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Biomarkers of epileptogenesis and pharmacoresistance in epilepsy

Habilitation thesis

(Collection of previously published scholarly works with commentary)

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I hereby declare that I wrote this habilitation thesis on my own, using the relevant resources listed in the references.

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Signature

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Content of the work

Commentary

Despite of advances in the diagnosis and treatment of epilepsy remain approximately 30%-40% of the patients pharmacoresistant. The International League Against Epilepsy (ILAE) defines drug-resistant epilepsy as "failure of adequate trials of two tolerated, appropriately chosen and used antiepileptic drug schedules (whether as monotherapies or in combination) to achieve sustained seizure freedom". Only parts of these patients can be offered curative epileptosurgical solutions or other non-pharmacological treatment options (vagus nerve stimulation, ketogenic diet, etc.).

The pharmacoresistance to antiseizure drugs (ASD) per se is not a possible therapeutic target, as ASDs themselves do not prevent the development of epilepsy, merely blocking the seizures as they arise. Moreover, there is no evidence that they influence the course of epilepsy. There are no clinical tools or guidelines for predicting therapeutic response in individual patients, leaving them no choice other than to try all antiseizure drugs available as they suffer debilitating seizures with no relief.

Current therapy is limited to suppressing the symptoms of the disease- epileptic seizures, and does not allow the elimination of the cause (except epileptosurgery) or its prevention during the proces of epileptogenesis (in the patients who are at risk due to genetic predisposition or after brain insult).

The aim of research in the epileptology is discovery of the drugs that would not only suppress seizures, but ideally work as medication which prevent or modify the process of epileptogenesis, the medication working as disease-modifying drugs (DMD) and the treatment due to progressive process of neurodegeneration, neuroinflammation and neuronal hyperexcitability leading to the development of pharmacoresistance in epilepsy.

The discovery of predictive biomarkers and early identification of pharmacoresistant patients and patients who are at the risk of development of epilepsy (biomarkers of epileptogenesis) is the highest priority of current epileptology research.

This habilitation thesis is conceived as a collection of 6 articles previously published by the author and her colleagues. It contains individual chapters dealing with the basic aspects of epileptologenesis and pharmacoresistance in epilepsy. Each chapter is followed by commentaries introducing the topic of each publication, describing the current state of

knowledge and how the author has contributed to knowledge in this field. The work is based on research activities at the authors' workplaces, the Department of Pediatric Neurology, University Hospital Brno; the Faculty of Medicine, Masaryk University; and Central European Institute of Technology.

In the future, new therapeutic procedures should offer a wide range of options, respecting the specifics of individual forms of epilepsy as well as individual differences between patients with regard to the development and prognosis of the disease.

Keywords: epilepsy, pharmacoresistance, biomarkers, epileptogenesis, disease-modifying drugs

1. Introduction

Epilepsy affects approximatelly 50 million people worldwide and about 2.4 million new cases are diagnosed annually. The clinical course is characterised by spontaneous recurrent seizures and often accompanied by debilitating cognitive impairment. Despite of advances in the diagnosis and therapy of epilepsy, approximately 30 to 40 % of patients remain pharmacoresistant. Only a part of these patients can be offered some curative treatment, in terms of epileptosurgery or other non-pharmacological treatment options (stimulation of the vagus nerve, ketogenic diet, etc.). Actual treatment of epilepsy is limited only to suppressing the symptoms of the disease, in the sense of epileptic seizures (anti-seizure medication; ASM) and does not allow the elimination of the cause (exept for epileptosurgery) or the prevention of its occurrence in the patients, who are at the risk due to genetic predisposition or after epileptogenic brain insult.

The aim of research in the epileptology is discovery of the drugs that would not only suppress seizures, but ideally work as medication which prevent or modify the process of epileptogenesis, the medication working as disease-modifying drugs (DMD) and the treatment due to progressive process of neurodegeneration, neuroinflammation and neuronal hyperexcitability leading to the development of pharmacoresistance in epilepsy (Loscher and Brand 2010).

1.1. Pharmacoresistance in epilepsy

The International League Against Epilepsy defines pharmacoresistant epilepsy as the failure of a patient's seizures to respond to at least two antiseizure medications that are appropriately chosen and used for an adequate period (Kwan et al 2010). The pharmacoresistance to antiseizure drugs (ASD) is not a possible therapeutic target, as ASDs themselves do not prevent the epileptogenesis (the process of the development of epilepsy), just blocking the seizures as they arise. Another challenge is the nature of the pharmacoresistance itself, as different intractability patterns can be present in individual patients (Bohošova et al. 2021).

The etiopathogenesis of the development of pharmacoresistance in epilepsy is still unclear. The results of some studies indicate that neuroinflammation plays a crucial role. Importantly, upregulation of many proinflammatory molecules, such as high mobility group box 1 (HMGB1), cyclooxygenase 1 (COX-1), cyclooxygenase 2 (COX-2), interleukin-1β (IL-1β), and nuclear factor kappa-lightchain-enhancer have been found in drug resistant epilepsy patients (Vezzani et al 2016). Some of them should become potential therapeuti targets.

We distinguish 3 types of pharmacoresistance (FR) in epilepsy: de novo FR, progressive FR and relapsing-remitting form (waxing and waning) FR. **De novo FR** means presentation of FR even before the use of antiepileptic medication. On the other hand, **progressive FR** means FR arising during the treatment of epilepsy, i.e. after a certain period when the patient responded to treatment. **Waxing and waning FR** is characterised by alternationg periods od drug resistance with remission when patients become seizure-free (Bohošova et al 2021).

Currently, there are two main hypotheses on the development of pharmacoresistance in epilepsy. Firstly, the **drug transporter hypothesis** states that pharmacoresistance to ASDs is caused by overexpression of multidrug cell transporter (ATP-binding casette subfamily member 1, P-glycoprotein), which pumps the drug out of the cells thereby blocking entry into

the brain leading to insufficient concentrations (van Vliet et al 2010, Volk 2005). On the other hand, **the drug target hypothesis** supposes that the change in the molecular structure of the drug target prevents it from binding the drug and thus leads to pharmacoresistance (Bauer et al 2008; Bohošova et al 2021; Zibell et al 2009).

1.2. Epileptogenesis

The term epileptogenesis is most commonly assosiated with symptomatic epilepsy, such as traumatic brain injury (TBI), stroke etc. On the other hand, some studies suggest that process of epileptogenesis also occurs in genetic epilepsies where affects developmental programming of the gene expression leading to abnormal circuitry during maturation (Zara et al 2009).

Epileptogenesis is a dynamic process leading to the formation of epileptogenic networks. Change in the balance between excitation and inhibition of neuronal networks is the underlying mechanism of epileptogenesis. It can be defined as the latency period between occurence of insult to the development of epilepsy. In the case of genetically linked epilepsy (such as TSC; tuberous sclerosis complex), this is the period between the genetic diagnosis and the development of epilepsy in the patient. In some patients, epilepsy manifests itself months to years after the initial epileptogenic insulin or identification of the genetic basis of epilepsy.

The processes of epileptogenesis include processes of neurodegeneration, gliosis, neurogenesis, neuroinflammation and disruption of the blood-brain barrier, which lead to reorganization of neuronal circuits and the development of abnormal excitability (Lukasiuk 2009). This progressive alters of neuronal excitability and decreasing of seizure threshold caused the emergence of the critical interconnections and probably structural changes before the first spontaneuos seizure occurs (Pitkanen & Lukasiuk 2011).

Recent studies have provided insight into molecular mechanisms of epileptogenesis including gene regulating of synaptogenesis, brain plasticity, proliferation, neuronal death and neuroinflammation response. These mechanisms would be potential targeted to provedent epileptogenesis (Pitkanen & Lukasiuk 2011).

During the latent period of the process of epileptogenesis, we do not have a treatment that would work as a prevention of the development of epilepsy.

Unfortunately, current epileptology offers only symptomatic therapy, which works only as a "prevention" of seizure (**ASM**: antiseizure medication), while therapy affecting the process of epileptogenesis (**AED**: antiepileptic drugs) is still unavailable.

The fundamental prerequisite for discovering of "prophylactic" therapy affecting the process of epileptogenesis is the identification of **biomarkers of epileptogenesis**. The research in epileptology is simultaneoulsy focusing on the identification of **biomarkers of pharmacoresistance**, which will allow early identification of patients in whom the treatment of epilepsy will be ineffective (pharmacoresistant patients). The verification of biomarkers of epileptogenesis and pharmacoresistance in epilepsy is crucial for development of disease modifying drugs (DMD) and discovery of individual "personalized" treatment in epilepsy.

2. Biomarkers-definition and classification

FDA-NIH (Food and drug administration and National Institute of Health) uses the BEST (Biomarkers, Endpoints, and other Tools) scheme to define and classify biomarkers. According to FDA is a biomarker defines as objectively measurable and evaluable indicator of normal and pathogenetic processes and/or response to therapy.

Biomarkers can be divided into diagnostic and predictive (prediction of disease dynamics and/or therapeutic response). We can further distinguish 3 categories of biomarkers: disease-defining biomarkers (susceptibility to disease, diagnostic, prognostic), therapeutic response biomarkers (predictive, pharmacodynamic response and safety) and treatment monitoring biomarkers. Furthermore, we can divided biomarkers by type into molecular, histological, radiological, electroencephalographic, psychological etc; Simonato et al 2021).

Table 1: Clasification of biomarkers according to FDA (Simonato et al. 2021, modified)

Monitoring biomarkers	using for assesment of the status of a disese or for evidence of exposure to some medication
Diagnostic biomarkers	using for detection and/or confirmation of the presence of the disease
Susceptibility risk biomarkers	biomarkers that indicate the potential for developing a disease or the medical condition

Prognostic biomarkers	using for identification of a clinical event, disease reccurence or it's progression
Predictive biomarkers	used to identify individuals who are more likely than similar individuals without the biomarkers to experience a favourable or unfavourable effect from exposure to a medical product o ran enviromental agent
Pharmacodynamic/response biomarkers	using to show biological response to some medication
Safety biomarkers	biomarkers measured before or after an exposure to medication to indicate adverse events or toxicity

The golden standard of biomarker study design includes a so-called discovery or explorative phase and validation phase. In the discovery phase, the main aim is to screen for potential biomarkers from a large pool of molecules, to provide a so-called expression profile.

In the next part of the work we will go through individual types of biomarker according to current state of art. Individual subchapters are supplemented by the author's contributions to the issue.

2.1. Molecular biomarkers

Molecular biomarkers can be considered as any molecule (proteins, metabolites, neucleic acid) in biofluids (e.g. blood serum, cerebrospinal fluid (CSF)), where thay can be free or bound to proteins. From many points of view are molecular biomarkers the most atractive biomarkers in terms of low cost andaccesibility in easily available compartment, such as CSF, blood, urine etc.

2.1.1. Proteins as molecular biomarkers of epileptogenesis and pharmacoresistance in epilepsy

Recent studies identify some brain-enriched proteins in the plasma or serum that could be potential biomarkers of epileptogenesis. Importantly, these proteints could reflect alteration in brain functions that are associated with epileptogenesis, such as neuronal hyperexcitability, axonal injury, dendritic remodelling, blood-brain barrier dysruption and neuroinflammation. Potential candidate biomarkers of epileptogenesis and pharmacoresistance in epilepsy has been identified. Potential promissing proteins include: neuronal specific enolase (NSE), ubiquitin C-terminal hydrolase I (UCH-L1), glial fibrilary acidic protein (GFAP), calcium binding protein (S100β), matrix metalloproteinase 9 (MMP-9), human mobility group box 1 (HMGB1) and biomarkers of neuroinflammation (Simonato et al 2021).

2.1.2 Biomarkers of neuroinflammation

Brain inflammation as a "biomarker" of epileptogenicity

Immune mechanisms are recognized as important factors contributing to refractory epileptic activity and status epilepticus. Excessive activation of inflammatory signaling pathways seems to be a hallmark of epileptogenesis. Experimental and clinical evidence have demonstrated the increased synthesis of specific inflammatory mediators, and the upregulation of their cognate receptors in the chronic epileptic brain (Vezzani et al 2011). Brain inflammatory pathways play a key role in seizure generation and exacerbation. The evidence provided by these studies highlights, that specific inflammatory pathways and molecules may be putative targets for novel therapeutic interventions in status epilepticus (Vezzani et al 2011; Vezzani et al 2015).

Understanding the complex role of inflammation in the generation and exacerbation of epilepsy is crucial for the identification of new molecular targets for therapeutic intervention.

The role of cytokines and chemokines in epileptogenesis and development of pharmacoresistance in epilepsy

In terms of epileptogenesis, cytokines participate in the modulation of ion channels and synapses and thus they can modify neuronal excitability (Vries et al 2016). Induction of inflammatory cytokines within the brain following status epilepticus is well documented in experimental animal models and cytokines released during seizures include proinflammatory cytokines such as interleukin IL-1beta, IL-6 and TNF-alpha, which enhance excitatory mechanisms (Vries et al 2016, Vezzani et al 2011). IL-10 and IL-1 receptor antagonist (IL-1RA) can inhibit IL-1beta signaling effect. In addition, recombinant variant of IL-1RA has not only shown strong anticonvulsant properties in animal models, but also dramatic seizure reduction in several human cases of refractory status epilepticus (Kenney-Jung DL 2016 et al).

Chemokines are a family of cytokines that guide migration of leukocytes and play role in cell interaction through specific chemokine receptors on targeted cells. Frequently studied chemokines in neuroinflammation are C-X-C and C-C motif ligand such as (CXCL)-8, -10, -13 and CCL-2 [10]. CXCL-8 and CCL-2 play role in blood brain barrier disruption, which may reinforce sustained epileptic activity and leads to status epilepticus (Kenney-Jung DL 2016 et al). In addition, increased levels of CCL2 and CXCL10 enhanced epileptic activity in animal models, and they have been shown to be elevated in humans with pharmacoresistant epilepsy (Cerri et al 2017; Cerri et al 2016). Increased levels of CXCL10 were also detected in various types of encephalitis, i.e. a condition that is frequently presented with seizures (Kothur et al 2016). Finally, the chemokine CXCL13 is the major chemoattractant for B cell migration to the CNS and seems to be a sensitive biomarker for different types of neuroinflammation (Irani 2010). There are limited studies indicating its role in epileptogenesis. Taken together, several studies provided on experimental models suggested that chemokines and their receptors play

roles in epileptogenesis and may represent new therapeutic targets for seizure control (Cerri et al 2017; Torri et al 2016).

Summary of mechanisms of influence of neuronal excitability by mediators of inflammation (cytokines, chemokines, blood-brain barrier breakdown; Aulická et al 2020)

IL-1β

- ↑ release and ↓uptake of glutamate in astrocytes, ↓ GABA neurotransmission→ neuronal hyperexcitability
- release of other pro-inflammatory cytokines: IL-6, IL-8- by gene transcription induction

TNFα

- ↑ release of glutamate in microglia; upregulation of AMPA receptors; organization of excitatory/inhibitory synapses

IL-6

- ↑ exposure to IL-6 in the prenatal period (possible transplancent transmission) leads to neurodegeneration of the hippocampus (disrupts the structure and morphology of hipoccampus mutualy) → hyperexcitability of hippocampus and progression of epileptogenesis (co-contribution ↑ IL-6 and ↑ IL-1β)

IL-1β, IL-6, IL-8

- development of febrile convulsions and febrile status epilepticus

Prostaglandins (PGE2)

- Binding of PGE2 to EP2 receptor (astrocytes) increases glutamate release from astrocytes (hyperexcitation) and leads to induction of apoptosis

TOLL-like receptors (TLR 1,2,3; transmembrane glycoproteins)

- stimulation of TLR inflammatory cascades leads to induction of cytokine secretion (IL-1β, IL-6, TNF-α) → development of neuro-inflammation and influence of neuronal excitability
- TLR3 activation leads to hyperexcitability in the hippocampus region

HMGB1 (high mobility group box 1; nuclear protein)

- The proconvulsant effect of HMGB1 is mediated by its binding to the NMDA receptor and its interaction with TLR4 (toll-like receptor 4)
- The inflammatory cascade HMGB1-TLR4 leads to the generalization of seizures

Chemokines

- affect neuronal excitability through modulation of voltage-gated ion channels

Blood-brain barrier disruption (BBD)

- leads to the induction of epileptogenesis and promotes the generalization of seizures

Author's contribution to this issue:

Brain inflammation represents a common substrate of pharmacoresistant epilepsy of different etiologies and it can directly affect neuronal excitability. Neuromodulatory properties of some proinflammatory molecules (cytokines, chemokines) may be responsible for hyperexcitability in neuronal networks. The relation between inflammation and epilepsy is reciprocal. The inflammatory processes in the brain may participate in initiating seizure activity and simultaneously they may be a consequence of the recurrence of the seizures. Pharmacological studies on experimental models focused on IL-1β/ IL-1R1, HMGB1/ TLR4 and COX-2/ prostaglandin systems demostrate that these inflammatory pathways significantly in triggering

and recurring seizure activity. Status epilepticus (SE) leads to development of inflammatory processes which can be detected in brain tissue, cerebrospinal fluid and blood serum. Prolonged seizures and SE lead to fast and prolonged activation of specific inflammatory pathways in brain areas accordant with the epileptogenic zone. Understanding of the complex role of inflammation in the generation and exacerbation of epilepsy and development of pharmacoresistance in epilepsy is crucial for the identification of new molecular targets for therapeutic intervention in these patients. The authors Aulická et al published in 2020 review article focusing on the role of neuroinflammation in development of pharmacoresistance in epilepsy and refractory status epilepticus. This work has become theoretical basis for other research activities on this field.

See annex 1 here.

2.1.3 Genetic biomarkers in epilepsy

Epilepsy can be described as a network disorder and changes in the properties which compose these networks constitutes a critical mechanism of disease development. There is an urgent need for valid predictive biomarkers to guide patient-tailored individualized treatment strategies in epilepsy (Weber et al 2014).

Large scale changes in gene expression and regulation is seen at the epigenetic, transcriptional and post-transcriptional levels. For this reason will this chapter focuse on genomic factors as part of an individual concept for AED treatment.

MicroRNA as biomarkers of epileptogenesis and pharmacoresistance in epilepsy

State-of-the-art

miRNAs as biomarkers of epilepsy

MicroRNA (miRNA) is a class of short non-coding RNAs (21-25 nucleotides long) functioning as important negative posttranscriptional regulators of gene expression (Bartel 2018).

Emerging evidence shows that epilepsy and epileptogenesis are regulated by the epigenetic factors that control multiple genes and proteins involved in neuronal death or dysfunction, gliosis, changes in ion channel function, neurogenesis, and neuroinflammation (Ji menez-Mateos EM & Henshall DCC 2013). The epilepsy is associated with wide ranging changes to miRNA levels in the brain. The research in animal models has provided strong evidence that acute or recurrent seizures change miRNA expression and function (Henshall et al 2016). Recent evidence has indicated that liquid biopsies could replace invasive surgical

biopsies because body fluids (CSF, blood) contain proteins, nucleic acids and other cellular components released by various brain lesions, reflecting current biological situation and allowing identification of clinically useful biomarkers e.g. miRNAs (Henshall et al 2016).

In cases of mesial temporal lobe epilepsy (MTLE), the most common aetiology of drug-resistant epilepsy in adults, the group of Eleonora Aronica discovered differentially expressed microglial, astrocytic, and neuron-specific genes in hippocampal tissue samples. In the class of small RNAs, they discovered specific miRNAs (e.g., let-7b-3p and let-7c-3p) related to MTLE (Mills et al 2020).

The miRNAs in biofluids (CSF and blood) might be useful biomarkers of brain injury. These miRNAs might originate from controlled release into exosomes or from damage or disruption of the blood–brain barrier, allowing passage of small quantities of brain-expressed miRNAs (Henshall et al 2016). Thus, altered miRNA profiles in biofluids may be potentially useful biomarkers of epileptogenesis. In summary, miRNAs represent an important layer of gene expression control in epilepsy with therapeutic and diagnostic potential (Henshall et al 2016).

The role of miRNAs in neuroinflammatory regulation

The expression of selected miRNAs known to be involved in the regulation of immune responses, such as differentiation of immune cells and the outcome of immune responses.

Key *pro-inflammatory* (**miR-155, miR-27b, miR-326**), *anti-inflammatory* (**miR-124, miR-146a, miR-21, miR-223**), and *mixed immunomodulatory* (**let-7 family**) miRNAs, which regulate neuroinflammation, were recognized in various neurological disorders, such as multiple sclerosis, ischemic stroke, epilepsy and others (Gaudet al 2017). For instance, upregulation of **miR-146a** (inflammation-associated microRNA) was identified in experimental and human temporal lobe epilepsy (Kothur et al 2016). The miR-146a is potential

endogenous regulator of Toll like receptor and cytokine receptor signaling (Tiwari et al 2018; Aronica et al 2010). The exact role of miR-146a in the modulation of the inflammatory response and associated pathogenic signaling in epilepsy is not completely clarified.
Understanding the role of miR-146a and other neuroinflammation associated miRNAs may be crucial for the development of new therapeutic strategies in epilepsy (Aronica et al 2010; O'Connell et al 2010).

Author's contribution to this issue – I am part of the research team of Prof. Ondrej Slaby and Prof. Milan Brazdil research group focusing on analysis of microRNAs profiling in patients with focal cortical dysplasia and mesial temporal epilepsy with hippocampal sclerosis.

MiRNAs are particularly abundant in the brain where they play numerous regulatory roles in both mature and developing neurons (e.g. neuronal differentiation and remodelling, neurotrophin signalling, synaptic plasticity, excitability, etc.; Saba et al 2010; Ye et al 2016).

Recent studies have demonstrated that miRNAs are critical for proper neural progenitor development during corticogenesis and the dysregulation of their expression could underlie the onset of complex human neurological disorders (Volvert et al 2021). For example, the pathogenesis and epileptogenesis of cortical dysplasia might be triggered by the disruption in mTOR (mammalian target of rapamycin) or LIS1 (lissencephaly 1) pathways (Lee et al 2014). The impairment in these pathways leads to the dysmorphism of the cells and the cortical dyslamination, respectively. These malformation of cortical development (MCD) show aberrant miRNA profiles (Lee at al 2014).

In our project we aim: (1) to detect FCD tissue-specific miRNA expression profile that is distinct in FCD compared to normal brain tissue and to MTLE/HS as an example of another kind of epileptogenic brain tissue; (2) to identify FCD-specific circulating miRNAs and to

describe FCD-specific expression profile of circulating miRNAs in paediatric and adult patients with FCD; (3) to identify miRNAs specific for FCD subtypes; (4) To analyse diagnostic potential of circulating miRNA in specific groups of patients with difficult-to-diagnose FCD, specifically MRI-negative FCD.

Detection of FCD-specific miRNAs may contribute to a more precise diagnosis in the following situations: (i) when MRI findings are inconclusive (so-called "MR-negative" FCD), (ii) after epilepsy surgery as an additional marker of completeness of surgical resection and (iii) as a possible marker of risk of seizure recurrence after epilepsy surgery. In addition, blood levels of circulating FCD-specific miRNAs might guide the process of withdrawal of anti-epileptic medication after epilepsy surgery for FCD. By comparing the miRNA profiles of adult and paediatric patients between different FCD types (I and II) we should be able to detect the age-dependent difference in expression profiles of circulating miRNA in adult and paediatric patients with FCD.

The proposed project could help to understand etiopathogenesis of development of FCD and most importantly may be the basis for a new therapeutical approaches in the treatment of FCD.

Importantly, our research group recently published review article (Aulicka Š as senior researcher and corresponding authors). The discovery of predictive biomarkers and early identification of pharmacoresistant patients is of the highest priority in this group.

MicroRNAs (miRNAs), a class of short noncoding RNAs negatively regulating gene expression, have emerged in recent years in epilepsy, following a broader trend of their exploitation as biomarkers of various complex human diseases.

We performed a systematic search of the PubMed database for original research articles focused on miRNA expression level profiling in patients with drug-resistant epilepsy or drug-resistant precilinical models and cell cultures. In this review, we summarize 17 publications concerning miRNAs as potential new biomarkers of resistance to antiseizure drugs and their potential role in the development of drug resistance or epilepsy.

Although numerous knowledge gaps need to be filled and reviewed, and articles share some study design pitfalls, several miRNAs dysregulated in brain tissue and blood serum were identified independently by more than one paper. These results suggest a unique opportunity for disease monitoring and personalized therapeutic management in the future.

See annex 2 here.

Transcriptomics as a biomarker of epileptogenesis and pharmacoresistance in epilepsy

A transcriptome is a collective term for all molecules created by genes present a time point in a cell. The major trancript that forms the template for protein formation is the messenger RNA (mRNA) molecule. With the growing availability of high-throughput sequencing methods, we have become increasingly capable of detecting subtle changes in gene structure and expression, beyond those observed on the DNA level. In the last decade, there has been a huge breakthrough in the field of RNA sequencing, which makes it possible to identify even a single cell transcriptome with great accuracy (Hong et al 2020). Transcriptomics can tell us which genes are currently active and extent which trascription is taking place and give us answers to exactly which genes play a key role in the processes of many different diseases.

The transcriptome, which is determined by increasingly sophisticated RNA sequencing methods, has been analyzed in the last decade, especially in tumor cells, as these show large differences in gene transcription compared to normally functioning cells. In many cases, detailed knowledge of the transcriptome can be a **diagnostic and prognostic marker**, but it can also give us a picture of tumor heterogeneity, their drug resistance or immunotherapy (Hong et al 2020). According to Hong et al., RNA sequencing is used in cancer research to detect differential gene expression and identify tumor markers, tumor heterogeneity and evolution, tumor pharmacoresistance, tumor microenvironment, immunotherapy and identification of neoantigens. High heterogeneity and transcriptomic diversity of the tumor often negatively correlates with the patient's prognosis (Hong et al. 2020). Transcriptomic analyses have shed new light on differential expression profiles of multiple tumour types, even when the same genes are affected (Bongaarts et al 2020).

In the last few years, scientific research has begun to focus on many other diseases. Preclinical and clinical neurological research includes Alzheimer's disease, multiple sclerosis or

Parkinson's disease. In addition to their use as markers, transcriptome analysis can provide information on the pathogenesis of neurological diseases, the molecular architecture of the nervous system, and potential treatment targeting.

Author's contribution to the issue: I am part of the research team of Prof. Ondrej Slaby and Prof. Milan Brazdil research group focusing on Transcriptomics And DNA Methylation Analysis In Patients With Focal Cortical Dysplasia (Project Ministry of Health NU21-04-00305).

Focal cortical dysplasia (FCD) is a subtype of malformations of cortical development (MCD) that represents the most common cause of drug-resistant epilepsy in children and the second most common cause of drug-resistant epilepsy in adults (Harvey et al 2008; Blumcke et al 2017). Despite of the formidable progress in our knowledge of FCD, we still lack the complete understanding of FCD pathogenesis and related epileptogenesis, and no pharmacological approach is yet capable of treating this disorder sufficiently. Intractable epilepsy in FCD patients along with side effects of ineffective treatments are associated with developmental delay, cognitive deficit, psychiatric, psychosocial and other serious comorbidities. These deleterious consequences of long-lasting epilepsy could be prevented by reliable early diagnosis and effective treatment. Detection of FCD at the time of epilepsy onset is however challenging since many discrete dysplastic lesions can be overlooked at conventional brain MRI and FCD-specific electrophysiological patterns have not yet been sufficiently studied. In addition, 38 % of patients with the diagnosis of FCD fail to achieve freedom from seizures two years after epilepsy surgery (Baud et al 2010).

Since the early 2000s, multiple studies have occurred that aimed to trace the origins of FCD. Ranging from perinatal insults (Kršek et al 2010) to a putative role of HPV infection (Chen et al 2012), multiple hypotheses attempted to explain the pathogenesis of FCD. With a growing

number of reports on the role of somatic and second-hit mutation events (Lim JS et al 2017; Lim JS et al 2015), recently there has been a consensus that FCD occurs as a result of somatic, germline and second-hit somatic mutation (Baldassari et al 2019; Sim et al 2019). However, despite a growing number of genetically-confirmed cases of FCD, there remain a significant proportion of patients without a detectable genetic cause of their FCD; genetic cause could not have been established in 71% and 37% of FCD type I and II cases, respectively (Baldassari et al 2019). Taken together, we obviously lack a clear understanding of the underlying disease biology of focal cortical dysplasia.

Apart from genetic (DNA-level) and transcriptomic (RNA-level) changes, there exists yet another level of genetic regulation that may contribute to FCD formation; the epigenetic changes, especially DNA methylation, serve as molecular "switches" that activate or suppress gene expression. Methylation profiling represents a vital diagnostic tool in cancer genomics, especially in brain tumour diagnostics (Kumar et al 2018; Stone et al 2018), where methylation profile significantly contributes to tumour diagnosis and resolves diagnostic uncertainties (Capper et al 2018). In a small cohort of FCD patients, methylation profiles were able to distinguish between FCD types I, IIa and IIb (Kobow et al 2019).

The main goal of our work is to characterize transcriptomic and methylation profiles of FCD subtypes and to identify novel signaling pathways that could become targets of personalized therapy in patients for whom surgery is not feasible.

In our project we aim to (i) characterize the distinctive transcriptomic and methylation patterns of FCD subtypes, (ii) combine the transcriptomic and methylation analysis data with the genetic data and to correlate them with the clinical course and prognosis of epilepsy surgery patients and (iii) to identify previously unrecognized signalling pathways involved in FCD formation that might in future serve as potential therapeutic targets, especially for patients in whom

epilepsy surgery is not possible. The proposed project will significantly contribute to the understanding of FCD pathogenesis, combine basic and clinical research methods and thus contribute to improved patient care in the Czech Republic and worldwide.

3. Neuroimaging biomarkers - state of art

Neuroimaging is a powerful tool to explore structural and functional brain alterations. Several modalities, such as MRI (magnetic resonance imaging), PET (positron emission tomography), SPECT (single-photon emission computer tomography) have already been applied as biomarkers of epileptogenesis, especially in animal models.

Remarkable advances in neuroimaging methods bring numerous possibilities to obtain information about neuronal activity, blood-brain barrier alteration, brain inflammation, and various molecular alteration during epileptogenesis or for prediction of pharmacoresistance in epilepsy. These advances allow us to study molecular, structural, and functional changes within the brain and use these methods as a biomarkers of epileptogenesis and pharmacoresistance in epilepsy. During the workshop on Neurobiology in Epilepsy (XIII WONOEP), organised in 2015 by the Neurobiology Commission of the International League against Epilepsy, the focus was on neuroimaging biomarkers. The **biomarkers of epileptogenesis** (the development of epilepsy), **ictogenesis** (the propensity to generate spontaneous seizures) and **pharmacoresistance** (non-response to treatment) were defined (Vliet et al 2017).

In this chapter, it will be provided an overview of the current knowledge of neuroimaging biomarkers for epileptogenesis, pharmacoresistance in epilepsy and ictogenesis related to brain inflammation, blood-brain-barrier dysfunction, epileptogenic lesions and seizure onset zone. Imaging method to be discussed will inculde PET and advanced MRI methods such as spectroscopy, contrast-enhaced imaging and T2 relaxometry in animal models and also in the human epileptic brain.

3.1. Positron emission tomography as biomarker od epileptogenesis, the epileptogenic zone, and status epilepticus

Positron emission tomography (PET) with 2-deoxy-2-[fluorine-18]fluoro-D-glucose (^{18}F-FDG), an analogue of glucose, provides valuable functional information about brain lessions, epileptogenic zone etc. FDG uptake from the blood to the brain is mediated by glucose transporters. PET allows neurotransmiter and metabolic functions.

The utility of FDG-PET in the patients with epilepsy was first describe in 1980 demonstrating focal ictal hypermetabolism and interital hypometabolism that co-localized with the epileptogenic zone (Vliet et al 2017). Recent sudies performed evidence that hypometabolism is present early during epileptogenesis, from 12 to 24 hours after status epilepticus (Guo et al 2009).

PET has been used in several studies to asses potential mechanisms of epileptogenesis. For example, the study of Shultz et al, who described thad combination of hippocampal surface and FDG-PET data predict development of epilepsy (epileptogenesis processes) after fluid percussion injury in rats (Shultz et al. 2013). In humans, PET has shown that the 5-HT$_{1A}$ receptores is reduced in temporal lobe foci (Toczek et al. 2003). PET can be also used in assesment of brain inflammation by using radioligant translocator protein (TSPO). Importantly, TSPO expression is increased in patients with mesial temporal sclerosis and focal cortical dysplasia. Also, TSPO studies in kainate-induced and electrically induced status epilepticus in rodents providet evidence that inflammation is associated with spontaneous reccurent seizures (Bertoglio et al. 2017). Overexpression of TSPO correlates with activation of microglia activation in status epilepticus model (Vliet et al 2017). Interestingly, positive correlation between TSPO expression and the number of seizures has beeh found in tha kainic-acid and post-status epilepticus model (Amhaoul et al 2014).

In conclusion, according to the recent studies, TSPO is non-invasive biomarker of epileptogenesis, brain inflammation, and/or ictogenesis. TSPO may also be biomarker of pharmacoresistance in epilepsy (drug response). For example, studies are focusing on investigation if antiinflammatory treatment oh chronic neuroinflammation in epilepsy model sis reflected by a reversal of TSPO expression. Preclinical studies show that TSPO can be also used as biomarker of pharmacoresitance in epilepsy. However, this preclinical result mast be confirm by clinical studies (Vilet et al 2017; Simonato et al 2021).

3.2 Single photon Emission Computed Tomography as important part of pre-epileptosurgery evaluation

SPECT (Single Photon Emission Computed Tomography) is one of the functional imaging methods that allow to capture the functional state of individual parts brain. It is used in neurological diagnostics various types of radiopharmaceuticals - ligands showing the level of regional perfusion are used in the epileptological and cerebrovascular indications

SPECT is standardly used in clinical practice, especialy in pre-epileptosurgery assesment of the potential candidates of epileptosurgery. Typically, we can see ictal hyperperfusion and interictal hypoperfusion that co-localized with the epileptogenic zone.

Ictal SPECT is the method with high sensitivity (90%) and specificity (80%). In terms of technical design, however, it goes about a very demanding method - requires the application of radiopharmaceuticals as soon as possible after the onset of the seizure and time of the application can fundamentally affect the results of examination. The application itself is performed on video-EEG monitoring unit and analyzed in the subsequent interpretation of the result.

3.3 MRI methods

Remarkable advances in the field of MRI methods increase their use in the diagnostic and treatment of epilepsy. Development of imaging methods in the recent years enabled a significant refinement of the diagnosis and classification of epilepsy and epileptic syndromes. Imaging methods used in epileptology can be divided into two basic groups:

1. examination showing the structure of brain tissue

2. methods of capturing the functional state of individual parts of the brain, so-called functional-imaging methods.

MRI is ideal for biomarker studies, as it is safe, non-invasive and translatable to clinical studies (Simonato et al 2021). MRI and MR spectroscopy provide tool for non-invasive detection of the abnormalities in neuronal networks during epileptogenic process both in experimental models and also in humans (Vliet et al 2017).

In this chapter, structural, functional and spectroscopy methods of MRI in accordance with epileptogenesis process will be discussed.

Structural MRI as a method for identifacation of biomarkers of epileptogenesis

In terms of identification of biomarkers of epileptogenesis, high resolution structural MRI has to be used, especially in vivo techniques (Vliet et al. 2017).

One of the most widespread and complex structural MRI approach is measurement of hippocampal volume and signal intensity (Simonato et al 2021). In an animal model of postraumatic epilepsy, hippocampal T1 ð was found to be a prognostic biomarker of the development of increased seizure susceptibility (Pitkanen et al 2014). On the other hand, in the human, an ongoing study known as FEBSTAT study, described development of acute

hippocampal injury (see as MRI T2 hyperintensity) in children after febrile status epilepticus (Lewis et al 2014). The final results will be compared with the animal models.

Importantly, T2 imaging of the amygdala aand thalamus could become promising biomarkers of epileptogenesis in experimental models (Vliet et al 2017).

Other possible MRI approach to the discovery of potential biomarkers of epileptogenesis is coritcal thickness measurement and diffusion tensor imaging tractography (Simonato et al 2021).

Diffusion tensor imaging (DTI) is used to visualisation of the progression of structural changes in different hippocampal subfields after induced status epilepticus (pilokarpine or kainic acid) in animal model. The method is based on restricted movement of water molecules with a high spatial resolution. Advanced diffussion MRI such as high angular resoultion difusion imaging (HARDI) is method used for detection of local magnetic susceptibility differences (Vliet et al. 2017).

Role of structural MRI in epilepsy patients – clinical work-up

The identification of seizure onset zone (SOZ) in potential candidates of epileptosurgery is critical step of pre-surgery evaluation. The most important first step of this evaluation is to obtain high-quality structural MRI using standard epilepsy protocol as suggested by ILAE (Epilepsy CoNotILA 1997). Despite of neuroimaging advances in recent years, 15-30 % of pharmaroresistant patients with focal epilepsy have no visible lession on MRI (MR negative focal epilepsy).

If no epileptogenic lession can be seen on MRI by expert in epilepsy imaging (neuroradiologist), further MRI methods acquision and postprocessing methods have to be used. These tools include quantification measurement of hippocampal architecture, volume and T2 signal using methods as MRI volumetry, T2 relaxometry, and shape analysis. Other new

acquisiton of postprocessing methods are voxel-based morphometry, voxel-based relaxometry, sulcal morphometry, computational modelling of cortical thickness, blurring, tissue intensity and diffusion imaging. These studies lead to 30-40 % improvement of lesion detection (Vliet et al 2017).

MR spectroscopy as a tool to identify metabolic biomarkers of epileptogenesis

MR spectroscopy (MRS) provides in vivo biochemical and cellular metabolite analyses of tissue. There are five predominant metabolite peaks in proton MRS: choline-containing compounds, which reflect membrane turnover; creatine, which represents energy synthesis and serves as an internal control for determining metabolite ratios given its relative stability; N-acetyl aspar-tate (NAA), which is found mostly in neurons but may also be found in glial cells and serves mostly as a marker of neuronal cells; lactate, which results from anaerobic metabolism and is seen in necrotic tumors and hypoxic or infarcted tissue; and lipid, which peaks when there are increased cellular and myelin breakdown products or nonviable necrotic tissue (Zarifi et al 2016). We can choose to use localised MRS in a preselected voxel of interest and generate a quantitative tissue neurochemical profile. Another possbility is to map larger portion of the brain with reduced number of meadurable neurochemicals (Vliet et al 2017).

The main importance of MRS is examination of metabolic abnormalities within the brain. Importantly, in fact that epileptogenesis-related metabolic abnormalities can be focal and mild, the voxel placement becomes critical to avoid false-negative findings. A recent consensus paper on the role of proton-MRS in clinical management recommended combined used of single voxel MRS and MR spectroscopy imaging (Oz et al 2014). The other recommendatation is that the localisation of the pathology shoud by done using MRI spectroscoping imaging and quantification of the focal neurochemical profile by short echo times (Vliet et al. 2017).

Identifiable molecules by using short echo times MRS can be diveded according to energy metabolism to follows (lactate, creatine, glutamate, glutamine, γ-aminobutyrate, glutsthione, taurine, myo-inositol, N-acetyl aspartate). Filibian et al. described progressive increase of myo-inositol and glutathione and simultaneously reduction of N-acetyl aspartate in the hippocampus of animal model during induced epileptogenesis (after kainic acid induced status epilepticus). These results suggest that those markers are biomarkers of epileptogenesis (Filibian et al. 2012).

MRS can also identified focal abnormalities in patients witg focal epilepsy. According to recent studies reduction of N-acetyl aspartate and elevation of cholin and creatine in ipsilateral hippocampus of patients with hipocampal sclerosis was described (Cross et al. 1996; Achten et al 1998). In case of temporal lobe epilepsy, abnormalities in metabolism of myo-inositol were shown by authors Wellard et al., in terms of elevation of myo-inosiotol in ipsilateral hippocampus. Increased myo-inositol/creatinin indicate gliosis. (Wellard et al. 2003).

The role of functional MRI for prediction od postoperative cognitive deficit after epileptosurgery-prognostic biomarkers

Functional magnetic resonance imaging (fMRI) is a modern imaging method used for functional brain imaging, respectively mapping the brain response to an external or internal stimulus. With the development of computer technology and statistical methods, the fMRI method is developing as a tool for visualizing the anatomical structures of the brain involved in the mechanisms of perception, motor control and thinking. It is different from standard magnetic resonance imaging by the ability to detect dynamic signal changes caused by local fluctuations in the ratio of oxyhemoglobin and deoxyhemoblologin depending on neuronal activity (BOLD, i.e. Blood Oxygenation Level Dependent). This is also the basis for its advantages and limitations in comparison with other methods of functional brain mapping. The

limits of individual methods are determined by the so-called temporal and spatial resolution. fMRI has a relatively high spatial resolution (order of millimeter units), time resolution is limited compared to EEG (electroencephalography) or MEG (magnetoencephalography). fMRI finds application mainly in neurophysiological research.

This method maps neuronal activity only indirectly, following a local change in oxygenation and perfusion of the cerebral cortex.

fMRI have a important role in the presurgical evaluation of epilepsy patients as a predictive biomarker of langualge and memory decline after anterior temporal lobe resection. For exmaple, verbal memory fMRI paradigm asess lateralization index of memory and associated language functions.

Contrast enhanced magnetic resonance imaging for the detection of blood-brain-barrier dysfunction

The blood–brain barrier (BBB) is a highly selective semipermeable border of endothelial cells that prevents solutes in the circulating blood from non-selectively crossing into the extracellular fluid of the central nervous system where neurons reside (Daneman et al. 2015). BBB play an important role in the homeostasis of the brain and protect the brain from potentially harmful substances and patogens. BBB dysfunction have improtant consequences in terms of neuronal excitability. For the detection of BBB dysfunction is used contrast enhaced MRI with gadolinium-DTPA (Gd-DTPA) as an intravenous contrast medium for magnetic resonance imaging. Importantly, when the BBB is disrupted, the intravenously administrated contrast leaks out of the blood vessels and accumulates in brain parenchyma. This implies that

changes in MRI signal can be used to localize BBB dysruption and to quatify the relative degree of BBB permeability (Vliet et al 2017). The dysruption (opening) of BBB leads to induction of epileptogenesis and promote the generation of seizures. Therefore, is BBB permeability cosider as biomarker of epileptogenesis (Vezzani et al 2011). Although, contrast enhancing MRI is using routinely in clinical practice, quantitative imaging of BBB permeability in humans has not be implemented because of complicate dynamic scane protocol (Vliet et al 2017).

Conclusion

Remarkable advances and the development of structural and functional neuroimaging techniques has provided significant insights into etiopathological mechanisms underlying epileptogenesis and help to discover new neuroimaging biomarkers.

In the future, I expect further developments that will improve on the sensitivty and specificity of imaging biomarkers od epileptogenesis and ictogenesis. Targets could include markers of BBB dysruption, neuroinflammation, glial proliferation, metabolic and neuronal dysfunction, synapses and receptors. Simultaneously I expect development of high resolution structural and functional MRI.

Recent advances in neuroimaging of epileptogenesis and ictogenesis significantly improved the diagnosis and treatment of epilepsy and help us to aim epileptological research to discover anti-epileptogenesis treatments and also personalised treatment of patients with epilepsy, based on knowledge of etiopatogenetic mechanisms lead to development of given epilepsy in given patient.

Author's contribution to this issue

We published case report of 18- years old patient with autosomal dominant temporal lobe epilepsy associated with heterozygous reelin mutation. 3 T brain MRI study with advanced neuroimaging methods were performed in the standard protocols to analyzse voxel-based MRI, cortical thickness, and functional connectivity.

Results: Morphometric MRI analysis (blurred grey-whitematter junctions, voxel-based morphometry, and cortical thickness analysis) did not provide any informative results. The functional connectivity analysis revealed higher local synchrony in the patient in the left temporal (middle temporal gyrus), left frontal (supplementary motor area, superior frontal gyrus), and left parietal (gyrus angularis, gyrus supramarginalis) regions and the cingulate (middle cingulate gyrus) as compared to healthy controls. See Fig 1 here.

Conclusions: Evidence of multiple areas of functional connectivity supports the theory of epileptogenic networks in autosomal dominant temporal lobe epilepsy. Further studies are needed to elucidate this theory.

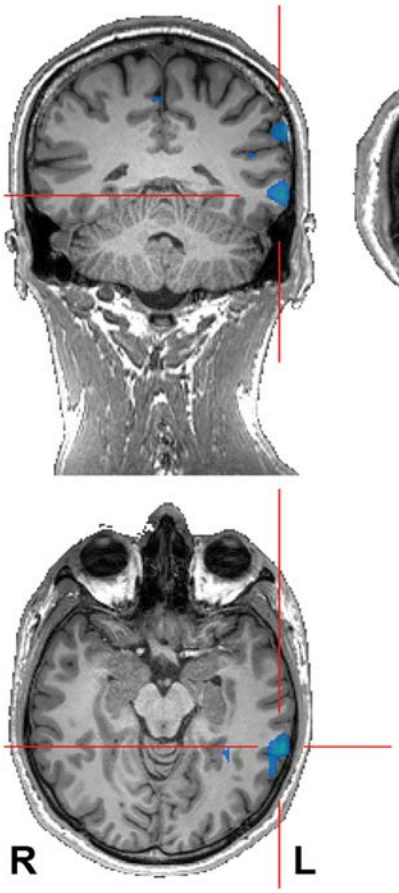

Table 1
The regions that show increased local synchrony in the patient as compared to the HC (N = 26; p < 0.001). The underlined regions are depicted in Fig. 1.

Region	# voxels	Z-value[a]	Coordinate[a] [mm]
L SMA	19	7.02	−6 18 72
L supramarginal g	14	6.12	−66 −30 30
L angular g	40	5.84	−48 −66 42
L middle temporal g	13	5.31	−66 −48 −6
L middle cingulate g	31	5.26	−6 12 36
R superior frontal g	5	4.29	24 36 54

[a] Cluster maximum; L — left; g — gyrus; SMA — supplementary motor area; voxel size = (6 × 6 × 6) mm^3.

Fig. 1. The regions showing increased local synchrony in the patient as compared to HC (N = 26; p < 0.001).

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See Annex 3 here

I participated at the DTI MR tractography research project during my intership at the University of Minnesota (Fulbright research program) in 2019.

In this study, we aim to evaluate and quantify the variability that arises from different protocols for bundle segmentation. Through an open call to users of fiber tractography, including anatomists, clinicians, and algorithm developers, 42 independent teams were given processed sets of human whole-brain streamlines and asked to segment 14 white matter fascicles on six subjects. In total, we received 57 different bundle segmentation protocols, which enabled

detailed volume-based and streamline-based analyses of agreement and disagreement among protocols for each fiber pathway.

Results show that even when given the exact same sets of underlying streamlines, the variability across protocols for bundle segmentation is greater than all other sources of variability in the virtual dissection process, including variability within protocols and variability across subjects. In order to foster the use of tractography bundle dissection in routine clinical settings, and as a fundamental analytical tool, future endeavors must aim to resolve and reduce this heterogeneity. Although external validation is needed to verify the anatomical accuracy of bundle dissections, reducing heterogeneity is a step towards reproducible research and may be achieved through the use of standard nomenclature and definitions of white matter bundles and well-chosen constraints and decisions in the dissection process. The results of this international stude were published in Neuroimage in 2021.

See Annex 4 here

4. Electroencephalography biomarkers

Electroencephalography (EEG) is a technique for recording brain electrical activity from the human (or animal) scalp for research and diagnostic purposes. EEG is golden standard in diagnosis of epilepsy and also in differential diagnostic of epileptic and non-epileptic events. In case of epilepsy it is a key method for detection of epileptiform abnormalities.

EEG has multiple advantages with respect to biomarkers. For the first, extremly high temporal resolution and relatively high spatial resolution (which can be improved by the use of high-density EEG). Secondly, the technique is non-invasive and available in all hospitals. Finally, EEG analysis can be highly quantitative, a key factor for the development of biomarkers. On the other hand, there are some disadvantages in particular the activities measured are mostly cortical (any sobcortical activities can be measures). Second limitation are artefacts, especially in children (Simonato et al 2021).

Preclinical and clinical studies identified some potential EEG biomarkers. Especially, in rodent models after brain injury or insult were detected: shortening of sleep spindles (Andrade et al 2017), loss of stability in theta power (Milikovsky et al 2017) and patological high frequency oscilations (Bragin et al 2016). The results of preclinical studies suggest that EEG has potential to be biomarker of epileptogenesis in humans.

Importantly, standard scalp EEG is limited by measurement of brain activities with frequency up to 100 Hz, however preclinical studies on animal models shows that activity-based biomarkers of epileptogenesis can be detected at frequencies above 100 Hz (Andrade et al 2017; Perruca et al 2019). High frequency oscilations (HFOs) are the category of brain activity between 80 and 500 Hz, subdivided into ripples (80-250 Hz) and fast ripples (250-500 Hz; Bragin et al., 1999). HFOs can be detected invasively using intracranial dept electrods

or with scalp EEG. Scalp HFOs were more specific than spikes in localizing the epileptogenic zone and predicting outcome (Noorlag et al 2022).

Other study has shown that loss of theta pover over time, event without seizure activity is highly predictive biomarker of epileptogenesis (Milikovsky et al 2017).

From a clinical point of view, early detection of epileptiform activity (epileptiform discharges, periodic discharges, rhytmic theta) and/or early seizures in the case of traumatic brain are associated with an increase risk of development of post-traumatic epilepsy (Tubi et al. 2018). Similary, early detection of epileptiform abnormalities in the patients with tuberous sclerosis can predict development of epilepsy (Wu et al 2016).

EEG flattering (decrease EEG signal amplitude accross frequecy bands) after neonatal hypoxia could predict later development of epilepsy (Jain et al 2017).

In conclusion, improvement of EEG method standardly use in clinical practice coud accelerate development of EEG-based biomarkers of epileptogenesis. In addition, use of tripolar EEG electrodes and HFO EEG will improve spatial resolutioin and enable a wider range of frequencies to bo monitored (Besio et al 2014).

Author's contribution to the issue:

I colaborate on a project focusing on prediction of Stimulation Efficacy in Epilepsy (PRESEnCE; Ministry of Health project number NV19-04-00343).

Identifying individuals who will benefit from stimulation as a method for epilepsy treatment prior to the implantation of the VNS/DBS device would improve patient selection, minimize unnecessary surgical procedures, and reduce associated financial expenses dramatically. Recent meta-analyses reveal greater benefits of chronic VNS therapy in pediatric

patients, in patients with generalized and post-traumatic epilepsies, and individuals with tuberous sclerosis relative to other patient groups (Englot el al 2011, 2016), but these studies waste their noticeable value on the level of an individual patient. The information of DBS efficacy prediction in epilepsy are much sparer, despite the fact that DBS is officially approved therapeutical option for drugresistant epilepsy treatment.

It is presumed that VNS increases seizure threshold by activating neuronal networks in the thalamus and other limbic structures (Theodore et al 2004; Alexander et al 2012) but the precise mechanism of VNS action is not yet understood fully. Desynchronization of the electroencephalogram (EEG) has been proposed as a possible mechanism behind the antiepileptic effect of VNS, (Jaseja et al 2010) and recent neurophysiological studies focusing on EEG parameters lend support to this: Fraschini et al. report a significant correlation between VNS-induced global desynchronization in gamma bands and positive clinical outcome in temporal lobe epilepsy patients (Fraschini et al 2013). Similarly, Bodin et al. revealed a lower level of global EEG synchronization in delta and alpha frequency bands during the ON phase of VNS in responders (Bodin et al 2015). Theoretically, differential alterations in brain rhythms from VNS therapy between responders and non-responders might reflect inter-individual variability in the (non-specific) susceptibility of EEG to be synchronized or desynchronized by external stimulation. It follows that differences in this susceptibility might underlie individual VNS efficacy. Recently, several authors have attempted to identify predictors of VNS outcome.

Study design: We determine the response to the VNS/DBS stimulation in each patient. The patients are categorized according to following rules: 1/ Stable responder vs. stable non-responder – characterized by seizure reduction ≥ 50% or < 50% for whole follow-up period 2/ Predominant responder vs. predominant nonresponder – characterized by seizure ≥ 50% or < 50% for more than half of follow-up period (this classification is used in patients who change

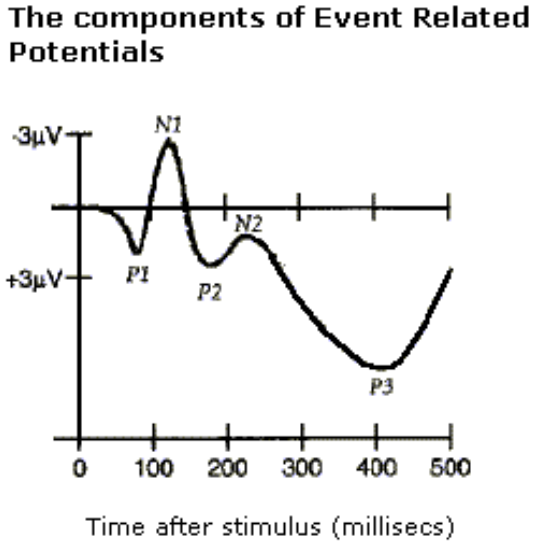
the classification category during follow-up period). Subsequently, the preimplantation EEG in a given patient is identified and processed.

5. Neurophysiological biomarker of network dysfunction

5.1 Methods of neurocognitive network research

5.1.1 Cognitive event related potentials (ERP)

Endogenous event-related potentials (ERPs) are thought to reflect the neurophysiologic correlates of cognitive processes. The P3 component of ERPs, which is a target detection response, has been one most studied. This long-latency waveform (300 milliseconds range) may represented various functions, such as closure of sensory analysis, cognitive closure of the recognition processing, the attentional and decisional processes and the update of working memory (Roesler et al, 1986; Verleger et al, 1994, 2005; Comerchero and Polich, 1999).



The main ERP components were identified by visual inspection and quantified by latency and amplitude measures. P3-like waves were identified in the 250-600 milliseconds latency range.

Author's contribution to the issue:

In our study (Rusnáková et al, 2011) the occurrence of the local generators of P3 like potentials, elicited by a noise-compatibility flanker test was used in order to study the processing of executive functions, particularly in the frontal and temporal cortices.

The test performed with arrows comprised a simpler congruent and a more difficult incongruent task. The two tasks activated the attention and several particular executive functions i.e. working memory, time perception, initiation and motor control of executed task. The incongruent task increased demand on executive functions, and beside the functions common for both tasks an inhibition of automatic responses, the reversal of incorrect response tendency, the internal ordering of the correct response and the initiation of the target-induced correct response was involved. In seven epilepsy surgery candidates (4 males and 3 females), ranging in age from 26 to 38 years, multi-contact depth electrodes were implanted in 590 cortical sites. We focused on local sources of P3-like potentials. Only the “phase reversal” and “steep voltage change” were considered to be generators of the studied potentials, because of their significance as the accepted signs of proximity to generating structure (Vaughan et al., 1986; Halgren et al., 1995a, b).

In the two tasks, the P3 like potential sources were displayed in the mesial temporal structures; the lateral temporal neocortex; the anterior and posterior cingulate; the orbitofrontal cortex and dorsolateral prefrontal cortex. The P3 like potentials occurred more frequently with the incongruent than with congruent stimuli in all these areas. This more frequent occurrence of P3 sources elicited by the incongruent task appeared significant in temporal lateral neocortex and orbitofrontal cortex.

Annex 5 here

5.1.2 Event-related synchronization and desynchronization (ERD/S)

Event- related synchronization and desynchronization (ERD/S) represents a quantitative nonlinear EEG signal analysis method that enables to evaluate the changes of the background activity in any frequency ranges. These changes are related to an external or internal stimulus and are linked to the brain activation. It is widely used in the neuroscience research as a form of functional brain mapping. Especially the intracerebral recording data analysis have a big importance.

In a previous intracerebral depth electrodes study (Bočková et al., 2007) the neurocognitive network in the frontal and lateral temporal cortices was investigated by a visual-motor tasks of writing of single letters. The first task consisted of copying letters appearing on a monitor. In the second task, the patients were requested to write any other letter. The cognitive load of the second task was increased mainly by larger involvement of the executive functions. The task-related Event Related Desynchronization/Synchronization (ERD/ERS) of the alpha, beta and gamma rhythms was studied. The alpha and beta ERD/ERS linked specifically to the increased cognitive load was present in the PFC, the orbitofrontal cortex and surprisingly also the temporal neocortex. Particularly the TLC was activated by the increased cognitive load. It was suggested that the TLC together with frontal areas forms a cognitive network processing executive functions. The test used in Bočková's study consisted from an original and rather complex task, with involvement of several executive and non-executive processes. In consequence, the interpretation was rather complex. In conclusion, in Bočková et al. cognitive intracerebral studies was documented using ERD/S methodology the involvement of the lateral temporal neocortex in the neurocognitive network of executive functions.

With the view of the confirmation of the suggested involvement of the temporal lateral neocortex in the central executive, we decided to perform the present study with a test that has been commonly used for studying executive functions.

Author's contribution to the issue:

We published the article:

"Subthalamic nucleus involvement in executive functions with increased cognitive load: A subthalamic nucleus and anterior cingulate cortex depth recording study";

The essence of signal processing obtained from intracerebral electrodes and externalized DBS electrodes was a quantitative nonlinear method of analysis - event related desynchronization and synchronization (ERD / ERS). ERD in the alpha, beta and ERS frequency bands is considered to correlate the activation of a certain area of the brain. For the correlate of deactivation resp. active inhibition of the relevant area of the brain is considered ERS in the lower frequency bands (delta, theta).

The group consisted of 4 patients with drug-resistant epilepsy with implanted intracerebral electrodes in the anterior cingulate cortex and 3 patients with Parkinson's disease with externalized DBS electrodes. All patients were insufficiently pharmacologically compensated and therefore indicated by the Epileptosurgery and Neuromodulatory Surgery Commission at St. Anne's University Hospital for epileptosurgery or DBS. The inclusion of each patient in the study was preceded by a detailed neuropsychological examination, which ruled out a disorder of executive functions. The protocol was approved by the Ethics Committee of St. Anne's University Hospital. All patients signed an informed consent.

We studied the appearance of broadband oscillatory changes (ranging 2-45 Hz) induced by a cognitive task with two levels of complexity. The event-related de/synchronizations (ERD/S) in the subthalamic nucleus (STN) and in the anterior cingulate cortex (ACC) were evaluated in an executive function test. Four epilepsy surgery candidates with intracerebral electrodes implanted in the ACC and three Parkinson's disease patients with externalized deep brain stimulation electrodes implanted in the STN participated in the study. A Flanker test (FT) with visual stimuli (arrows) was performed. Subjects reacted to four types of stimuli presented on the monitor by pushing the right or left button: congruent arrows to the right or left side (simple task) and incongruent arrows to the right or left side (more difficult complex task). We explored the activation of STN and the activation of the ACC while processing the FT. Both conditions, i.e. congruent and incongruent, induced oscillatory changes in the ACC and also STN with significantly higher activation during incongruent trial. At variance with the ACC, in the STN not only the ERD beta but also the ERD alpha activity was significantly more activated by the incongruent condition. In line with our earlier studies, the STN appears to be involved in activities linked with increased cognitive load. The specificity and complexity of task-related activation of the STN might indicate the involvement of the STN in processes controlling human behaviour, e.g. in the selection and inhibition of competing alternatives.

See Annex 6 here

5.1.3 Functional magnetic resonance (fMRI)

During the last decade occurred brisk development of the method of functional MRI which maps of regional changes of cerebral perfusion and indirectly assesses also the neuronal activation in the examined parts of the brain. It´s contribution to investigations of cognitive functions is not quite unequivocal so far. In the study of Brázdil et al. (2003) auditory "oddball" task examination was performed in 10 healthy volunteers using the method of "event-related" functional MRI (efMRI). The authors compared the assembled results with the results of previous efMRI and intracerebral ERP studies with the objective to evaluate the extent of agreement between areas with haemodynamically significantly different response to rare target stimuli and known intracerebral generator of the P3 potential. Both methods proved the activation of several areas in particular the parietal and frontal lobe (lobulus parietalis superior, inferior, gyrus supramarginalis, gyrus cinguli, of the lateral prefrontal cortex, gyrus temporalis superior and of the thalamus). Consistent with the assumed significant role of the neurocognitive network for directed attention in the course of detection of target stimuli in the majority of these structures a more marked haemodynamic response was observed on the right side. Against expectation in the presented experiment nor in any previous efMRI studies a significant haemodynamic response to target stimuli was not proved at the side of the most marked P3 generator in the amygdalohippocampal complex. Different results were also obtained on examination of further areas, e.g. rostral cingulum. Thus although the contribution of efMRI to recognition of the neuroanatomical correlate of mental processes is extremely high, it is unable to provide alone a complete map of activated cerebral areas in the course of cognitive operations. The reason is most probably the inability to reflect fully transient short-term mentary method and it´s results must be evaluated with maximum caution (Brázdil et al; 2003).

Conclusion

Intracranial and neuroimaging studies demonstrated a widespread distribution of cognitive ERPs in multiple cortical and subcortical regions in the human brain. The participation of the frontal, temporal and parietal cortices, in addition to the cingulate and mesial temporal regions, the basal ganglia and thalamus, has been shown with visual, auditory and somatosensory stimuli (Halgren et al., 1995 a,b, 1998; Clarke et al., 1999, 2003; Smith et al., 1990; Baudena et al., 1995; Lamarche et al., 1995; Brázdil et al., 1999, 2003; Rektor et al., 2001 a,b, 2004, 2007; Bočkova et al 2007; Rusnáková et al. 2011).

Based on other studies (Baláž et al, 2008; Rektor et al, 2009; Bočková et al.), even subthalamic nucleus (STN) is a part of widespread neurocognitive network. Cognitive activities in the STN could be explained by existence of hyperdirect cortico-STN pathway. Certain effect of deep brain stimulation (DBS) on cognitive performance is possibly caused by a direct influence on ´cognitive´ parts of STN (Rektor et al, 2009).

6. Future directions & perspectives in the treatment of epilepsy

6.1 Current potentials of therapeutic influence of neuroinflammation responses and experimental possibilities of immunomodulatory treatment

The immunotherapy is currently routinely used in the treatment of epilepsy with the expected immune-mediated pathogenesis, such as Rasmussen encephalitis, anti-NMDA encephalitis, NORSE/FIRES and others. Standard drugs used to influence the inflammatory response in epileptology include: corticosteroids and adrenocorticotropic hormone (ACTH), which belong to the first-line drugs in the treatment of infantile spasms (West syndrome).

The therapeutic effect has also been described in epileptic encephalopathies such as Lenox-Gastaut syndrome, Landau-Kleffner syndrome, epilepsy with a continuous spike-wave complexes during sleep, which are resistant to therapy with conventional antiepileptic drugs.

In acute cases (such as refractory SE type NORSE/FIRES) we use as the immunotherapy of the first-choice pulses of methylprednisolone, intravenous immunoglobulines or plasmapheresis. In second-line immunotherapy, tacrolimus, rituximab and cyclophosphamide (French et al 2017).

Anti-inflammatory effect of vagal nerve stimulation and ketogenic diet, but also cannabinoids was approved at the experimental level. It has been shown that KD leads to a decrease in the level of pro-inflammatory cytokines by an unclear mechanism. The influence of ketone bodies (eg. ß-hydroxy-butyrate) to mitochondrial target structures is assumed. KD also leads to ketone-induced discontinuation of inflammasomes (French et al 2017). Inflammasomes are cytosolic multi-protein oligomers of the innate immune system responsible for the activation of inflammatory responses.

The influencing of neuroinflammation through vagal nerve stimulationis is explained by regulating the release of cytokines through the so-called “reflex of inflammation”, in which it plays the vagus nerve plays a key role (Horak 2019). VNS leads to a reduction in the release of pro-inflammatory cytokines at the level of immune cells of the innate immune system (French et al 2017; Horak 2019).

In clinical practice, the effect of cannabinoids (tetrahydrocannabinol and cannabidiol) is also being tested. Their anti-inflammatory effect is explained by a decrease in the level of anti-inflammatory cytokines (IL-1α, IL-3, IL-6, IL-12, IL-17, TNFα, IFNß/γ), increased levels of pro-inflammatory cytokines (IL-10), by reducing COX2 activity, reducing microglia activity and reducing oxidative stress (French et al 2017).

A pitfall of anti-inflammatory treatment in the treatment of the epilepsy is the fact that inflammatory component in ictogenesis of individual types of epilepsy is different, also it is different at the different stages of the disease and simultaneously it is different in the course of the disease. For this reason, is so difficult to identify patients in whom is this inflammatory component "active" and therefore should be treated.

This fact points to the need to identify the so-called biomarkers of neuroinflammation (in the blood serum, CSF, and on the neuroimaging methods).

6.2 Experimental possibilities of immunomodulatory therapy

Various pharmacological studies have been conducted in animal models targeting IL-1ß /IL-1R1, HMGB1/TLR4, COX-2/prostaglandines and complement systems that are significantly involved in the onset and recurrence of the epileptic seizures (Vliet et al 1018; Vezzani et al 2015). Anti-inflammatory treatments that led to improved prognosis in experimental models shows table bellow.

Name of the drug	Mechanism of the action	Effect
Celecoxib, parecoxib	Inhibition of the COX-2	antiepileptogenic, DMD*
Aspirin	Inhibition oh the COX-1,2	DMD*
A4-integrin-specific antibody	leukocyte adhesion to the endothelium	Prevention of BBB dysruption
Erythropoetin	Broad spectrum	broad spectrum
Fingolimod	modulator of S1P receptor	anticonvulsive, antiepileptogenic, DMD*
Anakinra (Kineret)	Antagonist of IL-1β receptor (IL-1R1)	anticonvulsive, antiepileptogenic, DMD*
Anakinra+COX-2 inhibition	Antagonist of IL-1R1+inhibition of COX-2	anticonvulsive, antiepileptogenic, DMD*
Anakinra+TLR4 antagonist	IL-1R1+HMGB1 inhibition	anticonvulsive, antiepileptogenic, DMD*
Nrf2 gene treatment	↓oxidative stress, ↓ production of cytokines	neuroprotective
miRNA-146a	IL-1β/TLR4	anticonvulsive, DMD*
EP2 antagonist	EP2 receptor	antiepileptogenic, DMD*
Dexametasone	broad spectrum	broad spectrum
Statins	Inhibition of synthesis of prostaglandins	antiepileptogenic, DMD*

COX-2: cykloxygenase-2; IL-1R1: IL-1β receptor; HMGB1: high mobility group box 1; TLR4: Toll like receptor 4; EP2: receptor for prostaglandin E2, S1P: sfingosin-1-phosphate receptor; HEB: hematoencefalic barrier; DMD* (disease modifying drugs); NrF2: nuclear transkription factor 2 (Aulicka et al 2020; republished with permission)

Therapeutic regulation of the axis IL-1β/IL-1R1

Anakinra (non-glycolized human IL-1 receptor antagonist [IL-1ra]) has anti-inflammatory and immunomodulatory abilities and is currently used in biological treatment of rheumatoid arthritis. The main problem its use is the risk of hepatotoxicity. At the experimental level, the application leads to delayed onset, shorter duration and reduction of epileptiform activity in pilocarpine-induced SE (Vliet et al 2018).

In clinical practice, it has been successfully used in the treatment of NORSE/FIRES (new onset refraktory status epilepticus; febrile infection related epilepsy). In the literature was found only few case reports, so experiences are very limited (Česká at al 2018).

The most effective treatment for acute phase of status epilepticus (SE) at the experimental level remains the antagonist P2X7 receptor, which significantly reduced duration of SE, even within 1 hour after administration (Henshal et al 2013; Henshal et al 2015). The purinegic P2X7 receptor is membrane ion channel activated by extracellular ATP. Activation of this receptor leads to the activation of microglia and the release of IL-1ß, thereby leads to the development of inflammation and increases excitability of the brain. The P2X7 receptor can also directly modulate neurotransmission and promote incorporation of immune cells into the brain tissue. Recent studies identified that P2X7 receptor antagonist at animal model reduces seizure aktivity and lead to reducing of the neuronal damage (Henshal et al 2015). The combination of P2X7 receptor antagonist with benzodiazepines led to suppression of SE in the model of refractory SE (Henshall et al 2015). Importantly, P2X7 receptor is considered as a new potential target for the treatment of SE, which leads to the termination of SE by the reduction of inflammation pathways in the brain.

Therapeutic regulation of the axis HMGB1/ TLR4

The effect of HMGB1 is mediated through binding to the TLR4 receptor. HMGB1, together with IL-1β, decreases seizure threshold, leading to hyperexcitability of the brain. HMGB1 inactivation using an antagonist its TLR4 receptor significantly reduces seizure activity. At the experimental level, the application of anti-HMGB1 monoclonal antibodies is tested with reduction of the seizure activities (Viet et al 2018).

Therapeutic regulation of the axis COX-2/ prostaglandines

The essence of the action of non-steroidal anti-inflammatory drugs is the inhibition of inflammatory mediator production- prostaglandins. Binding of PGE2 to the receptor EP2 (on the surface of astrocytes) leads to increased glutamate release (hyperexcitation in neural networks) and to induce apoptosis. The EP2 receptor antagonist induces a reduction of the seizure activity and neuronal damage (Rana et al 2018). COX-2 is released at SE, we're talking about the COX-2 pathway of induction of inflammation during SE (Jiang et al 2018). The effect of COX-2 inhibitor therapy depends on the type of used inhibitor and the timing of the treatment (Rojas et al 2014).

Celocoxib was tested at the experimental level (selective COX-2 inhibitor). Application 1 day before induction and 28 days after SE induction led to a reduction in frequency and shortening of length duration of spontaneous seizures. Parecoxib applied after the end of SE led to the prevention of generalization seizures and prevented water levels from rising PGE2 (Vliet et al 2018; Aronica et al 2017).

In the patients with Sturge-Weber syndrome, the clinical effect of prophylactic administration of acetylsalicylic acid acid in terms of reduction of seizure activity has been demonstrated (91% of patients had a significant reduction in the frequency and duration of epileptic paroxysms; Vliet et al 2018). On the other hand, prophylactic administration of ibuprofen in pediatric patients with febrile seizures had no effect on its occurrence. Importanty, no clinical studies are available to monitor effect of selective inhibitor therapy COX-2 in patients with epilepsy (Vliet et al 2018).

The use of selective COX-2 inhibitors is considered as a new potential therapeutic option with antiepileptogenic and potentially DMD effect. This treatment (including EP2 receptor antagonist) has a significant neuroprotective effect and reduces mortality during SE in experimental model (Vezzani et al 2015). Studies in animal models point out the need for early intervention (application of anti - inflammatory drugs within 3-4 hours of the start of the SE) to allow a chance to achieve therapeutic success. These findings draw attention to the possibility of application in clinical medicine in the sense of blocking the activation of inflammatory cascades during SE, thus improving prognosis patients in de novo SE, but also in SE in chronic epilepsy (Vezzani et al 2015).

Retrospective studies further show that statins given to patients before development of SE have led to improvement of prognosis and reduction of the mortality (Rojas et al 2014). The effect of the statins is not based exclusively on their hypolipidemic effect, but also cholesterol-independent action, on the so-called pleiotropic effects. Between the most well-known pleiotropic effects include antiplatelet and anti-inflammatory action based to inhibit the synthesis of prostaglandins and thromboxanes. Experimental level studies demonstrated anti-inflammatory and anti-epileptogenic effect of statins (Sierra-Marcos A et al 2015).

6.3 Other immunomodulation options

Adalimumab (monoclonal antibody anti-TNFα): in clinical medicine trial used in the treatment of Rasmussen encephalitis. On the experimental models its use in the treatment of epilepsy with significant decrease of frequency and duration of the seizures (Vliet et al 2018).

Fingolimod - an immunomodulator with potential immunosuppressive properties used in the treatment of remitting multiple sclerosis. Mechanism effects lies in the modulation of sfingosin-1-phosphate (S1P) receptor on the surface of lymphocytes, astrocytes, oligodendrocytes and neurons in the sense of internalization of receptors, particulary acts as

functional S1P receptor antagonist. On the experimental model of temporal lobe epilepsy was proven its anticonvulsant, antiepileptogenic and the neuroprotective effect (Pitsch et al 2019).

Micro-RNA 146a acts as negative IL1R / TLR4 cascade regulator. On molecular level has been shown to have antiepileptogenic and the DMD effect (Vliet et al 2018).

Nuclear transcription factor 2 (Nrf2) gene therapy - Nrf2 is a gene regulating production a variety of antioxidant and anti-inflammatory proteins that protect the cell against oxidation damage. Studies on animal models demonstrated that induction of the Nrf2 pathway has neuroprotective effect in epilepsy and others neurodegenerative diseases (Vliet et al 2018; Carmona-Aparicio et al 2015).

Experimental and clinical data show that inflammatory changes in the brain during SE play important role in epileptogenesis by involved in increasing neuronal hyperexcitability, negatively affect neuronal plasticity networks and thus contribute to development and recurrence seizures. Increased synthesis of specific pro-inflammatory mediators and up-regulation of their receptors is involved in the development of epilepsy (Vezzani et al 2011). Examination of inflammation mediators and assessment balance of pro-/anti-inflammatory mechanisms are considered as potential biomarkers of epileptogenicity.

Conclusions

Treatment of epilepsy in the future should be focused not only on the suppression of seizures, but on the targeted treatment of the disease as such. One compelling challenge in the therapy of epilepsy is to develop anti-epileptogenic drugs with an impact on the disease progression.

The verification of biomarkers of epileptogenesis and pharmacoresistance in epilepsy is crucial for development of disease modifying drugs (DMD) and discovery of individual "personalized" treatment in epilepsy. Identification of these biomarkers has proceed comprehensively on the level of molecular, neuroimaging, electroencephalograpic, neurophysiological and others biomarkers. I believe that this is the right way to development of targeted personalized treatment of epilepsy that improve the prognosis of the patients with epilepsy. Also, I am in hope, that we are close to discovering new perspectives for epilepsy treatment. The aim of this work is to achieve this goal.

Abbreviations:

NSE: neuronal specific enolase

UCH-L1: ubiquitin C-terminal hydrolase I

GFAP: glial fibrilary acidic protein

S100β: calcium binding protein

MMP-9: matrix metalloproteinase 9

HMGB1: human mobility group box 1

ASM: antiseizure medication

ASD: antiseizure drugs

AED: antiepileptic drugs

CSF: cerebrospinal fluid

ILAE: International League against Epilepsy

COX-1: cyclooxygenase 1

COX-2: cyclooxygenase 2

IL-1β: interleukin-1β

TBI: traumatic brain injury

FR: pharmacoresistance

DMD: disease modifying drugs

FDA-NIH: Food and drug administration and National Institute of Health

BEST: Biomarkers, Endpoints, and other Tools

miRNA: micro RNA

MCD: malformation of cortical development

FCD: focal cortical dysplasia

MTLE/HS: mesiotemporal epilepsy with hippocampal sclerosis

MRI: magnetic resonance imaging

PET: positron emission tomography

SPECT: single-photon emission computer tomography

FDG-PET: 2-deoxy-2-[fluorine-18]fluoro-D-glucose (^{18}F-FDG) positron emission tomography

TSPO: translocator protein

DTI: diffusion tensor imaging

HARDI: high angular resoultion difusion imaging

SOZ: seizure onset zone

MRS: MR spectroscopy

fMRI: functional magnetic resonance imaging

BOLD: Blood Oxygenation Level Dependent

EEG: electroencephalography

COX-2: cykloxygenase-2

IL-1R1: IL-1β receptor

HMGB1: high mobility group box 1

TLR4: Toll like receptor 4

EP2: receptor for prostaglandin E2

S1P: sfingosin-1-phosphate receptor

HEB: hematoencefalic barrier; DMD* (disease modifying drugs)

NrF2: nuclear transkription factor 2

NORSE: new onset refraktory status epilepticus

FIRES: febrile infection related status epilepticus

SE: status epilepticus

ERP: event related potentials

ERD: event related desynchronisation

ERS: event related synchronisation

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List of Annexes

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Aulická Š., Česká K., Šána J., Loja T., Jabandžiev P., Papež J., Danhofer P., Vinohradská H., Doležalová I., Brázdil M., Štourač P., Ošlejšková H., Slabý O. The role of inflammation etiopatogenesis of pharmacoresistant epilepsy and refractory status epilepticus. Cesk Slov Neurol N 2020; 83/116(1): 8-13**.** Doi: 10.14735/amcsnn20208.

Annex 2

Bohošová J, Vajčner J, Jabandžiev P, Ošlejšková H, Slabý O, **Aulická Š.** MicroRNAs in the development of resistance to anti-seizure drugs and their potential as biomarkers in pharmacoresistant epilepsy. **Epilepsia** 2021; 62 (11): 2573-2588.

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Česká K*, **Aulická Š***, Horák O, Danhofer P, Říha P, Mareček R, Šenkyřík J, Rektor I, Brázdil M, Ošlejšková H. Autosomal dominant temporal lobe epilepsy associated with heterozygous reelin mutation: 3 T brain MRI study with advanced neuroimaging methods. Epilepsy & Behavior Case Reports. doi: 10.1016/j.ebcr.2018.10.003. (*contributed firt author)

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Annex 1

Aulická Š., Česká K., Šána J., Loja T., Jabandžiev P., Papež J., Danhofer P., Vinohradská H., Doležalová I., Brázdil M., Štourač P., Ošlejšková H., Slabý O. The role of inflammation etiopatogenesis of pharmacoresistant epilepsy and refractory status epilepticus. Cesk Slov Neurol N 2020; 83/116(1): 8-13**.** Doi: 10.14735/amcsnn20208.

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Role zánětu v etiopatogenezi farmakorezistentní epilepsie a refrakterního status epilepticus

The role of inflammation in etiopathogenesis of pharmacoresistant epilepsy and refractory status epilepticus

Souhrn

Zánět mozku představuje jeden z hlavních substrátů farmakorezistentní epilepsie různé etiologie a může přímo ovlivnit neuronální excitabilitu. Neuromodulační schopnosti některých prozánětlivých molekul (cytokinů, chemokinů) mohou být odpovědné za hyperexcitabilitu v neuronálních sítích. Vztah zánětu a epilepsie je reciproční. Zánětlivé procesy v mozku se mohou účastnit na spouštění záchvatové aktivity a zároveň mohou být následkem pokračujících záchvatů. Farmakologické studie na zvířecích modelech cílené na systémy IL-1β/IL-1R1, HMGB1/TLR4 a COX-2/prostaglandiny prokazují, že tyto zánětlivé kaskády mají významný podíl na spouštění a opakování záchvatové aktivity. Status epilepticus (SE) vede k rozvoji zánětlivých procesů, které mohou být detekovány v mozkové tkáni, mozkomíšním moku i séru. Prolongované záchvaty a SE vedou k rychlé a dlouhotrvající aktivaci specifických zánětlivých kaskád v těch oblastech mozku, které odpovídají epileptogenní zóně. Pochopení komplexní role zánětu při vzniku a exacerbaci epilepsie a rozvoji farmakorezistence je zásadním předpokladem možnosti identifikace nových molekulárních cílů, které by se mohly uplatnit v léčbě těchto pacientů.

Abstract

Brain inflammation represents a common substrate of pharmacoresistant epilepsy of different etiologies and it can directly affect neuronal excitability. Neuromodulatory properties of some proinflammatory molecules (cytokines, chemokines) may be responsible for hyperexcitability in neuronal networks. The relation between inflammation and epilepsy is reciprocal. The inflammatory processes in the brain may participate in initiating seizure activity and simultaneously they may be a consequence of the recurrence of the seizures. Pharmacological studies on experimental models focused on IL-1β/IL-1R1, HMGB1/TLR4 and COX-2/prostaglandin systems demostrate that these inflammatory pathways significantly in triggering and recurring seizure activity. Status epilepticus (SE) leads to development of inflammatory processes which can be detected in brain tissue, cerebrospinal fluid and blood serum. Prolonged seizures and SE lead to fast and prolonged activation of specific inflammatory pathways in brain areas accordant with the epileptogenic zone. Understanding the complex role of inflammation in the generation and exacerbation of epilepsy and development of pharmacoresistance in epilepsy is crucial for the identification of new molecular targets for therapeutic intervention in these patients.

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Klíčová slova

farmakorezistentní epilepsie – refrakterní status epilepticus – cytokiny – chemokiny – polymorfizmy

Key words

pharmacoresistant epilepsy – refractory status epilepticus – cytokines – chemokines – polymorphisms

Úvod

Zánět je stereotypní odpověď lidského organizmu na infekci nebo poškození tkání a reprezentuje klíčový homeostatický endogenní mechanizmus, který vede k uzdravení a reparaci. Pokud není tato zánětlivá odpověď dostatečně regulována endogenními protizánětlivými mechanizmy, může mít škodlivé účinky. Nedostatečná endogenní protizánětlivá odpověď byla prokázána také v mozku pacientů v průběhu rekurentních záchvatů a status epilepticus (SE) [1]. Zásadní otázkou v patofyziologických následcích zánětu indukovaného SE jsou možnosti promptní intervence, které jsou studovány na zvířecích modelech. Zjišťuje se, jakým způsobem daný zásah do zánětlivých kaskád ovlivní dlouhodobé následky SE (ztrátu neuronů, behaviorální změny, rozvoj epilepsie, mortalitu atd.). Hlavním smyslem těchto studií je skutečnost, že zánětlivé kaskády a molekuly jsou potenciálním cílem nového terapeutického postupu v léčbě SE.

Zánětlivé procesy v mozku se mohou účastnit na spouštění SE a na druhé straně mohou být následkem pokračujících záchvatů. Vztah zánětu a epilepsie je tedy reciproční. Na zvířecích modelech bylo prokázáno, že zánět, který se objeví v mozku v průběhu SE, hraje rozhodující úlohu v pokračování záchvatů a jejich dlouhodobých škodlivých následcích nezávislých na infekční či autoimunitní příčině. Zánět je následkem, ale i usnadněním pokračování záchvatu [1].

I přes pokroky v diagnostice a terapii epilepsie zůstává přibližně 30 % pacientů farmakorezistentních. Farmakorezistentní epilepsie je definována jako epilepsie, u níž se nepodaří dlouhodobě plně kompenzovat pacienta za použití dvou vhodně zvolených antiepileptik v adekvátních terapeutických dávkách – v monoterapii nebo kombinované terapii. Jenom části těchto pacientů lze nabídnout kurativní epileptochirurgické řešení či jiné možnosti nefarmakologické léčby (stimulace nervus vagus [SNV], ketogenní dieta [KD] atd.). Soudobá terapie se omezuje na potlačování symptomů onemocnění, tedy epileptických záchvatů, a neumožňuje odstranění příčiny (s výjimkou epileptochirurgie) nebo prevenci jeho vzniku u pacientů, kteří jsou rizikoví z důvodu genetické predispozice či prodělaného neurologického inzultu. Nové terapeutické postupy by v budoucnosti měly nabídnout širokou škálu možností respektujících specifika jednotlivých forem epilepsií i individuální rozdíly mezi pacienty s ohledem na vývoj a prognózu onemocnění [2].

Z těchto důvodů je cílem výzkumu v epileptologii snaha vyvinout léky, které by nejenom potlačovaly záchvaty, ale v ideálním případě fungovaly jako medikace tlumící proces epileptogeneze (proces vedoucí ke vzniku epilepsie), léky modifikující průběh onemocnění (disease-modifying drugs; DMD) a léky zabraňující postupujícímu procesu neurodegenerace či rozvoji farmakorezistence.

Aktuálně probíhají studie zkoumající tyto procesy na různých molekulárních úrovních (DNA, RNA, mikro-RNA, zánětlivých molekul a jejich kaskád, proteomiky atd.). Cílem této práce je poskytnout přehled problematiky a nastínit její význam pro budoucí rozvoj léčebných možností u epilepsie.

Tab. 1. Souhrn mechanizmů ovlivnění neuronální excitability mediátory zánětu (cytokiny, chemokiny, porucha HEB) [4–14].

IL-1β	↑ uvolňování a ↓vychytávání glutamátů v astrocytech, ↓ GABAerní neurotransmise → neuronální hyperexcitabilita;
	uvolnění dalších prozánětlivých cytokinů: IL-6, IL-8 cestou indukce genové transkripce
TNFα	↑ uvolňování glutamátů v mikroglii; upregulace AMPA receptorů; organizace excitačních/inhibičních synapsí
IL-6	↑ expozice IL-6 v prenatálním období (možný transplacentární přenos) vede k neurodegeneraci hipokampu (narušuje strukturu i morfologii HIPP bilat.) → hyperexcitabilita HIPP a progrese epileptogeneze (spolupodíll ↑ IL-6 a ↑ IL-1β)
prostaglandiny (PGE2)	vazba PGE2 na receptor EP2 (astrocyty) zvyšuje uvolňování glutamátu z astrocytů (hyperexcitace) a vede k indukci apoptózy
TLR 1,2,3; transmembránové glykoproteiny	stimulace TLR zánětlivých kaskád vede k indukci sekrece cytokinů (IL-1β, IL-6, TNF-α) → rozvoj neuro-zánětu a ovlivnění neuronální excitability;
	aktivace TLR3 vede k hyperexcitabilitě v oblasti HIPP
HMGB1 (jádrový protein)	prokonvulzivní efekt HMGB1 je zprostředkován jeho vazbou na NMDA receptor a interakcií s TLR4
	aktivace zánětlivé kaskády HMGB1-TLR4 může vést ke generalizaci záchvatů
chemokiny	ovlivnění neuronální excitability přes modulaci napěťově řízených iontových kanálů
porucha HEB	porucha HEB vede k indukci epileptogenze a podporuje generalizaci záchvatů

HEB – hematoencefalická bariéra; HMGB1 – high mobility group box 1; TLR – Toll-like receptory

Etiopatogenetické mechanizmy

Studie na experimentální i klinické úrovní prokazují, že v procesu epileptogeneze, spouštění/opakování záchvatové aktivity a rozvoji farmakorezistence epilepsie sehrávají významnou roli zánětlivé kaskády: IL-1β/IL-1R1, HMGB1/TLR4, COX-2/prostaglandiny, chemokiny a porucha hematoencefalické bariéry (HEB) (tab. 1).

Zánětlivá kaskáda systému IL-1β/IL-1RA

IL-1β je hlavní cytokin, který se podílí na zprostředkování zánětlivé odpovědi (uvolněním dalších prozánětlivých cytokinů) a má prokonvulzivní efekt. Jeho přirozený antagonista IL-1RA (antagonista receptoru IL-1) má naopak antikonvulzivní efekt a podílí se na protizánětlivé autoregulaci [3].

IL-1β vede cestou indukce genové transkripce k uvolnění dalších prozánětlivých cytokinů (IL-6, IL-8), které dále cestou intracelulární transdukce vedou k ovlivnění genové transkripce. Takto se spouští kaskáda zánětlivých procesů v CNS, které vedou ke zvýšené excitabilitě a rozvoji záchvatové aktivity. Za normálních okolností je IL-1RA uvolňován v mnohem vyšších koncentracích v poměru k IL-1β, a proto nedochází k masivnímu uvolnění prozánětlivých cytokinů, které pronikají přes HEB a vedou k hyperexcitabilitě mozku.

V případě abnormalit systému IL-1 (často na genetickém podkladě, tzv. polymorfizmy interleukinového systému) dochází k nízkému poměru IL-1RA/IL-1, a to mechanizmem masivního uvolnění IL-1β, inadekvátním uvolňováním IL-1RA a/anebo nedostatečnou vazbou IL-1RA k receptoru pro IL-1β [3].

K hyperexcitabilitě neuronů na podkladě nadměrné aktivace IL-1β dochází několika mechanizmy [3]:

- aktivace NR2B podjednotky NMDA (N-methyl-D-aspartát) receptoru → výrazný influx Ca^{2+};
- zvýšený influx Ca^{2+} do buňky přes aktivaci napěťově řízených Ca^{2+} kanálů;
- inhibice efluxu K^+ přes Ca^{2+}-K^+ kanály;
- inhibice influxu Cl^- přes kanály GABA.

Zánětlivá kaskáda systému HMGB1/TLR4

Marosso et al identifikovali v roce 2010 prozánětlivou kaskádu HMGB1/TLR4 (Toll-like receptor 4), která se podílí na generování a opakování záchvatové aktivity [4].

HMGB1 je jádrový protein uvolňovaný především buňkami glie, který je v průběhu záchvatu (nebo jiného neuronálního poškození) rychle uvolňován do cytoplazmy a následně do intersticiálního prostoru, čímž se spolupodílí na generování kaskády zánětu. Prokonvulzivní efekt HMGB1 je zprostředkován jeho vazbou na NMDA receptor a interakcí s TLR4 receptorem [4].

Toll-like receptory jsou transmembránové glykoproteiny. Záchvatová aktivita vede přes stimulaci TLR k aktivaci zánětlivé kaskády HMGB1/TLR4 s následnou indukcí sekrece cytokinů (IL-1β, IL-6, TNF-α), rozvoji neurozánětu, a tím ovlivnění neuronální excitability. Tímto mechanizmem vede aktivace TLR-4 ke snížení záchvatového prahu a tím k chronické hyperexcitabilitě v hipokampálních neuronálních sítích. Na experimentální úrovni byl prokázán antikonvulzivní efekt blokace TLR-4 receptoru. Blokátory TLR-4 receptoru a inhibice prozánětlivé kaskády HMGB-1/TLR-4 jsou tedy slibným terapeutickým cílem v léčbě epilepsie [4].

Zánětlivá kaskáda COX-2/prostaglandiny

Klíčovým enzymem syntézy prostaglandinů je enzym COX, který katalyzuje přeměnu kyseliny arachidonové na prostaglandiny. Podstatou působení nesteroidních antiflogistik je právě inhibice produkce mediátorů zánětu – prostaglandinů. Vazba PGE2 na receptor EP2 (na povrchu astrocytů) vede ke zvýšenému uvolňování glutamátu (hyperexcitace v neuronálních sítích) a k indukci apoptózy [5].

Chemokiny

Chemokiny jsou malé molekuly produkované krevnímu buňkami, které fungují jako chemický atraktant při migraci leukocytů. Nedávné studie prokazují, že chemokiny a jejich receptory jsou produkovány také mozkovými buňkami a účastní se na vzniku některých neurologických onemocnění, vč. epilepsie. Pozornost se soustředí na roli prozánětlivých chemokinů (CCL2, CCR2, CCR5, CX3CL1) a jejich receptorů v kontrole záchvatů [6]. Chemokiny fungují jako „neuromodulátory", ovlivňují neuronální excitabilitu přes modulaci napěťově řízených iontových kanálů, regulují průnik leukocytů přes HEB a uvolňování neurotransmiterů [6,7]. Podílejí se na neuronální migraci, proliferaci a modulaci synaptické aktivity v průběhu vývoje mozku. Probíhající studie zjišťují, zda chemokiny a jejich receptory sehrávají klíčovou úlohu v epileptogenezi a zda jsou možným terapeutickým cílem v léčbě epilepsie [6].

Porucha hematoencefalické bariéry

Klíčovou úlohu v zachování integrity HEB sehrávají 2 systémy: IL-1β/IL-1R1 a COX-2 (cyklooxygenáza 2)/EP2. Porucha HEB vede k indukci epileptogeneze a podporuje generalizaci záchvatů. Vyšetření poruchy HEB se považuje za pomocný biomarker epileptogeneze a prozánětlivé odpovědi mozku [8].

Klinická data

Role zánětu v etiopatogenezi status epilepticus (shrnutí experimentálních a klinických poznatků)

Epileptogenní efekt mediátorů zánětu (neuronální poškození, HEB) je zprostředkovaný aktivací receptorů pro vazbu IL-1β (IL-1R1), TLR4, receptoru pro pokročilou glykaci konečného produktu (RAGE pro HMGB1) a receptoru pro PGE2 (EP2). Toto jsou hlavní signalizační cesty zánětlivé kaskády v mozku po SE [9].

Chemicky nebo elektricky indukovaný SE vede k intenzivní zánětlivé kaskádě. Bylo prokázáno, že 30–60 min od začátku SE dochází k elevaci IL-1β, IL-6, TNF-α v hipokampu a předním mozku a současně narůstá hladina neuronálního COX-2 proteinu. Tyto změny doprovázejí i morfologické změny (aktivace mikroglie, reaktivace gliózy a infiltrace monocyty) [5,9].

Vliv zánětu na vyvíjející se mozek v průběhu SE byl studován na experimentálním modelu. Mediátory zánětu hrají roli v normálním vývoji mozku, regulaci neurogeneze a synaptogeneze. Přehnaná zánětlivá odpověď může ohrozit normální vývoj mozku [10].

Zánětlivé procesy mohou vést také k rozvoji febrilního SE (FSE) [11,12]. Autoři Gallentine et al [13] v rámci studie FEBSTAT 2017 zjistili, že nižší poměry IL-1RA/IL-1β, IL-1RA/IL-6 a IL-1RA/IL8 byly asociovány s rozvojem FSE. Dále prokázali signifikantně vyšší hladiny IL-6 a IL-8 a signifikantně nižší poměry IL-1RA/IL-6, IL-1RA/IL8 u dětí s T2 hyperintenzními změnami hipokampu po proběhlém FSE. Nízký poměr IL-1RA/IL-6 měl silně prediktivní hodnotu (95 % dětí) s T2 hyperintenzními změnami hipokampu po FSE, což může představovat potenciální biomarker hipokampálního poškození po proběhlém FSE [13]. Ztráta neuronů v oblasti hipokampu podporuje teorii neurodegenerativního efektu SE na vyvíjející se mozek [11]. U pacientů s FSE byla dále zjištěna elevace hladiny HMGB1 v likvoru a jeho přesun z jádra do cytoplazmy [14].

Dále byly prováděny studie hladin cytokinů v likvoru u refrakterního SE typu FIRES (epileptický syndrom vázaný na febrilní infekt), kde byla nalezena signifikantní elevace hladiny IL-6, IL-8, CXCL10 a HMGB1 v porovnání s pacienty s jiným zánětlivým onemocněním [14,15]. Několik hodin po SE dochází také k extravazaci albuminu následkem poruchy HEB v hipokampech a kortexu [11,13].

Role zánětu v etiopatogenezi farmakorezistentní epilepsie a jeho význam v epileptogenezi (shrnutí experimentálních a klinických poznatků)

Zánětlivé změny vedou často k rozvoji chronické epilepsie, a to mechanizmem snížení záchvatového prahu [1,16].

Role zánětu v generování záchvatů se předpokládá již dlouho, a to na základě klinických zkušeností, ve smyslu promptního efektu imunoterapie (kortikosteroidy, imunoglobuliny, adrenokortikotropní hormon a jiné) u některých epilepsií a epileptických syndromů refrakterních, ke konvenční antiepileptické terapii (jako je např. Westův syndrom, epilepsie s kontinuálními výboji hrot-vlna, epilepsie u Rasmusenovy encefalitidy, NORSE/FIRES [nově vzniklý refrakterní SE/epileptický syndrom vázaný na febrilní infekt] a jiné).

Tuto teorii podporují patologické a imunohistochemické analýzy resekátu pacientů po epileptochirurgii. V resekátech pacientů

po anteromediální temporální resekci byla prokázána masivní zánětlivá odpověď ve smyslu aktivace mikroglie a astroglie (intenzivní glióza), up-regulace neuronů produkujících IL-1β, HMGB1 a COX-2, aktivace komplementového systému a dalších zánětlivých mediátorů (prozánětlivé chemokiny CCL2, CCL3, CCL4). Dále byla nalezena extravazace albuminu následkem porušené HEB [17]. V astrocytech, monocytech a mikroglii z hipokampů u pacientů s TLE byl nalezen pohyb HMGB1 z jádra do cytoplazmy [4]. Disulfidická izoforma HMGB1 je považována na základě studií na experimentálních modelech za potenciální biomarker epileptogeneze, resp. predikce rekurence epileptické aktivity) [18].

Obdobné nálezy byly zaznamenány i u pacientů s epilepsií na podkladě fokální kortikální dysplazie (FCD) či tuberózní sklerózy (TS) [19]. Imunohistochemické analýzy resekátů pacientů s FCD prokazují aktivaci vrozené i získané imunity a odpovídajících zánětlivých kaskád [18]. V kohortě pacientů s FCD II. typu stupeň aktivace mikroglie signifikantně koreluje s trváním epilepsie a frekvencí záchvatů před epileptiptochirurgickým výkonem. Při porovnání FCD I. a II. typu byly nalezeny signifikantně vyšší hladiny IL-1β, chemokinů, HMGB-1 a aktivace mikroglie u FCD II. typu, což zdůrazňuje význam neurozánětu v etiopatogenezi FCD typu II. Aktivace obdobných zánětlivých kaskád jako u FCD II byla prokázána u epileptogenních kortikálních tuberů v rámci TS [19].

U pacientů s farmakorezistentní epilepsií byly v likvoru nalezeny vysoké koncentrace IL-1β, IL-6, IL-8, TNFα, IL-18, chemokinů (CCL2, CXCL10) a CCR2 (receptor pro CCL2) [1,7,8,18].

Na zvířecích modelech byla prokázána asociace mezi elevaci prozánětlivých cytokinů (IL-1β, IL-6), epileptogenezí a vznikem poškození hipokampu.

Buněčná složka imunitního systému a neurozánět u epilepsie

Pokud porovnáváme etiopatogenezi neurozánětu u epilepsie např. s RS, můžeme detekovat hlavní rozdíl v zapojení imunitních mechanizmů. V případě RS dochází k aktivaci buněk získané imunity (aktivace především B a T lymfocytů). V případě epilepsie se v zánětlivých procesech uplatňují především buňky vrozené imunity (aktivace mikroglie, astrocytů, ale také monocytů a makrofágů). Hlavní roli v biosyntéze a uvolňování zánětlivých molekul mají aktivované buňky glie. Na zvířecích modelech byla prokázána pozitivní korelace mezi aktivací mikroglie a astrocytů a počtem spontánních epileptických záchvatů [18]. K uvolňování zánětlivých molekul přispívají také endotelové buňky HEB. Důležitým poznatkem je, že zánětlivé molekuly (cytokiny, chemokiny) fungují nejenom jako efektor imunitního systému, ale také jako neuromodulátory (vazbou na jejich specifické receptory na povrchu neuronů přímo ovlivňují jejích excitabilitu). Mechanizmem snížení záchvatového prahu vedou k navození hyperexcitability mozku. Získaná imunita sehrává úlohu především u autoimunitně podmíněných epilepsií [18,20]. Popis autoimunitně podmíněných epilepsií a uplatnění mechanizmů získané imunity v etiopatogenezi epilepsie překračuje rámec předkládaného textu.

Neuroradiologické možnosti detekce neurozánětu – neuroradiologické biomarkery zánětlivých procesů

K objektivizaci zánětlivých změn v průběhu epileptogeneze lze použít MR a PET/SPECT. Aktuálně se preklinické a klinické studie koncentrují na zobrazení mikrovaskulární patologie v souvislosti s poruchou HEB, detekci časných změn v temporálních lalocích po FSE na MR/T2 zobrazení (a jejich korelace s intracelulární translokací HMGB1), neurozobrazování glie pomocí PET a astrocytů metodou PET/SPECT (biomarkery epileptogeneze) a jiné. Probíhá testování validity zobrazovacích biomarkerů v procesu epileptogeneze a vzniku farmakorezistence na preklinické a klinické úrovni [21]. Popis principu jednotlivých metod přesahuje rámec předkládaného textu.

Současné možnosti terapeutického ovlivnění zánětlivé odpovědi a experimentální možnosti imunomodulační terapie

Imunoterapie je v současnosti rutinně používána v terapii epilepsií s předpokládanou imunitně zprostředkovanou patogenezí, jako jsou např. Rasmussenova encefalitida, anti-NMDA encefalitida, NORSE/FIRES a jiné. Mezi standardně používané léky k ovlivnění zánětlivé odpovědi v epileptologii patří kortikoidy a adrenokortikotropní hormon (ACTH), které patří k lékům první linie v léčbě infantilních spazmů (Westův syndrom). Terapeutický efekt byl popsán také u epileptických encefalopatií jako jsou např. Lenox-Gastautův syndrom, Landau-Kleffnerův syndrom, epilepsie s kontinuální výboji hrot-vlna ve spánku, které jsou rezistentní k terapii konvenčními antiepileptiky. V akutních případech (jako je např. refrakterní SE typu NORSE/FIRES) používáme v imunoterapii první volby pulzní dávkování metylprednizolonu, intravenózní imunoglobuliny nebo plazmaferézu. V imunoterapii druhé linie pak takrolimus, rituximab a cyklofosfamid [22].

Na experimentální úrovni byl prokázán protizánětlivý efekt KD a VNS běžně používaných v rámci nefarmakologické léčby epilepsie, ale také např. kanabinoidů [22,23]. Bylo prokázáno, že KD vede ke snížení hladiny prozánětlivých cytokinů dosud nejasným mechanizmem. Předpokládá se vliv ketolátek (např. β-hydroxy-butyrátu) na mitochondriální cílové struktury. KD také vede ke ketony indukovanému přerušení inflamazomů [23]. Inflamazomy jsou cytosolové multiproteinové oligomery imunitního systému odpovědné za aktivaci zánětlivých odpovědí.

Ovlivnění neurozánětu prostřednictvím VNS se vysvětluje regulací uvolňování cytokinů přes tzv. reflex zánětu, v kterém sehrává klíčovou úlohu právě nervus vagus [22]. VNS vede k redukci uvolňování prozánětlivých cytokinů na úrovni imunitních buněk vrozeného imunitního systému [22].

V klinické praxi se zkouší také efekt kanabinoidů (tetrahydrokanabinol a kanabidiol). Jejich protizánětlivý efekt je vysvětlován snížením hladiny protizánětlivých cytokinů (IL-1β, IL-3, IL-6, IL-12, IL-17, TNFα, IFNβ/γ), zvýšením hladiny prozánětlivých cytokinů (IL-10), snížením aktivity COX2, snížením aktivity mikroglie a redukcí oxidačního stresu [22].

Hlavním úskalím protizánětlivé léčby v terapii epilepsie je skutečnost, že zánětlivá komponenta v iktogenezi jednotlivých typů epilepsie se liší, je odlišná v různých fázích průběhu onemocnění a je obtížné identifikovat pacienty, u kterých je tato zánětlivá komponenta „aktivní", a tudíž má být léčena. Tato skutečnost poukazuje na nutnost identifikace tzv. biomarkerů neurozánětu (v krvi, likvoru, na neurozobrazovacích metodách).

Dalším problémem je pozdější nástup účinku protizánětlivé léčby a přetrvávání jejího efektu po ukončení léčby (cca 1–2 týdny), čímž je obtížné odečíst její efekt. Tato skutečnost opět poukazuje na nutnost identifikace biomarkerů neurozánětu, které by nám pomohly zhodnotit úspěšnost léčby [22].

Experimentální možnosti imunomodulační terapie

Byly provedeny různé farmakologické studie na zvířecích modelech zacílené na sys-

témy IL-1β /IL-1R1, HMGB1/TLR4, COX-2/prostaglandiny a komplementový systém, které se signifikantně podílejí na vzniku a opakování akutních záchvatů [1,9,24]. Protizánětlivé léčby, které vedly ke zlepšení prognózy v experimentálních modelech SE, jsou uvedeny v tab. 2 [1,9,25].

Terapeutické ovlivnění kaskády systému IL-1β/IL-1R1

Anakinra (neglykolizovaný lidský antagonista receptoru pro IL-1 [IL-1ra]) má protizánětlivé a imunomodulační schopnosti a v současné době je používán v biologické léčbě revmatoidní artritidy. Hlavním problémem jeho použití je riziko hepatotoxicity.

Na experimentální úrovni vede aplikace IL-1ra u pilokarpinem indukovaného SE k opožděnému nástupu a kratšímu trvání SE a redukci hrotové aktivity na EEG [1,24]. V klinické praxi byl úspěšně použit v léčbě NORSE/FIRES (nově vzniklý refrakterní SE) – v literatuře nacházíme jenom několik kazuistických sdělení, zkušenosti jsou tudíž velmi omezené [25].

Nejúčinnější léčbou akutní fáze SE na experimentální úrovni zůstává antagonista P2X7 receptoru, který významně zkracuje délku trvání SE, a to dokonce do 1 h od podání [26,27]. Purinergní P2X7 receptor je membránový iontový kanál aktivovaný extracelulárním ATP. Aktivace tohoto receptoru vede k aktivaci mikroglie a uvolnění IL-1β, čímž vede k rozvoji zánětu a zvyšuje excitabilitu v mozku. P2X7 receptor může také přímo modulovat neurotransmisi a gliotransmisi a podpořit inkorporaci imunitních buněk do mozkové tkáně. Antagonista P2X7 receptoru na zvířecím modelu redukuje záchvatovou aktivitu, čímž vede k redukci neuronálního poškození. Kombinace antagonisty P2X7 receptoru s benzodiazepiny vedla k supresi SE u modelu refrakterního SE [26]. P2X7 receptor je považován za nový potenciální cíl léčby SE, který vede k ukončení SE mechanizmem redukce zánětu v CNS.

Terapeutické ovlivnění kaskády systému HMGB1/TLR4

Efekt HMGB1 je zprostředkován přes vazbu na receptor TLR4. HMGB1 spolu s IL-1β snižuje záchvatový práh, a tím vede k hyperexcitabilitě mozku. Inaktivace HMGB1 s využitím antagonisty jeho receptoru TLR4 signifikantně redukuje záchvatovou aktivitu. Na experimentální úrovni se také zkouší aplikace inaktivované anti-HMGB1 monoklonální protilátky [24].

Tab. 2. Protizánětlivé léčby, které vedly ke zlepšení prognózy v experimentálních modelech status epilepticus [1,9,25].

Název léku	Mechanizmus působení	Efekt
celecoxib, parecoxib	inhibice COX-2	antiepileptogenní, DMD
aspirin	inhibice COX-1,2	DMD
A4-integrin-specifická protilátka	adheze leukocytů na endotel	prevence poruchy HEB
erythropoetin	široké spektrum	široké spektrum
fingolimod	modulátor S1P receptoru	antikonvulzivní, antiepileptogenní, DMD
anakinra (Kineret)	antagonista IL-1R1	antikonvulzivní, antiepileptogenní, DMD
anakinra + COX-2 inhibice	antagonista IL-1R1 + inhibice COX-2	antikonvulzivní, antiepileptogenní, DMD
anakinra + TLR4 antagonista	IL-1R1 + HMGB1 inhibice	antikonvulzivní, antiepileptogenní, DMD
Nrf2 genová terapie	↓ oxidativní stres, ↓ produkce cytokinů	neuroprotektivní
miRNA-146a	IL-1β/TLR4	antikonvulzivní, DMD
EP2 antagonista	EP2 receptor	antiepileptogenní, DMD
dexametazon	široké spektrum	široké spektrum
statiny	inhibice syntézy prostaglandinů	antiepileptogenní, DMD

COX-2 – cykloxygenáza-2; DMD – disease modifying drugs; EP2 – receptor pro prostaglandin E2; HEB – hematoencefalická bariéra; HMGB1 – high mobility group box 1; IL – interleukin; IL-1R1 – IL-1β receptor; NrF2 – nukleární transkripční faktor 2; S1P – sfingosin-1-fosfatový receptor; TLR4 – Toll-like receptor 4

Terapeutické ovlivnění kaskády systému COX-2/prostaglandiny

Podstatou působení nesteroidních antiflogistik je inhibice produkce mediátorů zánětu – prostaglandinů. Vazba PGE2 na receptor EP2 (na povrchu astrocytů) vede ke zvýšenému uvolňování glutamátu (hyperexcitace v neuronálních sítích) a k indukci apoptózy. Antagonista receptoru EP2 navozuje redukci záchvatové aktivity a neuronálního poškození [19]. COX-2 je uvolňován u SE, hovoříme o COX-2 cestě indukce zánětu v průběhu SE [5]. Efekt terapie inhibitory COX-2 závisí na typu použitého inhibitoru a načasování terapie [25,28].

Na experimentální úrovni byl testován celocoxib (selektivní inhibitor COX-2). Aplikace 1 den před indukcí a 28 dní po indukci SE vedla ke snížení frekvence a zkrácení délky trvání spontánních záchvatů. Parecoxib aplikovaný po ukončení SE vedl k prevenci generalizace záchvatů a zabránil zvýšení hladiny PGE2 [20,24].

Na klinické úrovni byl prokázán efekt profylaktického podávání acetylsalicylové kyseliny na záchvatovou aktivitu u pacientů se Sturge-Weberovým syndromem (u 91 % pacientů došlo k signifikantnímu snížení frekvence a trvání epileptických paroxysmů) [20,24]. Na druhou stranu profylaktické podávání ibuprofenu u dětských pacientů s febrilními křečemi nemělo vliv na výskyt febrilních paroxysmů. Dosud není k dispozici klinická studie, která by sledovala efekt terapie selektivními inhibitory COX-2 u pacientů s epilepsií [24].

Použití selektivních inhibitorů COX-2 je zvažováno jako nová terapeutická možnost s antiepileptogenním a potenciálně DMD (průběh onemocnění modifikujícím) efektem [1]. Tato léčba (vč. antagonisty EP2 receptoru) má na experimentální úrovni signifikantní neuroprotektivní efekt a snižuje mortalitu v průběhu SE [9,28,29].

Studie na zvířecích modelech poukazují na nutnost časné intervence (aplikace antiflogistik do 3–4 h od začátku SE), aby byla šance na dosažení terapeutického úspěchu. Tyto nálezy upozorňují na možnost aplikace v klinické medicíně ve smyslu blokace aktivace zánětli-

vých kaskád v průběhu SE, a tím zlepšení prognózy pacientů u *de novo* vzniklého SE, ale také u SE v rámci chronické epilepsie [1].

Retrospektivní studie dále prokazují, že statiny podávané pacientům před rozvojem SE vedly ke zlepšení prognózy a snížení mortality [29]. Účinek statinu není založen výlučně jen na jejich hypolipidemickém účinku, ale i na cholesterolu nezávislém působení, na tzv. pleiotropních účincích. Mezi nejznámější pleiotropní účinky patří antiagregační a protizánětlivé působení založené na inhibici syntézy prostaglandinů a tromboxanů. Studie na experimentální úrovni prokázaly protizánětlivý a antiepileptogenní účinek statinů [30].

Další možnosti imunomodulace

Adalimumab (monoklonální protilátka anti-TNFα) – se v klinické medicíně zkouší v léčbě Rasmussenovy encefalitidy. Na experimentální úrovni se testuje jeho použití v terapii epilepsie, kde vede k snížení frekvence a délky trvání záchvatů [24].

Fingolimod – imunomodulátor s potenciálně imunosupresivními vlastnostmi používaný v léčbě remitentní formy RS. Mechanizmus účinků spočívá v modulaci sfingosin-1-fosfátového (S1P) receptoru na povrchu lymfocytů, astrocytů, oligodendrocytů a neuronů ve smyslu internalizace receptorů (t.j. působí jako funkční antagonista S1P receptoru). Na experimentálním modelu temporální epilepsie byl prokázán jeho antikonvulzivní, antiepileptogení a neuroprotektivní efekt [31].

Micro-RNA 146a funguje jako negativní regulátor kaskády IL1R/TLR4. Na molekulární úrovni byl prokázán její antiepileptogenní a efekt DMD [24].

Nukleární transkripční faktor 2 (Nrf2) genová terapie – Nrf2 je gen regulující produkci celé řady antioxidačních a protizánětlivých proteinů, které chrání buňku proti oxidačnímu poškození. Studie na zvířecích modelech prokázaly, že indukce Nrf2 dráhy má neuroprotektivní efekt u epilepsie i dalších neurodegenerativních onemocnění [24,32].

Závěr

Experimentální a klinická data prokazují, že zánětlivé změny v mozku v průběhu SE sehrávají důležitou roli v epileptogenezi tím, že se podílejí na zvýšení neuronální hyperexcitability, negativně ovlivňují plasticitu neuronálních sítí a přispívají tak k rozvoji a opakování epileptických záchvatů. Zvýšená syntéza specifických prozánětlivých mediátorů a up-regulace jejich receptorů se podílí na vzniku epilepsie, jak vyplývá z nálezů aktivovaných prozánětlivých kaskád u epileptických ložisek [8].

Vyšetření mediátorů zánětu a posouzení rovnováhy pro-/protizánětlivých mechanizmů jsou považovány za potenciální biomarkery epileptogenicity.

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Konflikt zájmů

Autoři deklarují, že v souvislosti s předmětem studie nemají žádný konflikt zájmů.

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Annex 2

Bohošová J, Vajčner J, Jabandžiev P, Ošlejšková H, Slabý O, **Aulická Š.**

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CRITICAL REVIEW – INVITED COMMENTARY

MicroRNAs in the development of resistance to antiseizure drugs and their potential as biomarkers in pharmacoresistant epilepsy

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Summary

Although many new antiseizure drugs have been developed in the past decade, approximately 30%–40% of patients remain pharmacoresistant. There are no clinical tools or guidelines for predicting therapeutic response in individual patients, leaving them no choice other than to try all antiseizure drugs available as they suffer debilitating seizures with no relief. The discovery of predictive biomarkers and early identification of pharmacoresistant patients is of the highest priority in this group. MicroRNAs (miRNAs), a class of short noncoding RNAs negatively regulating gene expression, have emerged in recent years in epilepsy, following a broader trend of their exploitation as biomarkers of various complex human diseases. We performed a systematic search of the PubMed database for original research articles focused on miRNA expression level profiling in patients with drug-resistant epilepsy or drug-resistant precilinical models and cell cultures. In this review, we summarize 17 publications concerning miRNAs as potential new biomarkers of resistance to antiseizure drugs and their potential role in the development of drug resistance or epilepsy. Although numerous knowledge gaps need to be filled and reviewed, and articles share some study design pitfalls, several miRNAs dysregulated in brain tissue and blood serum were identified independently by more than one paper. These results suggest a unique opportunity for disease monitoring and personalized therapeutic management in the future.

KEYWORDS

biomarkers, drug-resistant epilepsy, intractable epilepsy, noncoding RNA, refractory epilepsy

1 | INTRODUCTION

Epilepsy is a group of heterogeneous syndromes affecting more than 50 million people worldwide. Although many new antiseizure drugs (ASDs) have been developed in the past decade, approximately 30%–40% of patients remain pharmacoresistant (drug-resistant epilepsy [DRE], pharmacoresistant epilepsy, intractable epilepsy, refractory epilepsy). Furthermore, up to now, no effective treatment exists to prevent the development of epilepsy despite

increasing understanding of the underlying molecular and cellular mechanisms of epileptogenesis.[1]

The International League Against Epilepsy defines pharmacoresistant epilepsy as the failure of a patient's seizures to respond to at least two antiseizure medications that are appropriately chosen and used for an adequate period.[2] The pharmacoresistance to ASD per se is not a possible therapeutic target, as ASDs themselves do not prevent the development of epilepsy, merely blocking the seizures as they arise. Moreover, there is no evidence that they influence the course of epilepsy. Another challenge is the nature of the pharmacoresistance itself, as different intractability patterns can be present in individual patients. Often, drug resistance exists long before administration of the first ASD therapy (de novo drug resistance). However, some patients develop pharmacoresistance over time, although they are easily tractable at the beginning of the treatment, with pharmacoresistance developing years later during their life with epilepsy (progressive drug resistance). On top of that, patients with transient reversal of drug resistance with remission and relapses become seizure-free during trials of new ASDs (waxing and waning drug resistance).[3,4]

Although pharmacoresistance is a significant issue in the effective therapeutic management of epilepsy, exact ASD pharmacoresistance mechanisms are still unclear.[5] Some hints indicate that neuroinflammation plays a crucial role. Many proinflammatory molecules are typically upregulated in patients with DRE. Namely, interleukin-1β (IL-1β), high mobility group box 1 (HMGB1), cyclooxygenase 1 (COX-1), COX-2, and nuclear factor kappa-light-chain-enhancer of activated B cells all lead to neuronal loss.[6–10] Some of them are also potential therapeutic targets[11–14] or are directly associated with known mechanisms of pharmacoresistance.[8] There are currently two main hypotheses on the development of pharmacoresistance in epilepsy, whereas some other proposed mechanisms are yet to be supported by more convincing data.[15] The drug transporter hypothesis states that pharmacoresistance to ASDs is based on overexpression of multidrug cell transporter (P-glycoprotein, ATP-binding casette subfamily member 1), which pumps the drug out of the cell and thus prevents it from entering the brain in sufficient concentrations.[5,16–19]

On the other hand, the drug target hypothesis postulates that the change in the molecular structure of the drug target prevents it from binding the drug and thus leads to pharmacoresistance or is a predictive marker for drug failure.[20] The third and most recent resistance theory suggests that the drug refractoriness is not separate from ongoing epilepsy; instead, it is determined by its severity. Intrinsic severity of the seizures and their frequency at disease onset are the main determinants of the drug resistance risk,[21] although some discrepancies exist, such as patients with relatively mild disease onset who later become drug-resistant nonetheless.[21] However, some results show that none of the mechanisms stated above can explain drug resistance sufficiently, and therefore a fourth, epigenetics-based mechanism has been suggested.[5]

Key Points

- A literature review was conducted on the biomarker potential of miRNAs in the early assessment of therapy response
- A brief discussion of miRNA biology and the role of miRNAs in epilepsy and pharmacoresistance is provided
- The most significant results in tissue and serum profiling and studies on animal models and cell cultures are discussed in more detail
- Independently validated miRNAs are highlighted
- An overview of general study design pitfalls and suggestions for future research are provided

The development of targeted treatments for epilepsy depends on identifying biomarkers that would allow for individually tailored treatment.[1] This major clinical need has arisen in recent years, as the therapy options for epileptic patients have become wider but still not effective for some of them. Although the trial-and-error approach eventually rules out ineffective drugs, and patients are then indicated for surgery, it may take years of debilitating seizures that affect not only patients themselves but also their families and society as well. Identification of pharmacoresistance-related biomarkers could substantially improve the management of patients with epilepsy.

2 | MICRORNAS AND THEIR BIOMARKER POTENTIAL IN EPILEPSY

Change in the balance between excitation and inhibition of neuronal networks is the underlying mechanism of epileptogenesis. This multifactorial dynamic process associates with alterations of gene expression and brain network remodeling.[22] Neuronal death, inflammation, changes in the function of ionic channels, neurogenesis, and gliosis are typical for epileptogenesis.[23] Aberrant protein production with prominent transcriptional suppression creates a backdrop for these processes.[24,25]

MicroRNAs (miRNAs) are a class of short noncoding RNAs (21–25 nucleotides long) functioning as important negative posttranscriptional regulators of gene expression.[26,27] There are approximately 2300 distinct miRNAs in the human genome estimated,[28] of which about 600 are well studied, and many are strongly conserved among species.[29,30] The canonical pathway of miRNA biogenesis starts with the primary transcript with the hairpin loop structure originating in the nucleus. Two processing stages, first in the nucleus and then in the cytoplasm, occur until an miRNA-duplex structure is created. One of the duplex strands is selected and incorporated into a complex with the argonaute proteins. The resulting miRISC (miRNA-induced silencing complex) binds the potential mRNA target in its 3′ untranslated region (UTR) of sufficient complementarity. Mature miRNAs are known to be involved in all cellular processes; the most significant impact is on mRNAs with a longer 3′ UTR. Other additional factors are then recruited, which leads to degradation or repression of translation of the target mRNA.[31]

The miRNA structure and function mechanisms outline their potential as biomarkers of ongoing pathogenesis and a therapeutic target. Simple benchtop technology can be used for the detection of miRNA expression levels. Mostly polymerase chain reaction (PCR)-based kits and various modifications of RNA sequencing have been used in recent years.[32,33] miRNAs can be found either enclosed in exosomes[34] or bound in protein complexes,[35] thus being protected from degradation. Despite many freeze–thaw cycles and RNA isolation, their exceptional stability predestines them to be a perfect potential biomarker. However, different methods of sample handling and processing introduce some level of variability into miRNA profiles.[33,35–38] Stemming from their role as fine-tuners of gene expression, miRNA expression levels mirror the cell's processes. Therefore, we can observe temporospatial specificity of miRNA expression, creating a pattern particular for each organ,[39] tissue,[40,41] or cell type[42,43] in each moment,[44] not only in respective locations but also in body fluids,[45] if functionally associated with the process of interest. A unique opportunity arises to facilitate miRNAs as biomarkers of ongoing pathology and create novel, more precise clinical guidelines for assessing diagnosis, prognosis, and overall clinical outcome of patients with about any complex disease.

Although, up to this day, prominently in cancer,[46] miRNAs have been studied intensely also in other human diseases, including epilepsy. Not long after their discovery, it has been shown that serum miRNA levels are influenced by seizures.[47] As seizures often occur due to psychogenic nonepileptic disorders, a precise diagnostic approach is of the highest importance.[48] However, according to the latest results, miRNAs seem specific for epilepsy irrespective of seizure and brain activity.[42] Moreover, some miRNAs are enriched in the brain, with several miRNAs being almost exclusive to brain tissue.[42,49] As Organista-Juárez et al.[24] point out, more than 1000 miRNAs are differentially expressed in different brain regions of human and experimental epilepsy.[50] The expression patterns of miRNAs differ throughout brain locations,[51] such as a specific hippocampal miRNome unique for mesial temporal lobe epilepsy (mTLE) patients with hippocampal sclerosis, a common pathological finding in patients with temporal lobe epilepsy (TLE).[40] Supporting evidence for the central role of miRNAs in epileptogenesis is evident from studies on mice with induced epilepsy.[52] The observed reduction of Dicer levels in hippocampal tissue suggests that the loss of crucial components of canonical miRNA biosynthesis and the loss of miRNAs involved in regulating normal brain functioning may be features of the hippocampal sclerosis pathophysiology.

Based on miRNAs′ significance in epileptogenesis, manipulating the most dysregulated miRNAs could potentially alleviate epilepsy symptoms. The antiseizure effect of miR-134 is well documented and tested. Early administration of miR-134 antagonist after epilepsy-inducing insult leads to significant abolishment of seizures across multiple species.[53–56] Another recent study[44] identified potential miRNA involvement in the development of chronic epilepsy. miR-10a-5p, miR-21a-5p, and miR-142a-5p were all upregulated in hippocampal tissue of murine models. The potential role of these miRNAs in transforming growth factor beta signaling underlines the role of neuroinflammation in the origins of epilepsy. Their knockdown leads to antiseizure phenotypes, and their combined inhibition reduced seizures, suggesting novel treatment options for patients with epilepsy. However, the involvement of miRNA in the epileptogenesis and inflammatory response to the seizure is evident from many other results, such as the association of miR-15a upregulation with apoptosis and inflammation in TLE,[57] downregulation of miR-125a-5p shown in rat models with induced epilepsy,[58] impairment of IL-1 receptor type 1/Toll-like receptor 4 signal transduction using miR-146a,[59] and many others.[60]

Moreover, miR-22 has been identified as a potent regulator of the inflammatory response to the seizure. Deletions in miR-22 lead to suppression of inflammatory signaling in epileptic mice after status epilepticus.[61] miRNA is also known to interact with other noncoding RNAs, such as long noncoding RNAs, in an intricate regulatory network involved in inflammation and epileptogenesis.[62–64]

Regarding the potential of liquid biopsy, some previous efforts have been made to identify circulating biomarkers of epileptogenesis.[65] The main focus has been on proteins in peripheral blood, mainly molecules related to

inflammation not specific for epilepsy. Instead, they reflect a general inflammatory process associated with many conditions. On the other hand, protein-coding transcripts are sufficiently specific; they, however, suffer from instability in circulation and detection difficulty. Short RNAs offer a perfect combination of specificity and stability in body fluids, with a functional link to different aspects of epilepsy.[65]

3 | ROLE OF miRNAS IN PHARMACORESISTANCE TO ANTISEIZURE DRUGS

One of the two most significant mechanisms of pharmacoresistance in epilepsy is facilitated through overexpression of multidrug transporters such as multidrug resistance gene 1 (*MDR1*) coding a P-glycoprotein (P-gp) in the brain astrocytes, which prevents the accumulation of a drug in a sufficient concentration in the cells.[66,67] Existing evidence suggests that hypoxia-inducible factor 1α (HIF-1α) targets and modulates *MDR1* expression.[68] Studies show that *HIF-1α* and *MDR1* are coordinately overexpressed, which leads to a lower concentration of ASDs in brain.[69,70] HIF-1α is a potential therapeutic target, although we still lack a deeper understanding of its role in epilepsy.[71,72] Possible involvement of miRNAs in mechanisms of pharmacoresistance (summarized in Figure 1) through HIF-1α pathway has been outlined in a study by Ikemura et al.,[73] showing a role of miR-145 in the regulation of P-gp expression in intestinal cells. According to the results of this study, miR-145 negatively regulates the expression of P-gp through a repressive interaction with *MDR1* mRNA, leading to increased levels of P-gp. Another miRNA associated with *MDR1* regulation is miR-451,[24,74] a miRNA positively linked with the number of ASDs used for the treatment.[24] Another miRNA, miR-153, has been suggested as a regulator of the HIF-1α pathway and ASD pharmacoresistance in a study by Li et al.[75] and independently validated by Gong et al.,[76] confirming that the downregulation of miR-153 negatively correlates with upregulation of HIF-1α. Li et al.[75] started with miRNA expression profiling and then applied an *in silico* approach to identify downregulated miRNAs that are putative regulators of *HIF-1α*. Among others, miR-153, miR-543, miR-191, and miR-494 have been dysregulated. Previous studies have already shown that at least miR-153 and miR-494 can play a role in pharmacoresistance in epilepsy.[77,78] *In silico* analysis identified two binding sites for miR-153 in the *HIF-1α* mRNA sequence, which a luciferase assay has proved. In both studies, rat astrocytes were induced to overexpress *MDR1* gene and its translational product, P-gp, thus creating a pharmacoresistant in vitro model, which has been established and modified according to the previous work.[79] miR-153 overexpression in pharmacoresistant astrocytes inhibited the expression of *HIF-1α* in these pharmacoresistant cells.

However, some studies show that levels of some miRNAs can be influenced by ASDs. Several miRNAs decrease in response to phenobarbital or valproate[80,81]

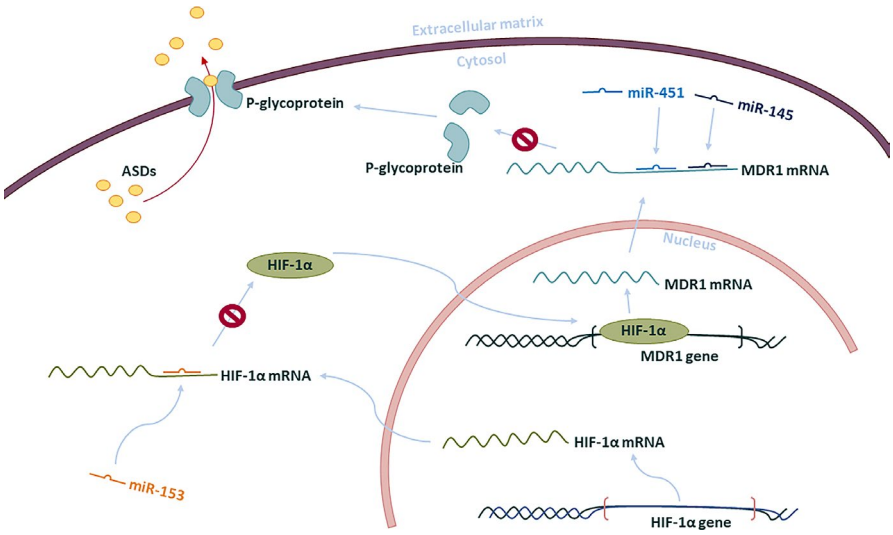

FIGURE 1 Role of microRNAs in pharmacoresistance to antiseizure drugs (ASDs). miR-153 binds to hypoxia-inducible factor 1α (HIF-1α) mRNA and blocks its translation. In decrease or absence of miR-153 regulation, HIF-1α mRNA is translated, and HIF-1α protein travels back to the nucleus, where it binds to the multidrug resistance gene 1 (MDR1) gene as its transcriptional regulator. MDR1 mRNA is then in cytoplasm translated into P-glycoprotein (P-gp), incorporated into the cell membrane as a multidrug transporter pumping ASDs out of the cell. When miR-145 and miR-451 reach physiological levels, they repress translation of MDR1 mRNA by binding to its 3′ untranslated region region, thus decreasing levels of P-gp

administration, which is also reflected in blood,[82] suggesting that although miRNA, in general, can be a potential diagnostic, prognostic, or predictive biomarker, some miRNAs would find their application rather as therapeutic targets. Moreover, the results highlight the need for cautious interpretation of results and diligent controlling for confounding factors. Nevertheless, studies on the feasibility of miRNAs as biomarkers, especially blood-based biomarkers, were just a matter of time.[83] To review the knowledge on the potential use of miRNAs as biomarkers and possibly also novel therapeutic targets in DRE, we searched the PubMed database for relevant studies published from inception to March 5, 2021. Using the search terms ("pharmacoresistant" AND "epilepsy" AND "miRNA") OR ("intractable" AND "epilepsy" AND "biomarkers") OR ("refractory" AND "epilepsy" AND "biomarkers") OR ("drug-resistant" AND "epilepsy" AND "biomarkers"), and excluding nonclinical studies, reviews, and papers on non-miRNA-based biomarkers, we identified 17 articles relevant for further discussion (summarized in Tables 1–3). Table 1 provides an overview of the results obtained using brain tissue of pharmacoresistant epilepsy patiens, and Table 2 summarizes all the results obtained using serum samples of pharmacoresistant patients. Table 3 provides a summary of miRNAs studied in preclinical models. The tables might overlap, as some papers contain both tissue and serum miRNA profiling or in vitro and in vivo functional tests.

4 | miRNA PROFILING IN BRAIN TISSUE OF PATIENTS WITH PHARMACORESISTANT EPILEPSY

Biomarker studies aiming to identify potential diagnostic, prognostic, or predictive miRNAs have emerged in recent decades along with the commercial success of next generation sequencing (NGS). The gold standard of biomarker study design includes a so-called discovery or explorative phase and a validation phase. In the discovery phase, the main aim is to screen for potential biomarkers from a large pool of molecules, to provide a so-called expression profile. Therefore, high-throughput methods such as different kinds of arrays and RNA sequencing have been the technologies of choice in this step. Both arrays and especially RNA sequencing allow for robust detection of thousands of molecules, usually in smaller cohorts of patients and controls, considering the still relatively high costs. Thus, the results suffer from small sample size error and high rates of false positive and false negative results and must be further validated using different methods. In the validation phase, quantitative PCR (qPCR) has been the gold standard, providing high sensitivity and reliability, albeit at higher cost and demanding greater laboratory work. Therefore, only a selected few candidate biomarkers are validated on larger patient cohorts, providing results with higher statistical power. Typically, one set of patients for the discovery phase and a different set for validation of the results have been used to further strengthen the results' statistical value. de Ronde et al. elaborated on a biomarker study design.[84]

Conventional miRNA profiling studies on tissue samples are not as numerous in pharmacoresistant epilepsy. The tissue biopsy specimen is available only in patients indicated for brain surgery as a last resort after all other therapy options fail. Therefore, miRNAs dysregulated in these patients lack a proper comparison with the samples of drug-responsive patients, as those remain seizure-free without the surgery. Moreover, the samples' scarcity prevents collection of cohorts large enough for two nonoverlapping independent groups—discovery and validation cohorts. Instead, presumably out of necessity, validation of high-throughput methods is usually carried out on the same cohort of patients extended for several new specimens. Another limitation lies in the timing of the specimen collection, which is typically after therapy failure, not before it. The potential influence of ASD administration in miRNA levels has been shown before, and it is difficult to distinguish from the effect of epilepsy itself.[80–82] Authors usually do not test the results with receiver operating characteristic (ROC) analysis and do not state the area under the curve (AUC), sensitivity, and specificity of potential biomarkers. Therefore, it is not easy to come to a useful conclusion on which miRNAs are more or less promising. Generally, the field lacks more extensive high-throughput profiling, preferably by NGS, which would provide a profile of miRNA specific for drug-resistant patients.

Out of the few tissue-profiling studies on patient tissue samples (summarized in Table 1), Zucchini et al.[85] provided the first profiling study on 14 paraffin-fixed formalin-embedded hippocampal or temporal lobe tissue specimens from DRE patients with hippocampal sclerosis. Although the research focused on granule cell pathology (GCP), it offered interesting initial insights into miRNAs' involvement in pharmacoresistance. GCP, which has been investigated in this study, has been associated with a favorable prognosis in postsurgery DRE patients. miR-487a has been identified as the most significantly dysregulated in patients with DRE with GCP. Of the several targets of miR-487a, a transmembrane protein, anthrax toxin receptor 1, seems to be interesting, as its increased levels in case of miR-487a dysregulation could favor granule cell dispersion.[85]

De Matteis et al.[86] provided a case report of significant upregulation of miR-301a-3p in a patient with drug-resistant mTLE who died of sudden unexpected death in

TABLE 1 Summary of studies on miRNA profiling in tissue samples of pharmacoresistant epilepsy patients

	Discovery cohort–expression profiling		Validation cohort–validation of the results, qPCR				
Ref.	Patients vs. controls, *n*; method; tissue original site	Significantly up-/downregulated miRNAs, *n*	Patients vs. controls, *n*; tissue original site	Significantly deregulated miRNAs, $p < .01$	Change in expression in patients	*p*, AUC	Biomarker type
85	5 DRE GCP vs. 5 DRE non-GCP; microarray; hippocampus, temporal lobe	6 up- and 6 downregulated	7 GCP vs.7 non-GCP (including discovery cohort); hippocampus, temporal lobe	miR−487a	↓	<.01, -	Predictive
75	32 DRE mTLE vs. 18 surgical controls; microarray; temporal lobe	29 up- and 65 downregulated	32 DRE vs. 18 surgical controls (discovery cohort); temporal lobe	miR−153 miR−494	↓ ↓	<.001, -, <.001, -	Diagnostic
92	9 DRE FCD vs. 8 controls; microarray; temporal lobe	138 up- and 11 downregulated	9 DRE FCD vs. 8 controls (including discovery cohort); temporal lobe	miR−4521	↑	.001, .813	Diagnostic
91	9 DRE FCD vs. 8 controls; microarray; temporal lobe	138 up- and 11 downregulated	13 DRE FCD vs. 13 controls (including discovery cohort); temporal lobe	miR−129–2-3p	↑	<.0001, .929	Diagnostic
40	16 DRE mTLE+HS vs. 8 controls; NGS; anteromesial temporal resections	107/47 differentially expressed miRNAs (depending on the statistical approach)	33 mTLE+HS vs. 9 controls (including discovery cohort); temporal lobe	miR−1260a miR−1260b miR−1275 mir−129–1-3p miR−129–2-3p miR−1298 miR−142-3p miR−142-5p miR−144-5p mR−150-5p miR−191-5p miR−193b−3p miR−339-5p miR−374b−5p miR−4443 miR−4454 miR−451a miR−4792 miR−6087 miR−874-3p miR−134-5p	↑ ↑ ↑ ↑ ↑ ↓ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↓	<.01, - <.01, - <.01, - <.01, - <.01, - <.01, - <.01, - <.01, - <.05, - <.01, - <.05, - <.01, - <.01, - <.05, - <.01, - <.01, - <.01, - <.01, - <.01, - <.05, - <.05, -	Diagnostic
90	9 DRE FCD vs. 8 controls; microarray; temporal lobe	138 up- and 11 downregulated	9 DRE FCD vs. 8 controls (including discovery cohort); temporal lobe	miR−323a−5p	↑	.0012, .944	Diagnostic

(Continues)

TABLE 1 (Continued)

	Discovery cohort–expression profiling		Validation cohort–validation of the results, qPCR				
Ref.	Patients vs. controls, *n*; method; tissue original site	Significantly up-/downregulated miRNAs, *n*	Patients vs. controls, *n*; tissue original site	Significantly deregulated miRNAs, $p < .01$	Change in expression in patients	*p*, AUC	Biomarker type
76	-	-	22 mTLE (DRE?) vs. 20 controls; temporal lobe	miR−153	↓	<.01	Diagnostic
86	-	-	1 TLE vs. 10 controls; hippocampus	miR−301a−3p	↑	<.05, -	Diagnostic
24	-	-	12 mTLE vs. 10 controls; temporal lobe	miR−451	↑	.0008, -	Diagnostic
				miR−1260	↑	.0062, -	
				mir−1275	↑	.04, -	
				miR−34a	↓	.0343, -	
				miR−1298	↓	.0161, -	

Note: Controls are nonepileptic subjects.

Abbreviations: AUC, area under the curve; DRE, drug-resistant epilepsy; FCD, focal cortical dysplasia; GCP, granule cell pathology; HS, hippocampal sclerosis; miRNA, microRNA; mTLE, mesial TLE; NGS, next generation sequencing; qPCR, quantitative polymerase chain reaction; Ref., reference; TLE, temporal lobe epilepsy.

epilepsy. Based on literature search, miR-301a-3p, miR-194-5p, miR-30b-5p, miR-342-5p, and miR-4446-3p were measured in tissue and plasma. Only miR-301a was significantly dysregulated in an epilepsy patient compared to 10 nonepileptic autopsies both in tissue and plasma.[86] However, as it is only a case report, the results should be taken with caution, although other studies have suggested this miRNA's role, providing conflicting results regarding the direction of dysregulation.[40,52,87] In a study by Bencurova et al.,[40] miR-301a-3p has been identified by NGS as well. However, qPCR validation on a notably larger cohort did not confirm this miRNA as differentially expressed. Postmortem changes, which could be another caveat in this small study, were also addressed by Bencurova et al.[40] The team checked for autopsy delay-related decrease in miRNA levels and did not observe any, suggesting high miRNA stability even 32 h postmortem. This study was the first to facilitate a high-throughput NGS approach in the tissue of DRE patients and identified some new differentially expressed miRNAs (miR-1260a, miR−1260b, and miR−4443, among others) that have been linked to other diseases, but not epilepsy. On the other hand, miRNAs widely studied in association with epilepsy (miR-146a, miR-132-3p, miR-132-5p, miR-134-5p) did not show expression changes. However, this could be ascribed to the uniqueness of the explicitly hippocampal profile in this study.

Conversely, Organista-Juárez et al.[24] later identified a positive correlation of miR-146a with the number of ASDs used for treatment and the frequency of seizures. miR-146a is recognized as a significant player in immunity[88] and known to be enriched in astrocytes, regulating neuroinflammation. Authors suggest that its expression probably increases as a compensatory mechanism to post-seizure inflammation.[24,89] Regarding the identification of drug refractoriness, however, it might not be the most adept as a biomarker, as it reflects general inflammation rather than nuanced differences between responding and nonresponding epilepsy patients.

Other miRNAs identified in Bencurova et al.[40] were in concordance with the literature, for example, miR-451, which is specifically enriched in neurons and has been shown to be upregulated in rodents and involved in the *MDR-1*-mediated mechanism of pharmacoresistance.[24,74] Organista-Juárez et al.[24] successfully validated miR-451; in this case, there was an association with the number of seizures, as in miR-146a.

Che et al.[90] provided a typical biomarker study with explorative expression profiling in brain tissue of patients with DRE caused by focal cortical dysplasia compared to eight controls indicated for surgery due for other reasons. Validation of miR-323a-5p on the same cohort achieved statistical significance. Based presumably on the same

TABLE 2 Summary of studies on miRNA profiling in serum samples of pharmacoresistant epilepsy patients

	Discovery cohort–expression profiling in serum		Validation cohort–validation of the results in serum, qPCR
Ref.	Patients vs. controls, *n*; method	Significantly up-/downregulated miRNAs, *n*	Patients vs. controls, *n*
65	30 DRE vs. 30 DSE; NGS	185 miRNAs differentially expressed	77 DRE vs. 81 DSE vs. 85 healthy
75	-	-	32 DRE vs. 18 controls
91	9 DRE FCD vs. 8 controls; microarray	138 up- and 11 downregulated	25 DRE vs. 25 controls (including discovery cohort)
92	9 DRE FCD vs. 8 controls; microarray	138 up- and 11 downregulated	30 DRE and control samples
90	9 DRE FCD vs. 8 controls; microarray	138 up- and 11 downregulated	30 DRE and 23 controls
93	14 mTLE vs. 13 FCD vs. 16 controls	Targeted profiling of miR−23a miR−31 miR−134	38 DRE vs. 27 DSE vs. 83 controls
76	-	-	22 DRE vs. 20 controls
86	-	-	1 TLE vs. 10 controls
94	-	-	40 DRE vs. 42 controls
95	-	-	16 DRE vs. 8 DSE vs. 8 controls
96	-	-	86 DRE vs. 76 DSE
97	-	-	10 DRE vs. 17 DSE (+ 20 controls)

Note: Controls are nonepileptic subjects.

Abbreviations: AUC, area under the curve; DRE, drug-resistant epilepsy; DSE, drug-sensitive epilepsy; FCD, focal cortical dysplasia; miRNA, microRNA; mTLE, mesial TLE; NGS, next generation sequencing; qPCR, quantitative polymerase chain reaction; Ref., reference; TLE, temporal lobe epilepsy.

study cohort, Sun et al.[91] found miR-129–2-3p to be upregulated in brain tissue ($p < .0001$). Another study based on this cohort[92] found miR-4521 to be upregulated in brain tissue. These three studies are the only exceptions in that they offered ROC analysis of the results, providing the AUC value and confidence interval, although the authors did not provide their biomarkers' sensitivity and specificity.

The involvement of miR-153 in the HIF-1α pathway and its role in refractory epilepsy has been proven independently by Li et al.[75] and Gong et al.[76] In both studies, the in vitro phase focused solely on miR-153. However, in the study by Li et al.,[75] there was also a discovery phase using a global microarray profiling of miRNA expression in DRE patients compared to nonepileptic surgical controls. The authors first compared its expression in mTLE patients to healthy controls' temporal cortex tissue and plasma along with the expression of HIF-1α. Whereas miR-153 was downregulated, HIF-1α was upregulated. In both studies,[75,76] using a luciferase vector, the authors also showed that miR-153 targeted the 3′ UTR of HIF-1α. Furthermore, overexpression of miR-153 in pharmacoresistant astrocytes has led to inhibition of HIF-1α. In contrast, miR-153 inhibition correlated with an increase in HIF-1α, suggesting an miR-153 role in HIF-1α regulation and the mechanism of pharmacoresistance discussed earlier.

5 | CIRCULATING miRNAS AND LIQUID BIOPSY

Compared to tissue profiling, much more attention has been paid to circulating miRNAs (Table 2). In this way, it is possible to also include therapy-sensitive patients in the study cohort. Moreover, a circulating biomarker is the only viable option for the prediction of therapy response. Indication for surgery comes after the failure of all available therapy options, which leaves the analysis of brain tissue profiles out of the question. However, most studies to date compared drug-resistant patients to healthy nonepileptic controls anyway, making any putative biomarker

Significantly dysregulated miRNAs, $p < .01$	Change in expression in patients	p	Sensitivity, specificity, AUC	Biomarker type
miR−194-5p	↓	<.0001	80.5%, 81.2%, .897	Predictive, diagnostic
miR−301a−3p	↓	<.0001	Panel 84.2%, 79.5%, .902	
miR−30b−5p	↓	<.0001		
miR−4446-3p	↓	<.0001		
miR−342-5p	↓	<.0001		
miR−153	↓	<.001	-	Diagnostic
miR−129–2-3p	↑	.0008	AUC .778	Diagnostic
miR−4521	↑	.0415	AUC .718	Diagnostic
miR−323a−5p	↑	.0320	AUC .742	Diagnostic
miR−134	↓	.00033 (only mTLE vs. controls)	75%, 58%, .671 (only mTLE vs. controls)	Diagnostic
miR−153	↓	<.01	-	Diagnostic
miR−301a−3p	↑	<.05	-	Diagnostic
miR−145	↓	.033	65%, 33%, .632	Diagnostic
miR−34c−5p	↓			Diagnostic, predictive
miR−134	↑	.010	AUC .617	Predictive
miR−146a	↑	.002	AUC .640	
miR−142	↑	.0008	-	Predictive
miR−223	↑	.00004	-	

a diagnostic one. Comparisons of drug-resistant patients with their drug-responsive counterparts would be more appropriate. Despite the apparent advantages of blood samples in DRE patients, not all tissue-profiling studies contain validation of miRNA profiles in blood,[24,40,85] although providing an extensive analysis of the tissue transcriptome[40] is assuredly a valuable contribution to the field. On the other hand, there is a subgroup of studies that do not follow the correlation of miRNA profiles in brain tissue and serum and only focus on miRNA levels in blood, verifying the existing results from other teams.[65,93–97] Nevertheless, levels of several miRNAs have been shown to be correlated in tissue and peripheral blood, namely miR-153,[75] miR-129–2-3p,[91] miR-4521,[92] miR-323a-5p,[90] and a case report from De Matteis on miR-301a-3p.[86] Moreover, mir-153 has been validated in serum by two independent teams as well.[75,76]

The first comprehensive study of miRNA profiles in serum samples has been provided by Wang et al.[65] The study cohort comprised 303 participants, and the study was designed as a biomarker study involving the explorative phase using a high-throughput NGS approach. Independent validation revealed that miR-194-5p, miR-301a-3p, miR-30b-5p, miR-342-5p, and miR-4446-3p were significantly downregulated in drug-resistant patients. miR-301a-3p showed the best diagnostic properties for the distinction between drug-resistant and drug-responsive patients. However, combining some of the validated miRNAs, miR-94-5p, miR-301a-3p, miR-30b-5p, and miR-4446-3p, performed very well in a predictive model. Association of miR-301a-3p with DRE was shown repeatedly in serum[65,86] and brain tissue.[40,86,87] However, as mentioned above, the results are conflicting regarding miR-301a-3p dysregulation. The discrepancies may be attributed to variations in the surgery selection protocol as well as study design. However, miR-301a deregulation is not specific for TLE, as its upregulation has been associated with immune response and inflammation similarly to miR-146a.[98] Authors have predicted targets of miR-301a-3p in silico, including mitogen-activated protein kinase 1, myeloid differentiation primary response 88, retinoblastoma-like protein 1, TNF receptor associated

TABLE 3 Overview of miRNAs studied in preclinical models of pharmacoresistant epilepsy

miRNA (change in expression)	Studied population	Sample type	Studied effect	Signaling pathway	Target molecule	Ref.
miR−206 (↓) miR−374 (↓) miR−142-5p (↓) miR−468 (↑)	Multidrug-resistant mice (valproate, levetiracetam)	Brain resection	Unique behavioral features of DRE mice	-	-	5
No difference	Chronic epilepsy rat model treated with phenobarbital	Hippocampal resection	Resistance to phenobarbital	-	-	112
miR−153 (↓)	Pharmacoresistant astrocytes	Cell culture	Resistance to ASDs	HIF-1α	HIF-1α	75 76
miR−34c−5p (↓)	Drug-resistant vs. drug-sensitive epileptic rats	Hippocampal resection	Resistance to ASDs	IL-1β/TNF-α	HMGB1	95

Abbreviations: ASD, antiseizure drug; DRE, drug-resistant epilepsy; HIF-1α, hypoxia-inducible factor 1α; HMGB1, high mobility group box 1; IL-1β, interleukin-1β; miRNA, microRNA; Ref., reference; TNF-α, tumor necrosis factor α.

factor 6, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta, and interferon alpha and beta receptor subunit 2, supporting a role in inflammation and apoptosis.[65]

The level of miR-146a along with miR-134 was explored in Leontariti et al.[96] in the serum of patients with DRE. Both miRNAs are central to the development of neurological conditions, as they are deeply involved in the regulation of neuroinflammation and synaptic plasticity.[88,99,100] Patients with elevated levels of miR-134 and miR-146 were at higher risk of developing drug resistance. After adjusting miR-134/146 levels for other clinicopathological factors, good results were achieved, which shows an independent prognostic significance of both miRNAs. However, the most recent study of De Benedittis et al.[97] did not find significant miR-146a dysregulation in DRE patients, although it had reasonable diagnostic potential for distinguishing epileptic patients from healthy controls.

On the other hand, Avansini et al.[93] provideed contradicting results concerning miR-134. Their study investigated plasma levels of three candidate miRNAs in patients with mTLE compared to focal cortical dysplasia patients and healthy controls. They aimed to find a noninvasive subsyndromic diagnostic biomarker of epilepsy. The three candidate miRNAs were previously reported as dysregulated in patients' tissue and preclinical models with different types of epilepsy.[100,101] Their results showed significant downregulation of miR-134 in patients of mTLE. However, no difference was identified between responsive and nonresponsive subcohorts, suggesting promising results for diagnostic purposes, but not for predicting therapy outcome. The authors acknowledge the lack of statistical significance due to the small study cohort and point out the need for more extensive studies. The decision to investigate the behavior of miR-134 during epilepsy more in-depth seems reasonable, as it has been studied extensively for its antiseizure effect.[42,54,55,100] However, research suggests that this miRNA is influenced by the ASD valproate, as it has been shown that miR-134 levels significantly decreased in epilepsy patients after administration of the drug.[82] The authors of the study proposed a possible mechanism of valproic acid effect on miR-134 levels through downregulation of brain-derived neurotrophic factor (BDNF), which in turn affects binding of miR-134 to LIM domain kinase 1 mRNA. miR-134 levels have also been associated with seizure duration prior to treatment and with seizure severity, suggesting that miR-134 would be feasible as a response-monitoring biomarker or novel therapy target rather than as a response-predictive marker.

Several smaller papers have provided validation of tissue expression profiling in serum (miR-129-2-3p,[91] miR-153,[75,76] miR-4521,[92] miR-323a-5p[90]); a hypothesis-driven approach, validating miRNAs previously associated with

epilepsy (miR-145[94]); or analysis of online NGS datasets and validation of candidate miRNA (miR-34c-5p[95]). The biomarker potential of miR-34c-5p was studied in the serum of children with DRE compared to drug-sensitive epilepsy patients and nonepileptic controls. Only this study focused on pediatric DRE patients. A general decrease of serum levels has been observed in epilepsy patients, with lower levels in the DRE group, whereas HMGB1 and IL-1β levels were higher.[95] Although the results are promising, the study cohorts are considerably small, and as in several other studies, independent validation with a larger cohort is necessary.

6 | FUNCTIONAL STUDIES ON CELL CULTURES AND PRECLINICAL MODELS

Papers focusing on miRNA involvement in pharmacoresistance as studied in preclinical models are summarized in Table 3. There are currently only four publications concerning miRNAs tested in animal or cell culture models, which might be surprising. As opposed to difficulties with patient samples and the absence of drug-sensitive control specimens, preclinical models can simulate drug-refractory and drug-responsive individuals. Several preclinical models are available, which can exhibit some degree of drug selectivity and refractoriness.[102–105] Although with some exceptions[106,107] most preclinical models can mimic resistance to two or three ASDs at once, the main issue of pharmacoresistance to ASDs in epileptic patients is the resistance to several, if not all, ASDs available regardless of the mechanism of action of different ASDs.[16,108] As the models' limited resistance is primarily driven by cost-effectiveness, it is likely that mechanisms of pharmacoresistance can be studied using these models.

Moreover, preclinical models allow us to observe behavior changes, frequency of seizures, and associated cellular changes and responses over time. For example, Moon et al.[5] reported a significantly altered profile of four miRNAs in multiresistant epileptic mice compared to control groups sensitive to levetiracetam or valproate. The test animals underwent a series of behavioral tests (open field, object exploration, elevated plus maze, and light–dark transition) focused on the assessment of anxiety and locomotor activity. Along with alteration of miRNA levels, a behavioral change has been observed in the multiresistant model animals, which showed heightened anxiety and impairment of risk assessment and decision-making abilities. This behavioral change is unlikely to occur in response to the ASD treatment. Therefore, the authors concluded that the behavioral change was due to pilocarpine-induced status epilepticus and can be influenced by the epigenetic mechanisms, considering the changes in miRNA levels and behavior of drug-responsive mice.[5,109] miR-206 was found in this study to reduce BDNF levels in the brain. BDNF is commonly increased after seizures and plays a vital role in epileptogenesis by increasing neuronal excitability.[5,110,111] However, the authors of the study found their results puzzling, because the miR-206 level was decreased in ASD-responsive mice. A similar pattern has been observed in the miR-371 level. miR-486 and miR-142-5p have been impressive because the resistant and responsive mice have shown an opposite level, suggesting a unique contribution to pharmacoresistance. However, the functions of these miRNAs are not fully understood yet. The authors of this study suggest that an epigenetic mechanism of pharmacoresistance stands separately from other suggested theories of pharmacoresistance, namely, the intrinsic severity hypothesis, drug transporter hypothesis, and target hypothesis.

An exception to the rule, the work of Haenisch et al.[112] on a chronic rat model of TLE treated with phenobarbital surprisingly found no difference in expression of miRNAs in the hippocampus in responders versus nonresponders. However, they observed a difference in the expression of 13 miRNAs when comparing preclinical rats after status epilepticus versus control rats, where the most significant differences are expected.

In addition to miR-153 involvement in the HIF-1α pathway repeatedly being shown in drug-resistant cell cultures,[75,76] another potentially major player in DRE is miR-34c-5p, which several papers have highlighted. Fu et al.[95] analyzed an online NGS dataset of a previously published profiling of 33 DRE patients and nine postmortem controls provided in the study of Bencurova et al.[40] mentioned above. Among other miRNAs, they found miR-34c-5p to be downregulated in DRE patients. After target gene analysis, the authors found 11 genes to be involved in the inflammation, among them HMGB1, a hallmark of DRE. HMGB1 plays a role in inflammation, as its higher levels are associated with an increase of IL-1β and tumor necrosis factor α in patients with DRE.[95,113] Thus, HMGB1 could act as a critical initiator of inflammation and generator of seizures.[114] Further analyses found a direct targeting of HMGB1 by miR-34c-5p. In DRE model rats' hippocampi, the expression of miR-34c-5p was reduced and was associated with hippocampal neuronal loss. The authors hypothesize that recurrent seizures in DRE patients could lead to neuronal apoptosis and hippocampal sclerosis along with focal cortical dysplasia, prevalent conditions in refractory epilepsy patients. As miR-34c-5p is known for its antiapoptotic effect, its decreased levels could expose the cells to higher apoptotic stress, leading to the activation of apoptotic pathways and neuronal loss observed in the DRE rat model in this study.[95]

7 | CONCLUSIONS AND FUTURE PERSPECTIVES

Refractoriness of the patient's epilepsy reveals itself after long-term treatment, often comprising several cycles of different therapies spanning across years, accompanied by repeated seizures that are not manageable by the therapy. Such conditions threaten patients' health, delay comprehensive treatment, and impose unnecessary economic, physical, health, and psychological burdens on patients, their families, and society.[94] Therefore, discovering predictive biomarkers and identifying pharmacoresistant patients is of high priority in this group.

As Enright et al.[49] concluded for blood-based miRNAs as biomarkers (which remains relevant for other sources of miRNAs), there are several knowledge gaps that need to be addressed in future research on biomarkers in epilepsy. The main issue shared among reviewed articles remains cohort size, sample collection, and adequate comparison of patients with pharmacoresistance to those responding to the therapy. Brain tissue is resected only in DRE patients during the indicated surgery, whereas those responsive to treatment are not indicated for surgery.[16] Besides missing "responding" tissue samples, the timing of the sample collection presents another limitation, as it comes only after all other therapeutic options have been exhausted. Considering the evidence that some miRNAs are influenced by ASDs,[80,81] it is difficult to draw any conclusion on the independent predictive value of the results gathered from the analysis of brain tissue. Unfortunately, the levels of some miRNAs in blood have been observed to react to ASD administration as well.[82] This situation could be overcome by appropriate preclinical models and cell cultures.

Despite the pitfalls, there are several promising miRNAs studied repeatedly in several articles, such as miR-34c-5p, miR134, miR-146a, miR-153, and miR-301a-3p, which are dysregulated not only in the tissue of drug-resistant patients but also in the serum, which presents an opportunity to bring a much searched-for predictive biomarker into the therapeutic management of epilepsy patients. Moreover, some of the studied miRNAs, namely miR-34c and miR-153, were also studied regarding their involvement in epileptogenesis and/or pharmacoresistance to ASDs, which further strengthens their position as potential biomarkers specific for drug-resistant patients, not only for epilepsy or general seizure activity. However, prospective studies on patient samples collected at the time of the diagnosis are sill missing in this field. The analysis of previously treated patients is of limited value for any meaningful biomarker discovery. As shown on miR-134, the direct influence of ASDs could be a significant confounding factor in miRNA level differences. Moreover, for any miRNA to be shifted forward to clinical practice, potentially collaborative efforts must be made to validate the results on larger cohorts, including not only drug-resistant patients but also drug-responsive controls and multiresistant preclinical models.

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CONFLICT OF INTEREST

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

AUTHOR CONTRIBUTIONS

All authors contributed to the concept, development, and evaluation of the manuscript and approved the final version.

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Annex 3

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Case Report

Autosomal dominant temporal lobe epilepsy associated with heterozygous reelin mutation: 3 T brain MRI study with advanced neuroimaging methods

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ABSTRACT

Purpose: Autosomal dominant lateral temporal epilepsy (ADLTE) is a genetic focal epilepsy syndrome characterized by focal seizures with dominant auditory symptomatology. We present a case report of an 18-year-old patient with acute onset of seizures associated with epilepsy. Based on the clinical course of the disease and the results of the investigation, the diagnosis of ADLTE with a proven mutation in the RELN gene, which is considered causative, was subsequently confirmed. The aim of this study was to use 3 Tesla (3 T) magnetic resonance imaging (MRI) and advanced neuroimaging methods in a patient with a confirmed diagnosis of ADTLE.
Methods: 3 T MRI brain scan and advanced neuroimaging methods were used in the standard protocols to analyzse voxel-based MRI, cortical thickness, and functional connectivity.
Results: Morphometric MRI analysis (blurred grey-white matter junctions, voxel-based morphometry, and cortical thickness analysis) did not provide any informative results. The functional connectivity analysis revealed higher local synchrony in the patient in the left temporal (middle temporal gyrus), left frontal (supplementary motor area, superior frontal gyrus), and left parietal (gyrus angularis, gyrus supramarginalis) regions and the cingulate (middle cingulate gyrus) as compared to healthy controls.
Conclusions: Evidence of multiple areas of functional connectivity supports the theory of epileptogenic networks in ADTLE. Further studies are needed to elucidate this theory.

1. Introduction

Autosomal dominant lateral temporal epilepsy (ADLTE), also known as autosomal dominant partial epilepsy with auditory features (ADPEAF), is a genetic focal epilepsy syndrome. It is characterized by focal seizures with or without a loss of consciousness, inconstantly with secondary generalization. Focal seizures are mainly characterized by auditory symptoms. Auditory auras are the most common symptom, and occur in isolation or precede some kind of receptive aphasia. Other symptoms following the auditory phenomena include vertigo, paroxysmal headache, déjà-vu, and epigastric discomfort [2]. Sensory symptomatology (e.g., visual, olfactory) and autonomic motor symptomatology are less common. Neurological findings and the mental status of patients are normal. The manifestation of the syndrome occurs between the ages of four and 50 years, with the maximal occurrence in the adolescent period [3]. Structural examinations of the brain (CT, MRI) at standard resolutions most often return normal findings. Routine and sleep electroencephalography (EEG) may be normal, but findings of focal/slow wave abnormality in the temporal areas are not uncommon, occurring in approximately 20% of patients [2,3]. The disease heredity is autosomal dominant with varying penetration (about 70%) [1]. The diagnosis is based on personal and family history, seizure semiology, and normal MRI brain scan. Approximately 33% of patients show a pathogenic variant in the LGI1 gene [2]. In a smaller percentage of ADLTE cases, mutation in the reelin (RELN) gene is shown in heterozygous

Abbreviations: ADLTE, Autosomal dominant lateral temporal epilepsy; ADPEAF, Autosomal dominant partial epilepsy with auditory features; LGI1, Leucine-rich, glioma inactivated 1; RELN, Reelin; MRI, Magnetic resonance imaging; CT, Computer tomography; EEG, Electroencephalography; CBZ, Carbamazepine; CLB, Clobazam; 3 T, three Tesla; 1.5 T, one Tesla; TLE, Temporal Lobe Epilepsy; HDEEG, high density resting-state EEG; GMC, grey matter concentration; GMV, grey matter volume; WMC, white matter concentration; WMV, white matter volume; LS, Local synchrony.

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form [2]. The RELN gene is primarily expressed in brain tissue. The protein product of the RELN gene is called reelin. Reelin regulates the correct formation of laminated structures during embryonic development and postnatally modulates dendritic growth and synaptic plasticity [2]. Homozygous variants of the RELN mutation cause lissencephaly with cerebellar hypoplasia, severe neuronal migration defects, delayed cognitive development, and epileptic seizures [5]. Heterozygous mutation of the RELN mutation can cause small changes in the cortex corresponding to neuronal migration disorders [2]. The prognosis of the disease is benign and, in most cases, there is a very good response to treatment with properly selected anti-seizure drugs (valproate, phenytoin, and carbamazepine are recommended).

2. Case report

We present a case report of an 18-year-old man who was admitted to the Department of Pediatric Neurology of the University Hospital Brno in 2017. According to his personal history, he was born in the 32nd week of gestation (the reason is unknown) and his psychomotor development was normal. The family history showed no neurological disease or epilepsy. At the age of 17, the patient suddenly began to experience epileptic seizures without clear provocation. The clinical manifestation was dominated by focal auditory seizures, without the loss of consciousness. The seizures (auras) were described as a short-term loss of hearing and simultaneous sensations of warmth lasting up to 30 s. The auras consistently progressed into focal seizures with right-handed facial-brachial motor symptomatology. Focal to bilateral tonic-clonic seizures occurred inconsistently. The seizures were daily, and the frequency at initial onset was very high. The auditory and vegetative auras occurred several times a day and convulsive seizures following auras occurred five to six times per week. On admission, the patient was already being treated with valproic acid monotherapy at a total dose of 1500mg per day with a suitable serum concentration. This medication had no significant effect. A CT and MRI (1.5 T) scan, performed at another institution, were described as normal. A routine EEG showed a non-specific finding of theta activity in the left fronto-centro-temporal region. The patient was admitted to our department urgently after a generalized tonic–clonic seizure. A complete neurological examination and routine laboratory tests as well as EEG returned normal findings. Due to the clinical course of the disease and the seizure semiology, a genetic examination with high suspicion for ADLTE (a requirement for the LGI1 gene and the RELN gene) was performed. For therapeutic purposes, the patient was switched from valproate to carbamazepine (CBZ), at a total dose of 600 mg per day, with a partial effect on seizures (there was a reduction in seizure intensity, not a reduction in frequency). Clobazam (CLB) was added to the carbamazepine at a dose of 40 mg per day, with a pronounced effect on seizures: convulsive seizures disappeared and auditory seizures decreased to 20%. Overall, the patient's quality of life improved, as reported by the patient and his family.

The causal mutation in the RELN gene (c.877G>A p. (Asp293Asn)) in the heterozygous state was confirmed. Despite the patient's negative family history, an investigation of the patient's parents' DNA was recommended. An identical mutation was found in the patient's mother through genetic testing. The patient's mother's EEG was normal and further treatment of the mother was not pursued.

3. Methods

The magnetic resonance data was acquired on a 3 T Siemens Prisma machine. The protocol contained: T1 MPRAGE, T2 FLAIR, T1 MP2RAGE; T2 TSE; T2 FLASH; T1 TIR; T2 TIRM; T1 TIR; T2 TIRM; and T2 TSE sequences.

The high density resting-state EEG (HDEEG) data was acquired using the GES 400 amplifier (Electrical Geodesics, Inc.) with a 256-channel EEG cap. The subject was instructed to sit still with closed eyes during 20 min of recordings.

4. Image analysis

4.1. Morphometry

The T1 MPRAGE and T2 FLAIR images were preprocessed using the SPM12 and CAT12 toolbox (http://dbm.neuro.uni-jena.de/cat/index.html) running under MATLAB (Mathworks, Inc.). We obtained images showing the spatial distribution of the local grey matter volume/concentration (GMV/GMC). The resulting GMV and GMC images were voxel-wise compared to a set of GMV/GMC images and white matter volume/concentration (WMV/WMC) images acquired with the same MR protocol and resulting from the same preprocessing process applied to data from healthy subjects (HC) (N = 48). The data were intensity normalized by estimating the total intracranial volume to correct for bias introduced by variability in head size [6]. The GMC and WMC images were used to localize abnormalities in grey/white matter junctions [7]. The patient's junction image was compared with the HC using a two-sample T-test with age and gender as nuisance covariates.

The CAT12 output contains an estimate of cortical thickness [13], which makes it possible to localize abnormalities in grey matter with higher sensitivity than VBM. The patient data were compared to HC data in the same way as junctions and GMC/GMV images.

4.2. Functional connectivity

The HDEEG data were segmented into 1 s epochs. We select 300 epochs with clean EEG. The sensor-space data from 256 channels were projected using sLORETA into the source space using Cartool [9]. Using the Corrected Imaginary Coherence metric, we estimated the spatial distribution of local synchrony (LS) in source-space. The increased local synchrony was shown to be a potential marker of epileptogenicity [10]. We compared the patient's LS image with LS images from 26 healthy controls using a two-sample T-test.

5. Results

5.1. MRI findings

Brain 3 T MRI was used with our patient. We found discrete changes (subtle cortical thickness in T2-weighted sequences and very mild decrease of signal intensity in T1-weighted sequences) in the left superior temporal gyrus on 3 T MRI. Subtle cortical dysplasia in this site was. Consequently, advanced neuroimaging methods (voxel-based 3D MRI analysis, cortical thickness analysis, and functional connectivity) were used. Morphometric MRI analysis (blurred grey-white matter junctions, voxel-based morphometry, and cortical thickness analysis) did not provide any informative results. The functional connectivity analysis revealed higher local synchrony in the left temporal (middle temporal gyrus), left frontal (supplementary motor area, superior frontal gyrus), left parietal (gyrus angularis, gyrus supramarginalis) region and the cingulate (middle cingulate gyrus) gyrus of the patient as compared to healthy controls (See Fig. 1 and Table 1).

6. Discussion

We present a case of an 18-year-old patient with an acute manifestation of severe epileptic seizures that had a significant impact on the quality of life of the patient and his family. The age at seizure onset, clinical course of the disease, seizure semiology and the results of the paraclinical examinations indicated possible ADLTE. The diagnosis was supported by genetic testing, which revealed a heterozygous mutation in the RELN gene. In 30–50% of cases, ADLTE is caused by a mutation in the LGI1 gene; around 17% of patients carry a mutation in the RELN gene [2,3]. Nearly 50% of patients are not tested for causal mutation. Due to the similar clinical course of the disease in both mutations, it is recommended to test for both genes for ADLTE in suspected patients

[2]. To our knowledge, seven families have thus far been identified with a proven mutation in the RELN gene [1,2]. Dazza et al. reported that the dominant type of focal auditory seizures is present in 71% of patients. However, these seizures occur mostly at a low frequency (weekly or yearly). Seizure freedom was achieved with the first antiepileptic drug in 63% of patients; 31% of patients continued to experience sporadic auditory auras on established antiepileptic therapy. Tonic–clonic seizures disappeared in all studied patients [1]. The patient in this case report was not seizure free even after trying several anti-seizure drugs. He continued to experience sporadic auditory auras. However, the quality of life of the patient and the whole family has improved. As a significant success of therapy, the patient reports that the seizure frequency has decreased to 20%, and the tonic–clonic seizures have disappeared on the combination of CBZ and CLB. The patient's EEG was abnormal only at the onset of the disease. Dazza et al. observed routine and/or sleep EEG revealing epileptiform abnormality or deceleration in 12 patients out of a total of 15 (80%); 20% of those patients had normal EEG or non-specific abnormalities [1]. The family history of some individuals with ADLTE may appear negative due to the early unrelated death of a parent, later manifestations of epilepsy (perhaps manifested in the 50th year of life), or reduced penetration. Approximately 33% of patients with a pathogenic variant of the gene remain asymptomatic [3]. If the genetic examination in the proband parents is negative, there are two explanations for the result: germinal mosaicism in the parents or de novo mutation in the proband. The possibility of de novo mutation in this type of epilepsy is assumed to be less than 1% [3].

The homozygous form of mutation in the RELN gene can cause serious brain damage, such as lissencephaly and cerebellar hypoplasia, as well as severe neuronal migration disorders. It can thus be assumed that in the heterozygous form of mutation, small changes in the cortex corresponding to neuronal migration disorders may result [2]. These

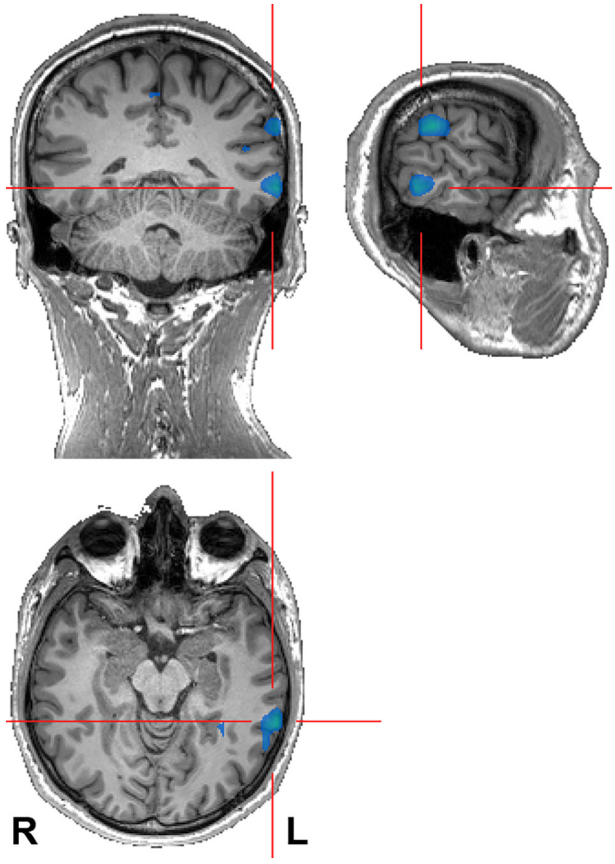

Fig. 1. The regions showing increased local synchrony in the patient as compared to HC (N = 26; p < 0.001).

Table 1
The regions that show increased local synchrony in the patient as compared to the HC (N = 26; p < 0.001). The underlined regions are depicted in Fig. 1.

Region	# voxels	Z-value[a]	Coordinate[a] [mm]
L SMA	19	7.02	−6 18 72
L supramarginal g	14	6.12	−66 −30 30
L angular g	40	5.84	−48 −66 42
L middle temporal g	13	5.31	−66 −48 −6
L middle cingulate g	31	5.26	−6 12 36
R superior frontal g	5	4.29	24 36 54

[a] Cluster maximum; L – left; g – gyrus; SMA – supplementary motor area; voxel size = (6 × 6 × 6) mm^3.

subtle changes cannot be detected on commonly available low resolution MRI devices. Brain 3 T MRI and advanced neuroimaging methods (voxel-based MRI analysis, cortical thickness analysis, and functional connectivity) were used with our patient. The 3 T MRI findings in the left superior temporal gyrus were felt to be insignificant . Advanced neuroimaging methods including morphometric MRI analysis (blurred grey-white matter junctions, voxel-based morphometry, and cortical thickness analysis) did not provide any informative results. The functional connectivity analysis revealed higher local synchrony in the left temporal, left frontal, and left parietal regions and the cingulate when the patient was compared to healthy controls (see Fig. 1 and Table 1).

The evaluation of brain networks using functional connectivity fMRI is a relatively new technique that has been used successfully to identify brain networks in several conditions, including autism, depression, and schizophrenia [11]. Evidence of multiple areas of functional connectivity confirms the theory of epileptogenic networks in ADTLE. Connectivity abnormalities have potential for clinical relevance and correlation. They may assist with diagnosis, they may provide insights into neurologic deficits associated with TLE, and they may benefit invasive treatments through a more accurate understanding of the functional anatomy of TLE [11]. The epileptogenic network concept is a key factor in identifying the anatomic distribution of the epileptogenic process, which is particularly important in the context of epilepsy surgery [12].

Ethical publication statement

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Disclosure

None of the authors has any conflict of interest to disclose.

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Annex 4

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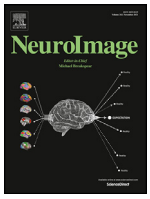

Tractography dissection variability: What happens when 42 groups dissect 14 white matter bundles on the same dataset?

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ABSTRACT

White matter bundle segmentation using diffusion MRI fiber tractography has become the method of choice to identify white matter fiber pathways *in vivo* in human brains. However, like other analyses of complex data, there is considerable variability in segmentation protocols and techniques. This can result in different reconstructions of the same intended white matter pathways, which directly affects tractography results, quantification, and interpretation. In this study, we aim to evaluate and quantify the variability that arises from different protocols for bundle segmentation. Through an open call to users of fiber tractography, including anatomists, clinicians, and algorithm developers, 42 independent teams were given processed sets of human whole-brain streamlines and asked to segment 14 white matter fascicles on six subjects. In total, we received 57 different bundle segmentation protocols, which enabled detailed volume-based and streamline-based analyses of agreement and disagreement among protocols for each fiber pathway. Results show that even when given the exact same sets of underlying streamlines, the variability across protocols for bundle segmentation is greater than all other sources of variability in the virtual dissection process, including variability within protocols and variability across subjects. In order to foster the use of tractography bundle dissection in routine clinical settings, and as a fundamental analytical tool, future endeavors must aim to resolve and reduce this heterogeneity. Although external validation is needed to verify the anatomical accuracy of bundle dissections, reducing heterogeneity is a step towards reproducible research and may be achieved through the use of standard nomenclature and definitions of white matter bundles and well-chosen constraints and decisions in the dissection process.

1. Introduction

Diffusion MRI fiber tractography (Xue et al., 1999, Conturo et al., 1999) offers unprecedented insight into the structural connections of the human brain. In a process that parallels post-mortem microdissection, tractography – in combination with a set of rules, constraints, and procedures to dissect and segment major white matter fascicles of the brain – allows noninvasive visualization and quantification of the shape, location, connectivity, and biophysical properties of white matter bundles. This process of *in vivo* "virtual dissection" (Catani and Thiebaut de Schotten, 2008, Catani et al., 2002), also called *bundle segmentation*, has led to new insight into how structural connectivity underlies brain function, cognition, and development, in addition to dysfunction in neurological diseases, mental health disorders, and aging (Le Bihan and Johansen-Berg, 2012). Additionally, bundle segmentation is used routinely to provide critical clinical information in both pre-operative and intra-operative mapping of brain tumor resections (Essayed et al., 2017, Vanderweyen et al., 2020).

Despite widespread use in clinical and research domains, there are a large number of variations in workflows for bundle segmentation that have been adopted by the neuroimaging community (Fig. 1). Normally, workflows either generate bundles of streamlines, i.e., digital representations of fiber trajectories, or dissect subsets of streamlines from an ensemble of streamlines throughout the whole brain. These protocols typically differ in the rules and constraints used to isolate a given pathway, ranging from manual delineation of inclusion and exclusion regions of interest, to fully automated segmentations based on shape, location, or connectivity. Contributing to this variability, agreements on the anatomical definitions of pathways in the human brain are far from settled (Forkel et al., 2014, Mandonnet et al., 2018, Panesar and Fernandez-Miranda, 2019, Bajada et al., 2015), in part hindered by the lack of a consistent framework for defining tracts. Descriptive tract definitions have traditionally focused on the shape and area of convergence of axons deep in the white matter, but may also focus on the specific regions to which these fibers connect (Mandonnet et al., 2018, Bajada et al., 2015, Bajada et al., 2017, Carpenter and Sutin, 1983, Nieuwenhuys et al., 2008, Schmahmann et al., 2007). Consequently, and coming full circle, differences and disagreements in anatomical definitions and their interpretation may lead to further variations in protocols used in the virtual dissection process.

For these reasons, the process of bundle segmentation has been described as existing somewhere between science and art (Schilling et al., 2019). Variation in protocols can result in different segmentations which can lead to different scientific conclusions or clinical decisions (Pujol et al., 2015). This inter-protocol variability adds "noise" to the literature when it comes to the process of bundle segmentation (Rheault et al., 2020, Botvinik-Nezer et al., 2020), a variability that prevents a direct comparison of the outcomes of different studies, and hinders the translation of these techniques from the research laboratory to the clinic. Yet, an estimate of the variability that exists across different protocols remains unclear. In order to ultimately harmonize the anatomical definition of tracts and standardize the bundle segmentation process, we propose a first step is to quantify this variability, and understand the similarities and differences in bundle segmentation results across protocols.

There have been many works that benchmark or validate the anatomical accuracy of tractography, typically comparing against simulated data (Daducci et al., 2014, Neher et al., 2015, Maier-Hein et al., 2017), physical phantoms (Guevara et al., 2012, Perrin et al., 2005), animal tracer studies (Schilling et al., 2019, Donahue et al., 2016, Girard et al., 2020, Grisot et al., 2021, Schmahmann and Pandya, 2006), or cadaveric dissections (Forkel et al., 2014, Lawes et al., 2008, Sarubbo et al., 2013, Maffei et al., 2018, Hau et al., 2017). These have led to insight into the challenges and limitations of tractography, including the presence of false positive and false negative pathways and subsequent sensitivity/specificity tradeoff in accuracy (Maier-Hein et al., 2017, Schilling et al., 2019, Thomas et al., 2014, Aydogan et al., 2018, Knösche et al., 2015), and the presence of biases (Rheault et al., 2020) due to pathway shape and location (Yeh et al., 2016), anatomy (Schilling et al., 2018, Reveley et al., 2015), and processing decisions (Girard et al., 2014). Importantly, differences and variability in results are expected due to differences in acquisition (Ambrosen et al., 2020), pre-processing (Maier-Hein et al., 2017, Cote et al., 2013), orientation reconstruction (Li et al., 2012), and the tractography approach/algorithm (Donahue et al., 2016, Cote et al., 2013, Smith et al., 2020, Smith et al., 2012, Bastiani et al., 2012). However, variability due to differences in protocols for segmenting specific white matter pathways has not been thoroughly investigated. Here, we ask "what happens when many groups attempt to dissect the same white matter bundles on the same tractography dataset" in order to isolate and quantify variability in the tractography dissection process. This variation represents differences that may occur when different groups segment and study the *same* major white matter pathways of the brain, even if all other sources of variation are removed.

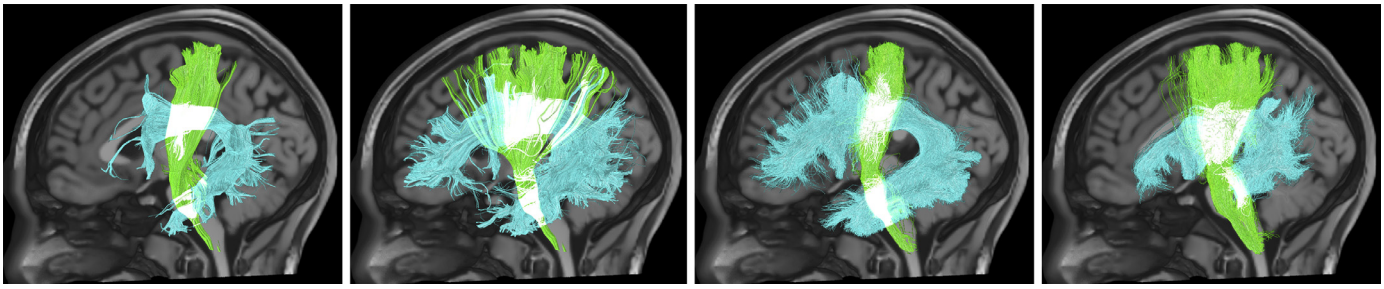

Fig. 1. Variation in white matter bundle segmentation. Four example segmentations of the corticospinal tract (green) and arcuate fasciculus (cyan) show variability in the size, shape, densities, and connections of these reconstructed white matter pathways.

Towards this end, the aims of this study are twofold: (1) to understand how much variability exists across different protocols for bundle segmentation, and (2) to quantify which fascicles exhibit the most agreement/disagreement across protocols. To do this we take a "many analysts, one dataset" approach previously used to study workflows for diffusion analysis (Jones et al., 2007), hippocampus segmentation (Boccardi et al., 2011), fMRI analysis (Botvinik-Nezer et al., 2020, Poline et al., 2006), and psychology research (Silberzahn et al., 2018). Through an open call to the community, we invited collaborations from expert scientists and clinicians who use tractography for bundle segmentation, provided them all with the same sets of tractography streamlines, and gave them the task of segmenting 14 white matter pathways from each dataset. This enabled streamline-based and volume-based quantification of inter-protocol agreement and disagreement for each fiber pathway and the results highlight the problem of variation of definitions and protocols for bundle segmentation.

2. Results

2.1. Submissions

We surveyed the protocols for bundle segmentation of 14 white matter bundles: Superior Longitudinal Fasciculus (SLF), Arcuate Fasciculus (AF), Optic Radiation (OR), Corticospinal Tract (CST), Cingulum (CG), Uncinate Fasciculus (UF), Corpus Callosum (CC), Middle Longitudinal Fasciculus (MdLF), Inferior Fronto-Occipital Fasciculus (IFOF), Inferior Longitudinal Fasciculus (ILF), Fornix (FX), Anterior Commissure (AC), Posterior Commissure (PC), and Parieto-Occipital Pontine Tract (POPT).

To isolate the effects of bundle segmentation from all other sources of variation, we directly provided six sets of whole-brain streamlines (both deterministic and probabilistic) to all collaborators, derived from 3 subjects with scan-rescan data acquired from the Human Connectome Project test-retest database (Glasser et al., 2016). Collaborators were given the choice of utilizing streamlines generated from one of two commonly used tractography methods, a deterministic or a probabilistic algorithm, which are known to generate different representations of white matter bundles and have different uses and applications as described in the literature (Pestilli et al., 2014, Sarwar et al., 2019).

In total, this collaborative effort involved 144 collaborators from 42 teams (Fig. 2, top). 57 unique sets of protocols were submitted, of which 28 submissions used the deterministic streamlines and 29 used probabilistic. A total of 3138 bundle tractograms were submitted. Because collaborators did not have to submit all bundles, pathways showed varying representation across submissions (Fig. 2, bottom), ranging from as low as 16 protocols for the PC, up to 50 protocols for the CST.

A detailed description of all protocols, submitted by each of the 42 groups is provided as a Supplementary Table.

2.2. Qualitative results

Example visualizations of randomly selected segmentations from a single subject are shown for exemplar projection, association, and commissural pathways (CST, AF, CC) in Fig. 3. These are visualized as both streamlines directly, and also as 3D streamline density maps. The primary result from this figure is that there are many ways to segment these structures that result in qualitatively different representations of the same white matter pathways. These examples demonstrate visibly apparent variations in the size, shape, and connectivity patterns of streamlines. In contrast, different protocols result in similar patterns of high streamline density in the deep white matter and midbrain, with similar overall shape and central location. Similar visualizations, for all submitted pathways, both probabilistic and deterministic, are provided in supplementary documentation. These observations apply to all dissected pathways, however the commissural AC and PC contained very few streamlines, with little-to-no agreement across protocols.

2.3. Pathway-specific results

To understand the variability that exists across protocols for a given pathway, we visualize volume-based and streamline-based overlaps among the protocols and show boxplots of agreement measures that quantify inter-protocol, intra-protocol, and inter-subject variation. The volume overlap is displayed as the volume of voxels in which a given percent of protocols agree that the voxel was occupied by a given pathway, where a streamline overlap is displayed as the individual streamlines in which a given percent of protocols agree that streamline is representative of a given pathway. For quantitative analysis, we use several measures to describe similarity and dissimilarity of streamlines, streamline density, and pathway volume (Fig. 4). This includes (1) v*olume Dice overlap* which describes the overall volume similarity, (2) *density correlation* which describes insight into similarity of streamline density, (3) *bundle adjacency* which describes the average distance of disagreement between two bundles, and (4) *streamline Dice* which describes the overlap of streamlines common between protocols (which can only be calculated because bundles come from the same original set of streamlines). We calculate geometric measures of pathways including number of streamlines, mean length, and volume, as well as microstructural measures of the average fractional anisotropy (FA) of the entire pathway volume and the FA weighted by streamline density (wFA).

For simplicity, we show results of the CST, AF, and CC. Analysis was conducted on all tracts, and results are provided in supplementary documentation.

2.3.1. Corticospinal Tract (CST)

Fig. 5 shows the results for the CST, and Appendix A summarizes the descriptive definitions and decisions made in the bundle segmentation workflow. Looking at the volume of agreement on a single subject, nearly all methods agree on the convergence of axons through the internal capsule and midbrain, with some disagreements on cortical terminations, and only a minority of protocols suggesting lateral projections of this tract. Streamline-based agreements show similar trends. The most striking result is that there were not any streamlines which were common to at least 75% of either the deterministic or probabilistic protocols.

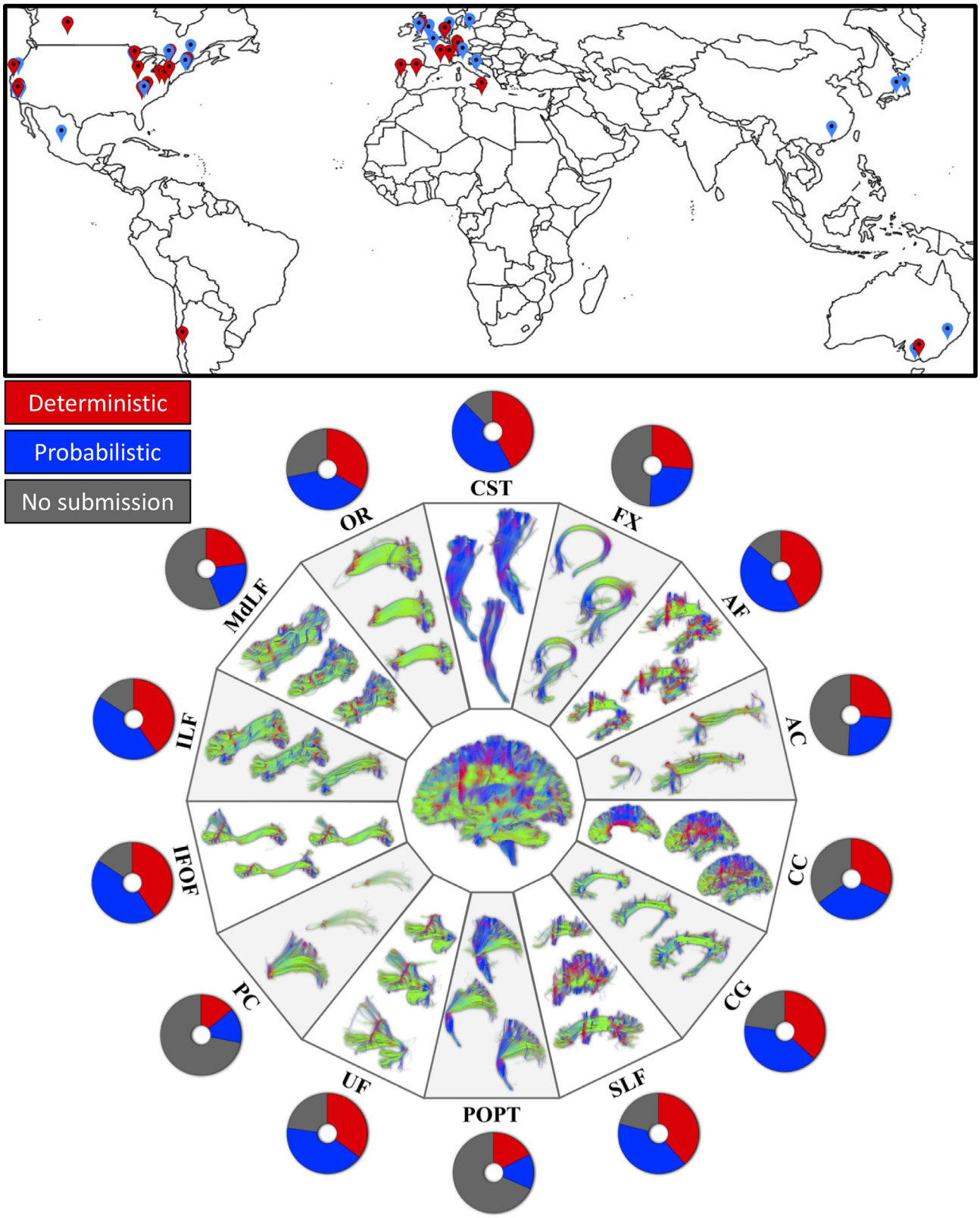

Fig. 2. Summary of teams and submissions. Location of the teams' affiliated lab (top). In total, 42 teams submitted 57 unique sets of bundle dissections, 28 utilized the provided deterministic streamlines, and 29 utilized probabilistic. Map icons are colored based on the set of streamlines utilized, with the same color-scheme as bar plots. Example submissions are shown for 14 pathways (bottom) along with a pie chart indicating the number of submissions for each bundle. Acronyms: see text.

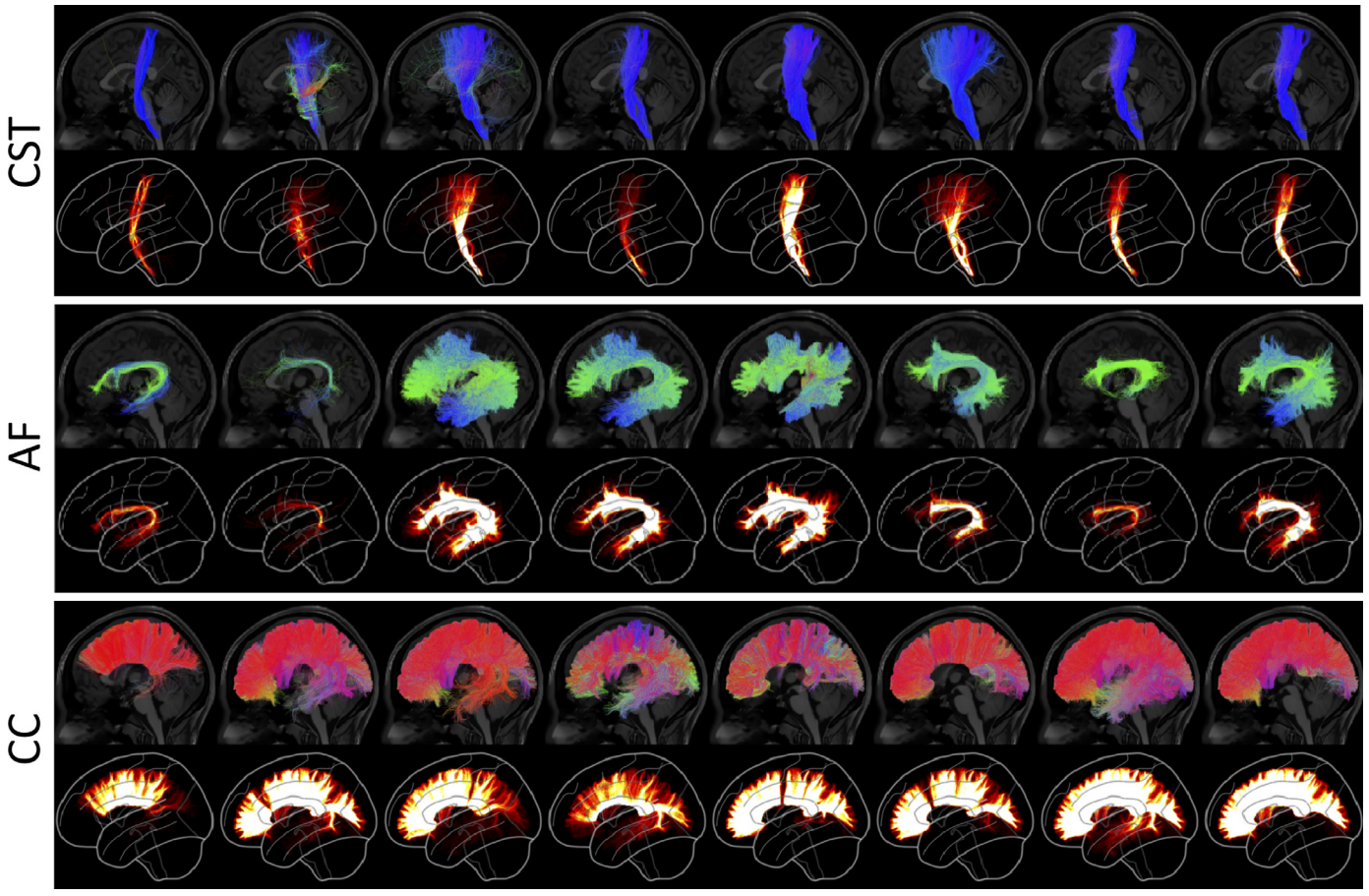

Fig. 3. Variation in protocols for bundle segmentation of example pathways (CST, AF, and CC) on the same subject from the same set of whole-brain streamlines. Eight randomly selected bundle segmentation approaches for each pathway are shown as segmented streamlines and rendered as 3D streamline density maps. Variations in size, shape, density, and connectivity are qualitatively apparent. Probabilistic streamlines are shown, see supplementary material for Deterministic submissions. Random selections generated independently for each pathway. Streamlines are colored by orientation and all density maps are windowed to the same range.

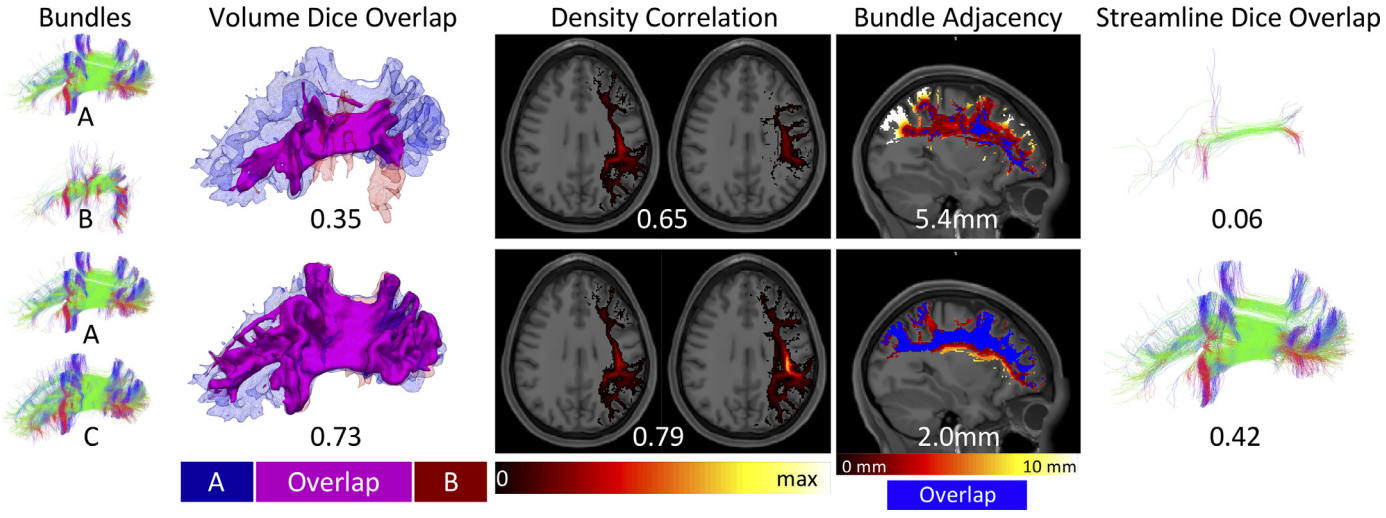

Fig. 4. Similarity and dissimilarity metrics to assess reproducibility. Example SLF datasets are used to illustrate a range of similarity values between bundles A and B (top) and between bundles A and C (bottom). Dice overlap is a volume-based measure calculated as twice the intersection of two bundles (magenta) divided by the union (red and blue). Density correlation is calculated as the correlation coefficient between the voxel-wise streamline densities (shown as a hot-cold colormap ranging from 0 to maximum streamline density) of the two bundles being compared. Bundle adjacency is calculated by taking the average distance of disagreement (not including overlapping voxels in blue) between bundles (distances shown as hot-cold colormap). Finally, streamline Dice is taken as the intersection of common streamlines divided by the union of all streamlines in a bundle and requires input bundles to be segmented from the same set of underlying streamlines (intersection shown in figure).

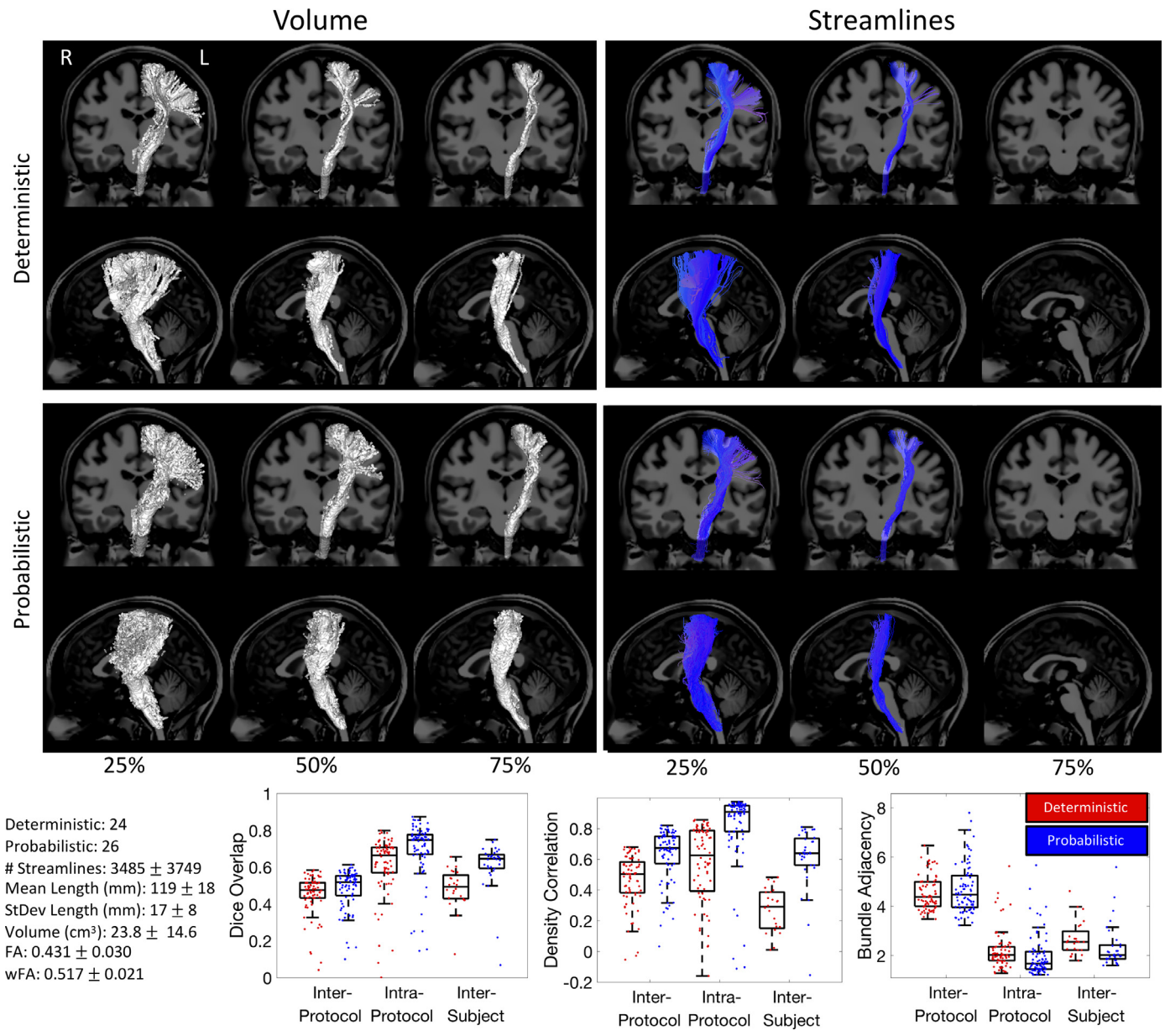

Fig. 5. Corticospinal Tract (CST) inter-protocol variability. Renderings show 25%, 50%, and 75% agreement on volume and streamlines for deterministic and probabilistic tractograms. Box-and-whisker plots of Dice overlap, density correlation, and bundle adjacency quantify inter-protocol, intra-protocol, and inter-subject variability (deterministic: red; probabilistic: blue). Each data-point in the plots is derived from the summary statistic of a single submission. Note that there were no streamlines which were common to at least 75% of the protocols.

Quantitative analysis indicates fairly low agreement across protocols. Inter-protocol Dice overlap coefficients largely fall between 0.4 and 0.6 (median Dice of 0.47 and 0.51 for probabilistic and deterministic, respectively), with a larger tail towards much lower Dice values indicating some outlier protocols that are substantially different from others. Protocols show moderate density correlation coefficients (median correlations of 0.51 and 0.67), and an average difference between protocols of >4mm (median bundle adjacency of 4.3mm and 3.9mm). Reproducibility within protocols is much higher, resulting in higher Dice coefficients, higher density correlations, and lower bundle adjacency. The variation across protocols is even greater than the variation across subjects when quantified using Dice overlap. However, the density correlation across protocols is higher than that across subjects, indicating that while the volume overlap decreases, measures of bundle density are more consistent across protocols. Finally, bundle adjacency is higher for inter-protocol analysis than inter-subjects, suggesting that volume-based differences across protocols are greater than volume-based differences across subjects. The quantitative index FA shows a coefficient of variation across protocols of 7% relative to its average value and the density weighted FA shows a variation of 4%.

2.3.2. *Arcuate Fasciculus (AF)*

Fig. 6 shows the results of the inter-protocol analysis for the AF, and Appendix B summarizes the descriptive definitions and decisions made in the bundle segmentation workflow. A majority of the extracted bundles agree on the volume occupied by the bundle, with both deterministic and probabilistic submissions showing the characteristic arching shape as the pathway bends from the frontal to temporal lobes. The volume of the 75% agreement is significantly smaller and much more specific than that of the 25% of agreement, occupying only the deep white matter core of this trajectory. Similar results are shown for streamlines. Very few streamlines were agreed upon by 75% of protocols for deter-

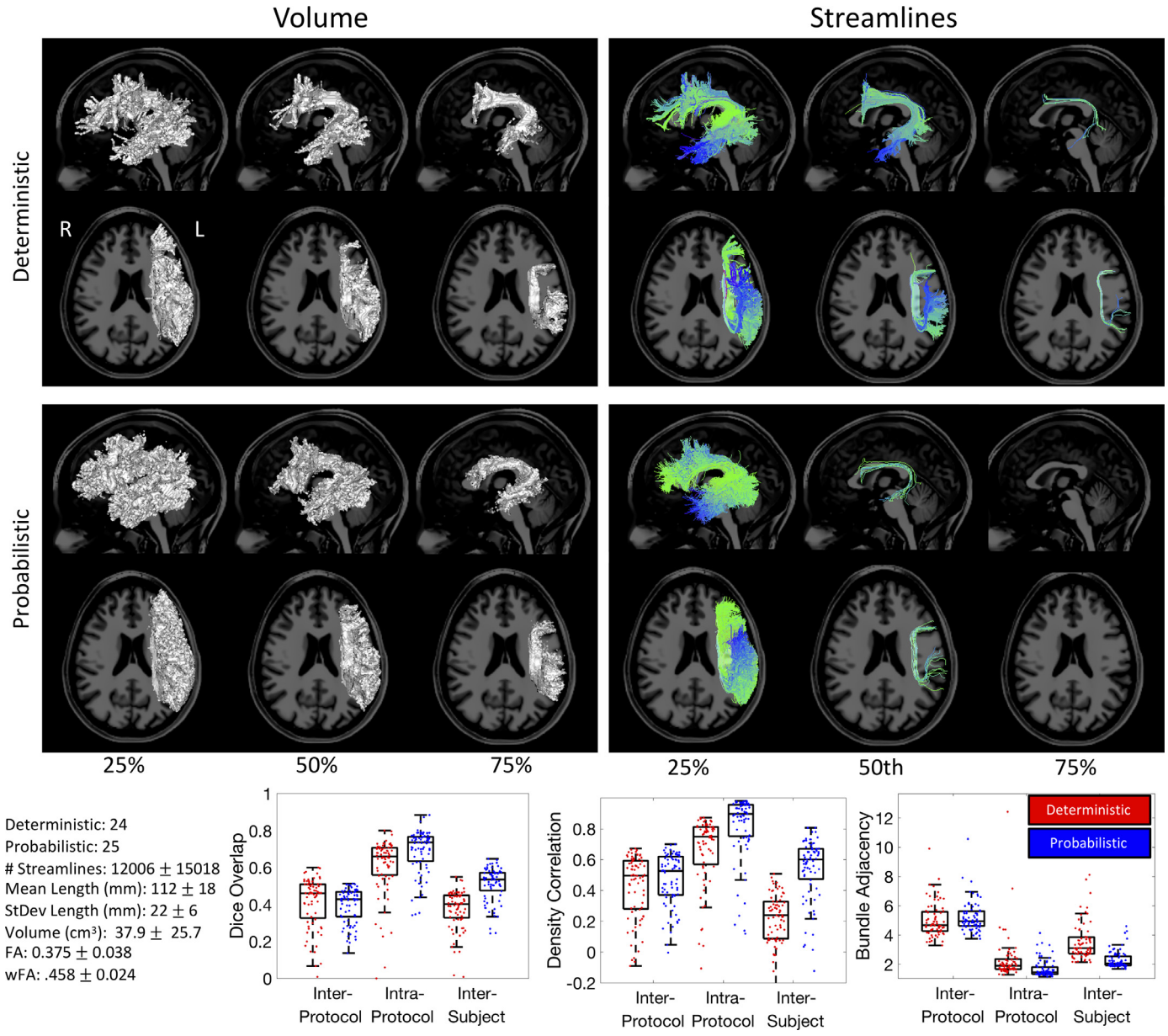

Fig. 6. Arcuate Fasciculus (AF) inter-protocol variability. Renderings show 25%, 50%, and 75% agreement on volume and streamlines for deterministic and probabilistic tractograms. Box-and-whisker plots of Dice overlap, density correlation, and bundle adjacency quantify inter-protocol, intra-protocol, and inter-subject variability (deterministic: red; probabilistic: blue). Note that there were no streamlines which were common to at least 75% of the protocols.

ministic tractography, and no single streamline was observed in 75% of probabilistic submissions. Cortical connections show significant variation. Qualitatively, as we become more strict with agreement, the connections become much more refined to the frontal and temporal lobes only, with fewer connections to the parietal cortex.

Quantitative analyses of similarity and agreement closely follow that of the CST. The Dice overlap indicates relatively poor inter-protocol agreement (median values 0.46 and 0.43 for probabilistic and deterministic, respectively), with a much higher intra-protocol agreement (median of 0.66 and 0.74). However, the inter-protocol overlap is similar to the variation across subjects (0.40 and 0.53). Similar trends are observed for density correlations. In this case, the inter-subject variation is lower than inter-protocol for deterministic, but higher for probabilistic, although both measures are lower than within protocol agreement. Finally, differences across protocols are on average >5mm of distance, whereas the disagreement is much less within protocols and even between subjects. Finally, the coefficient of variation of FA and wFA across protocols is 10% and 5% that of the average FA and wFA, respectively.

2.3.3. *Corpus callosum*

Fig. 7 shows the results of inter-protocol analysis of the CC, and Appendix C presents a summary of the descriptive definitions and decisions made in the bundle segmentation workflow. Most protocols generally agree that this structure takes up a large portion of the cerebral white matter in both hemispheres. While many streamlines were consistent across methods, when looking at the 75% agreement, many submissions do not include lateral projections – although they exist within the dataset – as well as fibers of the splenium (or forceps major) connecting to the occipital lobe and connections to temporal cortex.

Quantitative analysis shows much higher reproducibility than for the AF and CST, with mean Dice values across protocols of 0.66 and 0.72, which are again lower than intra-protocol reproducibility, but in this

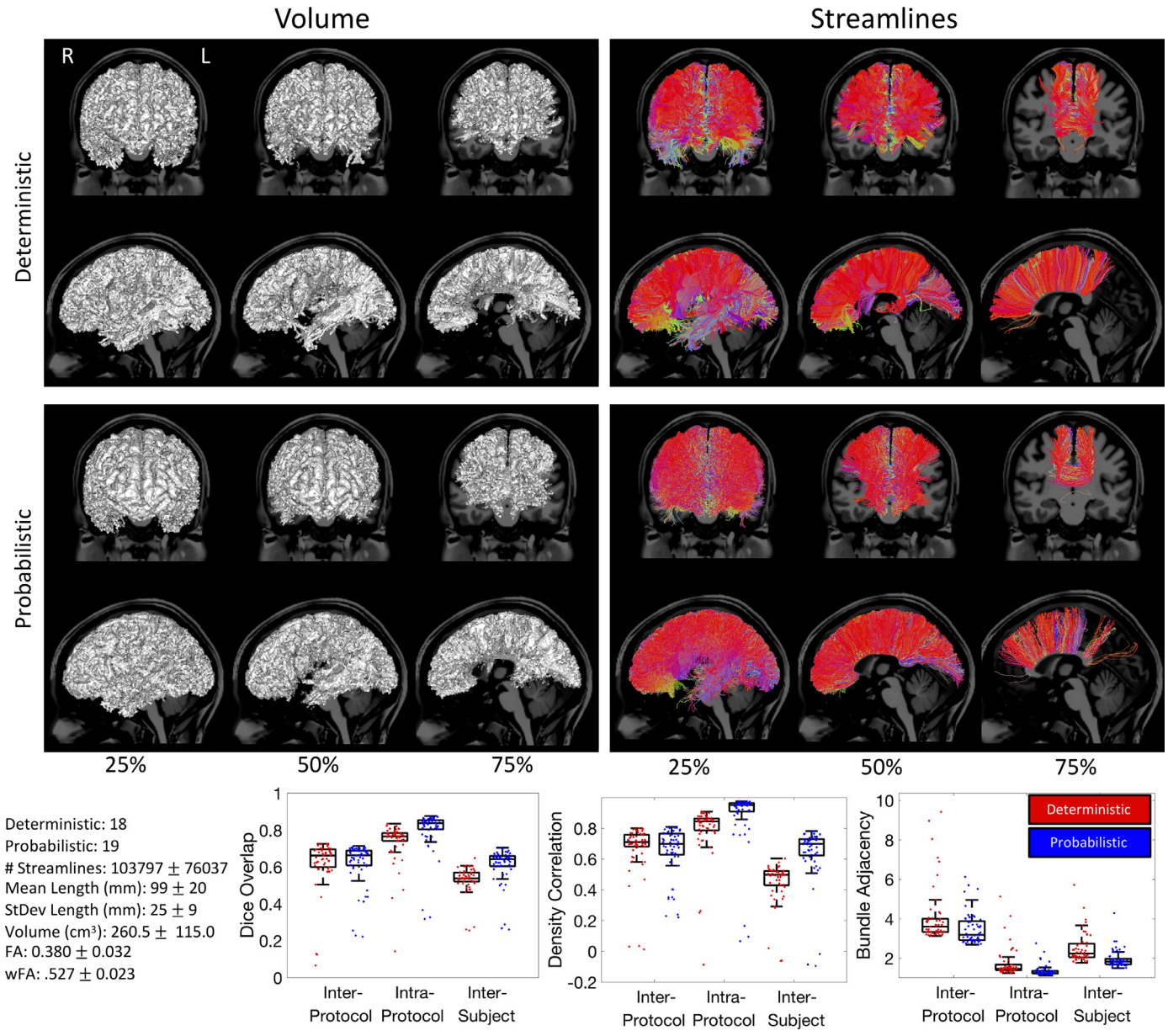

Fig. 7. Corpus callosum (CC) inter-protocol variability. Renderings show 25%, 50%, and 75% agreement on volume and streamlines for deterministic and probabilistic tractograms. Box-and-whisker plots of Dice overlap, density correlation, and bundle adjacency quantify inter-protocol, intra-protocol, and inter-subject variability (deterministic: red; probabilistic: blue).

case, both slightly higher than that across subjects. The density correlation shows similar trends. Finally, bundle adjacency is higher across protocols than across subjects, with measures indicating that disagreement is generally 3mm or greater across protocols. Even though this structure is quite expansive throughout the white matter, variation across quantitative FA measures are still on the order of 8% and 4% for FA and wFA, respectively.

2.4. Inter-protocol variability

To understand which pathways exhibit the most agreement/disagreement across protocols, intra-protocol volume-based variation measures of Dice overlap, density correlation, bundle adjacency, and Dice streamlines are plotted in Fig. 8.

There is a fairly large variation across pathways in the overall protocol agreement as measured by Dice volume overlap (Fig. 8A). Volume-wise, the most reproducible were the CC, the CST, and the IFOF. Reproducible results from the CC were expected due to its large size and unambiguous location of the CC proper, while the CST is arguably one of the most well-studied tracts. The IFOF, while one of the more controversial fasciculi (Forkel et al., 2014, Mandonnet et al., 2018, Altieri et al., 2019, Sarubbo et al., 2019), likely results in higher overlap because it is a long anterior-posterior directed pathway spanning from the occipital to frontal lobe, passing through the temporal stem, a tight and small bottleneck region (Hau et al., 2016) and most protocols agree that nearly any streamline spanning this extent through a ventral route, will belong to this pathway. In all cases, the overlap across protocols is fairly low, with median values of the CC of 0.66 and 0.72 being the highest among all pathways studied.

The least reproducible structures are those of the commissures, AC and PC, which are largely defined only by a single location along the midline with very little information on their routes or connections. The FX represented a unique case. Many groups submitted the left FX as expected, while others considered the left and right FX as a single struc-

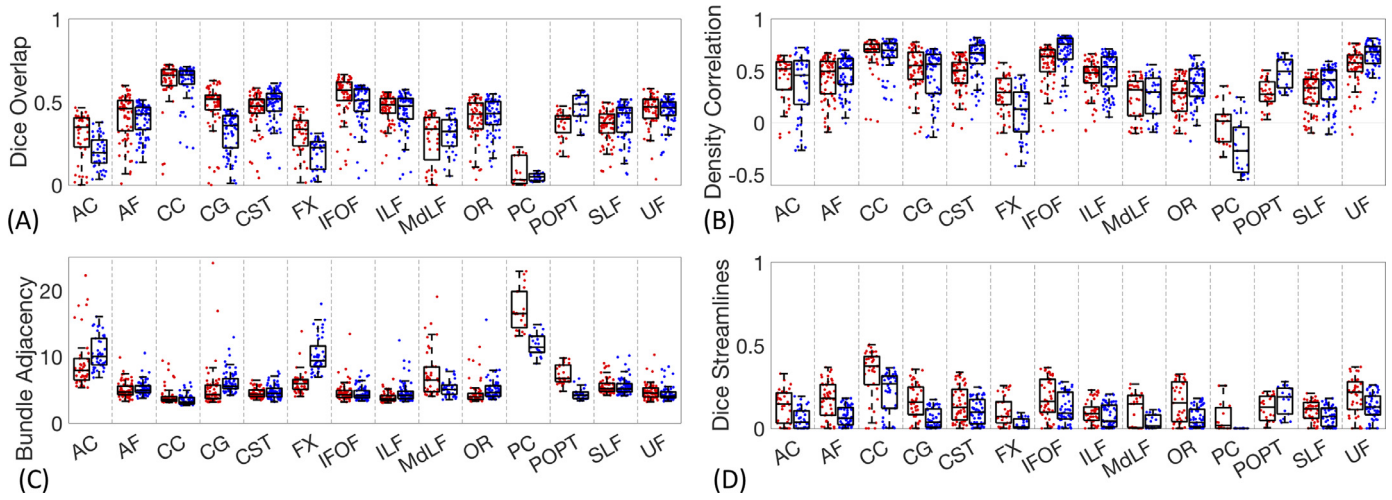

Fig. 8. Inter-protocol variability. Dice overlap coefficients, density correlation, bundle adjacency, and Dice streamlines for all studied pathways. Deterministic results shown in red, probabilistic in blue.

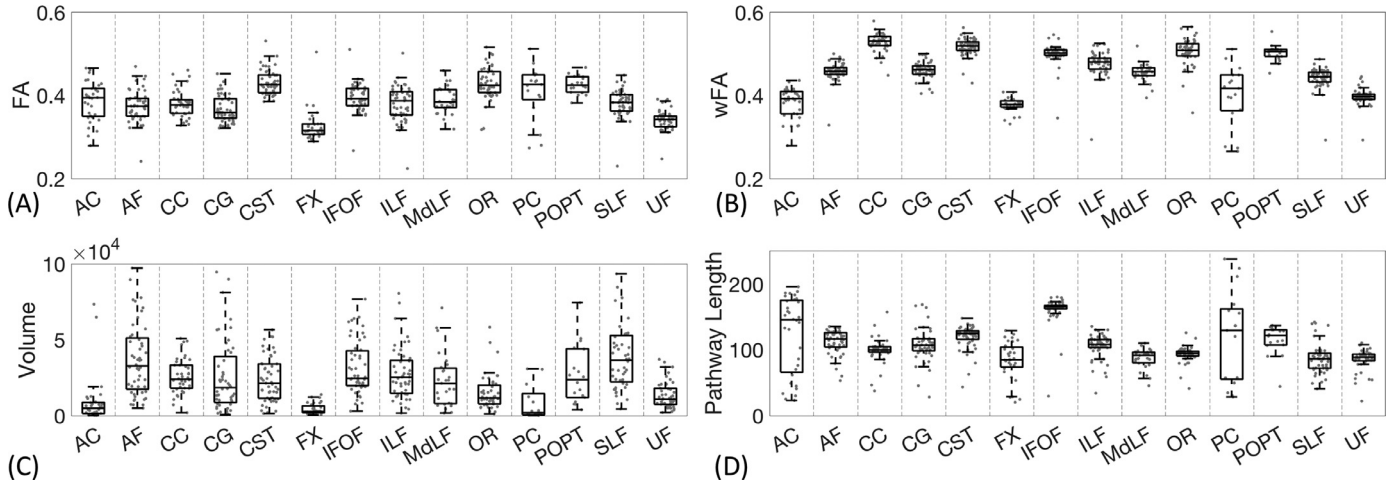

Fig. 9. Inter-protocol variation in mean FA, weighted-FA, volume (mm^3), and pathway length (mm) for all studied pathways. Note that CC volume is an order of magnitude larger than all other pathways and is shown on a 10^3 mm^3 scale.

ture due to its commissural component. Thus, while it is indeed a small structure, the quantitative value of overlap is overly critical based on qualitative observations.

In agreement with qualitative results, the density correlations (Fig. 8B) are moderate to high for most pathways, meaning that areas of high streamline density and low streamline density are generally in agreement across protocols. Pathways such as the CC, IFOF, CG, CST, and UF have high agreement in streamline densities, whereas pathways with generally lower number of streamlines and hence lower densities (i.e., PC, and FX) show lower density correlations.

Similar results are observed for dissimilarity (Fig. 8C). Again, AC, PC, show very large distances of disagreement, along with the FX and in this case the MdLF. For nearly all pathways, the range of disagreements across protocols are most typically on the order of 4-6mm. Looking at Dice overlap of the streamlines (Fig. 8D), it is immediately apparent that the overlap is very low in all cases, much lower than overlap of volume. For all pathways, a large majority of all comparisons yield streamline Dice coefficients less than 0.2, with many indicating no overlap at all. A trend observed in the streamline comparisons is that the overlap is generally greater for deterministic than probabilistic algorithms.

Fig. 9 shows protocol variability for pathway-specific measures of the mean fractional anisotropy, weighted fractional anisotropy, pathway volume, and pathway length across all protocols. In agreement with results on the CST, AF, and CC, the FA derived from different protocols varies by more than 8-12%, an effect greater than that observed in the literature across study cohorts (Landman et al., 2011, Farrell et al., 2007, Landman et al., 2007). Weighted-FA (wFA), however, varies much less across protocols (4-7%) and is of greater overall magnitude than the unweighted metric. The volume measurements show that different protocols can result in an order of magnitude difference in pathway volume, an effect observed for all pathways. Finally, pathways with more variation in average streamline length (Fig. 9) agree well with those with more variation in overlap measures. For example, AC, PC, and FX result in large differences in average length, while protocols on the IFOF consistently agree on the length of this structure.

2.5. Variability within and across pathways

To assess similarity and differences in submissions without a priori user-defined metrics of similarity, we utilized the Uniform Manifold Approximate and Projection (UMAP) (McInnes and Healy, 2007) technique to visualize all bundle segmentation techniques in a low-dimensional space. The UMAP is a general nonlinear dimensionality reduction that is particularly well suited for visualizing high-dimensional datasets, in this case, on a 2D plane. Fig. 10 shows all submissions, for all pathways, pro-

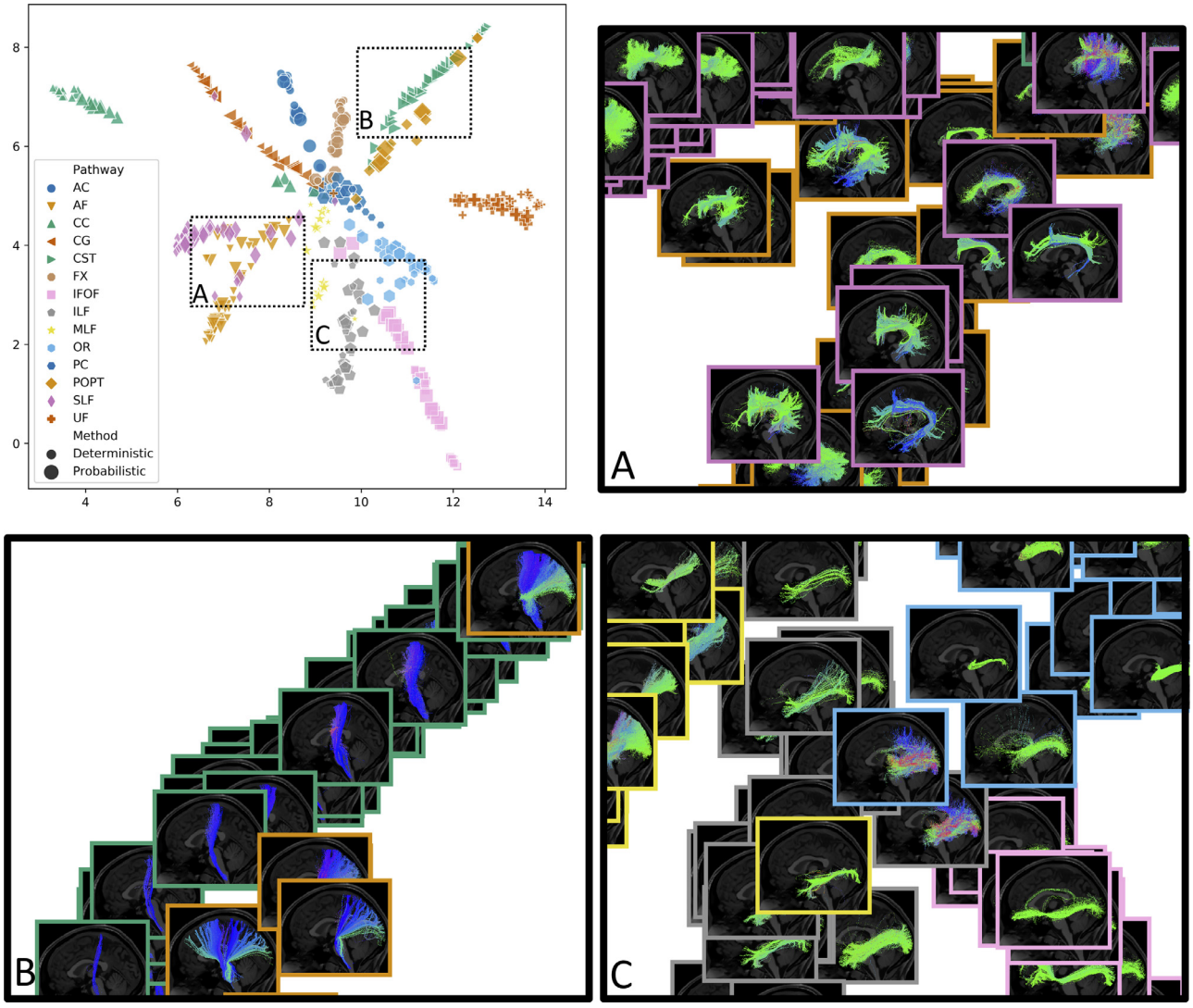

Fig. 10. UMAP dimensionality reduction projected bundles onto an un-scaled 2D plane. Object color and shape represent pathways, and object size designates deterministic/probabilistic. While variation exists within pathways and within deterministic/probabilistic streamlines, the white matter pathways generally cluster together in low dimensional space. Insets visualize data points as streamline renderings, and highlight areas where similarity and/or overlap is shown across different pathways.

jected on a 2D plane. While there are differences across protocols for a given pathway, all submissions for a given pathway generally cluster together and show similar low-order commonalities, for both probabilistic and deterministic. However, overlap between different pathways does occur in some instances, for example between the SLF and AF (Fig. 10, A), POPT and CST (Fig. 10, B), and MLF, ILF, and OR (Fig. 10, C). This suggests similar low-order representation of some submissions in these pathways.

3. Discussion

These results identify and quantify differences and the significant heterogeneity of white matter structures introduced by the use of different protocols for bundle segmentation with tractography. This variability may present difficulties interpreting differences in bundle segmentation results obtained by different labs, or meta-analyses extending and comparing findings from one study to other studies. Additionally, this variation in protocols can lead to variability in quantitative metrics that are greater than true biological variability across populations or subjects and may hinder translation of these techniques from the research laboratory to the clinic.

We propose that a major source of this variation stems from a lack of consensuses on the anatomical definition of pathways (Forkel et al., 2014, Mandonnet et al., 2018, Panesar and Fernandez-Miranda, 2019, Bajada et al., 2015). There is no standard framework for defining a tract, with some descriptive definitions focusing on the shape and locations of convergence of axons in the deep white matter, while others may focus on specific regions to which fibers connect (Mandonnet et al., 2018, Bajada et al., 2015, Bajada et al., 2017, Carpenter and Sutin, 1983, Nieuwenhuys et al., 2008, Schmahmann et al., 2007). Consequently, differences, misconceptions, and ambiguities in anatomical definitions and their interpretation may lead to different rules used in the dissection process. For example, workflows used to dissect a bundle range from manual to automated delineation of regions through which streamlines must pass, to shape-based, signal-based, or connection-based methods of segmentation. Importantly, the appropriateness and usefulness of the chosen reconstruction method is application dependent, and no single method is clearly wrong and/or better than the others.

This study was not intended to detract from the value of tractography and bundle segmentation, but rather the aim was to clearly define a current inherent problem and its scope. Looking forward, with a number of well-validated and valuable tools, pipelines, software, and processes

at our disposal, it becomes fairly straightforward to modify bundle segmentation protocols to match what we would ultimately strive for in a "consensus definition" of white matter bundles. Thus, instead of describing these results as revealing a problem, we see this as an opportunity, or a call-to-action to harmonize the field of bundle segmentation – both in the nomenclature and definition of white matter pathways, and in the best way to virtually segment these using tractography. Moreover, optimistically, it may be quite useful to have a supply of tools available to dissect and investigate the same white matter bundle in different ways depending on the research question, or the anatomy or functional system under investigation. We note that collaborative efforts have proven valuable to identify successes and limitations of tractography (Schilling et al., 2019, Pujol et al., 2015, Daducci et al., 2014, Maier-Hein et al., 2017, Schilling et al., 2019), and facilitate future improvements. Here, we pursue a different approach, focusing specifically on variability of the tractography dissection process when performed by different groups, rather than comparisons against simulations, phantoms, tracers, or prior knowledge.

3.1. What happens when 42 groups dissect the same dataset?

Our first main result is that *the inter-protocol agreement is generally poor across protocols for many pathways*, with limited agreement on the brain volume occupied by the pathway. With few exceptions, the average Dice coefficients from both deterministic and probabilistic streamlines were below 0.5, with many considerably lower. For most streamlines, the inter-protocol bundle adjacency is between 4-6 mm, meaning that when protocols disagree, they do so by an average of ~3-5 voxels. Shape and geometry-based measures (i.e., length and volume) of the streamline bundles vary by an order of magnitude across protocols. Consequently, quantitative metrics calculated based on this volume will vary, for example the average FA within a bundle varies by ~8-12% across protocols. Because our analysis was based on the same set of streamlines, these results represent a best-case measure of inter-protocol agreement, and would almost certainly result in increased variability if participants performed their own reconstruction and streamline generation procedures.

Our second main result is that *bundle segmentation protocols have better agreement in areas with high streamline densities*. Measures of streamline density correlation coefficients across submissions are on average >0.5, with few exceptions, which suggests that high density areas in tractograms generally correspond to high density areas of other tractograms, while low density areas correspond to low-density areas (or, in fact, regions with no streamlines). This agrees with observations of 3D density maps where areas of high streamline density are consistently observed in the same location across submissions. These areas of higher streamline density correspond to the core or stem of most of the bundles, generally located in the deep white matter of the brain. Because of this, weighting quantification by streamline density will reduce variability across protocols, for example, wFA varied by ~4-7% across protocols.

Third, we find that the *variability across protocols is greater than the variability within protocols*, and more importantly, similar to (or greater than) the variability across subjects. These results are in agreement with previous studies showing high overlap, high density correlations, and low disagreements *within* a protocol (Wakana et al., 2007, Nath et al., 2019, Rheault et al., 2020). Most importantly, in our study, this represents a worst-case intra-protocol measure. It includes sources of variability related to acquisition (and associated noise and artifacts), registration, reconstruction, and streamline generation – sources of variation which are shown to be still smaller than that across protocols. Thus, while there is little consensus on bundle dissection protocols, a study that uses a consistent protocol has been shown to have the power to reliably detect consistent differences within and across populations; however, there may be limitations in how the findings from a given study can be extended, applied, or compared to others with different protocols.

Fourth, we find that there is *variability per bundle in how much agreement there is across protocols*. The commissural CC has a higher reproducibility due to its large size and very clear anatomical definition, despite more ambiguous definitions of its cortical terminations. However, the PC and AC commissures showed very poor agreement, despite having a very clear location along the midline. This is in part due to smaller sizes, but also scarce literature on the location and connections of the bundles that pass through these regions. CST and IFOF also show moderate agreement across protocols, in part due to their length and at least one location that is moderately specific to these bundles (i.e., the pyramids of the medulla for the CST and the floor of the external capsules for the IFOF). Even here, the Dice overlap across protocols is 0.6 or less, on average. The MdLF and CG show relatively poor agreement. The MdLF is much less studied, and a relatively recent addition to the literature (Seltzer and Pandya, 1986, Makris et al., 2013), with some disagreement on parietal terminations (Bajada et al., 2015). The CG is a tract that is likely composed of both longer fibers extending throughout the whole tract, as well as multiple short fibers across its structure which may be both hard for tractography to entirely delineate the long fibers, and hard to capture and constrain segmentation of the shorter fibers that enter and leave throughout (Jones et al., 2013, Heilbronner and Haber, 2014). The POPT showed relatively higher agreement. This bundle was included as a relatively ambiguous nomenclature (seen in the literature) of pontine tracts. Whereas both occipito-pontine and parieto-pontine fibers exist, they are not usually defined as a specific tract or fasciculus. Finally, some of the more commonly delineated structures (OR, ILF, SLF, UF) show inter-protocol variabilities somewhere in between, but still exhibit poor-to-moderate volume and streamline overlaps.

For many applications, end-users of bundle segmentation technologies are interested in gross differences in connectivity and location, and what matters is not so much that tracts are reconstructed in their entirety, but that they are not confused with one another. For example, misunderstanding or inapt nomenclature, and/or non-specific constraints in the bundle segmentation process could lead to misidentification of the desired pathway (possibly as another pathway or subset of another pathway) and would lead to confusion in the literature. Based on our results, an experienced neuroanatomist or neuroimager can easily classify the submitted pathways based on visual inspection of the streamlines. Thus, these *inter-protocol bundle segmentations represent the same basic structure*, even if some variability in spatial extent and connections is observed. This is confirmed using an unsupervised data exploration tool for dimensionality reduction, where within-pathway submissions are clearly clustered (for both probabilistic and deterministic algorithms) in low dimensional space. However, there are a few exceptions. Notably, several AF and SLF submissions overlap significantly, which is not unexpected because these have often been defined and/or used interchangeably in the literature (Dick and Tremblay, 2012). Relatedly, several submissions of the POPT contain a subset of streamlines often assigned as CST, which is again expected because both are often (or can be) described as having parietal connections in common. Finally, several ventral longitudinal systems of fibers (MdLF, OR, ILF, and IFOF) are not clearly separated in this space, suggesting that in many instances they share similar spatial overlap and densities of streamlines across submissions.

Finally, while there is low volume-based agreement, streamline-based agreement is lower still. In fact, many protocols did not agree on a single streamline belonging to a pathway of interest. Protocols agreed on consistently 20% or less of deterministic streamlines and less than 10% of probabilistic streamlines. Put another way, given a set of streamlines from which to select, very few streamlines were consistently determined to be a part of a given pathway across all groups performing the segmentation. With the wide variety of workflows to select streamlines, few streamlines met inclusion criteria associated with cortical connectivity, shape and spatial location, and survived possible exclusion criteria such as filtering based on length, curvature, or diffusions signal, as well as personal preference of the person performing dissection (for example

eliminating streamlines to reduce complexity of manual segmentation). Thus, the final main result is that *the measured variability depends on the scale upon which the variability is analyzed*. Protocols show little-to-no agreement in assigning individual streamlines to a pathway, whereas protocols show higher agreement in assessing spatial overlap of pathway, and even higher agreement when taking into account density of streamlines over a volume. This means that while selected streamlines may occupy the same volume, the streamlines that make up this volume are different.Thus, *the effects of this variability are dependent upon how these bundles are ultimately utilized in practice*, and there are a number of ways in which these bundles are used and applied. For this reason, we state that no submissions are inherently "wrong", and instead emphasize that they are simply "different from one another".

3.2. Sources of variability

We have identified variability in the protocols for bundle segmentation, which parallels variability in the literature of other techniques that have been used to elucidate the structure and function of the brain for the last 20 years. These types of disagreements and the challenge in advancing science beyond them are not new to computational neuroanatomy. Indeed, as we look at the history of brain science differences in opinions and associated results can be traced back a long way. Key examples of the inherent variability in anatomical and functional definitions and associated disagreements include the definition and functional specialization of cortical areas (Tootell and Hadjikhani, 2001, Weiner and Grill-Spector, 2012, Winawer et al., 2010). Hence, our findings here highlight the complexity of the scientific concepts and the difficulty in making progress towards understanding. The fact that the engineering of new methods needs to be refined because we still have (and have had for over hundreds of years in neuroanatomy) substantial variability in results does not necessarily mean that science is not progressing.

We postulate that the problem stems from two sources (1) the anatomical definition of a white matter pathway and (2) the constraints used to dissect this pathway. The descriptions of the white matter pathways given in the appendix highlight the problem of "definition". Pathways may be defined by their shape, their endpoints, or by regions through which they pass. Descriptions and definition approaches may vary based on the pathway itself (i.e., some may lend themselves more easily to descriptions of shape rather than endpoints), by the system or functions under investigation, by the training and/or occupation of the researcher/clinician, or by the modality used to define the tract. For example, cadaveric microdissection may facilitate description of fascicular organization and regional descriptions over highly specific lobular connectivity descriptions provided by histological tracers. Further, definitions do not always facilitate binary decision making in the bundle dissection process due to biological reasons. The brain is a complex structure, there are not always hard or unique borders between cortical or subcortical regions, and the location of endpoints or regions may not always be precisely determined. The goal of tractography bundle segmentation then is to recreate these definitions in the bundle dissection process (Schilling et al., 2020); however, certain algorithms, software packages, and manual pipelines lend themselves more naturally to one type of constraint than the other, and may implement them in different ways or with different levels of precision. Even if a definition has been entirely met, a sensitivity/specificity tradeoff is possible, influenced by potentially every step in the fiber tractography process from acquisition and reconstruction to the final constraints and streamline filtering techniques (Schilling et al., 2019, Thomas et al., 2014, Knösche et al., 2015).

3.3. The 'problem' and 'solution'

The question becomes "whose problem is this?". We propose that there may be shared responsibility on the part of classical anatomists, those developing tractography algorithms, and those implementing or performing segmentations. The endeavor to digitally segment the white matter is predicated upon there being some consensus of what structures are there to be segmented, this is the task of classical neuroanatomists. Next, tractography providers must endeavor to create candidate tractomes that resemble the white matter of the brain as closely as possible, as the resultant tractomes must contain viable anatomy for extraction. Finally, those who perform digital segmentations must decide an appropriate level of precision (sensitivity/specificity) and be clear and precise as they describe the methods of their segmentations as this will permit comparison and refinement between segmentations. This must be an iterative process, utilizing orthogonal information in the form of non-human model brains, micro-dissection, and alternative neuroimaging contrasts, in order to validate the existence and location or connections of a pathway, validate the rules and constraints that allow accurate dissection of this pathway, then iteratively refining the location and/or connections based on knowledge gained through the bundle segmentation process. Thus, we hope that this paper acts as a call to action on two efforts of consensus: both standardization of the anatomical definition (in addition to nomenclature) and the adoption of protocols to fulfill this definition.

Even without a consensus, there could be a convergence towards appropriate, or more specific, nomenclature and clustering of streamlines, or alternative accepted definitions. Additionally, a consensus on the healthy, young adult, individual may not lead to satisfactory results on developing, aging, or diseased populations. The effect of protocols and their adherence to definitions should be investigated in the presence of tumors, on the pediatric and elderly populations, and also with varying acquisition, reconstruction, and streamline generation conditions. Convergence upon protocols may come from isolating and operationalizing similarities and differences in definitions and protocols, as done in image segmentation literature (Boccardi et al., 2015), in order to slowly converge upon a consensus and/or guidelines. This may include: (1) exploring relationships between automated, semi-automated, and manual methods, (2) nomenclature and methodology based on volumetric characteristics (locations, shapes, orientation) versus connectivity characteristics (origins and terminations) (DN et al., 2021), and (3) studies of various constraints to best replicate nomenclature.

While we cannot currently give a recommended dissection protocol for a given pathway, we can recommend good practices to be used in all studies. First, we suggest transparency and explicit descriptions of pathway definition, dissection protocol, and ROIs (Catani and Thiebaut de Schotten, 2008, Fekonja et al., 2019). Second, understanding and quantifying the intra-protocol variability, for both automatic and manual approaches, is a necessary prerequisite to determine quantification variability and subsequent statistical power. Third, with the knowledge that the dense core of the pathway is consistent across protocols, weighting by density (or a focus on deep white matter, as is common in many statistical analyses (Smith et al., 2007, Yeatman et al., 2012)) will be more appropriate for evaluating inter-subject difference in microstructural properties, given its smaller inter-site and inter-lab differences. Finally, the results obtained by (and inferences made from) tractography must be interpreted with appropriate level of coarseness, by considering the existence of inter-protocol variability and coarse spatial scale of diffusion MRI measurements. Since some of statistical properties of tractography (streamline counts and densities, and geometry/volume of tracts) have dependency on method selections at this point, it is important to encourage studies by independent groups testing how much conclusions in a single original paper can be generalizable to a different segmentation protocol or datasets.

3.4. Limitations

This study has several limitations which constrain the generalizability of the results. First, there is a low number of subjects and low number of repeats. While automated methods can be run on several

hundred subjects using only CPU-hours, this study would have become prohibitive for manual or semi-automated methods with more than 14 pathways over six datasets (84 total possible dissections), and many of these methods would have been under-represented. Next, we did not include a number of pathways with functional relevance in the literature, but chose a sample representative of the commonly studied projection, association, and commissural bundles, and, again, a compromise was made between the number of pathways requested and expected time and effort. Future studies should consider studying pathway sub-divisions specifically, as well as additional major white matter pathways and superficial U-fibers (Guevara et al., 2020). Further, because we wanted to isolate the effect of bundle segmentation protocols, we forced the use of our own generated streamlines. This may not be optimal for a given segmentation process where streamlines are generated using different parameters or propagation methods, and filtered or excluded in various ways. However, allowing the creation of different streamlines would only increase the variability seen across protocols. Finally, there is no "right" measure to quantify variability (Rheault et al., 2020). No single measure can paint a complete picture of the similarities and differences of this complex technology across all applications. The measures used in this study were chosen as intuitive quantifications of volume-based, voxel-wise, and streamline-based agreement, as well as measures based on binary volumes and streamline densities. We also quantified measures of geometry which are often used in quantification or to modulate connectivity measures, as well as measures of microstructure within pathways (both weighted and unweighted by densities). The best measure of bundle variability is ultimately dependent on how the bundle is used.

Future studies may investigate which protocols (and which features of those protocols) result in bundles that are more or less similar to other protocols, and more importantly, quantify how well different protocols result in bundles that match the desired anatomical definition. This could be done using tools (Wassermann et al., 2016) to query text descriptions of volume, location, and connectivity to determine whether streamlines agree with the definition of a bundle. Finally, similar efforts with international and multi-disciplinary teams must apply evidence-based approaches pooling knowledge gathered from tracers, dissections, and functional contrasts from in vivo and ex vivo specimens in order to ultimately reach a consensus on tract descriptions (Yang et al., 2021, Bullock et al.,), and the best way to virtually dissect these tracts using fiber tractography.

4. Materials and methods

We surveyed the protocols for bundle segmentation of 14 white matter bundles, chosen to represent a variety of white matter pathways studied in the literature, including association, projection, and commissural fibers, fibers with clinical and neurosurgical relevance, as well as covering a range from frequently to relatively infrequently studied and/or described in the literature.

We made available the same datasets to be analyzed by a large number of groups in order to uncover variability across analysis teams. To isolate the effects of bundle segmentation from all other sources of variation, we directly provided six sets of whole-brain streamlines (both deterministic and probabilistic) to all collaborators, derived from 3 subjects with scan-rescan data acquired from the Human Connectome Project test-retest database (Glasser et al., 2016). We extended invitations for collaboration, disseminated data and the protocol with clearly defined tasks, and received streamlines from collaborators for analysis. In addition to streamlines, we requested a written "definition" of the pathways and a description of the constraints used to dissect it. Importantly, this dataset allows us to quantify and compare variability across protocols (inter-protocol), variability within protocols (intra-protocol), and variability across subjects (inter-subject). Detailed procedures are provided in supplementary material.

4.1. Data and protocol

The diffusion data for this study were selected from the Human Connectome Project test-retest database (Glasser et al., 2016). A total of three subjects (HCP IDs: 144226, 103818, 783462) were chosen that had repeat diffusion MRI scans, resulting in six high-quality datasets, free of any significant artifacts. This dataset was chosen as a compromise between quantification and inclusivity - the use of this small database still provides enough information to detect and quantify the variability among results with great enough participation across laboratories and scientists.

Collaborators were not informed that the six datasets represented only three subjects in order to not bias intra-protocol analysis. Distortion, motion correction and estimation of nonlinear transformations with the MNI space was performed using the HCP preprocessing pipelines (Glasser et al., 2016). Whole-brain tractograms were generated using the DIPY-based Tractoflow processing pipeline (Theaud et al., 2020, Garyfallidis et al., 2014), producing both deterministic and probabilistic sets of streamlines to be given to participants. Importantly, to be as inclusive as possible to all definitions and constraints, streamlines were not filtered in any way. Streamlines were separated into left, right, and commissural fibers in order to minimize file sizes. Also provided were the b0 images, Fractional Anisotropy (FA) maps (Jenkinson et al., 2012), directionally-encoded color maps (Jenkinson et al., 2012), T1 weighted images, and masks for the cerebrospinal fluid, gray matter, and white matter (Jenkinson et al., 2012).

The task given to collaborators was (see supplementary material) to dissect 14 major white matter pathways on the left hemisphere on the six diffusion MRI datasets provided. Collaborators were free to choose either deterministic or probabilistic streamlines, and free to utilize any software they desired. In order to maximize the quality of submitted results, investigators did not have to provide segmentations for all pathways if they did not have protocols or experience in some areas.

4.2. Submissions

For submission, we asked for a written definition of the white matter bundles, a description of the protocol to dissect these pathways, all code and/or temporary files in order to facilitate reproducibility of methods, and finally the streamline files themselves. Quality assurance was performed on file organization, naming conventions, and streamline spatial attributes, and visual inspection was performed for all streamlines of all subjects. Tools for quality assurance (QA) can be found at (https://github.com/scilus/scilpy).

4.3. Pathway-specific analysis

For all pathways, we focused on quantifying volume-based and streamline-based similarities and differences in the dissected bundles across protocols. Qualitatively, we assessed volume overlap and streamline overlap. Volume overlap was displayed as the volume of voxels in which 25%, 50%, and 75% of all protocols agreed that a given voxel was occupied by the pathway under investigation. Similarly, we viewed the individual streamlines in which 25%, 50%, and 75% of all protocols agreed that this streamline is representative of a given pathway. These qualitative observations were shown as volume-renderings or streamlines visualizations directly.

Next, quantitative analysis used three voxel-based measures (based on volume and streamline density) and one streamline-based measure (Rheault et al., 2020). The Dice overlap coefficient, density correlation coefficient, bundle adjacency, and streamline Dice overlap are illustrated in Fig. 4. Dice overlap measures the overall volume similarity between two binarized bundles (i.e., all voxels that contain a streamline), by taking twice the intersection of two bundles divided by the union of both bundles. A value of 1 indicates perfect overlap, a value

of 0 indicates no overlap. The density correlation coefficient is a measure of the Pearson's correlation coefficient obtained from the streamline density maps. This provides insight into not only overlap, but also agreement in streamline density. Bundle adjacency is a volume-based metric that describes the average distance of disagreement between two bundles. This was calculated by taking all non-overlapping voxels from one bundle, and calculating the nearest distance to the second bundle (and repeating from the second to the first bundle) and taking the average of these distances. By defining this metric, we are using a convenient symmetric distance between two binary volumes, which is a modification of the Hausdorff distance. A value of 3mm, for example, indicates that when the bundles disagree, they are an average of 3mm apart. Finally, streamline Dice is the streamline-equivalent of Dice overlap. Because all submissions for a given subject were derived from the same set of whole-brain streamlines, we had the ability to quantify whether an individual streamline was common to both submitted bundles. Streamline Dice was calculated by taking the total amount of streamlines common to both protocols (i.e., intersection) divided by the total number of unique streamlines in both bundles (i.e., union). Again, a value of 1 indicates that all streamlines are exactly the same, a value of 0 indicates no overlap in streamlines. Note that this final measure can be calculated only for datasets that are derived from the same original set of streamlines.

4.4. Quantifying variability across protocols

The measures introduced above were used to quantify variability across protocols (inter-protocol), variability within protocols (intra-protocol), and variability across subjects (inter-subject), with separate analyses for deterministic and probabilistic results. Below, we describe these three levels of variability assuming there were "N" submissions for a given pathway.

For inter-protocol variability, each bundle was compared to its counterpart as produced by each of the other N-1 protocols, and the results averaged, representing the average similarity/dissimilarity of that protocol with all others. This was done for all N submissions, for all 3 subjects, resulting in Nx3 data-points for each pathway.

For intra-protocol variability, we aimed to compare the same protocol performed on the same subject. For each of the N submissions, we calculated the similarity/dissimilarity measures with respect to the same submission on the repeated scan. This was repeated for all subjects, resulting in again Nx3 data-points for each pathway. A "precise" measure of intra-protocol variability would have been possible if the same set of streamlines had been provided twice for each subject. Instead, the study used scan/re-scan data to measure not only intra-protocol variability, but *the variability of everything up to, and including protocol*. Thus, this measure includes acquisition variability (i.e., noise and possible artifacts), registration (to a common space), reconstruction, and generation of whole brain streamlines.

For inter-subject variability, we sought to characterize how similar/dissimilar a bundle is across subjects within a single protocol. All streamlines were normalized to MNI space using nonlinear registration (*antsRegistrationSyn)* (Avants et al., 2008) of the T1 image to the MNI ICBM 152 asymmetric template (Fonov et al., 2011). For each of N protocols, the agreement measures were calculated from subject 1 to subject 2, from subject 2 to subject 3, and from subject 1 to subject 3, again resulting in Nx3 data-points for each pathway.

Finally, to visually assess differences across bundles and across protocols,we utilized the Uniform Manifold Approximate and Projection (UMAP) (McInnes and Healy, 2007) technique (https://github.com/lmcinnes/umap; release 0.4.1), which is particularly suited for visualizing clusters or groups of high-dimensional data and their relative proximities. UMAP input was the 3D density maps of all bundles for all submission, while the output was projection of all bundles onto the 2D space. We note that any dimensionality reduction technique and subsequent visualization could have been used, for example t-SNE (Hinton and Roweis, 2002), for qualitative analysis of tractograms grouped across bundles and protocols. Hyperparameters and algorithm initialization are known to influence results for these nonlinear dimension reduction techniques (Kobak and Linderman, 2021), but for our purposes (qualitative visualization of local and global clusters without an explicit user-defined scalar measure of agreement/disagreement) we have implemented this with all default parameters of distances, metrics, and components.

Data and Code Availability

The diffusion data for this study were selected from the Human Connectome Project test-retest database. A total of three subjects (HCP IDs: 144226, 103818, 783462) were used in this study. Code for bundle variability analysis is available at (https://github.com/scilus/scilpy).

Credit author statement

Conceptualization, Project administration: KS, FR, BL, MD.

All authors contributed to Methodology, Data analysis, Writing (review and editing).

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.neuroimage.2021.118502.

Appendix A. Cortico Spinal Tract (CST)

The CST is the major descending tract that mediates voluntary skilled movements (Jang, 2009, Wiesendanger, 1969). At its most basic, this tract is a pathway of fibers coursing primarily from the motor cortex down the spinal cord. Despite this apparent simplicity, dissecting this tract can be quite variable. Moderately increasing the complexity of the definition, the CST can be (unanimously) described as starting from the cortex, traveling through the corona radiata, converging into the internal capsule, continuing into the brainstem through the medulla, and finally extending to the spinal cord. Decisions to be made include choosing specific cortical terminations (which span both frontal and parietal lobes) and how these are delineated, selecting regions through which the streamlines must pass ("cortex to medulla" or "cortex to lower brainstem" or "motor cortex to medulla"), and implementing additional inclusion and exclusion regions throughout the extent of the pathway to further refine where it goes and where it does not go. Adding further ambiguity, the CST together with the corticobulbar tract make up the pyramidal tract, and because these are not easily (or not possibly) separated due to inherent tractography limitations and field of view restrictions, these have sometimes been used interchangeably and/or incorrectly in the literature. In this study, the CST was divided into precentral and postcentral divisions based on endpoints, hand-foot-face divisions based on regions of interest, anterior-posterior-central-cingulate divisions based on endpoints, combined/separated with ascending pathways with thalamic synapses, as well as combined/separated with the peri-Rolandic component based on endpoints, and divided into lateral and anterior components based on definition (but not dissected).

Appendix B. Arcuate Fasciculus (AF)

The AF plays a key role in language processing. This is an association tract that is well-understood to connect Wernicke's area (somewhere in the posterior temporal lobe) to Broca's area (located in the inferior frontal lobe). It gets its name (Latin for *curved* bundle) from the distinctive arch shape it makes as it curves from the anterior-posterior direction in the frontal-parietal cortex ventrally into the temporal cortex around the Sylvian fissure (lateral sulcus) (Catani and Mesulam, 2008, ten Donkelaar et al., 2018). This description of the AFs shape is generally agreed upon. A third area (inferior parietal lobule) is also traditionally included in this tract's connections, representing the pathway that Geschwind postulated to be damaged in conduction aphasia (Catani and Mesulam, 2008). For this reason, many descriptions include multiple segments of the AF - a direct pathway traversing the entire tract from temporal to frontal lobes, and an indirect pathway of shorter fibers connecting temporal to parietal to frontal lobes. Consequently, the AF can be described as connecting a number of areas of the perisylvian cortex of the frontal, parietal, and temporal lobes. To further complicate the literature, because the AF is a dorsal longitudinal system of tracts, it is occasionally considered to be part of the SLF system of tracts (Dick and Tremblay, 2012, Thiebaut de Schotten et al., 2012) and considered synonymous or used interchangeably in the literature (Dick and Tremblay, 2012). For these reasons, we hypothesized that we would see large variability when giving collaborators the task to "segment the arcuate fasciculus". Variability is observed due to differences in defining the location and method of delineating Wernicke's and Broca's areas, or selection of regions to capture the arch-like shape. Approximately 1/5 of submissions indicated dividing the AF into the long direct segment (often described as more medially located), and the anterior and posterior indirect segments (described as laterally located shorter segments).

Appendix C. Corpus Callosum (CC)

The CC is the largest, and arguably most easily recognizable, white matter structure of the brain. This structure is not a single tract, but rather a commissure, composed of axons coursing in the left-right orientation at the midline, and interconnecting the cerebral cortex of the two hemispheres. Many subdivisions of the CC have been proposed (Hofer and Frahm, 2006) with most partitioning the CC based on axon location in the mid-sagittal section. Most commonly, subcomponents are rostrum, genu, body, isthmus, splenium, and (sometimes) tapetum, although others include genu, splenium, and callosal body, or anterior, mid-anterior, central, mid-posterior, and posterior based on (FreeSurfer) parcellation schemes. Alternative subdivisions included separating according to the major lobes of the brain (frontal, parietal, occipital, and temporal) or numerical subdivisons (ranging between 5 and 12) based on cadaveric and histological dissections (Witelson, 1985), or homologous connections, or clusters of fibers. Common to all protocols is the large, easily distinguishable region near the midline. Constraints, decisions, and filters include choices of where these bundles cannot go (various temporal lobe regions, through or near subcortical structures, cingulum and parahippocampal gyri, etc), filtering by connection regions or lengths, or rules enforcing homologous connections.

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Annex 5

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The Executive Functions in Frontal and Temporal Lobes: A Flanker Task Intracerebral Recording Study

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Abstract: The occurrence of the local generators of P3-like potentials elicited by a noise-compatibility flanker test was used to study the processing of executive functions, particularly in the frontal and temporal cortices. The test performed with arrows comprised a simpler congruent and a more difficult incongruent task. The two tasks activated the attention and several particular executive functions, i.e., working memory, time perception, initiation, and motor control of executed task. The incongruent task increased demand on executive functions, and besides the functions common for both tasks, an inhibition of automatic responses, the reversal of incorrect response tendency, the internal ordering of the correct response, and the initiation of the target-induced correct response were involved. In seven epilepsy surgery candidates (four men and three women), ranging in age from 26 to 38 years, multicontact depth electrodes were implanted in 590 cortical sites. In the two tasks, the P3-like potential sources were displayed in the mesial temporal structures, the lateral temporal neocortex, the anterior and posterior cingulate, the orbitofrontal cortex, and dorsolateral prefrontal cortex. The P3-like potentials occurred more frequently with the incongruent than with congruent stimuli in all these areas. This more frequent occurrence of P3 sources elicited by the incongruent task appeared significant in temporal lateral neocortex and orbitofrontal cortex. The executive functions are processed in a widespread frontotemporal neurocognitive network. This study confirms the involvement of the temporal neocortex in the executive functions.

Key Words: Generator of P3, Temporal neocortex, Orbitofrontal cortex, Flanker test, Neurocognitive network.

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Cerebral activities related to processing of executive functions were studied via intracerebrally recorded event-related potentials (ERPs). ERPs were evoked by two modifications of a cognitive task. The following question was raised: which parts of the human cortex are engaged by task performance with elevated load on the executive functions?

Endogenous ERP are believed to reflect the neurophysiologic correlates of cognitive processes. An ERP in the 300 milliseconds range, i.e., the P3 component, which is a target detection response, has been the one most studied. It has been linked to both orienting and memory mechanisms (Squires et al., 1975). This long-latency waveform may represent various functions—the closure of sensory analysis, the update of working memory, the attentional and decisional processes, and, in a motor task, the facilitation of motor pathways (Comerchero and Polich, 1999; Rosler et al., 1986; Verleger et al., 1994, 2005; Wood et al., 1985). The shape of intracerebral potentials frequently varies, and it is often difficult to identify the equivalents of individual components recorded on the scalp. We prefer to state only that in the studied task, a cognitive process that shares critical features with the scalp-recorded P3 wave (Halgren et al., 1998) very probably elicited the ERP in this study. On the basis of earlier findings of task-specific cognitive ERPs, we hypothesized that different tasks would modify differently the brain network eliciting P3-like potentials (Rektor et al., 2007).

The executive functions are described as higher-level processes that exert control over elementary mental operations. A central position of the prefrontal cortex (PFC) and its cortical and subcortical connections in processing the executive functions have been suggested (Badgaiyan, 2000; Stuss and Benson, 1986). Ventromedial PFC is involved in decision-making processes; the dorsolateral portion has a role in working memory, planning, and sequencing of behavior. The caudal PFC is reported to be involved in attentional mechanism (Bruce and Goldberg, 1985). This hypothesis was reviewed by Parkin (1998) who criticized the concept of the central position of the PFC in the executive functions. He suggested instead a pattern of extensive heterogeneity with different executive tasks associated with different neural substrates. In fact, several studies have documented the diversity of executive functions and related anatomy (Godefroy, 2003).

We were particularly interested in the role of the temporal lateral cortex (TLC). In a previous intracerebral depth electrodes study (Bocková et al., 2007), the neurocognitive network in the frontal and lateral temporal cortices was investigated by visual-motor tasks of writing of single letters. The first task consisted of copying letters appearing on a monitor. In the second task, the patients were requested to write any other letter. The cognitive load of the second task was increased mainly by larger involvement of the executive functions. The task-related event-related desynchronization/synchronization (ERD/ERS) of the alpha, beta, and gamma rhythms was studied. The alpha and beta event-related desynchronization/synchronization linked specifically to the increased cognitive load was present in the PFC, in the orbitofrontal cortex, and surprisingly also in the temporal neocortex. In particular, the TLC was activated by the increased cognitive load. It was suggested that the TLC together with frontal areas forms a cognitive network processing executive functions. The test used in Bocková's study consisted of an original and rather complex task, with involvement of several executive and nonexecutive processes. In consequence, the interpretation was rather complex. To confirm the suggested involvement of the TLC in the central executive, we decided to perform this study with a test that has been commonly used for studying executive functions. We have chosen the flanker te-st (FT) in which subjects must react to direction of central arrow in congruent and incongruent tasks (Eriksen and Eriksen, 1974; Kopp et al., 1996; Praamstra et al., 1998).

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MATERIALS AND METHODS

Subjects

Seven patients (four men and three women; all right-handers), ranging in age from 26 to 38 years, (average age 34.1 years), all with pharmacoresistant epilepsy, participated in the study. The patients were all candidates for epilepsy surgery who were recommended by special commission for intracranial exploration. A neuropsychologic examination excluded severe cognitive disturbances and dementia in each patient. All the patients had normal motor performance, normal hearing, and vision.

Depth electrodes were implanted to localize the seizure origin before surgical treatment. All patients received 6 to 11 orthogonal platinum electrodes in the investigated brain lobes using the methodology of Talairach and Bancaud (Talaraich and Tournoux, 1967) to localize the epileptogenic foci before a surgical treatment. Standard semiflexible intracerebral electrodes, diameter 0.8 mm, contact lengths 2 mm, and intercontact interval 1.5 mm, were used for invasive EEG monitoring. The electrodes were implanted in three patients in the right hemisphere and in one patient in left hemisphere; three were recorded bilaterally (see patient characteristics, Table 1).

The exact position of the electrodes and their contacts in the brain were verified using postimplantation MRI with electrodes *in situ*. The recordings from lesional anatomic structures and the cortex showing interictal or ictal epileptiform potentials were not included in the analysis. A total of 592 sites in the temporal (284), frontal (260), and parietal (48) lobes were recorded by means of 73 multicontact depth electrodes for experimental analysis.

All the subjects were informed about the character of this study, and informed consent was obtained. The study received approval from the local ethical committee of the St. Anne's Hospital.

Procedure and EEG Recording

Subjects were half-sitting comfortably in the monitoring bed, in a quiet room, with constant temperature. Each patient was asked to minimalize movement, to keep the eyes fixed to the monitor, and to concentrate on the task. The monitor was situated at the same place for all the subjects, 1.5 m in front of their eyes, at the end of the monitoring bed.

Before the recording session, each subject was given a short practice period. All recordings were under video and examiners' visual control; the failed trials were removed.

The EEG signal was recorded from various cerebral structures using the intracerebral electrodes. The electromyograms (from the dominant-hand m. flexor carpi radialis), electrooculography, and the scalp EEG (Cz, Pz, Fz electrodes) were recorded simultaneously. All recordings were monopolar, with a linked earlobe reference.

The Oddball Paradigm

The flanker task with arrows was performed (Eriksen and Eriksen, 1974). The visual stimuli were black arrows against a white background that pointed to the left or right side (Fig. 1).

Subjects were required to focus on the central arrow and to signal whether the direction of this arrow was "congruent" or "incongruent" with the direction of arrows in the other fields. On congruent and incongruent trials, the flankers were arrows pointing in the same or different direction, respectively, relative to the central arrow. Subjects were instructed to respond to the target stimuli as quickly and as accurately as possible by pressing a microswitch button in the dominant hand. They were asked to react to four types of stimuli presented from the monitor by pushing the right or left button—congruent arrows to the right or left side and incongruent arrows to the right or left side.

The duration of the stimulus exposure was 200 milliseconds. The interstimulus interval was fixed to 4 seconds, the number of stimuli was 400, and the ratio of the congruent to incongruent arrows was 80% to 20%. In the trigger channel, the stimuli and reactions and their duration were recorded. The sampling rate was 256 Hz. Standard antialiasing filters were used.

Data Analysis

Data were analyzed using ScopeWin and ScopeMat software. Data were segmented because of the stimulation trigger onset at first, and the trials were visually inspected to eliminate EEG segments containing any artifact activity or/and epileptic activity.

The main ERP components were identified by visual inspection and quantified by latency and amplitude measures. P3-like waves were identified in the 250 to 600 milliseconds latency range. We focused on local sources of P3-like potentials. Only the "phase reversal" and "steep voltage change" were considered to be generators of the studied potentials, because of their significance as the accepted signs of proximity to generating structure (Halgren et al., 1995a,b; Vaughan et al., 1986).

We observed incorrect responses to stimuli only exceptionally; therefore, the errors were not rated separately. We suppose that it was caused by the fact that our patients were young, without important cognitive deficit, and the interstimulus interval was long (4 seconds).

We analyzed reaction time for each stimulus separately in term of congruent and incongruent conditions in all subjects. Summary of mean values is given in Table 2.

In an attempt not to atomize the results, we handled the recordings in both hemispheres together. The low density of sites could bias a lateralization study. Large studies with various stimulus modalities showed bilateral occurrence of P3-like cerebral potentials

TABLE 1. Subjects Characteristics

	Subject 1 (N.M.)	Subject 2 (Č.P.)	Subject 3 (A.P.)	Subject 4 (M.M.)	Subject 5 (H.M.)	Subject 6 (H.R.)	Subject 7 (F.J.)
Age (years)	35	36	33	34	26	37	38
Sex	Female	Female	Female	Male	Male	Male	Male
Seizure onset zone	R T-P-O junction	L mesio-T	R F	R mesio-T	L mesio-T	L mesio-T	RF
Therapy	CBZ, LEV, PGB	CBZ, LEV	VPA, LTG	CBZ, LEV	VPA, CBZ, PGB	VPA, PGB	LEV, CBZ
MR	R mesio-T sclerosis	L mesio-T Sclerosis	Normal	R mesio-T sclerosis	L mesio-T Sclerosis	L mesio-T Sclerosis	R F dysplasia
Implanted sites	LT, RT, RF, RP	LT, RT, LP	LT, RT, LF, RF	RF, RT	LF, LT	RT, RF, RP	RT, RF, RP
No. sites	71	77	97	66	107	94	80

MR, magnetic resonance imaging; CBZ, carbamazepine; LEV, Levetiracetam; PGB, Pregabalin; VPA, Valproic acid; LTG, Lamotrigine; F, frontal; L, left; O, occipital; P, parietal; R, right; T, temporal.

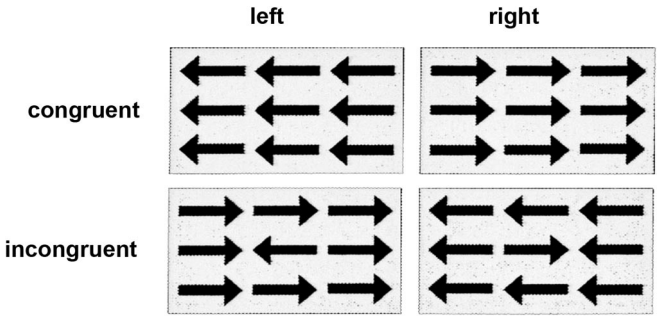

FIGURE 1. The four stimuli used in the task—Flanker paradigm. The visual stimuli were black arrows against a white background that pointed to the left or right side.

TABLE 2. P3 Latency and Amplitude

	Latency (Millisecond)	Amplitude (μV)	Reaction Times (Millisecond)
Congruent condition	345 ± 41	15 ± 6	650 ± 30
Incongruent condition	390 ± 30	19 ± 9	800 ± 25

(Baudena et al., 1995; Brázdil et al., 1999, 2001; Halgren et al., 1995a,b, 1998; Rektor et al., 2001a,b, 2004, 2007). When checking our data, we could not observe any difference between the recordings from the left and the right hemisphere. We do not think that omitting the lateralization aspect could bias the studied questions.

Statistical Analysis

The Wilcoxon-signed rank test was applied to test the differences in relative counts of generators of P3 between the two tasks in different cortical regions. The statistical significance of the differences between the two tasks was expressed as a point probability (pp) value. The differences were considered as significant when pp value was <0.05.

RESULTS

Local sources of the generators of P3 component of ERPs were found in a large number of investigated brain areas. The P3 generators were found in the orbitofrontal cortex (Brodmann area [BA] 11) the dorsolateral PFC (BA 9, 46), the mesial temporal structures (hippocampus), the anterior and posterior cingulate, and frequently in the lateral temporal neocortex (BA 21, 22); see Table 3.

Orbitofrontal Cortex (BA 10, 11)

The orbitofrontal cortex was explored in five subjects, in total 50 sites. Five electrodes were implanted in the right side and one in the left side. We found 6 P3 generators with simple task and 17 generators during incongruent task. This difference is significant (pp = 0.031).

Dorsolateral Prefrontal Cortex (BA 9, 46)

The dorsolateral PFC was explored in four subjects, in total 35 sites. Two electrodes were implanted in the right side and two in the left side. The generators of the P3 were found in two sites during simple task and in eight sites during more complex task. This difference is nonsignificant (pp = 0.350).

Cingulate (BA 24, 31, 32)

The cingulate was investigated in five subjects, in total 26 sites. Five electrodes were placed in the right anterior cingulate (BA 24, 32), three in the left anterior cingulate cortex, and two in posterior cingulate (BA 31) bilaterally.

We found 2 generators during simple task and 10 generators during more complex task. This difference is nonsignificant (pp = 0.125).

Temporal Neocortex (BA 21, 22)

The bilateral temporal neocortex was the most extensively investigated cerebral area. Six of seven subjects had explored temporal neocortex bilaterally, in total 120 sites. Thirteen electrodes were implanted in the left side and 17 in the right side. In the majority of the electrodes, we found generators of the P3 with significant prevalence during the second more complex task (pp = 0.016). We found 15 generators during simple task and 48 generators during complex task.

Temporal Pole (BA 38)

Two electrodes were implanted in temporal pole bilaterally; there were no generators of P3 found in this area.

Hippocampus

The hippocampus was explored in five subjects, in total 29 sites. Six electrodes were placed in the left side and five in the right side. We found 13 generators during simple task and 25 generators during more complex task. This difference is nonsignificant (pp = 0.125).

Lobulus Parietalis Inferior

The lobulus parietalis inferior (Brodmann area 40) was explored in four subjects; the recordings were obtained from four electrodes passing from the parietal convexity through the white matter in the parietal mesial cortex, altogether from 48 contacts. We found no generators of P3 in this area.

TABLE 3. Summary of Occurrence of P3 Generators in Individual Brain Areas

Cortex	Brodmann Area	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6	Subject 7
Orbitofrontal	BA 10, 11	++	+, ++	—	+, ++	++	—	+, ++
Prefrontal	BA 9, 46	—	—	—	—	++	+, ++	+, ++
Temporal	BA 21	++	+, ++	++	+, ++	+, ++	++	—
Temporal	BA 22	+, ++	++	+, ++	++	+, ++	++	—
Cingulate	BA 23, 24 BA 31, 32	++	++	—	—	++	+, ++	—
Hippocampus	—	+, ++	++	—	++	+, ++	+, ++	—

+: Occurrence of P3 generators in individual brain areas—congruent task.
++: Occurrence of P3 generators in individual brain areas—incongruent task.

TABLE 4. Statistical Analyses—Summary of Point Probability in Individual Cortical Regions

Cortical Regions	Point Probability
Orbitofrontal cortex	0.031
Dorsolateral prefrontal cortex	0.350
Cingulate	0.125
Temporal neocortex	0.016
Hippocampus	0.125

The comparison of the occurrence of P3 generators in both tasks is given in Table 3. The significant difference was found in temporal neocortex and orbitofrontal cortex (Table 4). As expected, the reaction time with incongruent condition was longer, as well as the P3 latency and its higher amplitude (Table 2).

DISCUSSION

Previous intracranial studies demonstrated a widespread distribution of cognitive ERPs in multiple cortical and subcortical regions in the human brain. The participation of the frontal, temporal, and parietal cortices, in addition to the cingulate and mesial temporal regions and the basal ganglia and thalamus, has been shown with visual, auditory, and somatosensory stimuli (Baudena et al., 1995; Brázdil et al., 1999, 2003; Clarke et al., 1999; Halgren et al., 1995 a,b, 1998; Lamarche et al., 1995; Rektor et al., 2001a,b, 2004, 2007; Smith et al., 1990). A variable and task-dependent internal organization of corticosubcortical systems generating the ERPs was suggested. The small regions in each field were active or inactive in relation to the nature of the task. This complex and largely distributed activation, comprising various cortical areas, reflects the complexity of even quite simple cognitive activities (Rektor et al., 2004, 2007).

In this study, the occurrence of the local generators of P3-like potentials, elicited by a noise-compatibility FT, was used to study the processing of executive functions, particularly in the frontal and temporal cortices. The FT was performed by arrows (Fig. 1), comprising a simpler congruent and an incongruent, more difficult task (Kopp et al., 1996; Praamstra et al., 1998). We supposed that both tasks activated the attention and several particular executive functions, i.e., working memory, time perception, and initiation and motor control of executed task. The incongruent task increased demand on executive functions, and besides the functions common for both tasks, an inhibition of automatic responses, the reversal of incorrect response tendency, the internal ordering of the correct response, and the initiation of the target-induced correct response were involved (Falkenstein et al., 2006).

The FT has been largely used also for the research of error detection (Falkenstein et al., 2006; Heldmann et al., 2008; Ullsperger et al., 2006). We preferred to use a paradigm with a long interstimulus interval that did not produce sufficient number of errors, as we were not specifically focused on errors in this study.

We evaluated the human event-related EEG signal recorded via intracerebral depth electrodes. The intracerebral recordings were performed in epileptic patients, and we cannot fully exclude pathologic process influence on the recorded electrical activity. To minimize the risk of such bias, we excluded recordings from brain tissue with pathologic activity: the cortex showing interictal or ictal epileptiform potentials and lesions were not implicated in the analysis. The limited number of recording sites did not enable mapping of all cortical areas. The electrode positioning is determined by clinical intention—not all structures can be fully explored. Some areas remained unexplored, e.g., the occipital cortex. In some other areas, only a few electrode contacts were located, e.g., in the temporal pole. In consequence, there may be parts of the executive function network that were not identified by this study. On the other side, the depth electrodes are submerged in the brain tissue and record from their immediate vicinity; the data are obtained directly from cortical structures; some of them almost inaccessible by scalp or subdural measurement. The quite limited spatial resolution that is typical for intracerebral recordings could be compensated for by a large number of recording sites (in this study, 592 sites; Figs. 2a and 2b).

We investigated which parts of the frontal and temporal cortex were activated during performance of the two tasks and searched for differences between the two tasks (Tables 3 and 4). In the two tasks, the P3-like potential sources were displayed in the mesial temporal structures (hippocampus); the lateral temporal neocortex (BA 21, 22); the anterior and posterior cingulate; the orbitofrontal cortex (BA 11); and dorsolateral PFC (BA 9, 46). Our results are partially supported by the functional MRI studies, which commonly confirm the central role of the PFC in FT with more variable observations in other parts of brain (Garavan et al., 2006). An important function elicited by the FT is a cognitive inhibition of learned routine. The control of nonroutine actions depends on a specific system, the supervisory system. The supervisory system is believed to be supported by the PFC and is assumed to operate in novel, conflicting or complex situations (Godefroy, 2003). Dorsolateral and ventrolateral PFC and frontal operculum, predominantly on the right hemi-

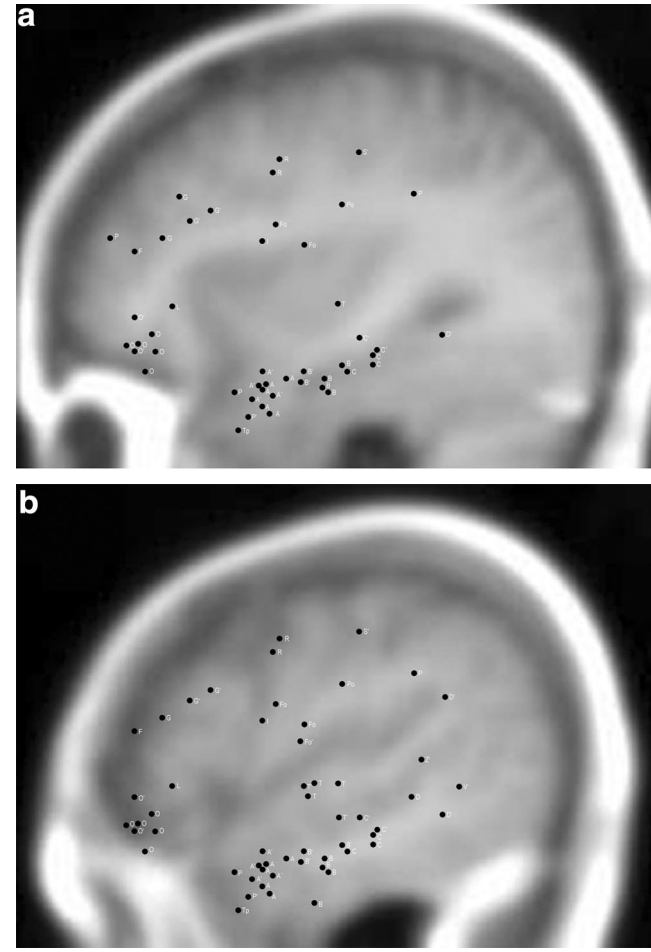

FIGURE 2. **a**, Paramedial sagittal section with electrode positions—overlapping MRI scans of all subjects. **b**, Lateral sagittal section with electrode positions—overlapping MRI scans of all subjects.

sphere, and parietal cortex, the anterior cingulate, the presupplementary motor area, and subcortical structures are thought to be implicated in motor response inhibition (Aron and Poldrack, 2006; Hazeltine et al., 2000). In a combined ERP and functional MRI study, two cortical systems, one involving right prefrontal and parietal areas and the other involving regions of the cingulate, underlying the inhibitory control were described (Garavan et al., 2006).

The P3-like potentials occurred more frequently with the incongruent than with congruent stimuli in all investigated areas except in the temporal pole and the lobulus parietalis inferior, where no P3-like generators were recorded. A trend to significance was observed in the cingulate and in the hippocampus but not in the dorsolateral PFC—low number of recordings in this areas might bias these results, because the P3 sources occurred there eight times with the incongruent task and only two times with the congruent task. Not surprisingly, the increased activation with the incongruent task was significant in orbitofrontal cortex (Fig. 3). The inferior frontal/orbitofrontal cortex is believed to be crucial for inhibitory control, according to repetitive transcranial magnetic stimulation and functional MRI studies (Chambers et al., 2007). The rostral orbitofrontal region (BA 11), which is primarily linked with the anterior medial temporal limbic region and lateral prefrontal cortical areas, is involved in the process of encoding new information (Frey and Petrides, 2000).

In the TLC, the region of interest was defined by the sites where the orthogonally implanted depth electrodes targeted into amygdalohippocampal complex entered the temporal lobe, namely in the central and anterior parts of the middle temporal gyrus (BA 21) and in adjacent parts of the superior and inferior temporal gyrus (Figs. 2a and 2b).

The significant increase of activation in the temporal neocortex might appear as more surprising, but it is in line with our earlier observation (Bocková et al., 2007). Little is known about the specific functional contribution of the human TLC, with regard to cognitive processing. Moreover, a recent article about the TLC contains in its title "The myth of silent cortex" (Devinsky et al., 2005). The temporal neocortex is traditionally connected with auditory and visual functions. The function of the temporal neocortex is fundamental for learning, memory storage, retrieval and consolidation, object recognition, and semantic

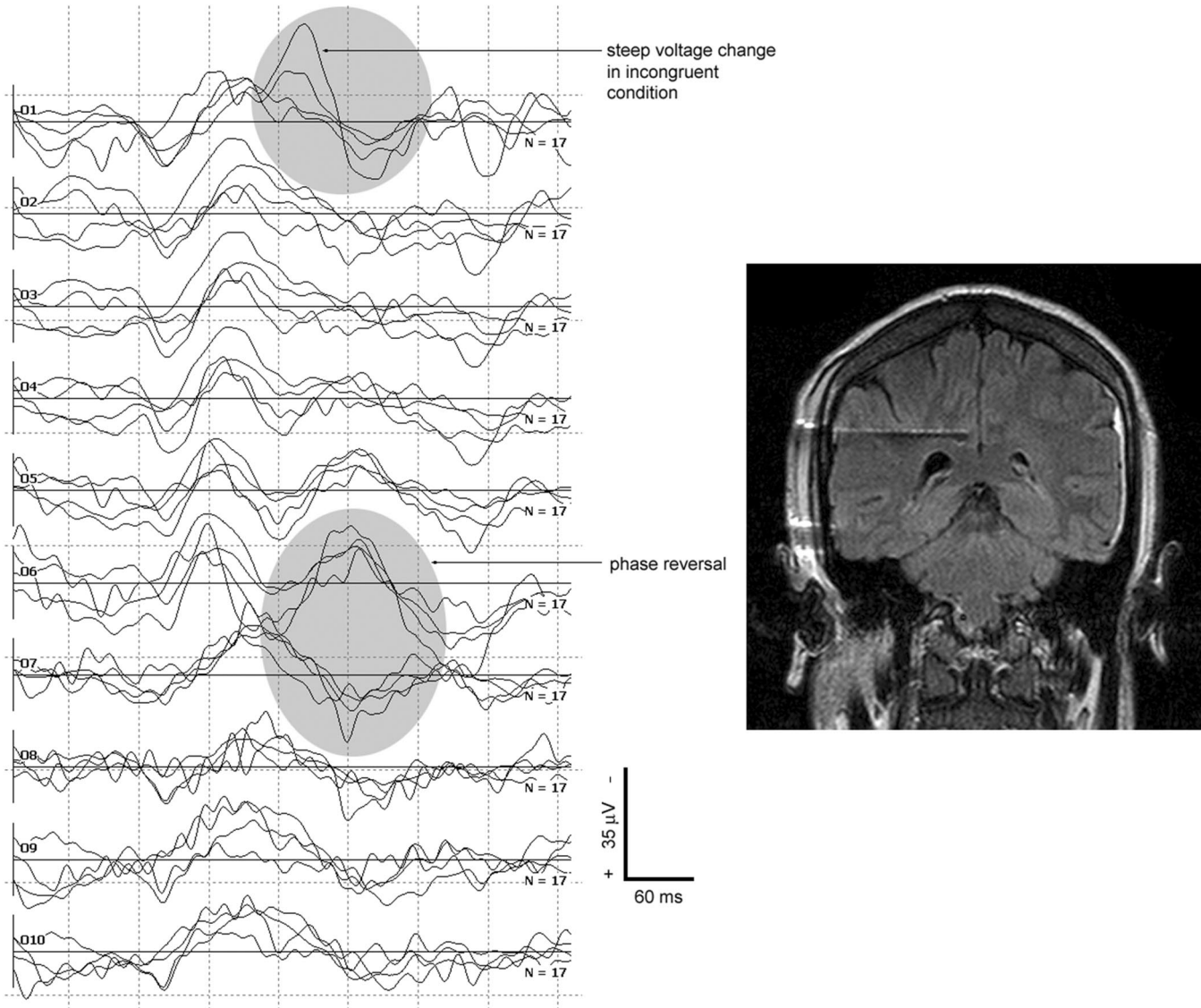

FIGURE 3. Steep voltage change in cingulate during incongruent condition (contact O1). Phase reversal in congruent as well as incongruent condition in orbital gyri (contacts O6, O7).

cognition (Gotman and Levtova, 1996; McClelland & Roger, 2003; Wiltgen et al., 2004; Zatorre, 2004). The temporal cortex might be a part of a system that envelopes cognitive contextual integration (Halgren et al., 1998). A larger activation of the temporal neocortex with the oddball P3 (rare stimulus, appearance uncertain) when compared with a P3 evoked by a frequent and predictable stimulus elicited by a S1-S2 task was observed. The lateral temporal cortex together with PFC, the amygdalo-hippocampal complex, and the cingulate formed a distributed network processing the target stimuli in the oddball protocol (Rektor et al., 2007). This network is implicated also in the appearance of P3-like potentials linked with the processing of the flanker task; its activation is enhanced by an increase of the cognitive load with the incongruent stimuli.

The TLC was activated in several studies involving the executive functions (Garavan et al., 2006), without evoking a greater attention. The results of this study are in line with the study by Bocková et al., (2007), who demonstrated with a letter writing task that the lateral temporal cortex together with frontal areas forms a cognitive network processing executive functions. This confirms the role of the temporal neocortex, using a task that has been traditionally used for executive functions testing. As the temporal activation could be demonstrated with two different studies with different tasks, it is probable that temporal involvement is not (only) related to a specific task but (also) reflects a constant involvement of the temporal cortex in executive processing.

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Annex 6

Aulická Š, Jurák P, Chládek J, Daniel P, Halámek J, Baláž M, Bočková M, Chrastina J, Rektor I. Subthalamic nucleus involvement in executive functions with increased cognitive load: a subthalamic nucleus and anterior cingulate cortex depth recording study. Journal of Neural Transmission, Wien: SPRINGER WIEN, 2014, roč. 121, č. 10, s. 1287-1296. ISSN 0300-9564. doi:10.1007/s00702-014-1191-5.

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Subthalamic nucleus involvement in executive functions with increased cognitive load: a subthalamic nucleus and anterior cingulate cortex depth recording study

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Abstract We studied the appearance of broadband oscillatory changes (ranging 2–45 Hz) induced by a cognitive task with two levels of complexity. The event-related de/synchronizations (ERD/S) in the subthalamic nucleus (STN) and in the anterior cingulate cortex (ACC) were evaluated in an executive function test. Four epilepsy surgery candidates with intracerebral electrodes implanted in the ACC and three Parkinson's disease patients with externalized deep brain stimulation electrodes implanted in the STN participated in the study. A Flanker test (FT) with visual stimuli (arrows) was performed. Subjects reacted to four types of stimuli presented on the monitor by pushing the right or left button: congruent arrows to the right or left side (simple task) and incongruent arrows to the right or left side (more difficult complex task). We explored the activation of STN and the activation of the ACC while processing the FT. Both conditions, i.e. congruent and incongruent, induced oscillatory changes in the ACC and also STN with significantly higher activation during incongruent trial. At variance with the ACC, in the STN not only the ERD beta but also the ERD alpha activity was significantly more activated by the incongruent condition. In line with our earlier studies, the STN appears to be involved in activities linked with increased cognitive load. The specificity and complexity of task-related activation of the STN might indicate the involvement of the STN in processes controlling human behaviour, e.g. in the selection and inhibition of competing alternatives.

Keywords ERD/S · Anterior cingulate cortex · Subthalamic nucleus · Flanker test · Executive functions

Abbreviations

ACC	Anterior cingulate cortex
AC–PC line	Anterior commissure–posterior commissure line
BA	Brodmann area
CBZ	Carbamazepine
CLZ	Clonazepam
CT	Computer tomography
dACC	Dorsal anterior cingulate cortex
DBS	Deep brain stimulation
EEG	Electroencephalography
EMG	Electromyography
EOG	Electrooculography
ERPs	Event-related potentials
ERD/S	Event-related de/synchronizations

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EOG	Electrooculography
FLE	Frontal lobe epilepsy
FT	Flanker test
IF	Individual frequency
LTG	Lamotrigine
LVT	Levetiracetam
fMRI	Functional magnetic resonance imaging
MTLE	Mesiotemporal lobe epilepsy
PGB	Pregabalin
PD	Parkinson's disease
RT	Reaction time
rTMS	Repetitive transcranial magnetic stimulation
SEEG	Stereo-electroencephalography
STN	Subthalamic nucleus
subgACC	Subgenual anterior cingulate cortex
TOP	Topiramate
TFA	Time–frequency analysis
VPA	Valproic acid
vACC	Ventral anterior cingulate cortex

Introduction

Neuropsychiatric disturbances observed in deep brain stimulation (DBS) indicate that the STN is involved in cognitive impairment as well as in personality changes, apathy, aggression, depression or mania, and impulse control disorder (Baunez et al. 2011; Peron et al. 2013; Rodriguez-Oroz et al. 2011). The involvement of the STN in a broad spectrum of various cognitive functions has been reported: attention (Bočková et al. 2011; Mallet et al. 2007), executive functions (Baláž et al. 2008, 2011; Rektor et al. 2009), impulse control (Rodriguez-Oroz et al. 2011), conflict resolution (Brittain et al. 2012), and verbal learning (Coulthard et al. 2012), as well as in emotion (Kühn et al. 2005; Peron et al. 2013). The STN is a part of the cortico-basal ganglia-thalamocortical circuitry that targets the ACC as well as other cortices (Alexander et al. 1986). A quite direct method for studying the function of the STN is the recording of event-related electrical activities via DBS electrodes. Other studies have also presented modifications of STN electrical activities using non-motor activities, i.e. movement observation, emotional stimuli, and impulse control disorder (Ballanger et al. 2009; Kühn et al. 2005; Marceglia et al. 2009).

ACC activation was observed in studies of cognition. The findings from electroencephalography (EEG) studies of a focal area of negativity in scalp electrodes following an error response led to the idea that the ACC might be the brain's error detection and correction device (Bush et al. 2000; Ullsperger and Cramon 2003). This supports the role of the ACC in performance monitoring. In particular, the key functions of the ACC revolve around shortfalls from some standard (Nieuwenhuis et al. 2001), anticipation and preparation before task performance, regulation of emotions, and detection of errors. The ACC appears to have a crucial function in processing the incongruent FT (Botvinick et al. 2004).

We studied the appearance of broadband oscillatory changes induced by an executive function task with two levels of complexity. The event-related de/synchronizations (ERD/S) induced by the FT in the STN and the ACC were studied in the frequency range of 2–45 Hz. Spatial mapping of ERD/ERS is widely used to study the dynamics of cortical activation patterns (Pfurtscheller 2001). A decrease in frequency power indicates ERD; an increase in frequency power indicates ERS (Pfurtscheller et al. 2003). We focused on the oscillatory changes in the alpha and beta bands that appeared significant; see below.

Generally, the ERD of the alpha and beta rhythms is interpreted as a correlate of an activated cortical area with increased excitability. The ERS in the alpha and lower beta bands can be interpreted as a correlate of a deactivated cortical area, i.e. active inhibition or cortical idling (Pfurtscheller 2001).

Several recent studies were devoted to the relationship between ERD/ERS and various cognitive processes. Their results support the theory that oscillatory changes associated with cognitive processing are task specific and may depend on modality of stimulus. For instance, alpha ERD with parieto-occipital maximum is typical after visual stimuli; alpha ERS after auditory stimulation is connected with memory and cognitive processing. This means that the term "inhibition" in the context of alpha ERS is not absolute. Alpha ERD occurs during cognitive processing (Pfurtscheller and Klimesch 1992; Yordanova et al. 2001). Alpha ERS was described as an activity important for functional coupling between prefrontal cortical areas and more posterior sites in working memory tasks (Sauseng et al. 2005), internally directed attention and increased task demands (Cooper et al. 2003), and active task-relevant processing (Mo et al. 2011; Palva and Palva 2011).

We chose the FT in which subjects must react to the direction of a central arrow in congruent and incongruent trials (Eriksen and Eriksen 1974; Kopp et al. 1996; Praamstra and Stegeman 1998). The FT was performed with visual stimuli (arrows) (see Fig. 2), with a simple congruent and a more difficult incongruent condition (Kopp et al. 1996). We expected that both conditions would activate the attention and several particular executive functions, i.e. working memory, time perception, initiation, and motor control. The incongruent task increased demand on executive functions; besides the functions common for both tasks, an inhibition of automatic responses, the

reversal of incorrect response tendency, the internal ordering of the correct response, and the initiation of the target-induced correct response were involved (Falkenstein and Willemssen 2006). In this study, we explored the activation of STN and the activation of the ACC while processing the FT.

We raised two questions

1. Is the STN involvement in executive function related to the complexity of the task?
2. Is the STN involvement in the executive functions specific, i.e. does it differ from the role played by the ACC?

Methods and materials

Subjects

Four epilepsy surgery candidates with intracerebral electrodes implanted in the ACC and three PD patients with externalized DBS electrodes implanted in the STN participated in the study (see Table 1). All patients were insufficiently controlled by therapy or were pharmacoresistent and indicated for epilepsy surgery and DBS surgery by the commissions for Epilepsy Surgery and Neuromodulation Surgery in Brno. All the subjects were informed about the character of this study and gave their informed consent. The study received the approval of the local ethics committee. The measurements were performed during the patient's "on" state in PD, approximately 1 h after morning dopaminergic medication dose. The medication was stable in all subjects. A neuropsychological examination excluded severe cognitive disturbances and dementia in each patient. All the patients were right-handed and had normal hearing and vision.

Table 1 Exact position of the electrodes in the MNI coordinate system

Patient no.	Electrode contacts	Electrode positions in MNI coordinates	Recording BA
1	G1	7; 33; 17	BA 32 (vACC)
1	G2	10.6; 33.1; 16.8	BA 32 (vACC)
1	G3	14.1; 33.2; 16.6	BA 32 (vACC)
2	O1	6; 36; −17	subgACC
2	O2	9.6; 36; −17	subgACC
3	G′1	−5; 25; 24	BA 32/24 (vACC)
3	G′2	−8.6; 24.8; 23.7	BA 32/24 (vACC)
3	G′3	−12.1; 24.6; 23.3	BA 32/24 (vACC)
3	Po′1	−8; −63; 26	BA 31 (dACC)
3	Po′2	−11.6; −63; 25.9	BA 31 (dACC)
4	F1	5; 29; 28	BA 32 (vACC)
4	F2	8.5; 29; 27.7	BA 32 (vACC)
4	F3	12; 29; 27.3	BA 32 (vACC)
4	G1	7; 41; 12	BA 32 (vACC)
4	G2	10.5; 41.2; 11.7	BA 32 (vACC)
4	G3	14; 41.4; 11.3	BA 32 (vACC)

vACC ventral anterior cingulate cortex, *dACC* dorsal anterior cingulate cortex, *subgACC* subgenual anterior cingulate cortex

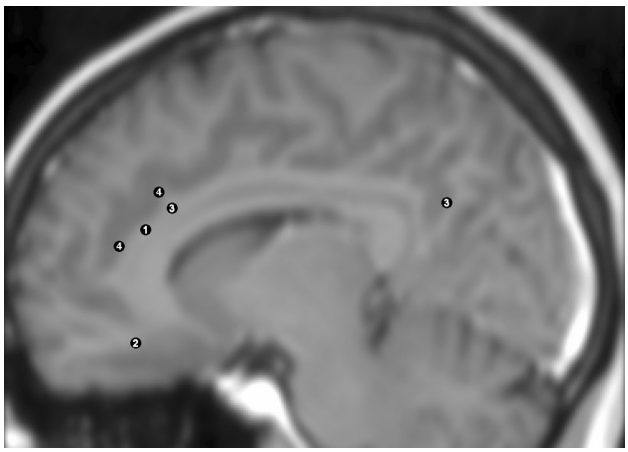

Fig. 1 Paramedial sagittal section with electrode positions in the ACC, overlapping MRI scans of all epilepsy surgery candidates (see patient numbers 1–4; Table 1)

Surgical procedure in candidates of epilepsy surgery

Depth electrodes were implanted to localize the seizure origin prior to surgical treatment. All patients received 6–11 orthogonal platinum electrodes in the investigated brain lobes using the methodology of Talairach et al. (1967) in order to localize the epileptogenic foci before surgical treatment. Standard semi-flexible intracerebral electrodes (ALCIS), diameter 0.8 mm, contact lengths 2 mm, inter-contact interval 1.5 mm, were used for invasive stereo-electroencephalography (SEEG) monitoring. The exact position of the electrodes and their contacts in the brain were verified using post-implantation magnetic resonance imaging with electrodes in situ. The recordings from lesional anatomical structures and epileptogenic zones were not included in the analysis. A total of 355 sites in the temporal (119), frontal (211), and parietal (25) lobes were recorded by means of 38 multi-contact depth electrodes for experimental analysis. We focused on the recordings from the ACC, especially the ventral cingulate gyrus (Brodmann area; BA 32, BA 24; 12 sites) and dorsal cingulate gyrus (BA 31; 2 sites). In patient 2, the subgenual ACC was explored (2 sites). Finally, 2 sites in the dorsal cingulate gyrus (in patient 2) were excluded because of poor-quality data.

The exact positions of the electrodes in the ACC are shown in Table 1 (Fig. 1).

Surgical procedure in PD patients

The stereotactic frame used during the surgical procedure was the Leibinger open frame with the Praezis Plus software and the Talairach diagram. The STN coordinates used were in respect to the anterior commissure–posterior commissure (AC–PC) line: 12.0 mm laterally, 5.0 mm below, and 3.0 mm behind the midpoint of the AC–PC line. The implantation procedure was performed in two steps. First, the stimulation leads (Medtronic, Inc.) were implanted bilaterally into the targeted structure by stereotactic MRI-guided technique under local anaesthesia. The lead placement was confirmed by microelectrode recordings. The motor part of the STN was identified by recording the patterns of neuronal activity and background activity, and by following motor responsiveness to intraoperative stimulation. Once the final target coordinates were determined, permanent quadripolar DBS electrodes (model 3389, with 1.5 mm contact length and 0.5 mm inter-contact intervals) were implanted. The electrode position was verified by the intraoperative use of fluoroscopy comparing the position of the microrecording electrode trajectories with the definitive quadripolar macroelectrode trajectory and also by a post-operative computer tomography (CT) scan.

In the second phase, the electrode cables were internalized and a neurostimulation device (Itrel III or Kinetra, Medtronic Inc., Minneapolis, USA) was implanted. The period between the two steps of the operation ranged from 3 to 4 days. This period served for functional assessment and testing of efficacy during the external stimulation. We also used this period of time for measuring task-related ERD/ERS.

Procedure and EEG recording

Subjects were seated half-reclining comfortably in the monitoring bed, in a quiet room with a fixed temperature. Each subject was asked to minimize movement, to keep their eyes fixed on the monitor, and to concentrate on the task. The monitor was situated at the same place for all subjects: 1.5 m in front of their eyes, at the end of the monitoring bed.

Before the recording session, each subject was given a short-practice period. All recordings were under the examiner's visual control, and failed trials were removed.

Recordings were made with respect to a reference, with a ground lead placed on the earlobes.

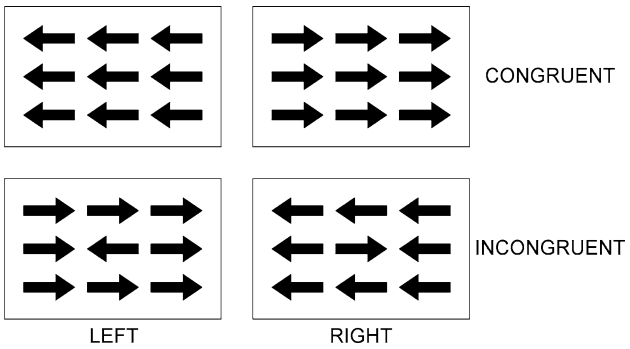

Fig. 2 The four stimuli used in the task—Flanker paradigm

The EEG signal was recorded from various cerebral structures using the intracerebral electrodes. The electromyography (EMG; from the dominant-hand m. flexor carpi radialis), electrooculography (EOG), and scalp EEG (Cz, Pz, Fz electrodes) were recorded simultaneously. ACC activity was recorded via the 128-channel TruScan (Alien, Czech Republic) with a sampling rate of 256 Hz. Because of the modernization of the EEG unit, PD subjects were recorded on the 192-channel Brainscope (M&I, Czech Republic) with a sampling rate of 1,024 Hz. Standard anti-aliasing filters were used.

Flanker task (FT)

The FT with arrows was performed (Eriksen and Eriksen 1974). The visual stimuli are indicated by black arrows against a white background that pointed to the left or right (see Fig. 2). The duration of the stimulus exposure was 200 ms. The interstimulus interval was fixed to 4 s, the number of stimuli was 200, and the ratio of congruent to incongruent arrows was 80–20 %. We used PRIME software for presenting trials. The screen size was 15″ (LCD monitor), the distance between eyes and screen was 70 cm, visual angel was 30°, and refresh rate 60 Hz. During interstimulus intervals, a white background was presented on the screen. Randomization was performed by PRIME software.

Subjects were required to focus on the central arrow and to react to four types of stimuli presented on the monitor by pushing the right or left button according to the direction of the central arrow. We distinguished four types of stimuli: congruent arrows to the right or left side and incongruent arrows to the right or left side (see Fig. 2). A congruent stimulus means that the flanking arrows are in the same direction as the target central arrow. An incongruent stimulus means that the flanking arrows are in the opposite direction from the central arrow. The response (by pressing the button to the right or left side) is determined by direction of the central arrow.

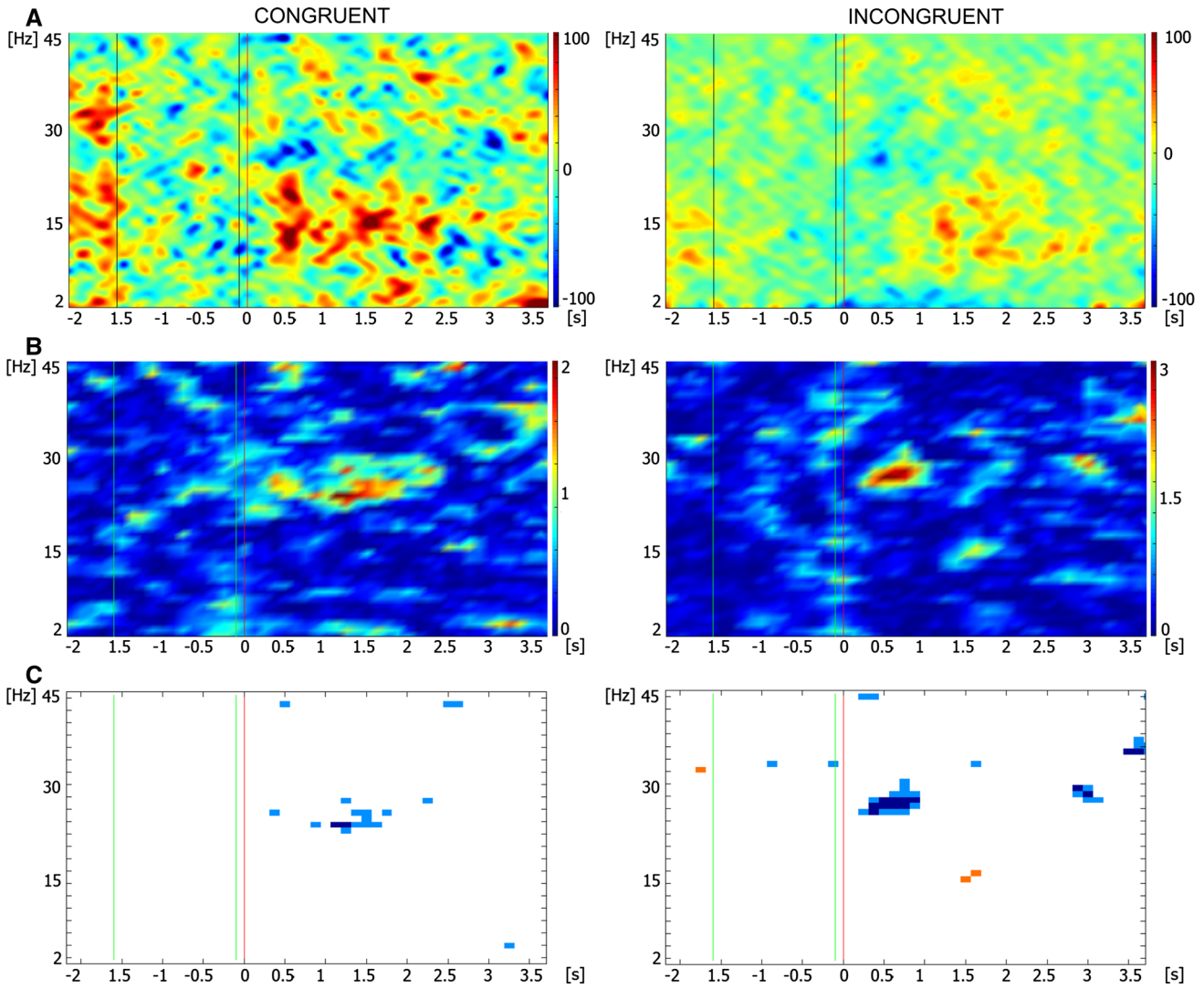

Fig. 3 TFA matrices from all selected ACC contacts in congruent and incongruent tasks and their differences in time–frequency interpretation. **a** Time–frequency representation of ERD/ERS in 2–45 Hz frequency range and time interval −2 and 3.5 s before and after stimuli. Time frequency (TFA) matrices were computed as a grand average from all selected ACC contacts and all epileptic subjects. The +100 value (*red*) means a doubling of instantaneous power with respect to the baseline region; the −100 value (*blue*) means a drop by half. This procedure provides a comparable colour interpretation of ERS and ERD. **b** TFA significance: statistical significance between the mean power at baseline region and the mean power in the window moving over whole trial. Statistical significance is presented in colours, where the more significant changes are *green*, *yellow* and *red* (*red* 2 or 3, it means $p < 0.01$ or $p < 0.001$). *Blue colour* here represents less significance. The Bonferroni correction was used to reduce p value. **c** TFA statistical significance from 3B with identification ERD and ERS, *red colour* identifies a significant power increase (ERS) with $p < 0.05$, *dark blue* a significant power decrease (ERD) with $p < 0.01$, *light blue* a significant power decrease (ERD) with $p < 0.05$

Data analysis

The data were processed and analysed off-line using ScopeWin and ScopeMat software and segmented according to the stimulation trigger onset. The trials were visually inspected to eliminate EEG segments containing any artefact activity or mistaken response. In each segment, the linear trend was eliminated.

Time–frequency analysis (TFA) (Akay 1997) with eliminated phase-lock signals was used to determine the event-related de/synchronizations (ERD/S) in 2–45 Hz frequency ranges. TFA produces a matrix in which each row represents the over trials averaged signal power envelopes in a 4 Hz frequency band width (x axis represents time; y axis represents frequency); see Figs. 3 and 4. The frequency step between two rows was 1 Hz. In the baseline, normalized TFA matrix, ERS is represented by positive values (red) and ERD by negative values (blue). Normalization with a baseline was done according to the equation:

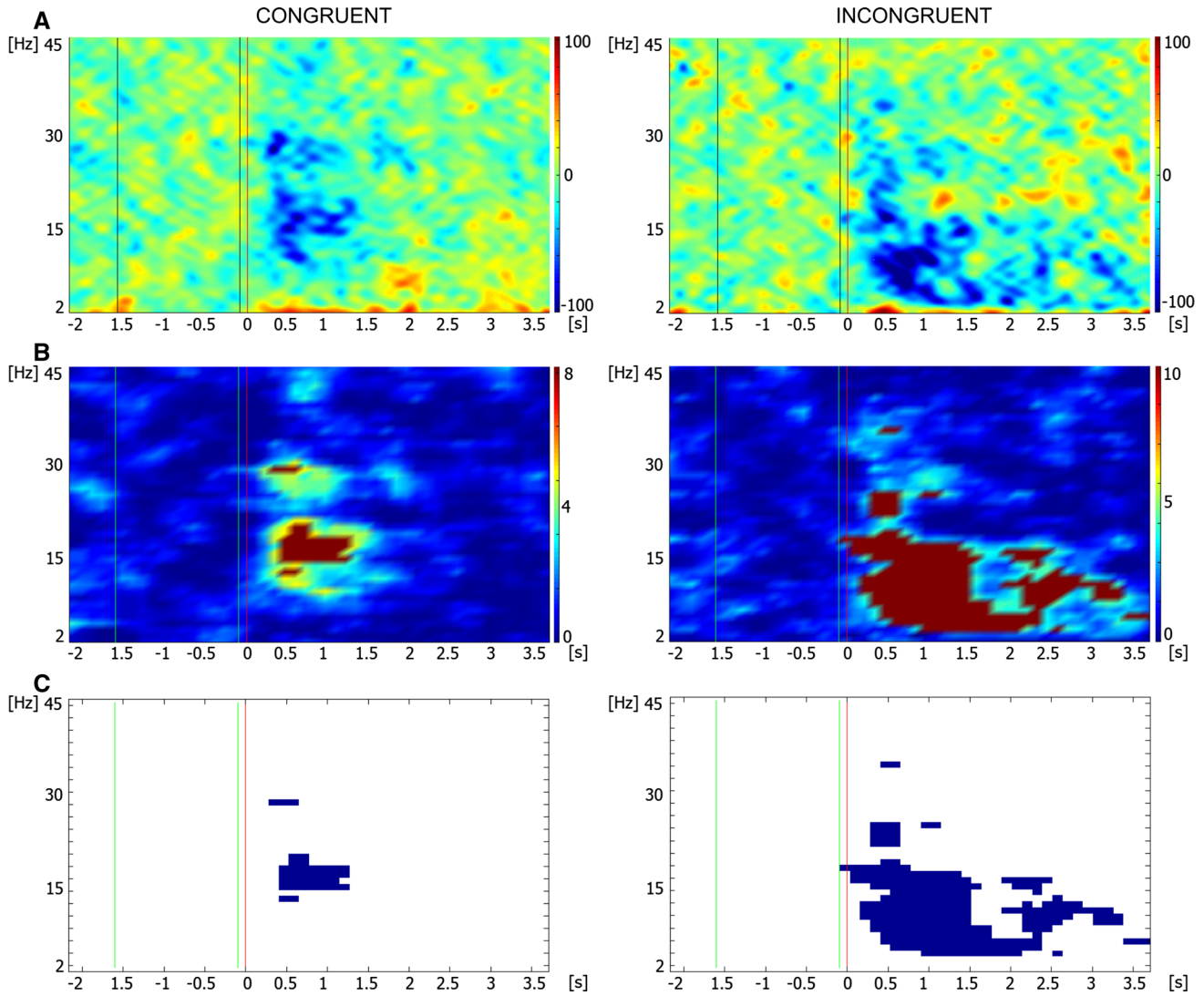

Fig. 4 TFA matrices from all selected STN contacts in congruent and incongruent tasks and their differences in time–frequency interpretation. **a** Time–frequency representation of ERD/ERS in 2–45 Hz frequency range and time interval −2 and 3.5 s before and after stimuli. Time frequency (TFA) matrices were computed as a grand average from all selected STN contacts and all PD subjects. The +100 value (*red*) means a doubling of instantaneous power with respect to the baseline region; the −100 value (*blue*) means a drop by half. This procedure provides a comparable colour interpretation of ERS and ERD. **b** TFA significance: statistical significance between the mean power at baseline region and the mean power in the window moving over whole trial. Statistical significance is presented in colours, where the more significant changes are *green*, *yellow*, and *red* (*red* = 8 or 10, it means $p < 1E{-}8$, or $p < 1E{-}10$). *Blue colour* here represents less significance. The Bonferroni correction was used to reduce p value. **c** TFA statistical significance from **b** with identification ERD, *blue colour* identifies a significant power decrease (ERD) with $p < 0.001$, there was no significant ERS

$$\mathrm{ERS} = 100 \times (\mathrm{PW}(t)/\mathrm{PWbaseline} - 1), \quad \text{when } \mathrm{PW}(t)/\mathrm{PWbaseline} \geq 1$$

$$\mathrm{ERD} = 100 \times (1/(\mathrm{PW}(t)/\mathrm{PWbaseline}) - 1), \quad \text{when } \mathrm{PW}(t)/\mathrm{PWbaseline} < 1$$

PWbaseline—mean power from baseline region (1,600–100 ms prior stimuli), PW(t)—instantaneous power. The limitation of ±100 of normalized values is used. The scale in the TFA matrix in the figures is +100, −100. The +100 value (red) means a doubling of instantaneous power with respect to the baseline region; the −100 value (blue) means a drop by half. This procedure provides a comparable colour interpretation of ERS and ERD.

The statistical significance of ERS/ERD was analysed from the differences over trials between the mean power at baseline and the mean power in the window moving over segment. Statistical significance is presented in a matrix where the significant changes to baseline are dark red, light red, dark blue, and light blue. The Bonferroni correction was used. The p coefficient was multiplied by 100, as TFA

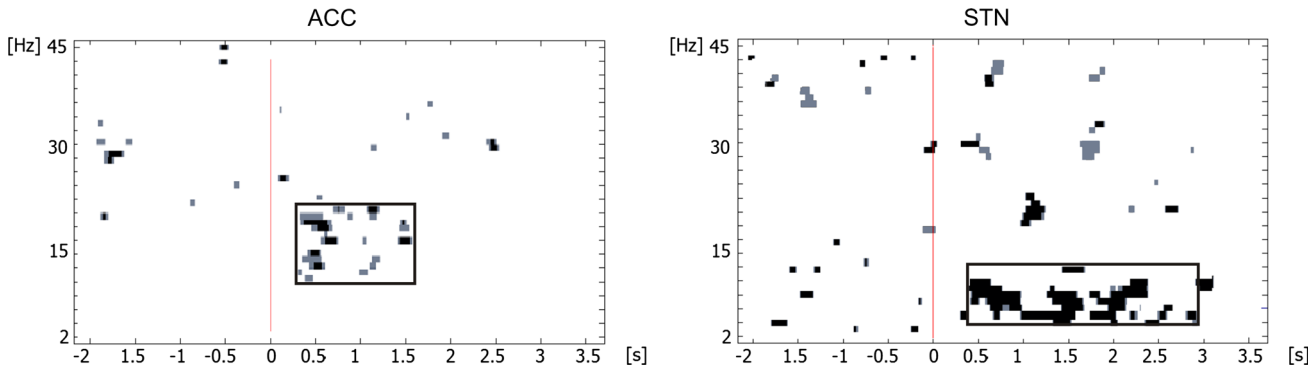

Fig. 5 *Black* and *white* matrices identify differences between two different stimuli (congruent, incongruent) in time–frequency interpretation. Each point of the matrix represents the significance of differences between tasks in the corresponding time and frequency position. *Dark grey* represents significance level $p < 0.005$ and *light grey* $p < 0.01$. In ACC, the differences between stimuli are mostly located in frequency region 8–24 Hz and time location 0.5–1.5 s. In STN differences between stimuli are most significant in low-frequency region 4–14 Hz and time location 0.5–3 sm

matricesmatrices have 40 lines and data are used two times: significance of single tasks and significance of differences between tasks. Dark red identifies a power increase when $p < 0.01$, light red a power increase when $p < 0.001$ (Fig. 3), dark blue a power decrease when $p < 0.01$, and light blue a power decrease when $p < 0.001$. White regions of this matrix identify non-significant changes to baseline.

To identify differences between two different stimuli in time–frequency interpretation, we computed an interstimulus statistical significance matrix; see Fig. 5. Each point of the matrix represents the significance of differences between tasks in the corresponding time and frequency position. In each time–frequency position, we tested the differences of two vectors. Each vector represents the values defined as the difference between the power envelope value in the appropriate frequency range and the time position and mean power envelope in the baseline region. Two vectors include values over all selected trials from congruent and incongruent tasks. We used a non-paired *t* test to determine significance. In Fig. 5, dark grey represents $p < 0.005$ and light grey $p < 0.01$.

Results

We evaluated the signal recorded from 16 contacts placed in the ACC and 18 contacts placed in the STN. The total number of trials of the incongruent task in the ACC was 280 and in the STN was 390. The number of trials with congruent stimuli was randomly reduced to be equal to the number of trials with incongruent stimuli. Time–frequency analyses (TFA) of ERD/ERS in 2–45 Hz were performed. TFA matrices were computed as a grand average from all selected ACC contacts (see Fig. 3) and STN contacts (see Fig. 4). Finally, statistical analyses of differences between congruent and incongruent stimuli were performed (see Fig. 5).

This figure demonstrates the presence of ERD in the 22–26 Hz frequency range with latency of 1–2 s with congruent stimuli. With incongruent stimuli, there is more localized and compact ERD in the 26–30 Hz frequency range with latency of 0.5–1 s. Although there is a large area of red (ERS) in Fig. 3a, ERS is not significant. This is because the incidence of higher power after stimulation is not regular and variable in amplitude (Table 2).

The figure demonstrates the presence of single small ERD core in the 14–20 Hz frequency range with latency of 0.5–1.5 s with congruent stimuli. With incongruent stimuli, there is a large ERD area in the 4–20 Hz frequency range with latency from 0.2 s up to 3 s.

Behavioural results in the FT

In the next step, we evaluated behavioural results in the FT, especially the quantification of the effect of conflict (RT incongruent–RT congruent) and the effects of conflict on error rate (error rate during congruent vs. error rate during incongruent trials). Finally, we evaluated RT and error rate differences across conditions to demonstrate that patients performed the FT correctly. A summary of the behavioural results is presented in Table 3.

Mean errors occurrence in congruent task is 7.05 ± 1.6 % and in incongruent 16.05 ± 9.7 %. Error rate differences between congruent and incongruent task in the paired *T* test are significant ($p < 0.01$).

Mean reaction time in congruent task is 762 ± 212 ms and in incongruent 967 ± 311 ms. Reaction time differences between congruent and incongruent task in the paired *T* test are significant ($p < 0.01$).

In accordance with our expectations, we demonstrated that the error rate is higher and reaction time is longer during incongruent task performance. These differences were found to be significant.

Table 2 Subject characteristics

Number	No. 1 (AP)	No. 2 (NM)	No. 3 (HM)	No. 4 (FJ)	No. 5 (SP)	No. 6 (HM)	No. 7 (AJ)
Age	33	35	26	38	49	60	50
Sex	Female	Female	Male	Male	Male	Female	Male
Diagnosis	Right non lesional FLE	Right MTLE	Left MTLE	Right FLE	PD	PD	PD
Target	ACC	ACC	ACC	ACC	STN	STN	STN
Medication	VPA, LTG	CBZ, LVT, PGB	VPA, CBZ, PGB	CBZ, LVT	L-DOPA, entacapone	L-DOPA, entacapone	L-DOPA, entacapone
MR	Normal	Right mesiotemporal sclerosis	Left mesiotemporal sclerosis	Right frontal dysplasia	Normal	Normal	Normal

FLE frontal lobe epilepsy, *MTLE* mesiotemporal lobe epilepsy, *LTG* lamotrigine, *CBZ* carbamazepine, *TOP* topiramate, *LVT* levetiracetam, *VPA* valproic acid, *PGB* pregabalin, *CLZ* clonazepam, *PD* Parkinson's disease, *STN* subthalamic nucleus, *ACC* anterior cingulate cortex

Table 3 Summary of behavioural results in the FT (errors and average reaction times with standard deviation separately for congruent and incongruent tasks)

Patient no.	Errors congruent %	Errors incongruent %	Reaction time congruent, mean ± SD (ms)	Reaction time incongruent, mean ± SD (ms)
1	5.3	7.4	734 ± 125	822 ± 120
2	7.1	11.5	860 ± 284	1,011 ± 444
3	7.2	10.4	530 ± 121	623 ± 113
4	7.1	11.8	579 ± 79	624 ± 92
5	4.7	11	758 ± 234	1,190 ± 416
6	9	32	1,175 ± 525	1,489 ± 640
7	9	28	703 ± 318	1,010 ± 424

Discussion

We studied the activation of the STN and the activation of the ACC while processing the FT. The ACC appears to have a crucial function in processing the incongruent FT (Botvinick et al. 2004). In our study, we observed that the two modalities of the FT, i.e. the congruent and incongruent, are processed in the ACC. Nevertheless, the activation by the incongruent was higher than by the congruent. The beta ERD was significantly more activated by the incongruent condition. This supports the role of the ACC in performance monitoring. We observed that the STN is also involved in processing of the two modalities of the FT, with significantly higher activation by the more difficult incongruent condition than by the simpler congruent condition. In contrast to the ACC, in the STN, the ERD beta and the ERD alpha activities were significantly more activated by the incongruent condition. We conclude that the STN is more involved in processing tasks with increased cognitive loads.

The incongruent task increased demand on executive functions, and in addition to the functions common for both tasks, it involved an inhibition of automatic responses, the reversal of incorrect response tendency, the internal ordering of the correct response, and the initiation of the target-induced correct response (Falkenstein and Willemssen 2006).

The STN role in controlled processing is often seen as the inhibition of competing alternatives or the inhibition of automatic stimulus–response associations. Task complexity increased beta desynchronization and gamma synchronization before and during the movement (Oswal et al. 2013). These authors provided evidence for two patterns of reactivity in the STN in the beta band: one that is anticipatory and has previously been linked to the likelihood of an upcoming action, and one that is perimovement in timing and is partially modulated by task complexity as determined by the need for more controlled behaviour and active stimulus–response remapping (Oswal et al. 2013; Jenkinson and Brown 2011).

The involvement of the STN in the modulation of cognitive activities is related to the cognitive load. We observed in our previous studies that STN involvement in the cognitive functions is task dependent (Baláž et al. 2008, 2010; Rektor et al. 2009; Bočková et al. 2011). It appears that the STN was involved in complex tasks with increased cognitive loads but not in simpler cognitive tasks (Baláž et al. 2008; Rektor et al. 2009; Bočková et al. 2011). In a dual task, the STN generated event-related potentials (ERPs) in a modified oddball protocol with postponing the dates, at variance with a standard oddball protocol (Baláž et al. 2008, 2010). Imaging studies showed that the effects of STN-DBS are task specific and depend on the particular networks involved in those specific tasks (Kalbe et al. 2009; Mallet et al. 2007; Coulthard et al. 2012). Probably, the tasks modulated by the STN are determined by the selective involvement of cortical neuronal populations that are interconnected with the STN. Repetitive transcranial magnetic stimulation (rTMS) on the inferior prefrontal

cortex but not over the dorsolateral prefrontal cortex shortened the latency of potentials evoked by a dual task in the STN (Baláž et al. 2010). The selectivity and degree of the STN involvement in cognitive functions might be linked with its role in regulating human behaviour. STN plays a role in the behavioural adjustment (Peron et al. 2013). The involvement of the STN in complex cognitive behavioural functions (Peron et al. 2013; Baunez et al. 2011) has been proposed.

In a previous study, we reported that not only the ACC but also the widespread cortical areas are involved in processing the FT (Rusnáková et al. 2011). The STN may act also in cooperation with cortical areas other than the ACC, for example with the inferior frontal cortex, which is linked with the STN via the hyperdirect pathway (Baláž et al. 2010). The STN acts as an integrator with the input from the hyperdirect pathway from the cortex and the indirect pathway from the cortico-basal ganglia-thalamo-cortical circuitry.

Our study aimed to answer whether the STN involvement in executive function reflects the complexity of the task. There are several limitations of our study that prevented studying the oscillations in more detail, notably the low number of investigated subjects. This is the result of the limited willingness and capability of patients to co-operate during the short period between the implantation of the DBS electrode and its internalization.

Conclusion

This study confirmed the role of the STN in complex cognitive activities. As in our earlier studies, the STN appears to be more involved in activities linked with increased cognitive loads. The specificity and complexity of the task-related activation of the STN might indicate the involvement of the STN in human behaviour. Further studies are needed to elucidate the complex role of the STN in higher-order brain activities and human behaviour.

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Commentary to habilitation thesis

Despite of advances in the diagnosis and treatment of epilepsy remain approximately 30%-40% of the patients pharmacoresistant. The International League Against Epilepsy (ILAE) defines drug-resistant epilepsy as "failure of adequate trials of two tolerated, appropriately chosen and used antiepileptic drug schedules (whether as monotherapies or in combination) to achieve sustained seizure freedom". Only parts of these patients can be offered curative epileptosurgical solutions or other non-pharmacological treatment options (vagus nerve stimulation, ketogenic diet, etc.).

The pharmacoresistance to antiseizure drugs (ASD) per se is not a possible therapeutic target, as ASDs themselves do not prevent the development of epilepsy, merely blocking the seizures as they arise. Moreover, there is no evidence that they influence the course of epilepsy. There are no clinical tools or guidelines for predicting therapeutic response in individual patients, leaving them no choice other than to try all antiseizure drugs available as they suffer debilitating seizures with no relief.

Current therapy is limited to suppressing the symptoms of the disease- epileptic seizures, and does not allow the elimination of the cause (except epileptosurgery) or its prevention during the proces of epileptogenesis (in the patients who are at risk due to genetic predisposition or after brain insult).

The aim of research in the epileptology is discovery of the drugs that would not only suppress seizures, but ideally work as medication which prevent or modify the process of epileptogenesis, the medication working as disease-modifying drugs (DMD) and the treatment due to progressive process of neurodegeneration, neuroinflammation and neuronal hyperexcitability leading to the development of pharmacoresistance in epilepsy.

The discovery of predictive biomarkers and early identification of pharmacoresistant patients and patients who are at the risk of development of epilepsy (biomarkers of epileptogenesis) is the highest priority of current epileptology research.

This habilitation thesis is conceived as a collection of 6 articles previously published by the author and her colleagues. It contains individual chapters dealing with the basic aspects of epileptologenesis and pharmacoresistance in epilepsy. Each chapter is followed by commentaries introducing the topic of each publication, describing the current state of knowledge and how the author has contributed to knowledge in this field. The work is based

on research activities at the authors' workplaces, the Department of Paediatric Neurology, University Hospital Brno; the Faculty of Medicine, Masaryk University; and Central European Institute of Technology.

In the future, new therapeutic procedures should offer a wide range of options, respecting the specifics of individual forms of epilepsy as well as individual differences between patients with regard to the development and prognosis of the disease.

Annex 1

AULICKA, S., K. CESKA, J. SANA, T. LOJA, P. JABANDZIEV, J. PAPEZ, P. DANHOFER, H. VINOHRADSKA, I. DOLEZALOVA, M. BRAZDIL, P. STOURAC, H. OSLEJSKOVA a O. SLABY. The role of inflammation in etiopathogenesis of pharmacoresistant epilepsy and refractory status epilepticus. *Ceska a Slovenska Neurologie a Neurochirurgie* [online]. 2020, **83**(1), 8–13. ISSN 1210-7859. Dostupné z: doi:10.14735/amcsnn20208

Experimental work	Supervision	Manuscript	Research direction
30%	-	70%	30%

Annex 2

BOHOSOVA, Julia, Jiri VAJCNER, Petr JABANDZIEV, Hana OSLEJSKOVA, Ondrej SLABY a ***Stefania AULICKA *(corresponding author)*.*** MicroRNAs in the development of resistance to antiseizure drugs and their potential as biomarkers in pharmacoresistant epilepsy. *Epilepsia* [online]. 2021, **62**(11), 2573–2588. ISSN 0013-9580. Dostupné z: doi:10.1111/epi.17063

Experimental work	Supervision	Manuscript	Research direction
-	70%	30%	30%

Annex 3

CESKA, Katarina, ***Stefania AULICKA *(corresponding author)****, Ondrej HORAK, Pavlina DANHOFER, Pavel RIHA, Radek MARECEK, Jan SENKYRIK, Ivan REKTOR, Milan BRAZDIL a Hana OSLEJSKOVA. Autosomal dominant temporal lobe epilepsy associated with heterozygous reelin mutation: 3 T brain MRI study with advanced neuroimaging methods. *Epilepsy & Behavior Case Reports* [online]. 2019, **11**, 39–42. ISSN 2213-3232. Dostupné z: doi:10.1016/j.ebcr.2018.10.003

Experimental work	Supervision	Manuscript	Research direction
20%	60%	50%	30%

Annex 4

SCHILLING, Kurt G., Francois RHEAULT, Laurent PETIT, Colin B. HANSEN, Vishwesh NATH, Fang-Cheng YEH, Gabriel GIRARD, Muhamed BARAKOVIC, Jonathan RAFAEL-PATINO, Thomas YU, Elda FISCHI-GOMEZ, Marco PIZZOLATO, Mario OCAMPO-PINEDA, Simona SCHIAVI, Erick J. CANALES-RODRIGUEZ, Alessandro DADUCCI, Cristina

GRANZIERA, Giorgio INNOCENTI, Jean-Philippe THIRAN, Laura MANCINI, Stephen WASTLING, Sirio COCOZZA, Maria PETRACCA, Giuseppe PONTILLO, Matteo MANCINI, Sjoerd B. VOS, Vejay N. VAKHARIA, John S. DUNCAN, Helena MELERO, Lidia MANZANEDO, Emilio SANZ-MORALES, Angel PENA-MELIAN, Fernando CALAMANTE, Arnaud ATTYE, Ryan P. CABEEN, Laura KOROBOVA, Arthur W. TOGA, Anupa Ambili VIJAYAKUMARI, Drew PARKER, Ragini VERMA, Ahmed RADWAN, Stefan SUNAERT, Louise EMSELL, Alberto DE LUCA, Alexander LEEMANS, Claude J. BAJADA, Hamied HAROON, Hojjatollah AZADBAKHT, Maxime CHAMBERLAND, Sila GENC, Chantal M. W. TAX, Ping-Hong YEH, Rujirutana SRIKANCHANA, Colin D. MCKNIGHT, Joseph Yuan-Mou YANG, Jian CHEN, Claire E. KELLY, Chun-Hung YEH, Jerome COCHEREAU, Jerome J. MALLER, Thomas WELTON, Fabien ALMAIRAC, Kiran K. SEUNARINE, Chris A. CLARK, Fan ZHANG, Nikos MAKRIS, Alexandra GOLBY, Yogesh RATHI, Lauren J. O'DONNELL, Yihao XIA, Dogu Baran AYDOGAN, Yonggang SHI, Francisco Guerreiro FERNANDES, Mathijs RAEMAEKERS, Shaun WARRINGTON, Stijn MICHIELSE, Alonso RAMIREZ-MANZANARES, Luis CONCHA, Ramon ARANDA, Mariano Rivera MERAZ, Garikoitz LERMA-USABIAGA, Lucas ROITMAN, Lucius S. FEKONJA, Navona CALARCO, Michael JOSEPH, Hajer NAKUA, Aristotle N. VOINESKOS, Philippe KARAN, Gabrielle GRENIER, Jon Haitz LEGARRETA, Nagesh ADLURU, Veena A. NAIR, Vivek PRABHAKARAN, Andrew L. ALEXANDER, Koji KAMAGATA, Yuya SAITO, Wataru UCHIDA, Christina ANDICA, Masahiro ABE, Roza G. BAYRAK, Claudia A. M. Gandini WHEELER-KINGSHOTT, Egidio D'ANGELO, Fulvia PALESI, Giovanni SAVINI, Nicolo ROLANDI, Pamela GUEVARA, Josselin HOUENOU, Narciso LOPEZ-LOPEZ, Jean-Francois MANGIN, Cyril POUPON, Claudio ROMAN, Andrea VAZQUEZ, Chiara MAFFEI, Mavilde ARANTES, Jose Paulo ANDRADE, Susana Maria SILVA, Vince D. CALHOUN, Eduardo CAVERZASI, Simone SACCO, Michael LAURICELLA, Franco PESTILLI, Daniel BULLOCK, Yang ZHAN, Edith BRIGNONI-PEREZ, Catherine LEBEL, Jess E. REYNOLDS, Igor NESTRASIL, Rene LABOUNEK, Christophe LENGLET, Amy PAULSON, **Stefania AULICKA,** Sarah R. HEILBRONNER, Katja HEUER, Bramsh Qamar CHANDIO, Javier GUAJE, Wei TANG, Eleftherios GARYFALLIDIS, Rajikha RAJA, Adam W. ANDERSON, Bennett A. LANDMAN a Maxime DESCOTEAUX. Tractography dissection variability: What happens when 42 groups dissect 14 white matter bundles on the same dataset? *Neuroimage* [online]. 2021, **243**, 118502. ISSN 1053-8119. Dostupné z: doi:10.1016/j.neuroimage.2021.118502

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Experimental work	Supervision	Manuscript	Research direction
30%	10%	30%	30%

Annex 5

RUSNAKOVA, Stefania, Pavel DANIEL, Jan CHLADEK, P. JURAK a Ivan REKTOR. The Executive Functions in Frontal and Temporal Lobes: A Flanker Task Intracerebral Recording Study. *Journal of Clinical Neurophysiology* [online]. 2011, **28**(1), 30–35. ISSN 0736-0258. Dostupné z: doi:10.1097/WNP.0b013e31820512d4

Experimental work	Supervision	Manuscript	Research direction
50%	-	80%	30%

Annex 6

AULICKA, Stefania Rusnakova, Pavel JURAK, Jan CHLADEK, Pavel DANIEL, Josef HALAMEK, Marek BALAZ, Martina BOCKOVA, Jan CHRASTINA a Ivan REKTOR. Subthalamic nucleus involvement in executive functions with increased cognitive load: a subthalamic nucleus and anterior cingulate cortex depth recording study. *Journal of Neural Transmission* [online]. 2014, **121**(10), 1287–1296. ISSN 0300-9564. Dostupné z: doi:10.1007/s00702-014-1191-5

Experimental work	Supervision	Manuscript	Research direction
50%	-	80%	30%