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Levetiracetam resistance: Synaptic signatures & corresponding promoter SNPs in epileptic hippocampi

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ABSTRACT

Pharmacoresistance to antiepileptic drugs (AEDs) is a major clinical problem in patients with mesial temporal lobe epilepsy (mTLE). Levetiracetam (LEV) represents a unique type of AED as its high-affinity binding site, the synaptic vesicle protein SV2A, is a component of the presynaptic release machinery. LEV often leads to full seizure control even in previously refractory patients. However, approximately 30% of LEV-treated mTLE patients do not show a significant response to LEV from the beginning of the pharmacotherapy and are therefore classified as a priori non-responders. This unexpected phenomenon prompted genetic studies, which failed to characterize responsible SV2A sequence alterations.

Here, we followed a different approach to study the mechanisms of LEV pharmacoresistance by screening for mRNA signatures specifically expressed in LEV a priori non-responders in epileptic brain tissue and subsequent promoter analyses of highly altered transcripts. To this end, we have used our unique access to analyze hippocampal tissue from pharmacoresistant TLE patients who underwent epilepsy surgery for seizure control ($n = 53$) stratified according to a priori LEV responders versus patients with impaired LEV-response. Transcriptome (Illumina platform) and subsequent multimodal cluster analyses uncovered strikingly abundant synapse-associated molecule mRNA signatures in LEV a priori non-responders. Subsequent promoter characterization revealed accumulation of the single nucleotide polymorphism (SNP) rs9305614 G-allele in a priori non-responders to correlate to abundant mRNAs of phosphatidylinositol N-acetylglucosaminyltransferase (PIGP), i.e. a key component of the Wnt-signaling pathway. By luciferase assays, we observed significantly stronger activation by the LBP-1 transcription factor of the rs9305614 G-allele PIGP promoter. The present data suggest an abundance of transcripts encoding for key synaptic components in the hippocampi of LEV a priori non-responder mTLE patients, which for PIGP as proof of concept can be explained by a particular promoter variant. Our data argue for epigenetic factors predisposing for a priori LEV pharmacoresistance by transcriptional 'overexposure of targets'.

Introduction

Resistance to antiepileptic pharmacotherapy (antiepileptic drug; AED) represents a major obstacle in patients with focal epilepsies. Seizures frequently originate in the mesial temporal lobe (mesial temporal lobe epilepsy; mTLE). General concepts of pharmacoresistance relate to (*a*) increased expression of multidrug efflux transporters (Heinemann et al., 2006; Luna-Tortos et al., 2008; Schmidt and Löscher, 2009) or (*b*) switch of properties of respective drug targets (Remy and Beck, 2006; Remy et al., 2003), i.e. ion channel subunits.

Levetiracetam (LEV), one of the most frequently administered AEDs in mTLE patients, often excellently controls seizures even in previously refractory patients (Lynch et al., 2009). With synaptic vesicle proteins, i.e. so far SV2A, LEV has unique target structures (Lynch et al., 2004). However, a subgroup of approximately 30% LEV-treated TLE patients does not display significant responses to LEV from the initiation of treatment (Lynch et al., 2009) and can be classified as a priori non-responders. Furthermore, the chance of a seizure reduction upon LEV after initial failure is very low (Lee et al., 2013). Taken together, these observations suggest the presence of a priori non-responders, prompting the idea of a genetic basis of this a priori pharmacoresistance. Respective genetic studies, however, did not reveal significant risk variants of e.g.

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SV2A in epileptic patients so far (Dibbens et al., 2012; Lynch et al., 2009). LEV appears to have effects on voltage-gated calcium and potassium channels as well as on $GABA_A$-receptors under specific conditions (Surges et al., 2008). Experimental studies, however, have produced conflicting results, and LEV's major mechanism of action is controversially discussed. In view of the specific binding affinity of LEV to SV2A, the most promising candidate mechanisms include effects on the presynaptic release machinery.

Functional analyses stress the role of SV2A and the synaptic compartment as target structures for LEV, i.e. SV2A knockout mice have an epileptic phenotype with reduced responsiveness to LEV (Kaminski et al., 2009). Intriguingly, under experimental conditions the action of LEV depends on the available concentrations of SV2A (Nowack et al., 2011). In addition to that, expression levels of SV2A in focal epilepsies due to brain tumors showed a correlation of the anticonvulsant response upon LEV treatment (de Groot et al., 2010, 2011; Winden et al., 2011). LEV has clear effects on synaptic function such as activity-dependently increasing inhibitory transmission (Meehan et al., 2012). Functionally, SV2A is strongly connected with many other molecules in the presynaptic release machinery modulating synaptic plasticity (Baldelli et al., 2007; Janz et al., 1999; Winter, 1999). Recently, it was suggested that LEV exerts its function in synapses after vesicular entry and acts on the intravesicular binding site of SV2A (Meehan et al., 2012).

We hypothesize that distinct transcriptomic signatures based on allelic promoter variants contribute to an individual predisposition of the anticonvulsant efficacy of LEV. Capitalizing on our human hippocampal tissue bank of TLE patients who underwent epilepsy surgery for seizure control, we carried out a genome wide expression array analysis after carefully stratifying patients in a five-tiered classification system according to *initial* LEV-response. Our results suggest differential hippocampal gene expression patterns in LEV a priori non-responders, i.e. a striking abundance of synapse-associated transcripts. By subsequent promoter analyses, we identified individual single nucleotide polymorphisms (SNPs) with strong impact on corresponding mRNAs as for PIGP, a key molecule for Wnt-signaling (Shao et al., 2009), differentially distributed according to LEV responses.

Materials and methods

Surgical specimens and patient stratification with respect to LEV response

Biopsy specimens were obtained from Caucasian patients ($n = 53$) with chronic pharmacoresistant mTLE and a clear history of LEV treatment who underwent surgical treatment in the Epilepsy Surgery Program at the University of Bonn Medical Center. A combination of presurgical invasive and non-invasive procedures revealed onset of seizures in the mesial temporal lobe in all patients (Kral et al., 2002). Surgical removal of the hippocampus was clinically indicated in every case. All procedures were conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of the University of Bonn Medical Center. Informed written consent was obtained from all patients. Comprehensive clinical characteristics of patients were available for our analyses as described before (Supplemental Table 1) (Pernhorst et al., 2011).

Table 1
Classification of TLE patients according to levetiracetam-responsiveness.

Group	Definition	Patients (53)	Maximal LEV dosage (mg/day)
1	No effect of LEV	**6**	**2810.6**
2	Only short effect of LEV > 3 month seizure-free	2	2911.8
3	Effect of LEV, seizures less frequent	12	3004.5
4	Clear effect, almost seizure-free	4	2875.0
5	Clear effect, >6 month seizure-free	**16**	**2633.6**
0	Not treated with LEV	13	0

Obviously, definition of parameters for pharmacoresistance represents a major critical issue in mTLE patients. Our classification system is based on the current definition of the ILAE (Kwan et al., 2010) which includes a failure of an 'appropriate' and 'adequate' intervention of at least two antiepileptic 'drug schedules'. These criteria are fulfilled in all patients used in this study (Supplemental Table 1). The patient collective of our present study meets particularly high standard criteria for clinical categorization with respect to detailed dosage of LEV administration over a longstanding time span of observation and short interval seizure frequency monitoring. For classification of LEV-treated patients we used a five-tiered system of pharmacoresponse to LEV to stratify the above-mentioned series of patients according to criteria given in Table 1. Importantly, group 1 comprises patients that a priori did not show any effect with respect to seizure activity on LEV treatment. Great care was taken in order to include only patients to whom a full dosage of LEV was administered, i.e. 2.0 g/day as minimum dose to be classified as a priori non-responders (Mbizvo et al., 2012). Conversely, group 5 contains mTLE patients that represent *primary full responders* to LEV for at least 6 months. Naturally, hippocampal biopsy specimens are only available from epileptic patients that develop pharmacoresistance at some point. Tissue samples were neuropathologically analyzed by experienced neuropathologists (AJB and PN) following international standards and only hippocampi with representation of the individual subfields were included (see Supplemental Table 1) (Blümcke et al., 2007).

RNA isolation and cDNA synthesis

mRNA was isolated from frozen specimens processed into sections up to 20 μm for complementary cDNA preparation and real-time quantitative (q)RT-PCR confirmation experiments using the Dynabeads mRNA Direct Micro Kit (Dynal, Oslo, Norway) according to the manufacturer's protocol. cDNA was prepared using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA) by reverse transcription following the manufacturer's protocol.

Real-time quantitative (q)RT-PCR

Quantification of *PIGP* and *synaptophysin* by real-time (q)RT-PCR was performed using Taqman Gene Expression Assays (*synaptophysin*: Hs00300531_m1; *PIGP*: Hs00212704_m1; Life Technologies, Carlsbad, Ca, USA). Synaptophysin was used as endogenous reference gene for normalization of the analyzed mRNAs as described before (Becker et al., 2008; Chen et al., 2001). We used the ABI Prism 9700HT sequence detection system (Life Technologies) and the relative $\Delta\Delta C_t$ quantification paradigm.

Microarray analyses

Briefly, sample preparation, hybridization to HumanHT-12v4 Expression BeadChip (Illumina, San Diego, CA), staining and scanning with the Illumina iScan BeadArray Reader were performed according to the manufacturer's protocol. RNA was isolated from 20 μm tissue samples using the AllPrep DNA/RNA Mini Kit (Qiagen, Hilden) according to the manufacturer's protocol. Ambion Illumina TotalPrep-96 RNA Amplification Kit (Life Technologies Corporation, Carlsbad, CA, USA) was used for cDNA synthesis and in vitro transcription to biotin-labeled cRNA. A total amount of 750 ng cRNA was hybridized to Illumina HumanHT12v4 Expression BeadChips with the Illumina Direct Hybridization Assay Kit (Illumina, San Diego, CA) following the manufacturer's instructions. Preparation of cRNA samples and BeadChips was carried out separately in BeadChip Hyb Chambers and the incubation was performed overnight to hybridize the labeled cRNA strand to the beads containing respective complementary gene-specific sequences. To detect differential signals on the BeadChip, Cy3-streptavidin was incorporated to bind to the hybridized probes. The Illumina BeadArray Reader