

Personalized translational epilepsy research - novel approaches and future perspectives Part II: Experimental and translational approaches.

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Authors: Sebastian Bauer^{1,2}, Natascha van Alphen¹, Albert Becker³, Andreas Chiocchetti⁴, Ralf Deichmann⁵, Thomas Deller⁶, Thomas Freiman¹, Christine M. Freitag⁴, Johannes Gehrig⁷, Anke M. Hermsen¹, Peter Jedlicka⁶, Christian Kell⁷, Karl Martin Klein^{1,2}, Susanne Knake², Dimitri M. Kullmann¹⁶, Stefan Liebner⁸, Braxton A. Norwood², Diana Omigie⁹, Karl-Heinz Plate⁸, Andreas Reif¹¹, Philipp S. Reif¹, Yvonne Reiss⁸, Jochen Roeper¹², Michael W. Ronellenfitsch¹⁰, Stephanie Schorge¹⁶, Gerhard Schratt¹³, Stephan W. Schwarzacher⁶, Joachim P. Steinbach¹⁰, Adam Strzelczyk^{1,2}, Jochen Triesch¹⁴, Marlies Wagner¹⁵, Matthew C. Walker¹⁶, Frederic von Wegner¹, Felix Rosenow^{1,2}

Affiliations:

- (1) Epilepsy Center Frankfurt Rhine-Main, Department of Neurology, Center of Neurology and Neurosurgery, Goethe University Frankfurt, 60528 Frankfurt, Germany
- (2) Epilepsy Center Marburg, Department of Neurology, Philipps-University Marburg, 35043 Marburg, Germany
- (3) Institute for Neuropathology, University Bonn, 53105 Bonn, Germany
- (4) Department of Child and Adolescent Psychiatry, Psychosomatics and Psychotherapy, Goethe University Frankfurt, 60528 Frankfurt, Germany
- (5) Brain Imaging Center (BIC) Frankfurt, Department of Neurology, Center of Neurology and Neurosurgery, Goethe University Frankfurt, 60528 Frankfurt, Germany
- (6) Institute of Clinical Neuroanatomy, Dr. Senckenberg Anatomy, Neuroscience Center, Goethe University Frankfurt, 60590 Frankfurt, Germany
- (7) Emmy-Noether Group Kell, Department of Neurology, Center of Neurology and Neurosurgery, Goethe University Frankfurt, 60528 Frankfurt, Germany
- (8) Edinger-Institute Frankfurt, Neuroscience Center, Goethe University Frankfurt, 60590 Frankfurt, Germany
- (9) Max-Planck-Institute for Empirical Aesthetics, 60322 Frankfurt, Germany
- (10) Dr. Senckenberg Institute for Neurooncology, Center of Neurology and Neurosurgery, Goethe University Frankfurt, 60528 Frankfurt, Germany
- (11) Department of Psychiatry, Psychosomatics and Psychotherapy, Goethe University Frankfurt, 60528 Frankfurt, Germany
- (12) Institute of Neurophysiology, Neuroscience Center, Goethe-University Frankfurt, 60590 Frankfurt, Germany
- (13) Institute of Physiological Chemistry, Philipps-University Marburg, 35043 Marburg, Germany
- (14) Frankfurt Institute for Advanced Studies (FIAS), 60438 Frankfurt, Germany
- (15) Institute of Neuroradiology, Center of Neurology and Neurosurgery, Goethe University Frankfurt, 60528 Frankfurt, Germany
- (16) Institute of Neurology, University College London (UCL), London, WC1E 6BT, United Kingdom

Summary (260 words): Despite the availability of more than 15 new “antiepileptic drugs”, the proportion of pharmacoresistant epilepsy patients has remained constant at about 20-30%. Furthermore, no disease-modifying treatments shown to prevent the development of epilepsy following an initial precipitating brain injury or to reverse established epilepsy have been identified to date. This is likely in part due to the polyetiologic nature of epilepsy, which in turn requires personalized medicine approaches. Recent advances in imaging, pathology, genetics and epigenetics have led to new pathophysiological concepts and the identification of monogenic causes of epilepsy. In the context of these advances, the First International Symposium on Personalized Translational Epilepsy Research (1st ISymPTER) was held in Frankfurt on September 8th 2016 to discuss novel approaches and future perspectives for personalized translational research. These included new developments and ideas in a range of experimental and clinical areas such as deep phenotyping, quantitative brain imaging, EEG/MEG-based analysis of network dysfunction, tissue based translational studies, innate immunity mechanisms, mircoRNA as treatment targets, functional characterization of genetic variants in human cell models and rodent organotypic slice cultures, personalized treatment approaches for monogenic epilepsies, blood-brain-barrier dysfunction, therapeutic focal tissue modification, computational modeling for target and biomarker identification, and cost analysis in (monogenic) disease and its treatment. This report on the meeting proceedings is aimed at stimulating much needed investments of time and resources in personalized translational epilepsy research. This Part II includes the experimental and translational approaches and a discussion of the future perspectives, while the diagnostic methods, EEG network-analysis, biomarkers and personalized treatment approaches were addressed in Part I [1]

Key Words: Treatment targets, Personalized medicine, Precision medicine, Biomarkers, New treatment targets

Human biopsies and animal models to study mesial temporal lobe epilepsy

Temporal lobe epilepsy (TLE) is the most common focal seizure disorder in adults. Generally, seizures do not start at birth but develop later in life – often after transient insults. In many patients, such transient brain insults, including status epilepticus (SE), are followed by a latent period of *epileptogenesis*, preceding the emergence of clinical seizures. Mechanisms of epileptogenesis obviously cannot be studied in human brain tissue but require the use of animal models (reviewed in [2]). A major component of translational epilepsy research addresses the association of pathomechanisms in human epilepsy tissue and corresponding animal models.

In hippocampal biopsies of pharmacoresistant TLE patients, the CA1 pyramidal cell layer often shows pronounced neuropathological changes including degeneration and functional hyperexcitability. Based on these precedents, we aimed to characterize key pathomechanisms that render CA1 pyramidal neurons chronically hyperexcitable after a transient brain insult. In a recent study in experimental animals, we demonstrated transcriptional upregulation of Ca_v3.2 T-type Ca²⁺-channels, resulting in an increased propensity for burst discharges of hippocampal CA1 pyramidal neurons, to represent an important trigger for epileptogenesis [3]. We further demonstrated that the metal-regulatory transcription factor 1 (MTF1) mediates the increase of Ca_v3.2 mRNA and intrinsic excitability consequent to a rise in intracellular Zn²⁺. Adeno-associated viral (rAAV) transfer of MTF1 into murine hippocampi led to increased Ca_v3.2 mRNA. Conversely, rAAV-mediated expression of a dominant-negative MTF1 abolished SE-induced Ca_v3.2 mRNA upregulation and attenuated epileptogenesis. Finally, data from resected human hippocampi surgically treated for pharmacoresistant TLE support the Zn²⁺-MTF1-Ca_v3.2 cascade to be active also in human TLE tissue. As a perspective from ‘bench-to-bedside’, we suggest that pharmacological interventions targeting the Zn²⁺-MTF1-Ca_v3.2 cascade may provide a novel approach for the treatment of pharmacoresistant TLE.

Innate Immunity – Cytokines and Toll-like Receptors in pathophysiology and as treatment targets

Mechanisms of innate immunity play an important role in the development of acquired epilepsies. Components of the innate immune system, e.g. cytokines or Toll-like receptors (TLRs), can mediate tissue remodelling, leading to network changes that eventually result in increased excitation and synchronization, as well as reduced inhibition. In addition, immune mediators also have a direct influence on neuronal excitability via post-translational modification of ion channels [4,4]. In this respect, interleukin-1 (IL-1) has been studied intensely in animal models of epilepsy. Much data from epilepsy models confirmed both pro-convulsive/pro-epileptogenic actions of IL-1 and protective effects of IL-1 antagonization/inhibition. Translation of these experimental results to clinical research led to the development of a randomized, double-blind phase IIa trial of VX-765, a selective inhibitor of IL-1 converting enzyme (ILE) [5]. This study showed a favorable safety profile and a reduction of seizure frequency of 8.6% in the treatment group vs. placebo. Surprisingly, a

follow-up trial was terminated by the manufacturer. Nevertheless, this trial marked an important step towards developing immunomodulatory drugs for epilepsy therapy, potentially with disease-modifying activity.

Translating findings from animal models of epilepsy to the clinic is not a simple process. Unpublished data from our group show, for example, opposite regulation of intracellular TLRs in two rat models of epilepsy, although both exhibit a very similar phenotype of mesial temporal lobe epilepsy with hippocampal sclerosis (mTLE-HS). Such findings highlight the need for personalized approaches in both basic research and patient treatment above and beyond the current standard, general methods. One helpful tool for personalization is cerebral microdialysis. This technique allows repetitive sampling of extracellular molecules at consecutive time points within the same individual. Applying a stereotaxic approach, the target area can be targeted with extreme precision. We used this technique to establish the time course of hippocampal cytokine release in a rat model of mTLE-HS over the course of several months (unpublished data). These results will be helpful for precise timing of immunomodulatory interventions for epilepsy therapy. In fact, cerebral microdialysis is increasingly used as part of multimodal monitoring in humans with severe neurological diseases that are often accompanied by status epilepticus. The technique will undoubtedly also allow additional insight into the mechanisms responsible for seizures and epilepsy.

MicroRNAs as novel targets in personalized translational epilepsy research

A fine balance between inhibitory and excitatory synaptic strength is critical for the proper functioning of neural networks and therefore a prerequisite for cognition. Defects in the inhibitory/excitatory (E/I) balance on the other hand can lead to neurological diseases, such as epilepsy. An important mechanism to control excitatory synapse function is homeostatic scaling, which regulates synaptic strength in a manner opposite to the stimulus to counteract potentially detrimental changes in the E/I balance. Homeostatic downscaling in response to chronic overexcitation of networks has been discussed as a pathophysiological mechanism in the context of epilepsy, but the underlying gene regulatory programs are little understood.

One hypothesis is that microRNAs (miRNAs), a large class of small regulatory non-coding RNAs, play an important role in the regulation of homeostatic downscaling during epilepsy. In previous studies, we have characterized an activity-regulated miRNA, miR-134, that is required for the morphological and functional downscaling of excitatory synapses by downregulating the RNA-binding protein Pumilio-2 [6]. Intriguingly, inhibition of miR-134 had been previously shown to have an anti-epileptogenic function in the kainate epilepsy model in mice, suggesting that miRNA-regulated homeostatic plasticity might be a maladaptive response during epileptogenesis.

More recently, we have performed unbiased screening for miRNAs regulated during synaptic downscaling in vitro using small RNA sequencing. Thereby, we identified eight miRNAs that are upregulated by chronic network activation ([7]. One of these new candidates, miR-129-5p, was subsequently studied in more detail. Inhibition of miR-129-5p using antisense

oligonucleotides interfered with synaptic downscaling in cultured neurons, suggesting that miR-129-5p upregulation is necessary for this process. Mir-129-5p expression was upregulated in several rodent epilepsy models in vivo and in the hippocampus of human TLE patients. Injection of miR-129-5p inhibitors in mice largely abolished kainate-induced epileptic seizures, as well as associated increases in neuronal firing rates and hippocampal cell death. We further identified two novel miR-129-5p target mRNAs, encoding for the calcium extrusion channel *Atp2b4* and the microtubule-associated protein *Doublecortin (Dcx)*. Overexpression of *Atp2b4* and *Dcx* interfered with synaptic downscaling in cultured neurons, suggesting that miR-129-5p mediated downregulation of these proteins is important during the scaling response. Moreover, miR-129-5p and *Atp2b4* levels were inversely correlated in the hippocampus of TLE patients, indicating that the miR-129-5p/*Atp2b4* interaction might also be relevant in the epileptic brain. Besides regulating *Atp2b4* and *Dcx*, we found that miR-129-5p further inhibits expression of synaptic genes by directly targeting the positive regulatory RNA-binding protein *Rbfox*. Therefore, miR-129-5p employs a dual mechanism to efficiently inhibit the expression of critical excitatory synaptic proteins during scaling. Previous findings regarding increased seizure activity in *Rbfox* knockout mice are consistent with an involvement of the miR-129-5p module in epilepsy ([7]).

Taken together, our studies suggest an important and widespread contribution of miRNA-dependent regulation of homeostatic synaptic plasticity to epileptogenesis. miRNAs might therefore be used as biomarkers for a precise classification of different types of epilepsy and to predict therapy outcome. The knowledge about specific miRNA profiles in epilepsy might further inform potential therapeutic strategies that target miRNAs or related pathways.

Isogenic cell models to functionally characterize genetic variants in epilepsy

In epilepsy, the penetrance of identified genetic variants is often incomplete and the functional impact uncharacterized. Therefore, these discoveries are of limited translational value. Thus, functional characterization using cost and time efficient model systems is of great importance.

Acknowledging that no model is perfect, we need to weigh between feasibility, effectiveness, and translatability of observable phenotypes: Animals have the great advantage to allow studying phenotypes at all levels of interest, i.e. molecular, cellular and organismal level. However, major limitations are: i) generation of genetically engineered animals for each identified variant is not feasible, ii) the non-human background may hamper translatability of the phenotype, and iii) the genetic background or noise varies across strains and generations. Currently, a good alternative at in-vitro level is using human isogenic neuronal cell models. These cell systems allow studying the molecular, cellular and neurophysiological effect of a mutation and the respective wild-type gene in the same genetic background. In addition, new methods are being developed to study complex cellular interactions at an organoid level [8].

Isogenic neuronal cell lines can be generated by inducing a mutation into human neuronal progenitor cells (HNPCs), or by repairing the respective mutation in patient derived induced pluripotent stem cells (iPSCs). Both cell types can then be differentiated into mature neurons. Currently, CRISPR/Cas9 technology has proven to be the most effective tool to insert genetic modifications at sequence level. This technique allows complete gene-knock-out by insertion of premature stop codons as well as precise site directed mutagenesis or gene correction within 2-3 months (e.g. correction of SCNA1 mutation in iPSCs [9]), making this system cost and time-effective.

HNPCs, in contrast to iPSCs, are more cost effective and can directly be differentiated into neuronal subtypes (i.e. glutamatergic and GABA-ergic or dopaminergic) without any intermediate steps. Furthermore, only little intra-individual variation has been reported for hNPC in contrast to iPSC derived neurons [10]. However, only iPSCs permit the study of the genetic effect within the patient's specific genetic makeup.

In summary, CRISPR/Cas9 in combination with iPSC or HNPC cell lines is currently the most cost-and time effective alternative to animal models, making them a useful tool to screen for the pathogenic effect of a potentially disease causing variant.

Organotypic slice cultures of rodent brains as a tool to study epilepsy

Organotypic slice cultures of rodent brains have been used for over 25 years to study fundamental questions of neuroscience [11]. With the advent of mouse genetics, viral transduction techniques, time-lapse imaging and multielectrode array recordings, the versatility of these culture preparations has been enormously expanded and they have been used singly or in complex preparations to address many biological questions. These technological advances have also made it possible to use the organotypic culture technique more extensively for disease- and therapy-related research. Thus, organotypic slice cultures are now used to (1) study general pathological mechanisms, such as brain lesion/regeneration, denervation/collateral sprouting, or neuroinflammation, (2) identify specific pathogenetic mechanisms of diseases such as Parkinson's disease or Alzheimer's disease, (3) investigate the role of disease genetics, and, (4) test pharmacological and non-pharmacological therapies (e.g. [12]; [13]; [14]. In short, the organotypic slice culture technique has come of age and is now one of the methodological pillars of neuroscientific research.

The use of organotypic slice cultures in the context of epilepsy research is versatile and well-documented (e.g., [15]). Epilepsy models have been transferred to the in vitro situation using organotypic cultures of rat or mouse brain. Seizure-like events can be induced in entorhino-hippocampal slice cultures using different approaches, e.g., pilocarpin-treatment, changes in ion concentrations, or potassium channel blockers. The pattern of damage seen in these preparations is similar to the pattern of damage seen in the intact animal. This similarity with the in vivo situation opens up many possibilities: Using the modern tools now available to study hyperactivity-induced changes directly, e.g. time-lapse imaging, patch-clamp

recordings or multielectrode recordings (e.g., [16]; [12]), the role of genetic modifiers and risk factors for the course of the disease as well as for its therapy can be elucidated. Furthermore, it has been pointed out that these cultures may be particularly effective for studying pharmacoresistant seizures and could be used for the identification of new antiseizure compounds [17]. Computational approaches complement the battery of experimental tools and generate predictions and hypotheses that can in turn be tested in the dish. Eventually, however, data obtained using organotypic slice cultures will require in vivo verification. Epilepsy is a systems disease and the state of the system as well as its functional input and/or output may have major modifying effects in intact animals. Such a combined use of in vitro, in vivo (e.g., [18]) and in silico epilepsy models may be used to understand the role of modifier genes carried by affected individuals in the context of epileptogenesis and may thus contribute to novel personalized therapeutics.

Blood-brain barrier regulation by the Wnt/ β -catenin pathway in epilepsy - closing the barrier as a potential approach to treat epilepsy

Endothelial Wnt/ β -catenin signalling is necessary for developmental angiogenesis of the central nervous system (CNS) and differentiation of the BBB. In the adult Wnt/ β -catenin maintains BBB characteristics of endothelial cells (ECs) at the neuro-vascular unit (NVU), formed by ECs, pericytes, astrocytes, neurons and perivascular microglia.

Under pathological conditions such as traumatic brain injury, stroke, brain tumor as well as Alzheimer's dementia, the functional homeostasis of the NVU is disturbed, leading to increased vascular permeability.

In the last few years, we and others have shown that Wnt/ β -catenin is a master regulator of vascular barrier function in the CNS and moreover, we have shown that in glioma, ECs of the CNS partially lose their barrier phenotype, indicated by destabilized intercellular junctions and down regulated metabolic genes such as ABC transporters and cytochrome P450 enzymes. The stabilization of β -catenin in glioma ECs resulted in a more quiescent and normalized vessel phenotype with reduced permeability, stabilized junctions and increased mural cell investment. Preliminary data show that systemic, pharmacological activation of the Wnt/ β -catenin pathway with an FDA-approved drug can mimic the vascular normalization observed in the transgenic mouse models for glioma, suggesting that the Wnt/ β -catenin pathway might be a promising target for re-sealing the BBB.

The loss of BBB properties of ECs is also well documented in epilepsy in patients and in rodent models. However, if a hampered BBB is the cause or the consequence of SE is largely unknown. Interestingly, it has recently been shown that a rare, monogenetic form of seizures known as incontinentia pigmenti (Bloch-Sulzberger Syndrome) is primarily caused by endothelial apoptosis and barrier dysfunction due to a mutation in IKK/Nemo of the NF- κ B pathway [19].

Although the interaction of the NF- κ B and the Wnt/ β -catenin pathway has been documented, its relevance in ECs and specifically for the etiology and progression of epilepsy is currently not known. Additional strong evidence for the involvement of the Wnt/ β -catenin pathway and the BBB in the occurrence of seizures comes from the recently published adult deletion of β -catenin specifically in ECs (iCKOEC) of transgenic mice. Induced deletion of β -catenin resulted in severe seizures, neuronal injury, haemorrhages and postictal death [20].

Based on these striking findings, we hypothesize that endothelial Wnt/ β -catenin signaling is hampered in SE and that therapeutic activation of the Wnt pathway may counteract the BBB loss and hence the initiation and/or progression of the disease. Consequently, we are currently investigating the BBB status of SE patients as well as of SE mouse models. Furthermore, we will explore the potential benefit of sealing the BBB in epileptic mice via Wnt/ β -catenin pathway activation in an inducible, conditional -catenin gain-of-function mouse model.

In conclusion, sustained and reinforced Wnt/ β -catenin signaling promotes vessel stabilization, which might prove to be a valuable therapeutic target for anti-epileptic therapy. Nevertheless, further investigation is required to better understand the regulation of the Wnt/ β -catenin pathway in epilepsy.

Focal tissue modification in personalized translational epilepsy treatment

Presently, the main strategy for focal modification of brain tissue to treat epilepsy is surgical ablation. Although this can be effective (epilepsy surgery can render 70% seizure free), the destruction of tissue severely limits this approach, as it is important to avoid eloquent cortex. An alternative approach is to modify neuronal excitability, whilst preserving function. In recent years, there have been considerable developments in the tools that can help us achieve this. In particular, advances in viral vector technology have led to potentially safe and efficacious ways of transfecting specific populations of neurons in a focus [21].

Such gene therapies are an attractive approach to the treatment of neurological disease, as they avoid the widespread destruction of epilepsy surgery, but permit specific focal tissue modification. We developed and characterized a model of focal motor seizures, which mimics *epilepsia partialis continua* in humans, in order to test the efficacy and safety of our gene therapies [22]. This model has a well-defined epileptogenic region in eloquent cortex where there are frequent bursts of epileptiform activity that are associated with a focal motor phenotype. We took advantage of lentiviral and adeno associated viral vectors which are being used in treatment studies of other human disease to transfect excitatory pyramidal cells.

Using a lentivirus vector we overexpressed the potassium channel Kv1.1 in pyramidal cells in the focus [22]. This approach reduced the excitability of pyramidal cells without affecting motor function. Nevertheless, overexpressing Kv1.1 progressively reduced the epileptiform discharges over a period of 20 days as the transgene was expressed [22]. Although the

results are very promising, the route to translation is somewhat confounded by gene dose. There is a danger that too much Kv1.1 could interfere with function and too little would be ineffective.

We therefore turned to two alternative approaches in which it is possible to titrate the effect once the transgene is expressed. First, we used optogenetics, expressing halorhodopsin in pyramidal cells [22]. This is a chloride pump that on exposure to yellow/green light pumps chloride into the neuron, so hyperpolarizing and decreasing the excitability of the neuron. By using different strengths and durations of light, it is possible to regulate the excitability of groups of neurons. We transfected pyramidal cells in the focus and placed a fibre optic in the focus to deliver light. With this method, we could use the light to decrease the excitability of the focus and so decrease seizure activity without interfering with function [22]. Such an approach has now been used by others in closed loop devices in which the detection of seizure activity activates the light. Although effective, this approach is complicated by having not only to target the viral vector to the focus but also to get light into the focus. Moreover, halorhodopsin is a foreign protein, raising the concern that it could generate an immune reaction, even though the brain is relatively immune privileged.

Lastly we used a technology termed designer receptors exclusively activated by designer drugs (DREADDs). In pyramidal cells in the focus, we expressed a modified “inhibitory” muscarinic receptor hM4Di, which is no longer sensitive to acetylcholine, but instead is activated by a selective, normally inactive drug clozapine-N-oxide (CNO) [23]. Systemic administration of CNO was able to suppress focal seizures evoked by chemoconvulsants and also had a robust anti-seizure effect in our model of focal neocortical epilepsy [23]. This technology has the advantage of being able to adjust the effect of the gene therapy by titrating the dose of drug that specifically activates the receptor. CNO has no other effect than on the neurons in which the receptor is expressed, so avoiding systemic and CNS side-effects. The receptor expressed is a modification of an endogenous receptor, so reducing the potential for immunogenicity. DREADDs seem to have many of the characteristics that would enable translation into a therapy for human focal epilepsy.

Computational neuroscience to identify treatment targets

There is a growing interest in how brain circuits are changing during epileptogenesis. Cell loss, altered connectivity, and inflammatory responses have all been implicated in epileptogenesis, but it's often unclear which changes are primary causes and which are just consequences of other changes. Unraveling these cause and effect relationships is of central importance when searching for new treatment methods. The development of computational models can be a great tool for understanding such cause and effect relationships, because a computational model facilitates a “dissection” of the system and so enables the study of the specific role of each component or mechanism for the overall system. Not surprisingly, therefore, a number of computational models have investigated how specific structural changes to a circuit can make it more prone to exhibit seizure-like activity patterns. This is an important first step, but it stops short of answering the harder question of what causes the

changes to the structure of these circuits in the first place. We argue that in order to fully understand these mechanisms of pathologic circuit changes, it is important to understand how these circuits are normally constructed and maintained under basal conditions. Remarkably, the structure of cortical and hippocampal circuits is highly dynamic, with new synapses constantly being formed and others being removed. Recent computational modeling work suggests that the formation and maintenance of brain circuits in the presence of this constant turnover of synaptic connectivity can be viewed as a process of self-organization fuelled by the interaction of a number of plasticity mechanisms, some of which are homeostatic [24]. Such models have successfully explained how the distribution and fluctuation patterns of synaptic connection strengths are rooted in fundamental plasticity mechanisms [25]. The challenge lying ahead is now to investigate what mechanisms could be primary causes of the observed circuit changes. The list of candidates is long and none of them are mutually exclusive. Aberrant neuronal properties, dysregulated plasticity mechanisms, an exhaustion of homeostatic mechanisms, or vicious cycles of neuro-immune interactions [26] could all be involved in the process of epileptogenesis. A combination of highly detailed, biologically realistic computer modeling [27] and simplified, mathematically tractable computational modeling [24,26] is needed to help identify how these mechanisms may contribute to epileptogenesis.

Future directions

Given the fact that the term “epilepsy” refers to a variety of different entities, a high demand of personalization in epileptology is obvious. Ultimate goals of personalized translational epileptology are

- to determine the individually contributing patterns of genetic factors and various disease mechanisms (e.g. dysregulation of miRNAs, inflammation, blood-brain barrier dysfunction) in each single patient, and
- to tailor specific etiology-related therapies which take account of particular disease characteristics in the respective individual (e.g. pharmacogenetics in drug treatment, functional anatomy of epileptogenic networks in invasive therapies).

Tight translational cooperation between basic and clinical researchers is necessary to achieve these goals. Hence, future emphasis will be directed to diagnostic methods that can be used likewise in both preclinical and clinical research, e.g. new MRI-based techniques or cerebral microdialysis. Development and validation of preclinical disease models should be addressed more intensely, because selection of valid models for distinct disease entities will avoid limitations of research impact on the respective human condition and, therefore, will save time, labor and costs.

Information flow between bench and bedside is bidirectional. Clinical diagnostics and resective therapy in epilepsy patients provide the respective electrophysiological data or tissue that is required to investigate dysfunction (and, partially, function) of the human brain.

Tissue banks are currently established and will become one foundation of interdisciplinary multi-method research on disease mechanisms. Thorough phenotyping using advanced techniques such as quantitative MRI is a prerequisite for interpreting the results. Analysis of EEG data from intracranial recordings will massively profit from the ever-increasing computational power and from cooperation between neuroscientists and mathematicians.

Personalized therapies using new techniques like DREADDs and optogenetics are currently still at the preclinical stage. In contrast to resective surgery, these methods offer reversible functional inhibition of specific neurons in the epileptogenic zone rather than irreversible destruction of brain tissue, and may therefore contribute to a future increase in treatment safety.

Preclinical testing of new therapies is labor-intensive and bears a high risk of failure. For this reason, the preclinical development is called “the valley of death” by pharmaceutical companies [28]. Concepts and methods of personalized translational research, as described above, have the potential to substantially shorten the walk through this unedifying area.

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Conflicts of interest

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