leaves + Flowers Barks Roots	Decoction Infusion	Epilepsy, convulsions, Insomnia, agitation Headaches, Malaria fever Anxiety, schizophrenia		Sedative	Humid tropical areas
Clerodendron thomsoniae	Leaves Roots	Decoction	Convulsions, head aches Parasitic diseases		effect on purinergic neurotransmission	Cameroon, India
Daniellia oliveri	Barks Roots		Epilepsy, Migraine, head aches Epilepsy, anxiety, schizophrenia			Angola, Cameroon Sudan, West Africa Central Africa
Datura stramonium	Fruits Leaves		Epilepsy Coughs, asthma, pains	Alkaloids, atropine		Africa, Asia, America, Europa
Detarium microcarpum	Leaves Barks Roots	Decoction	Dizziness, schizophrenia, paralysis malaria, diarrhoea Epilepsy, Pains Paralysis			West Africa Central Africa
Euphorbia hirta	Whole plant	Decoction	Convulsions, Insomnia Diarrhoea, amoeba, asthma, coughs, pains	Alkaloids, tannins	Anxiolytic	Africa continent
Flacourtia indica	Sterm barks Fruts Leaves		Epilepsy, headache, fever, stomach-ache, diarrhoea Sleep disorders	beta-sistosterol butyrolactone, steroids, flacourtine, flavonoids, coumarine, terpenoids, polyphenols	Antiplasmodial Protection against liver toxicity	

Name of the plant	Part of the plant used	Form of the medicine	Diseases	Chemical characterization	Pharmaco-logical properties	Country
Hymenocardia acida	Leaves Barks Roots	Infusion Powder	Headaches, fever, hypotension, diabetes, sickle cells Epilepsy, schizophrenia			Cameroon, Central Africa West Africa
Jatropha gossypiifolia	Leaves + Roots Roots		Convulsions, fever, hypertension Convulsions,			Cameroon, Central Africa, West Africa
Khaya senegalensis	Leaves, Barks Roots	Decoction	Headaches, schizophrenia, malaria Fever	Saponins, tannins, triterpenes	Antiinflamm-atory	Cameroon
Mentha cordifolia	Leaves	Infusion	Insomnia, muscle relaxant,		Antioxydant	Cameroon
Prosopis Africana	Leaves Barks	Decoction	Epilepsy, insomnia, anxiety states, headaches, migraine, agitation, fever Vermifuge, fever		Antitrypano-somal	Cameroon West Africa
Ricinus communis	Leaves + flowers	Decoction	Epilepsy, convulsions, headaches, diarrhea, asthma	ricin	Neuroleptic like properties	Central Africa, West Africa
Securidaca longepeduncul ata	Barks Roots, Leaves		Epilepsy, schizophrenia Pains, Rheumatisms		Anxiolytic	Central Africa, West Africa
Senna singueana	Leaves Leaves and flowers Barks and Roots,		Fever, Conjunctivitis, Convulsions, gonorrhoea, bilharzias, stomach-aches, constipation, Epilepsy, syphilis,	7-Methylphyscion Cassiamin A		Cameroon, Mali, Soudan, East and South Africa.
Terminalia glaucescens,	Leaves Barks Roots	Decoction Decoction Macera-tion	Malaria, stomach-aches, leucorrhoea, Hepatitis, leucorrhoea Epilepsy, diarrhoea, leucorrhoea	Terminalin A Glaucinoic Acid	antimicrobial	
Terminalia mollis	Roots		Epilepsy			Central Africa,
Tetrapleura tétraptera	Barks Fruits Roots	Decoction	Epilepsy Convulsions Fevers, malaria	Saponins, tannins	Anticonvulsant	Angola, Cameroon, Sudan, West Africa, Central Africa
Trichilia emetica	Roots Barks		Epilepsy, anti-parasitic diseases Head aches	Tannins, sterols		Savannah belt, open woodland in Africa
Vitelaria paradoxa	Leaves Leaves + Barks	Decoction	Convulsions, Epilepsy, headaches, stress Head aches	Saponins, alkaloids, tannins, cadiac glycosides	Antimicrobial	Cameroon, Brazil

Table 1. Parts of the plant, form of the medicine and diseases treated in traditional medicine. Adeyemi et al., 2010; Adjanohoum et al., 1984; Adjanouhoun et al., 1996; Adzu et al., 2003; Agassounon et al. 2008; Anete et al., 1998 ; Anuradha et al., 2008; Arbonnier, 2000; Berhaut, 1975; Brenan, 1959 ; Dimo et al., 2002; El-Mahmood et al., 2008; Ezugwu & Odoh, 2003; Gusman-Gutierez & Navarrete, 2009; Iwu, 1993; Joyner, 2004; Lompo et al., 1998; Malgras, 1992; Mutasa et al., 1990; Nazneen et al., 2009; Ogundiya, 2009; Ojewole, 2005; Palgrave, 2003; Pathak et al., 2010; Pousset, 1989; Satyarayana et al., 1996; Sunday et al., 2009; Saulnier, 1998; Seema Zareen, 2006; Worapan et al., 2009.

with these plants, very few were done to study their sedative and anticonvulsant properties. This study was undertaken to evaluate the anticonvulsant and sedative properties of these plants used in the treatment of insomnia and epilepsy in traditional medicine in Africa, particularly in Cameroon.

2. Materials and methods

2.1 Animals

Adult male mice (*Mus musculus* Swiss; 22 ± 2 g; 6 or 8 per group) were used for this study. The animals were housed in standard cages at 25°C, on a 12/12 h light-dark cycle. They were supplied with food and water *ad libitum*.

Drugs were administered in a volume of 10 ml/kg of mice body weight. The study was conducted in accordance with the nationally (N°.FWA-IRB00001954) and internationally accepted principles for laboratory animal use and care. In diazepam or sodium thiopental-induced sleep tests, mice were divided into negative control group that received distilled water and four test groups that received different doses of the plant extracts. In anticonvulsant tests, there was one more group that received a known anticonvulsant compound and served as a positive control.

2.2 Plant material

A voucher specimen of each plant was authenticated by a botanist, Professor Mapongmetsem Pierre Marie, Department of Biological Sciences, University of Ngaoundéré and deposited at the National Herbarium of Cameroon in Yaoundé.

2.3 Preparation of the extracts

2.3.1 Decoction

10 g of each plant material were macerated for 1 h in an amount of distilled water (25, 50, 75, 100 or 150 ml) according to the plant. The mixture was boiled for 20 min. After cooling, the supernatant (decoction) was collected and filtered. The decoction of each plant was diluted in distilled water to obtain less concentrated solutions. In another experiment, the decoction was dried and the w/w yield of the extract was calculated (table 2). The decoctions were prepared according to the methods close to the ones used in traditional medicine.

2.3.2 Maceration

10 g of dried fruits of *Datura stramonium* were macerated in 50 ml of distilled water. After 1 h the supernatant was collected, filtered and used in mice. The w/w yield of the extract was obtained (table 2).

2.4 Anticonvulsant tests

2.4.1 N-methyl-D-aspartate (NMDA) test

Six groups of 6 or 8 mice received different treatments. Group I (negative control) was treated with distilled water. Groups II to V (test groups) were treated with 4 doses of the plant extracts. Group VI (positive control) was treated with 3 mg/kg of CGP 37849 i.p. or 33 ηmol/kg of D-AP7 i.p. Mice were injected subcutaneously with NMDA, 75 mg/kg 1 h after administration of the different treatments. They were observed for 30 min. Animals

that did not exhibit turning behaviour within the 30 min of observation were declared protected. Turning behaviour was characterised by two consecutive 360° cycles fulfilled by the same animal (Croucher et al., 1982; Ngo Bum et al., 2001; 2009a; 2009b; Schmutz et al., 1990).

2.4.2 Strychnine (STR) test

Six groups of 6 or 8 mice received different treatments as above, except that group VI (positive control) was treated with clonazepam (3 mg/kg, i.p.). Convulsions followed by death were induced in mice by the i.p. injection of 2.5 mg/kg STR nitrate 1 h after administration of the different treatments. The animals which survived more than 10 min after strychnine injection were qualified protected (Ngo Bum et al., 2001, 2009a).

2.4.3 Picrotoxine (PIC) test

Six groups of 6 or 8 mice received different treatments as above, except that group VI (positive control) was treated with clonazepam (0.4 mg/kg, i.p.). Clonic seizures were induced in mice by the i.p. injection of 7.5 mg/kg PIC 1 h after administration of the different treatments. The animals which did not convulse within the 15 min of observation after PIC injection were qualified protected (Lehmann et al., 1988; Ngo Bum et al., 2001).

2.4.4 Pentylenetetrazol (PTZ) test

Six groups of 6 or 8 mice received different treatments as above, except that group VI (positive control) was treated with clonazepam (0.1 mg/kg, i.p.). Clonic seizures were induced in mice by the i.p. injection of 70 mg/kg PTZ 1 h after administration of the different treatments. The animals that did not convulse within the 10 min from the injection of PTZ were qualified protected (Ngo Bum et al., 2001, 2009a, 2009b).

2.4.5 Isonicotinic hydrazide acid (INH) test

Six groups of 6 or 8 mice received different treatments as above, except that group VI (positive control) was treated with diazepam, 10 mg/kg (per os). Animals were injected i.p. with INH 250 mg/kg 1 h after the administration of the different treatments. The time to the onset of clonic or tonic seizures was recorded. (Bernasconi et al., 1988; Ngo Bum et al., 2001).

2.5 Diazepam or sodium thiopental-induced sleep in mice

Five groups of 6 or 8 mice received different treatments. Group I (negative control) was treated with distilled water and groups II to V (test groups) were treated with 4 doses of the plant extracts.The methods described by Beretz et al., (1978) and modified by Rakotonirina et al., (2001) were used. Sleep potentiating effects of the plant were studied in mice that received sodium thiopental or diazepam at a dose of 50 mg/kg (i.p.) 1 hour after the administration of the different treatments. The time between the loss of the straightening reflex and the regain of this reflex measured the sleeping time. The loss or the regain of the straightening reflex was measured by stimulating the external ear. When the mouse anterior paw does not move after stimulation with horsehair, the animal is sleeping. When the mouse is awakened, it moves and shakes its paw.

Name of the plant	Part of the plant used	Quantity plant powder (g)	Quantity of water (ml)	Yield (%)	Root of administration
Annona muricata	Fresh leaves	10	50	6	i.p.
Annona senegalensis	Dried leaves	10	75	5	i.p.
Bidens pilosa	Fresh leaves	10	25	3.5	i.p.
Bryophyllum pinnatum	Fresh leaves	10	25	7	i.p.
Citrus sinenis	Fresh leaves	10	50	5	i.p.
Clerodendron thomsoniae	Dried leaves	10	50	6.7	i.p.
Daniellia oliveri	Dried barks	10	50	9.9	p.o.
Datura stramonium	Dried fruits	10 (macerate)	50	7	i.p.
Detarium microcarpum	Dried roots	10	50	7.43	p.o.
Euphorbia hirta	Fresh plant	10	50	7	i.p.
Flacourtia indica	Dried barks	10	100	10	p.o.
Hymenocardia acida	Fresh leaves	10	25	2.19	i.p.
Jatropha gossypiifolia	Dried leaves	10	50	7	i.p.
Khaya senegalensis	Dried leaves	10	75	5	i.p.
Mentha cordifolia	Fresh leaves	10	50	7	i.p.
Prosopis Africana	Dried leaves	10	50	5.6	i.p.
Ricinus communis	Fresh leaves	10	50	6	p.o.
Securidaca longepedunculata	Dried roots	10	150	10	i.p.
Senna singueana	Dried roots	10	50	8	p.o.
Terminalia glaucescens	Dried roots	10	100	7.6	p.o.
Terminalia mollis	Dried roots	10	50	7.1	p.o.
Tetrapleura tétraptera	Dried barks	10	50	4.2	i.p.
Trichilia emetic	Fresh roots	10	50	6.3	p.o.
Vitelaria paradoxa	Fresh leaves	10	150	12.6	i.p.

i.p. (intraperitoneal), p.o. (per os).

Table 2. Quantities of plants powder and distilled water, and part of the plant used to prepare the decoctions.

2.6 Statistical analysis

Three parameters were measured: the protection against chemically-induced seizures, the latency to the onset of seizures (min) in INH test, the latency to the onset of sleep and the sleeping time (min) in the sleep potentiation test. Data of the control groups were compared to data of groups treated with the plants extracts and to data of the positive control groups. The statistical analysis were done using Fisher exact test and Anova followed by Dunnett (REGWQ). $P<0.05$ was considered significant.

2.7 Chemicals

D-2-amino-7-phosphonoheptanoate, Clonazepam, Isonicotinic hydrazide acid, N-methyl-D-aspartate, penthylenetetrazol, picrotoxine, sodium thiopental and strychnine are from Sigma Chemical, USA. Diazepam is from Roche, France.

3. Results

3.1 Sedative properties

The extracts of twenty one plants increased in a dose-dependent manner the sleeping time induced by sodium thiopental or diazepam. The most potent was *Datura stramonium*. it multiplied by a factor of 5 the sleeping time of the control group (from 16 ± 7 to 94 ± 25 min at a dose of 70 mg/kg), but this extract was very toxic for animals. The decoctions of eight plants multiplied by a factor of 4 the sleeping time of their control group: *Annona senegalensis* (from 19 ± 4 to 89 ± 29 min at a dose of 67 mg/kg), *Clerodendron thomsoniae* (from 19 ± 3 to 94 ± 30 min at a dose of 134 mg/kg), *Daniellia oliveri* (from 20 ± 8 to 81 ± 13 min at a dose of 198 mg/kg), *Hymenocardia acida* (from 20 ± 11 to 85 ± 21 min at a dose of 87.6 mg/kg), *Securidaca longepedunculata* (from 18 ± 3 to 78 ± 14 min at a dose of 66.7 mg/kg), *Terminalia mollis* (from 17 ± 1 to 84 ± 15 min at a dose of 70 mg/kg), *Tetrapleura tetraptera* (from 19 ± 3 to 91 ± 15 min at a dose of 84 mg/kg) and *Trichilia emetica* (from 17 ± 1 to 84 ± 10 min at a dose of 126 mg/kg). The sleeping time of the control groups were multiplied by a factor of 3 by six plants: *Flacourtia indica* (from 16 ± 12 to 49 ± 3 min at a dose of 100 mg/kg), *Jatropha gossypiifolia* (from 11 ± 5 to 43 ± 15 min at a dose of 140 mg/kg), *Prosopis Africana* (from 19 ± 3 to 61 ± 26 min at a dose of 112 mg/kg), *Senna singueana* (from 24 ± 2 to 86 ± 5 min at a dose of 20 mg/kg),*Terminalia glaucescens* (from 37 ± 13 to 120 ± 21 min at a dose of 76 mg/kg), and *Vitellaria paradoxa* (from 25 ± 4 to 84 ± 20 min at a dose of 84 mg/kg). The decoctions of five plants multiplied by a factor of 2 the sleeping time of their control group: *Annona muricata* (from 31 ± 11 to 71 ± 15 min at a dose of 120 mg/kg), *Bidens pilosa* (from 31 ± 2 to 80 ± 2 min at a dose of 140 mg/kg), *Detarium microcarpum* (from 20 ± 6 to 52 ± 12 min at a dose of 111.45 mg/kg), *Euphorbia hirta* (from 56 ± 16 to 145 ± 10 min at a dose of 140 mg/kg) and *Mentha cordifolia* (from 10 ± 2 to 24 ± 3 min at a dose of 140 mg/kg). *Bryophyllum pinnatum* induced a slight increase of the sleeping time. Only *Citrus sinenis* and *Kaya senegalensis* could not increase the total sleep time of mice (table 3). Some of those plants also reduced the onset time of sleep (Table 4).

3.2 Anticonvulsant properties

3.2.1 On PTZ- induced convulsions

78.3% of plants extract were effective against PTZ-induced convulsions. *Annona muricata, Annona senegalensis, Bidens pilosa, Clerodendron thomsoniae, Daniellia oliveri, Datura stramonium, Detarium microcarpum, Euphorbia hirta, Flacourtia indica, Hymenocardia acida, Mentha cordifolia, Ricinus communis, Securidaca longepedunculata, Senna singueana, Terminalia glaucescens, Terminalia mollis, Tetrapleura tétraptera, Trichilia emetica* and *Vitellaria paradoxa* protected mice against convulsions induced by PTZ (table 5).

3.2.2 On STR- induced convulsions

The percentage of plants extracts that protected mice against STR-induced convulsions was *77.8%. Annona muricata, Bidens pilosa, Daniellia oliveri, Detarium microcarpum, Flacourtia indica, Hymenocardia acida, Jatropha gossypiifolia, Khaya senegalensis, Mentha cordifolia, Prosopis Africana, Securidaca longepedunculata, Senna singueana, Terminalia mollis, Trichilia emetica* protected mice against STR- induced convulsions (table 5).

3.2.3 On PIC- induced convulsions

The percentage of plants extracts that protected mice against PIC-induced convulsions was *87.5%. Clerodendron thomsoniae, Flacourtia indica, Mentha cordifolia, Securidaca longepedunculata,*

Senna singueana, Terminalia glaucescens and *Vitellaria paradoxa* protected mice against convulsions induced by PIC (table 5).

			Doses of the plants in mg/kg			
Daniellia		**CON**	**49.5**	**99**	**148.5**	**198**
oliveri	DIAZ	9 ± 3	6 ± 1	6 ± 1	5 ± 2	3 ± 1**
Detarium		**CON**	**37.15**	**47.3**	**111.45**	**148.6**
microcarpum	DIAZ	9 ± 3	7 ± 2	6 ± 3	4 ± 2*	6 ± 3
Flacourtia		**CON**	**10**	**25**	**50**	**100**
indica	DIAZ	4 ± 2	8 ± 6	4 ± 4	11 ± 6	4 ± 4
Hymenocardia		**CON**	**8.7**	**21.9**	**43.8**	**87.6**
acida	DIAZ	9 ± 3	7 ± 3	6 ± 1*	5 ± 1**	3 ± 1***
Mentha		**CON**	**14**	**35**	**70**	**140**
cordifolia	DIAZ	3 ± 1	2 ± 1	2 ± 1	6 ± 3	4 ± 1
Securidaca		**CON**	**10**	**20**	**50**	**66.7**
longepedunculata	DIAZ	6 ± 1	5 ± 1	5 ± 1	4 ± 1*	4 ± 1*
Senna		**CON**	**20**	**40**	**80**	**160**
singueana	DIAZ	15 ± 3	6 ± 1***	6 ± 1***	7 ± 1***	8 ± 1***
Terminalia		**CON**	**9.5**	**19**	**38**	**76**
glaucescens	DIAZ	4 ± 2	7 ± 4	5 ± 1	6 ± 3	2 ± 2
Terminalia		**CON**	**14**	**35**	**70**	**140**
mollis	DIAZ	7 ± 2	5 ± 1	2 ± 1**	3 ± 1**	4 ± 1*
Trichilia		**CON**	**12.6**	**33**	**66**	**126**
emetica	DIAZ	6 ± 1	5 ± 1	4 ± 1***	2 ± 1***	2 ± 1***

Data represent the onset time of sleep time. Values are means ± ESM. N = 6 or 8 per dose, *<p 0.05, **<p 0.01, ***<p 0.001 vs control, Anova followed by Dunnett (REGWQ). CON = distilled water, DIAZ = diazepam 50 mg/kg.

Table 3. The effects of the different plants on the onset time of sleep induced in mice by sodium thiopental or diazepam.

3.2.4 On NMDA- induced turning behaviour

The percentage of plants extracts that protected mice against NMDA-induced turning behaviour was 100%. *Annona muricata*, *Bidens pilosa*, *Bryophyllum pinnatum*, *Citrus sinenis*, *Euphorbia hirta*, *Khaya senegalensis* protected mice against turning behaviour induced by NMDA (table 5).

3.2.5 On MES- induced convulsions

The percentage of plants extracts that protected mice against MES-induced convulsions was 25%. *Securidaca longepedunculata* protected mice against convulsions induced by MES (table 5).

3.2.6 On INH- induced convulsions

The percentage of plants extracts that were effective against INH-induced convulsions in mice was 60%. *Ricinus communis*, *Securidaca longepedunculata*, *Senna singueana* delayed the onset of seizures in INH test (table 5).

3.2.7 Plants efficacy

Flacourtia indica, *Ricinus communis*, *Securidaca longepedunculata*, *Senna singueana*, *Terminalia glaucescens* showed very good anticonvulsant activities (80 to 100% of protection against PTZ, PIC or INH induced seizures). The other eighteen plants tested protected 50 to 75% of

			Doses of the plants in mg/kg			
Annona muricata	DIAZ	**CON** 31 ± 11	**12** 51 ± 26*	**30** 67 ± 6***	**60** 68 ± 2***	**120** 71 ± 15***
Annona senegalensis	DIAZ	**CON** 19 ± 4	**6.7** 52 ± 18***	**17** 72 ± 17***	**34** 79 ± 25***	**67** 89 ± 29***
Bidens pilosa	DIAZ	**CON** 31 ± 2	**14** 70 ± 2***	**35** 66 ± 3***	**70** 79 ± 5***	**140** 80 ± 2***
Bryophyllum pinnatum	DIAZ	**CON** 21 ± 2	**28** 32 ± 5**	**70** 26 ± 5	**140** 35 ± 1**	**280** 31 ± 6*
Citrus sinenis	DIAZ	**CON** 56 ± 24	**10** 50 ± 20	**25** 40 ± 10	**50** 45 ± 12	**100** 57 ± 10
Clerodendron thomsoniae	DIAZ	**CON** 19 ± 3	**13.4** 39 ± 9***	**33.5** 74 ± 22***	**67** 90 ± 16***	**134** 94 ± 30***
Daniellia oliveri	DIAZ	**CON** 20 ± 8	**49.5** 50 ± 3***	**99** 74 ± 8***	**148.5** 74 ± 7***	**198** 81 ± 13***
Datura stramonium	THIO	**CON** 16 ± 7	**3.5** 55 ± 15***	**7** 63 ± 21***	**35** 85 ± 23***	**70** 94 ± 25***
Detarium microcarpum	DIAZ	**CON** 20 ± 6	**37.15** 38 ± 10**	**47.3** 46 ± 13**	**111.45** 52 ± 12***	**148.6** 45 ± 9***
Euphorbia hirta	DIAZ	**CON** 56 ± 16	**14** 99 ± 24**	**35** 97 ± 21**	**70** 117 ± 26***	**140** 145 ± 10***
Flacourtia indica	DIAZ	**CON** 16 ± 12	**10** 15 ± 11	**25** 38 ± 25*	**50** 44 ± 4***	**100** 49 ± 3***
Hymenocardia acida	DIAZ	**CON** 20 ± 11	**8.7** 51 ± 12***	**21.9** 70 ± 15***	**43.8** 77 ± 23***	**87.6** 85 ± 21***
Jatropha gossypiifolia	DIAZ	**CON** 11 ± 5	**14** 29 ± 14*	**35** 27 ± 13*	**70** 32 ± 10***	**140** 43 ± 15***
Kaya senegalensis	DIAZ	**CON** 63 ± 15	**6.7** 52 ± 21	**17** 58 ± 25	**34** 58 ± 23	**67** 61 ± 23
Mentha cordifolia	DIAZ	**CON** 10 ± 2	**14** 16 ± 5*	**35** 21 ± 5**	**70** 21 ± 4**	**140** 24 ± 3**
Prosopis africana	DIAZ	**CON** 19 ± 3	**11.2** 52 ± 26*	**28** 57 ± 17**	**56** 31 ± 17	**112** 61 ± 26***
Securidaca longepedunculata	DIAZ	**CON** 18 ± 3	**10** 60 ± 27**	**20** 65 ± 14***	**50** 67 ± 27**	**66.7** 78 ± 14***
Senna singueana	DIAZ	**CON** 24 ± 2	**20** 86 ± 5**	**40** 77 ± 3***	**80** 44 ± 7**	**160** 29 ± 9***
Terminalia glaucescens	DIAZ	**CON** 37 ± 13	**9.5** 31 ± 15	**19** 79 ± 40*	**38** 86 ± 17***	**76** 120 ± 21***
Terminalia mollis	DIAZ	**CON** 17 ± 1	**14** 44 ± 7***	**35** 64 ± 11***	**70** 84 ± 15***	**140** 73 ± 8***
Tetrapleura tétraptera	DIAZ	**CON** 19 ± 3	**8.4** 39 ± 10**	**21** 67 ± 18***	**42** 82 ± 14***	**84** 91 ± 15***
Trichilia emetica	DIAZ	**CON** 17 ± 1	**12.6** 22 ± 2*	**33** 29 ± 4**	**66** 71 ± 7***	**126** 84 ± 10***
Vitelaria paradoxa	DIAZ	**CON** 25 ± 4	**12** 40 ± 13***	**21** 57 ± 6***	**42** 59 ± 8***	**84** 84 ± 20***

Data represent the total sleep time. Values are means ± ESM. N = 6 or 8 per dose, *p< 0.05, **p<0.01, ***p< 0.001 vs control, Anova followed by Dunnett (REGWQ). CON = distilled water, DIAZ = diazepam 50 mg/kg, THIO = sodium thiopental 50 mg/kg.

Table 4. The effects of the different plants on the total sleep time induced in mice by sodium thiopental or diazepam.

				Doses of the plants in mg/kg			
Annona		**CON**	**12**	**30**	**60**	**120**	**CP**
Muricata	PTZ	0	16	50*	33	50*	100***
	STR	0	12	0	12	50*	100***
	NMDA	0	16	16	33	50*	100***
Annona		**CON**	**6.7**	**17**	**34**	**67**	**CP**
Senegalensis	PTZ	0	12	37	50*	25	
100***							
Bidens		**CON**	**14**	**35**	**70**	**140**	**CP**
pilosa	PTZ	0	16	50*	33	50*	100***
	STR	0	16	50*	40	50*	100***
	NMDA	0	33	33	66**	50*	100***
Bryophyllum		**CON**	**28**	**70**	**140**	**280**	**CP**
pinnatum	PTZ	0	16	33	16	33	100***
	STR	0	0	10	0	16	100***
	NMDA	0	33	50*	50*	50*	100***
Citrus		**CON**	**10**	**25**	**50**	**100**	**CP**
sinenis	PTZ	0	25	25	12	0	100***
	STR	0	0	0	12	12	100***
	NMDA	0	50*	50*	75**	75**	100***
Clerodendron		**CON**	**13.4**	**33.5**	**67**	**134**	**CP**
Thomsoniae	PTZ	0	12	37	37	62*	100***
	PIC	0	0	25	50*	50*	100***
Daniellia		**CON**	**49.5**	**99**	**148.5**	**198**	**CP**
oliveri	PTZ	0	25	37	37	50*	100***
	STR	0	16	66**	50*	50*	100***
Detarium		**CON**	**37**	**47**	**111**	**148**	CP
microcarpum	PTZ	0	50*	37	0	0	100***
	STR	0	50*	33	33	16	100***
Euphorbia		**CON**	**14**	**35**	**70**	**140**	**CP**
hirta	PTZ	0	0	25	0	50*	100***
	STR	0	12	37	25	37	100***
	NMDA	0	33	33	50*	50*	100***
Flacourtia		**CON**	**10**	**25**	**50**	**100**	**CP**
indica	PTZ	0	80**	60*	40	40	100***
	STR	0	20	40	40	60*	100***
	PIC	0	40	60*	60*	80**	100***
	MES	0	40	0	0	40	100
	INH	36 ± 7	48 ± 5	36 ± 10	50 ± 12	49 ± 6	73 ± 11***
Hymenocardia		**CON**	**8.76**	**21.9**	**43.8**	**87.6**	**CP**
acida	PTZ	0	0	25	37	62*	100***
	STR	0	16	33	33	50*	100***
Jatropha		**CON**	**14**	**35**	**70**	**140**	**CP**
gossypiifolia	PTZ	0	0	25	37	0	100***
	STR	0	50*	50*	62*	25	100***
	PIC	0	0	0	0	0	100***
Khaya		**CON**	**6.7**	**17**	**34**	**67**	**CP**
senegalensis	PTZ	0	12	12	0	25	100***
	STR	0	25	50*	25	50*	100***
	NMDA	0	62*	33	33	50*	100***
Mentha		**CON**	**14**	**35**	**70**	**140**	**CP**
cordifolia	PTZ	0	16	33	66**	50*	100***
	STR	0	33	33	33	66**	100***
	PIC	0	16	50*	33	50*	100***
Prosopis		**CON**	**11.2**	**28**	**56**	**112**	**CP**
africana	PTZ	0	0	0	25	37	100***
	STR	0	62*	25	50*	50*	100***
Ricinus		**CON**	**12**	**30**	**60**	**120**	**CP**
communis	PTZ	0	37	50*	62*	87***	100***

	INH	31 ± 9	33 ± 6	36 ± 8	40 ± 7	56 ± 16*	77 ± 11**
Securidaca		**CON**	**10**	**20**	**50**	**66.7**	**CP**
longepedunculata	PTZ	0	67**	67**	83***	100***	100***
	STR	0	50*	67**	67**	67**	100***
	PIC	0	67**	100***	83**	83**	100***
	INH	46 ± 3	51 ± 5*	62 ± 12**	67 ± 20**	78 ± 21**	97 ± 20***
Senna		**CON**	**20**	**40**	**80**	**160**	**CP**
singueana	PTZ	0	80**	80**	40	40	100***
	STR	0	80**	80**	40	40	100***
	PIC	0	20	60*	0	20	100***
	MES	0	40	40	20	20	80**
	INH	21 ± 1	30 ± 1**	29 ± 6	32 ± 9**	37 ± 1**	42 ± 6**
Terminalia		**CON**	**9.5**	**19**	**38**	**76**	**CP**
glaucescens	PTZ	0	40	60*	40	100***	100***
	STR	0	40	20	0	0	100***
	PIC	0	20	60*	40	20	100***
	MES	0	20	40	0	40	80**
	INH	36 ± 7	41 ± 13	18 ± 5	42 ± 9	47 ± 13	85 ± 26***
Terminalia		**CON**	**14**	**35**	**70**	**140**	**CP**
mollis	PTZ	0	50*	37	25	37	100***
	STR	0	66**	33	33	50*	100***
Tetrapleura		**CON**	**8.4**	**21**	**42**	**84**	**CP**
tetraptera	PTZ	0	25	50*	50*	50*	100***
Trichilia		**CON**	**12.6**	**33**	**66**	**126**	**CP**
emetica	PTZ	0	25	50*	50*	50*	100***
	STR	0	12	25	50*	50*	100***
Vitelaria		**CON**	**12**	**21**	**42**	**84**	**CP**
paradoxa	PTZ	0	12	50*	62*	50*	100***
	PIC	0	0	12	50*	37	100***

Data represent the percentage of protected mice in different tests. N = 6 or 8 per dose, *p< 0.05, **p<0.01, ***p< 0.001 vs control, Anova followed by Dunnett (REGWQ). CON (negative control) = distilled water, CP (positive control) = clonazepam 0.1 mg/kg for PTZ test, clonazepam 0.4 mg/kg for PIC test, clonazepam 3 mg/kg for STR test, diazepam 10 mg/kg for INH test and D-AP7 33 ηmol/kg or CGP 37849 3 mg/kg for NMDA test.

Table 5. The effects of the different plants on the convulsions and turning behaviour induced in mice by INH, NMDA, PIC, PTZ and STR.

mice against the induced convulsions. 78% of plants protected both PTZ and STR-induced convulsions. 80.6% of plants protected both PTZ and PIC-induced convulsions. 80.8% of plants protected both STR and PIC-induced convulsions. Finally, 66.7% of plants at the same time protected PTZ, STR and PIC-induced convulsions.

3.2.8 Plants toxicity

Datura stramonium, *Ricinus communis and Securidaca longepedunculata* were also showed to be toxic. Their extract killed animal in 24h after their administration to mice.

4. Discussion and conclusions

The extracts of twenty one plants (91.3% of plants) increased the sleeping time induced by sodium thiopental or diazepam. The potentiation of the sleep time suggests the presence of sedative properties in the extracts of these plants (Rakotonirina et al., 2001; Ngo Bum et al., 2009a; 2009b). These sedative properties could be related to the presence of some components in the extracts activating the benzodiazepine, barbiturate and/or GABA

receptors in the $GABA_A$ receptor complex (Rang et al., 1999; Bonin & Orser, 2008; Olkkola & Ahonen, 2008). Diazepam (benzodiazepine) and sodium thiopental (barbiturate) all bind to the $GABA_A$ receptor complex. Diazepam potentiates GABA-mediated inhibition via the increase in the affinity of this inhibitory neurotransmitter to its recognition sites within the $GABA_A$ receptor complex, by increasing the opening frequency of the chloride ion channel which leads to the enhancement of influx of chloride anions into the neuron and subsequent hyperpolarisation (Czapinsky et al., 2005). While sodium thiopental that act on the barbiturate binding site directly gate the chloride ion channel of the $GABA_A$ receptor complex. The sedative properties found here could explain the use of the twenty one plants in traditional medicine in Africa, particularly in Cameroon in the treatment of insomnia. The first eight more potent plants to induced sedation were: *Datura stramonium* > *Clerodendron thomsoniae* > *Terminalia mollis* > *Trichilia emetica* > *Tetrapleura tétraptera* > *Annona senegalensis* > *Securidaca longepedunculata* > *Hymenocardia acida* > *Daniellia oliveri.* Two plants, *Citrus sinenis* and *Kaya senegalensis* did not show sedative properties. The results also showed that 95.6% of the tested plants possess anticonvulsant properties by inhibiting convulsions induced chemically or electrically. Five plants (*Flacourtia indica*, *Ricinus communis*, *Securidaca longepedunculata*, *Senna singueana*, *Terminalia glaucescens*) showed very good anticonvulsant activities against PTZ, PIC or INH induced seizures.

The effect was moderate for the rest of plants. *Tetrapleura tetraptera* one of the plants studied showed also anticonvulsant properties in fruits (Nwaiwu, 1986; Ojewole, 2005). The antagonism of INH, PTZ- and PIC-induced seizures suggests the interaction of these plants with the GABA-ergic neurotransmission (De Deyn et al., 1992; Doctor et al., 1982; Löscher & Schmidt, 1988; Salih & Mustafa, 2008; Perez-Saad & Buznego, 2008). GABA is the main inhibitory neurotransmitter substance in the brain and is widely implicated in epilepsy. Inhibition of GABA-ergic neurotransmission or activity has been shown to promote and facilitate seizures, while enhancement of GABA-ergic neurotransmission is known to inhibit or attenuate seizures (Gale, 1992; Li-Ping et al., 2008). Moreover, some studies indicated that PTZ diminishes the GABAergic tone (Mcdonald & Baker. 1977; Ahmadiani, 2003), probably by a competitive antagonist action on the BZD receptors (Rehavi et al., 1982). Correspondingly, drugs that enhance $GABA_A$-receptor neurotransmission, such as BZDs (White, 1997; Ahmadiani et al., 2003) can block seizures induced by PTZ. PIC is known to be a non competitive GABA antagonist exerting his effect by blocking the chloride channel in the $GABA_A$ receptor complex. Isoniazide can precipitate convulsions in patients with seizure disorders, and it is regarded as a GABA-synthesis inhibitor (Kale Shubhangi et al., 2010**)**. The antagonism of STR -induced convulsions suggests the presence of anticonvulsant effect through glycine-STR-sensitive receptors (Findlay et al., 2002). Few plants extract antatagonized MES induced convulsions, by probably prolonging neurons sodium channels inactivation (Holmes, 2007). The results show no difference in plants inhibiting convulsions induced by PTZ, PIC and STR. GABA and glycine-STR-sensitive neurotransmission are equally involved. But very few plants produced their anticonvulsant activities by prolonging neurons sodium channels inactivation. *Datura stramonium*, *Ricinus communis* and *Securidaca longepedunculata* were found toxic and therefore they are not suitable to be used to treat people. The toxicity of *Ricinus communis* could be related to the presence of a very toxic component named ricin (Iwu, 1993). The toxicity of *Datura stramonium* could be related to its delirants or anticholinergics compounds.

5. Conclusion

The purported anticonvulsant and sedative properties of the medicinal plants are scientifically shown. The ethnopharmacological study on Cameroon anticonvulsant and sedative medicinal plants is accurate in 90% of cases. A great amount of plants extract interacted through GABA and glycine-STR-sensitive neurotransmissions to inhibit convulsions. Many anticonvulsant plants also possess sedative properties. Twenty one plants possess sedative properties, but only eighteen plants could be used in traditional medicine in Africa in the treatment of insomnia. Eighteen plants possess at least moderate anticonvulsant effects, while five plants possess very good anticonvulsant properties. However only twenty medicinal plants could be used in the treatment of epilepsy. Three plants were found very toxic.

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9

The Potential Role of ATP-sensitive Potassium Channels in Treating Epileptic Disorders

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1. Introduction

Despite antiepileptic drug (AED) therapy, epilepsy remains uncontrolled in around one third of patients. The majority of current AEDs fall into two pharmacological classes, those that modulate neuronal voltage-gated sodium and calcium channels and those that modulate neurotransmitters. There is a need for new AEDs with novel mechanisms of action to serve as adjunct therapy for the treatment of intractable epilepsy. Among the diverse molecular targets, to selectively modify the excitability of neurons so that high frequency epileptic firing can be blocked without disturbing normal neuronal activity, potassium is a potential target. Potassium channels play a major role in the control of resting membrane potential, responsiveness to synaptic inputs, spike frequency adaptation and neurotransmitter release. Among them, the important metabolic coupler to electrical activity- ATP-sensitive potassium (K_{ATP}) channels provides a distinct link between the metabolic and electrical state of cells. We have demonstrated the role of K_{ATP} channels in epileptic seizures in diabetic hyperglycemia, providing the direct evidence that increases in extracellular glucose and intracellular ATP attenuate K_{ATP} channels, leading to a more excitable state. We also examined the K_{ATP} channel agonist mediating neuroprotection in diabetic individuals with status epilepticus. Here, we report how we investigated the diabetic hyperglycemia-related epileptic disorder from clinical observation to experimental studies, and review this potential novel mechanism underlying attenuating epileptic activities by opening K_{ATP} channels, especially related to metabolic syndrome.

2. Epileptic seizures in diabetic hyperglycemia: From clinical observation

More than 200 million persons worldwide will be diagnosed with diabetes (Mandrup-Poulsen, 1998). Epileptic seizures with diabetic hyperglycemia (DH) (Maccario et al., 1965; Venna and Sabin, 1981; Huang et al., 2005) are not uncommon and around one-fourth of DH patients have reported seizures (Venna and Sabin, 1981; Singh and Strobos, 1989). In more than half of these patients, seizures reveal previously undiagnosed diabetes (Venna and Sabin, 1981; Tiamkao et al., 2003; Harden et al., 1991).

Most seizures in DH are partial motor seizures (Singh and Strobos, 1989; Loeb, 1974; Tiamkao et al., 2003), while 15% present as status epilepticus (SE). The level of hyperosmolarity and hyponatremia, accompanied by a wide range of hyperglycemic symptoms, however, are inconsistent (Grant and Warlow, 1985). Previous case reports

suggest that DH-related epileptic seizures often develop at higher levels of glucose than non-DH-related seizures (Maccario et al., 1965; Venna and Sabin, 1981; Harden et al., 1991).
We conducted a prospective comparative follow-up study, focusing on newly diagnosed unprovoked seizures in adult patients, with and without DH, from 2000 to 2004 (Huang et al., 2008). We found that seizure clustering in initial presentation and in recurrence in the DH group was significantly higher than that in the non-DH group. Patients with poor glycemic control (HbA1c >9%) had significantly higher risk of seizure recurrence and clustering. Thus, DH might play a role in the severity of newly diagnosed adult epileptic seizures. Severe seizures might beget seizures in these patients. DH should be intensively investigated in adult patients with newly diagnosed seizures and aggressive blood sugar control might benefit seizure treatment in these patients, more than AEDs would.
The pathophysiology of epileptic seizures in DH is probably multi-factorial. Glucose itself could enhance synaptic transmission and propagation, leading to more excitable neurons (Tutka et al., 1998; Gispen and Biessels, 2000) and even epileptic seizures (Schwechter et al., 2003), regardless of the presence of organic lesions. Underlying focal ischemia has also been suggested as having a role in triggering these partial seizures (Singh and Strobos, 1989). Seizure susceptibility even in only moderate degrees of hyperglycemia has been reported in previous studies (Tiamkao et al., 2003; Brick et al., 1989); our study suggests that glucose itself is a pro-convulsant in DH.
In clinical observation, in the DH group, patients with recurrent seizures had more frequent SE at initial seizure presentation than those without, suggesting potential kindling during poorly controlled DH. In poorly controlled diabetes, neither the continued use of AEDs nor the presence of organic structural lesions affects seizure recurrence. Although simple partial seizures are more common in DH-related epileptic seizures, complex partial seizures, as the second most common in this study, and some rarer presentations, such as reflex, parieto-occipital, and sensory seizures (Brick et al., 1989; Huang et al., 2005, 2006, 2010; Lavin, 2005), should be carefully evaluated.
In animal experiments, a higher glucose level have facilitated amygdaloid kindling in rats (Priel et al., 1991) and decreased the time required for 50% of rats to recover sufficiently from a first maximal electroshock seizure (MES) to be able to have another MES (White et al., 1986). This is compatible with seizure clustering in the DH group observed in this study. This further suggests that kindling may continue and seizures may recur if blood glucose remains high.
Failure to identify the possible association between DH and seizures is common in clinical practice, potentially leading to inadequate treatment and seizure recurrences. Early recognition of the link will help early diagnosis and treatment, and prevent unnecessary interventions.

3. Epileptic seizures in experimental animals with diabetic hyperglycemia

As we all know, glucose plays a major role in metabolism and cerebral functions. However, the effects of hyperglycemia on the central nervous system (CNS) and neuronal excitability (Biessel et al., 1994; Stewart et al., 1999; Gispen and Biessel, 2000) are not fully understood. High glucose concentrations have been associated with a lower seizure threshold in an animal model with a single seizure (Schwechter et al., 2003) and neuronal excitability and seizures are related to rapid glucose utilization and glycolysis (Greene et al., 2003). Experimentally, the correlation between extracellular glucose concentration and excitability

(seizure) has been established in previous studies (Margineanu et al., 1998; Tutka et al., 1998; Schwechter et al., 2003).

In addition to increasing excitability, higher glycosylated hemoglobin values have been associated with moderate declines in motor speed and psychomotor efficiency (Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Study Research Group et al., 2007). In animal models of diabetes, spatial-learning impairments occur in association with distinct changes in hippocampal synaptic plasticity (Biessels et al., 1998; Kamal et al., 2000). On the other hand, cognitive impairments after SE have been reported in both clinical and experimental studies (Helmstaedter, 2007). Nevertheless, how synaptic plasticity change responds to SE in DH is currently unknown. In addition, it remains to be determined whether DH exaggerates the cognitive and pathological outcome of SE.

We examined whether SE in rats with DH caused acute neuronal damage in the hippocampus, and impaired learning and memory, and synaptic plasticity, to determine the behavioral, pathological, and electrophysiological effects of SE on diabetic animals. Adult male Sprague-Dawley rats (150-200 g) were first divided into groups with and without streptozotocin (STZ)-induced diabetes, and then into treatment groups given a normal saline (NS) (STZ-only and NS-only) or a lithium-pilocarpine injection to induce SE (STZ+SE and NS+SE). Serial Morris water-maze test and hippocampal pathology results were examined before and 24 hours after SE. We found the STZ+SE group had a significantly higher percentage of severe seizures and SE-related death and worse learning and memory performances than the other three groups. The STZ+SE group, followed by the NS+SE group, showed the most severe neuronal loss and mossy fiber sprouting in the hippocampal CA3 area.

Tetanic stimulation-induced long-term potentiation (LTP) in a hippocampal slice from these rats was recorded in a multi-electrode dish system (Huang et al., 2006a). LTP was markedly attenuated in the STZ+SE group, followed by the NS+SE group. We also used a simulation model to evaluate intracellular ATP and neuronal excitability and we found increased intracellular ATP concentration promoted action potential firing.

From our animal study, we found that compared with non-diabetic rats, diabetic rats were more susceptible to seizures, had higher SE-related mortality, performed significantly worse on hippocampus-dependent behavioral tests, lost more hippocampal neurons during the acute stage after SE, and exhibited an impaired LTP after SE. This finding suggests the importance of intensively treating hyperglycemia and seizures in diabetic patients with epilepsy (Huang et al., 2009).

SE-induced damage and network reorganization in lithium-pilocarpine-treated rats occurs as a consequence of neuronal loss and SE-induced sprouting (Lehmann et al., 2001). In addition to SE-related excitotoxicity (Curia et al., 2008), DH plus SE may amplify the adverse effects of hyperglycemia on neurons, both the direct effects and the indirect effects, such as diabetic oxidative stress (Vincent et al., 2007; Zupan et al., 2008), microvascular changes (Mraovitch et al., 2005; Qiu et al., 2008), and altered calcium homeostasis (Raza et al., 2004; Biessels et al., 2005). Because neuronal glucose uptake depends on the extracellular concentration of glucose, cellular damage can ensue after persistent episodes of hyperglycemia (Tomlinson and Gardiner, 2008). Diabetes may induce morphological changes in the presynaptic mossy fiber terminals that form excitatory synaptic contacts with the proximal CA3 apical dendrites (Magariños and McEwen, 2000).

Although impaired, synaptic plasticity is still present in animals with pilocarpine-induced epilepsy (Trudeau et al., 2004). The post-SE water maze analyses showed the STZ+SE group, followed by the NS+SE group, performed significantly worse than the other two groups. These findings again suggest the great negative impact of concomitant DH and SE on cognition, indicating that these two conditions interact in the brain. It has been suggested (Trudeau et al., 2004) that LTP deficits in diabetes might arise from dysfunction of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptors in the early stages of the disease. In addition, loss of LTP maintenance in STZ-treated rats has been suggested to be a result of disrupting the calcium-dependent process modulating post-synaptic alpha-amino-3-hydroxy-5-methylisoxazole propionic acid (AMPA) receptors (Chabot et al., 1997). The aggravating effect of SE on the glutamatergic system, supported an additional effect of DH on worsening LTP (Pitsch et al., 2007).

One of the empirically based clinical guideline for treating SE is to give glucose immediately (Chen and Wasterlain, 2006). Our study suggests that physicians pay attention to glucose management when encountering concomitant SE and DH. Because seizure clustering and SE in DH are frequently seen in clinical practice, we advocate intensively treating hyperglycemia and seizures in this special population.

4. Epileptic seizures in diabetic hyperglycemia: A mechanistic view

The mechanisms underlying hyperglycemia-increased neuronal excitability remains incompletely understood. As a coupling of metabolism to membrane electrical activity, Adenosine triphosphate (ATP)-sensitive K^+ channels (K_{ATP}) is an important regulator of neuronal excitability and neuroprotection in metabolic stress, such as DH (Liss and Roeper, 2001; Seino and Miki, 2003). The direct connection between the level of electrical activity and intracellular ATP concentration suggests K_{ATP} potential for an antiepileptic role. The physiological regulation of K_{ATP} during metabolic inhibition involves protein kinase C-mediated K_{ATP} internalization to lessen the action potential duration shortening (Hu et al., 2003). Whether the epileptic circuit in individuals with DH involves K_{ATP} functional adaptation need further investigation.

K_{ATP} exist in many excitable cells, including cardiac myocytes, pancreatic β cells, muscle cells, and neurons (Liu et al., 1999, Seino, 1999). In pancreatic β cells, these channels are known to couple cellular metabolism to electrical activity by opening and closing as the intracellular ATP/ADP ratio decreases and increases, respectively (Ashcroft and Gribble, 1998). They are octameric complexes composed of four pore-forming units with inward rectifying characteristics (Kir 6.1 or Kir 6.2) and four sulfonylurea (SUR) binding sites (SUR1, SUR2A, or SUR2B) (Shyng and Nichols, 1997), regulated by intracellular ATP as well as pharmacological agents (e.g., diazoxide). These channels are highly responsive to changes in intracellular ATP levels generated during glucose metabolism. Rising levels of intracellular ATP close the K_{ATP} leading to depolarization and firing (Rowe et al., 1996; Ashcroft and Gribble, 1998). When the [ATP]/[ADP] ratio decreases, SUR1 and Kir 6.2 interaction reduces the latter's affinity for ATP, thereby opening the K_{ATP}.

In pancreatic β cells, elevation in blood glucose and the closure of K_{ATP} trigger events leading to calcium influx, cellular depolarization, and insulin secretion (Miki and Seino, 2005). In the CNS, K_{ATP} exist in many tissues, particularly the hippocampus and neocortex (Dunn-Meynell et al., 1998). Except for a similar role in sensing central glucose in hypothalamic glucose-responsive neurons (Miki et al., 2001), they are not involved in

specific neuroendocrine functions. Therefore, a more general role for these channels, functionally expressed in neurons, needs be investigated. In the brain, cells containing Kir 6.2 mRNA are widely distributed (Dunn-Meynell et al., 1998). A striking overlap with SUR1 mRNA suggests that the Kir 6.2/SUR1 complex is the best candidate for the brain functional K_{ATP} (Zawar et al., 1999; Betourne et al., 2009).

Owing to the abundant expression of K_{ATP} in the brain (Hicks et al., 1994; Mourre et al., 1990), the activation of K_{ATP} during ATP-depleted conditions has become a subject of studies. Mice lacking Kir6.2 (Kir 6.2 (-/-) mice) are vulnerable to hypoxia, exhibiting a reduced threshold for generalized seizure (Yamada et al., 2001). Transgenic mice, overexpressing the SUR1 gene in the forebrain, show a significant increase in the threshold for kainate-induced seizures (Hernandez-Sanchez et al., 2001). However, with excessive extracellular glucose and ATP in the hippocampal neurons, how K_{ATP} react is still marginally understood.

We hypothesized increases in extracellular glucose and intracellular ATP would attenuate K_{ATP}, with cells becoming more depolarized, leading to a more excitable state in hippocampal neurons. Thus, we investigated the effects of higher extracellular glucose on hippocampal K_{ATP} channel activities and neuronal excitability by using the cell-attached patch clamp configuration on cultured hippocampal cells (H19-7 cells) and the multi-electrode recording system on hippocampal slices. We found that incremental extracellular glucose could attenuate the activities of hippocampal K_{ATP} channels. Glucose significantly attenuates K_{ATP} channel activity in a concentration-dependent manner, mainly through a decrease in open probabilities. Higher levels of extracellular glucose could enhance neuropropagation which could be attenuated by diazoxide, a K_{ATP} channel agonist. Additionally, we found high levels of intracellular ATP, enhanced the firing of action potentials in model neurons. The stochastic increases in intracellular ATP levels also demonstrated an irregular and clustered neuronal firing pattern. Thus, this phenomenon of K_{ATP} channel-attenuation could be one of the underlying mechanisms of glucose-related neuronal hyper-excitability and propagation (Huang et al., 2007) (Figure 1).

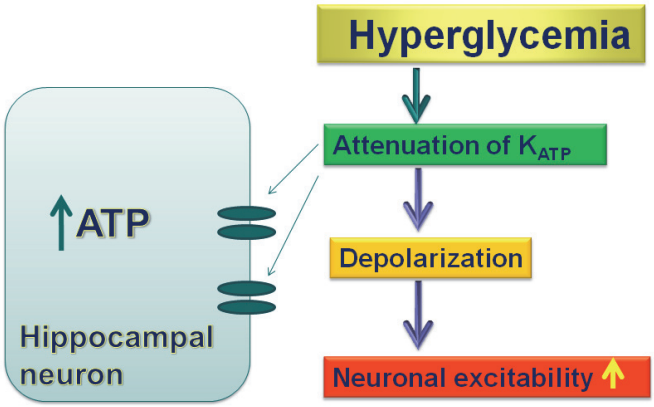

Fig. 1. The scheme of potential role of K_{ATP} underlying epileptic seizures in diabetic hyperglycemia.

From our study, we found glucose could enhance propagation through inhibition of K_{ATP} channel activity. The single-channel conductance, open-time, channel-bursting, ATP-sensitivity and voltage-insensitivity observed in these H19-7 cells were nearly identical to those described in native pancreatic β cells (Kir 6.2/SUR1) (Mukai et al., 1998). Despite the heterogeneous expression profiles of K_{ATP} channel subunits reported in hippocampal pyramidal neurons (Zawar et al., 1999), our study emphasized the role of β-cell type K_{ATP} channel in the hippocampus.

The effect of glucose on propagation has been suggested to be related to post-synaptic NMDA receptor activities (Abulrob et al., 2005). We implies the novel role of K_{ATP} channels. The increment in glucose leads to attenuation of K_{ATP} channel activities which would enhance field effects of EPSP, potentially caused by electrotonic spread of depolarization. Both pre- and post-synaptic K_{ATP} channels were involved in the electrical coupling effects in neurons (Matsumoto et al., 2002). Our study could support the role of post-synaptic K_{ATP} channel in neuropropagation, as there were more dominant effects on fEPSP with a relatively limited effect on pre-synaptic fiber volley and paired-pulse facilitation, in the presence of high glucose concentration.

The concentration-dependent attenuation of glucose on K_{ATP}-channel activity also aids in understanding the higher seizure susceptibility in higher degrees of hyperglycemia. Moreover, the stochastic simulation in this study suggested that in a state of intracellular ATP fluctuation, the neuronal firing pattern would show irregularity. This could be in parallel with the clinical situation where hyperglycemia-related seizures might develop as a result of paroxysmal action potential development in a steady state of hyperglycemia.

5. The therapeutic point-of-view on epileptic seizures in metabolic syndrome

As we investigated, the important metabolic couplers to electrical activity, K_{ATP} are potential mechanistic candidates when we treat these seizures (Figure 1). For clinical application, we started from the current available AEDs and we found there have been a few reports concerning the effects of Pregabalin (PGB), a newer AED, on some modulating effects on voltage-gated potassium channel (McClelland et al., 2004). Gabapentin-lactam, the derivative of gabapentin, has been found to exert an opening effect on K_{ATP} on the mitochondria (Pielen et al., 2004), which is a role in neuroprotection, added to the reduction of neuronal excitability. Moreover, it has been demonstrated that gabapentin can inhibit K^+-evoked [^{3}H]-noradrenaline release through the activation of K_{ATP} in both rat hippocampal and human neocortical cortex (Freiman et al., 2001). However, studies regarding the effects of PGB on K_{ATP} are still lacking.

We thus conducted an in vitro cellular study to investigate the effect of PGB on the activity of K_{ATP} present in H19-7 neurons. The inside-out configuration of the patch-clamp technique was employed to investigate K_{ATP} channel activities. Interestingly, PGB significantly opened these K_{ATP} channel activities in a concentration-dependent fashion with an EC_{50} value of 18 μM. There was a significant increase in the mean open-life time of K_{ATP} channels in the presence of PGB.

This study suggests that in differentiated hippocampal neuron-derived H19-7 cells, the opening effect on K_{ATP} channels could be one of PGB's underlying mechanisms in the reduction of neuronal excitability (Huang et al., 2006b). It's a novel finding regarding the mechanisms of PGB. Of interest, PGB applied to the intracellular surface of the excised

patches is able to activate K_{ATP} channels in these cells, suggesting that its binding side could be primarily on the intracellular leaflet.

The opening of K_{ATP} channels has been noted as neuroprotective, especially the β-cell-type K_{ATP} channels comprised of Kir6.2 and SUR1 (Yamada et al., 2001). Therefore, it is conceivable that the activation of K_{ATP} channels by PGB is anti-epileptic and potentially neuroprotective. PGB has been shown to rapidly penetrate the blood-brain barrier in pre-clinical animal studies (Ben-Menachem, 2004). In PGB treatment (600mg/d) for epilepsy, the usual therapeutic concentration range is around 2.8-8.2 mg/L (≒15-43 μM) at steady state (Berry et al., 2005). From our study, the EC_{50} for PGB in opening K_{ATP} channel activities is around 18 μM. From this point, it appears that the clinically relevant concentration would be similar to the concentration noted in our study.

Although we have shown (Huang et al., 2007) that, in *in vitro* hippocampal neurons, K_{ATP} agonists lead to membrane hyperpolarization and attenuate action-potential firing when extracellular glucose concentrations are high, there were no *in vivo* studies on whether K_{ATP} agonists protect against seizure severity and consequent SE-induced hippocampal damage in rats with DH. In addition, the functional relationship between Kir 6.2 and SUR1 in DH-related seizures remains unclear. We hypothesized diazoxide is protective in diabetic rats with SE, and, if so, whether the opening of K_{ATP} mediates this protection. K_{ATP} openers, including diazoxide, protect beta cells (Kir 6.2/SUR1) and preserve human and rat islets in high concentrations of glucose (Björklund et al., 2004; Maedler et al., 2004). Molecular and electrophysiological studies (Miki et al., 2001; Bancila et al., 2005; Sun et al., 2006; Huang et al., 2006b) report that Kir 6.2/SUR1 channels, like pancreatic beta cells, seem to be the dominant K_{ATP} isoform in the brain. It is thus reasonable to hypothesize that diazoxide protects CNS neurons.

In this study, adult male Sprague-Dawley rats (150-200g) were divided into two groups: the STZ-induced diabetes (STZ) group and the normal saline (NS) group. Both groups were treated with either diazoxide (DZX, 15 mg/kg, i.v.) (STZ+DZX, NS+DZX) or vehicle (STZ+V, NS+V) before lithium-pilocarpine-induced SE. We evaluated seizure susceptibility, severity, and mortality. The rats underwent Morris water-maze tests and hippocampal histopathology analyses 24 hours post-SE. Similar to previous studies, a multi-electrode recording system was used to study fEPSP. Seizures were less severe, post-SE learning and memory were better, and neuron loss in the hippocampal CA3 area was lower in the STZ+DZX than the STZ+V group. In contrast, seizure severity, post-SE learning and memory, and hippocampal CA3 neuron loss were comparable in the NS+DZX and NS+V groups. fEPSP was lower in the STZ+DZX but not in the NS+DZX group. In addition, RNA interference (RNAi) to knockdown Kir 6.2 in a hippocampal cell line was used to evaluate the effect of diazoxide, in the presence of high concentration of ATP. The RNAi study confirmed that diazoxide, with its K_{ATP}-opening effects, could counteract the K_{ATP}-closing effect by high dose ATP. We conclude that, by opening K_{ATP}, diazoxide protects against SE-induced neuron damage during DH (Huang et al., 2010).

We showed *in vivo* and *in vitro* evidence that diazoxide indeed protected STZ diabetic rats against SE-induced hippocampal damage. There are reports (Mattia et al., 1994; Yamada et al., 2001; Soundarapandian et al., 2007) that modulated K_{ATP} may alter the seizure threshold and epileptiform activity in hippocampal slices in rats. Nevertheless, this class of compounds has not yet been generally effective in some animal models, such as the maximal electroshock model, the kindling model (Wickenden, 2002), and in our NS rats.

[^{3}H] glyburide binding to SUR receptors in the brain appears to be generally upregulated in the state of hyperglycemia (Levin and Dunn-Meynell, 1998). In rats with DH, diazoxide opened K_{ATP}, which were frequently attenuated in a state of high extracellular glucose concentration (Huang et al., 2007). Diazoxide reduced glutamate release by opening presynaptic K_{ATP} Kir 6.2/SUR1 channels (Bancila et al., 2004). As diazoxide activates K_{ATP} by interacting with the SUR1 subunit (transmembrane domain 1 and nucleotide binding domain 1) (Nichols, 2006), opening K_{ATP} is potentially beneficial in a seizure during hyperglycemia.

The hippocampus is rich in Kir 6.2/SUR1-based channels (Thomzig et al., 2005; Sun et al., 2006). Hippocampal K_{ATP} are involved in processing new information at the mossy fiber CA3 synapses (Quinta-Ferreira and Matias, 2005). Reports (Zarrindast et al., 2006; Betourne et al., 2009) on the effect of diazoxide on contextual memory, however, are inconsistent. During learning, as the energy demand increases, mossy fiber K_{ATP} may sense a rise in intracellular ATP, which closes the K_{ATP} and increases glutamate release. Conversely, K_{ATP} that open immediately after intense electrical activity may also protect CA3 cells from glutamate-mediated excitotoxicity (Betourne et al., 2009). Based on our water-maze and pathology results, diazoxide -induced opening of K_{ATP} counteracted SE-related excitotoxicity in STZ rats.

6. Conclusion

Diabetic hyperglycemia might aggravate seizures. An aggressive search for diabetic hyperglycemia and intensive control of glucose in new onset seizures are helpful in management. The outcome of seizures is probably more deteriorating in diabetic patients with epilepsy. Because seizure clustering and status epilepticus in diabetic hyperglycemia are frequently seen in clinical practice, we advocate intensively treating hyperglycemia and seizures in this special population.

Our study provides more direct mechanistic evidence that increments of central neuron excitability, in a state of high glucose levels, can be attributed to K_{ATP} channel activity attenuation. K_{ATP} agonists are worth investigating as treatments for epileptic seizures in diabetic hyperglycemia.

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