

Publishable executive summary

**Full title: FUNCTIONAL GENOMICS AND NEUROBIOLOGY OF EPILEPSY:
A BASIS FOR NEW THERAPEUTIC STRATEGIES**

Acronym: EPICURE

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Project web-site: <http://www.epicureproject.eu>

Epilepsy is a serious and common neurological disorder that has major medical and social implications and affects currently 6 million of European citizens. More effective strategies are needed to prevent the epileptogenic process set in motion by genetic or environmental risk factors and to help the significant proportion of patients (60-70%) whose seizures are refractory to the presently available antiepileptic drugs.

To this aim the collaborative project EPICURE has been developed involving several groups from 13 European countries. The project is focused on the pathophysiological role of voltage-gated and ligand-gated ion channels dysfunctions due to mutations of the coding genes or to exogenous factors. The resulting epileptogenic alterations in structure and function of neuronal networks are investigated in animal and human tissue using complementary advanced methods. A main effort is devoted to investigate developmental aspects of epileptogenesis that account for the high frequency of epilepsies in children and for their often severe prognosis. Strictly related to the above research lines are the pharmacological approaches by which EPICURE aims at developing more effective antiepileptic strategies, at understanding the biological bases of pharmacoresistance and at evaluating the potential of innovative therapeutic approaches based on synaptic modulation by neurotrophic factors and cell cycle kinase inhibitors.

The main research lines are reflected by the titles of 5 subprojects (SP):

- SP1 *Genetics of human epilepsies*
- SP2 *Functional consequences of mutations in ion channel genes associated with idiopathic epilepsy and genetically determined pharmacoresistance*
- SP3 *Acquired channelopathy and neuronal network reorganisation underlying temporal lobe epilepsy*
- SP4 *Epilepsy and development*
- SP5 *pharmacogenetics of refractory epilepsy, mechanisms of drug resistance and new therapeutic strategies.*

During the first year of activity significant progress towards the planned objectives have been made.

SP1 The planned goals for the first 12 months have been reached. The Guidelines for sampling procedures and phenotyping protocols for Idiopathic Generalized epilepsies (IGE), Febrile seizures (FS) and photoparoxysmal response (PPR) traits have been developed. The original available sample collection has been further expanded. In more than half of the samples linkage analysis has already been completed. Stage-1 genome-wide association (GWA) study on IGE has been completed. The recruitment of additional 800 samples has

been currently in process to perform stage-2 GWA. The recruitment of 95 multiplex families for sequence analysis of candidate genes has been accomplished and about 20% of the mutational screening accomplished.

Clinical data and DNA samples are available for 269 IGE-multiplex families. We are in process to complete sampling of additional 67 IGE-multiplex families. Genotyping of genome-wide linkage STR panels was performed for 216 IGE-multiplex families collected by SP1 partners at our high-throughput genotyping facility. Non-parametric linkage IGE-loci in the chromosomal regions 3q26 (ZNPL = 3.38), 6p12 (ZNPL = 2.94) and 19q13 (ZNPL = 3.14). Parametric linkage showed linkage in the chromosomal regions 3q26.3 ($Z = 3.85$, $\theta = 0.15$), 19q13.31 ($Z = 3.38$, $\theta = 0.10$) and 2q34 ($Z = 3.02$, $\theta = 0.20$). None of the observed NPL hints reached significant evidence for a major IGE locus, suggesting a complex involvement of several susceptibility loci and substantial genetic heterogeneity. The proposed sample size of 500 IGE-multiplex families will be required to achieve conclusive results.

237 families with at least two siblings affected by FS were identified. Some of the large multigenerational families resemble GEFS+ but all known GEFS+ loci have been excluded. In total, 162 nuclear families with 2-3 affected siblings will be available. Sampling is completed for the majority of these families. Genotyping was performed for 93 FS-multiplex families. Linkage analyses of FS-multiplex families are in preparation.

Families with at least two siblings displaying PPR were identified. Clinical data and DNA samples are available for these families. Genotyping was performed in 75 PPR-multiplex families. NPL analysis in PPR families with 190 members exhibiting PPR type 3-4 revealed a novel locus for generalized PPR (type 3-4) in the chromosomal region 13q13.2 (PNPL = 6.8×10^{-5})

We reached our goal to complete Stage-1 of a genome-wide association (GWA) scan in a case-control sample of 704 Dutch/German IGE patients and 949 German population controls, using the novel Affymetrix Genome-Wide Human SNP array 6.0 that contains 906K SNPs and 946K non-polymorphic probes for the genome-wide evaluation of genomic copy number variations. For the IGE case-control samples, the Armitage's Trend Test (ATT) revealed 15 SNPs that showed statistically significant associations ($p < 0.5 \times 10^{-7}$). More than 20 significant associations were found in the IAE- and CAE-subsamples, whereas only one SNP achieved this significance level in the JME subsample. We are currently recruiting Stage-2 replication samples, consisting of 800 IGE patients and 800 ethnically matched controls, or alternatively parent-offspring trios. Sampling will be completed by March 2008.

We recruited 95 families with at least three individuals affected by Idiopathic Generalized Epilepsy (IGE). The family sample includes 328 individuals affected by definite IGE subsyndromes and 4 affected by unclassified IGE. In addition, 43 family members showed a form of epilepsy that was either non-IGE or non classifiable. The average number of individuals experiencing afebrile seizures per family is 3.95. The average number of individuals with IGE per family is 3.49.

Ninety nine candidate genes have been identified to be screened for IGE associated mutations, based both on existing knowledge on the role of various genes in mendelian forms of IGE and on plausible functional hypotheses emerging from functional studies and/or animal models of epilepsy. Genes that have been already investigated in IGE have been excluded from the study. Most of the selected genes encode for transmembrane proteins involved in the movement of ions. In particular 3 code for Voltage-gated chloride channels, 13 for Voltage-gated calcium channels, 4 Voltage-gated sodium channels, 2 for Voltage-gated Potassium channels, 8 for Inward rectifier potassium channels, 4 for Hyperpolarization-activated cyclic nucleotide-gated potassium channels (HCN channels), 4 for

Sodium/potassium pumps, 4 for Calcium pumps, 14 for GABA-receptors, 18 for Glutamate receptors and 2 for Glycine receptors. A common mutation report form has been developed for efficient pooling of results. At month 12 about 20% of the selected genes have been sequenced fulfilling the planned goals for the 12th month.

SP2 This subproject has a crucial role in characterizing the functional consequences of gene mutations identified by SP1 and SP5 with which it is strictly integrated. An important effort has been devoted to solve the problems in discriminating between +/+ from +/- genotypes of Nav1.1 knock-out (KO) mice. The success of this first part of the experimental work made it possible to characterize Na⁺ currents in hippocampal neurons from hetero and homozygous KO mice (thus confirming in preliminary experiments a specific effect of the deletion in interneurons). The effects of the GEFS⁺ R1648H mutation of Nav1.1 on the functional properties of the channel have been studied in neurons dissociated from transgenic mice, and other mutations of Nav1.1 are being investigated in transfected cells lines and neurons. An experimental model of febrile seizures has been improved and the experiments initially planned with transgenic mice will be done with R1648H knock-in mice, which have become available and are a better animal model of the disease. Furthermore, subcellular distribution of Nav1.1 & Nav1.2 WT and of Nav1.1-R1648H mutant channels and its age-dependent expression has been characterized by monoclonal antibodies in mouse brain slices.

Changes in GABAergic inhibition due to mutations of GABAA receptor subunit genes (that reduce GABAergic inhibition directly) or to mutations in the Cl⁻ channel gene CLCN2 (that can affect neuronal Cl⁻ homeostasis and so impair GABAergic transmission indirectly) are being investigated by electrophysiological and immunocytochemical techniques. Results on differential effects of two distinct GABA-A receptor gamma subunit (GABRG2) mutations on phasic, synaptic transmission (K289M) or on receptor trafficking and tonic activation of extra-synaptic GABA receptors (R43Q) are already in press.

Although this subproject is focused on functional characterization of channel gene mutations (genetically determined channelopathies) some experiments have also been carried out in acquired channelopathies thus establishing a strict interaction with SP3. Results of experiments performed epileptic tissue from patients with temporal lobe epilepsies (TLE) showed that molecules other than KCC2, including perhaps ClC-2 or Cl/HCO₃ and Na/HCO₃ exchangers, may be involved in regulating internal Cl/HCO₃ and thus the polarity of GABAergic signalling in TLE patients. This work is published.

To facilitate the expression of mutations in suitable expression systems, a new lipid-coated magnetic nanoparticles formulations specific for transfecting DNA has been developed, called NeuroMag. The results demonstrated successful and reliable in vitro transfection of primary neurons (see Fig 1).

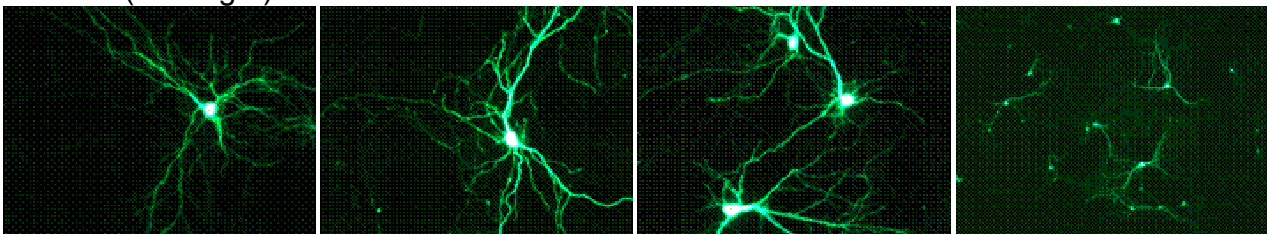


Figure 1. Primary rat hippocampal neurons were prepared in 24-well plates as described in the NeuroMag instruction manual. Cells were transfected after 14 DIV (Days In Vitro) using 1 µg / well of pEGFP plasmid and 3.5 µL of NeuroMag. Transfection efficiency was monitored by fluorescence microscopy 48 h post-transfection

Finally, characterisations of different ion channel mutations and genetic variants associated with idiopathic epilepsies and pharmacoresistance are being carried out and other variants will be selected and studied in collaboration with SP1 and SP5 partners.

SP3 The subproject is mostly focused on the role of GABA-ergic transmission in epileptogenesis. A particular attention is devoted to intrinsic, ion channel dependent, excitable properties of GABA-ergic interneurons. The results obtained in different animal models are correlated with those obtained on human tissue from surgical samples.

As recordings from interneurons in adult preparations are very difficult and therefore time consuming an indirect monitoring of interneuron function has been developed to test the strength and frequency dependence of feedback inhibition by recording from pyramidal neurons (Fig. 2).

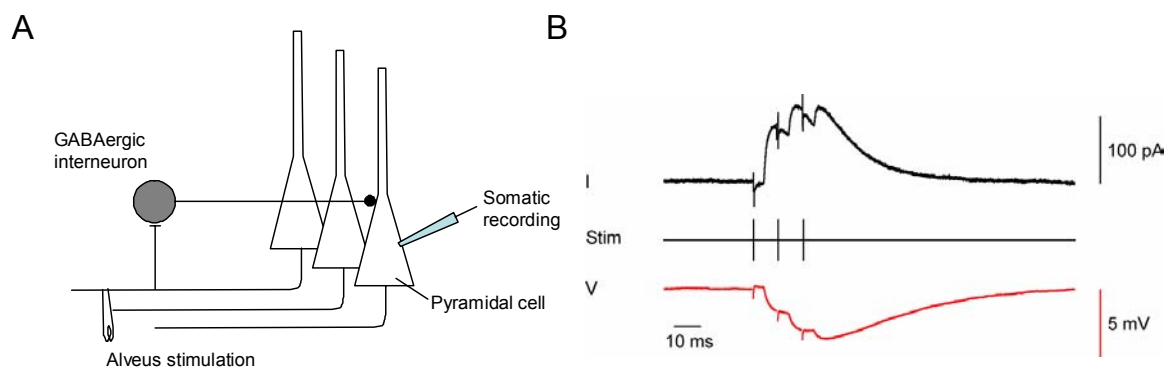


Fig. 2: Analysis of recurrent feed-back inhibition in the CA1 region. **(A)** Schematic diagram of the recording configuration. **(B)** Representative example of GABAergic IPSCs (black trace) or IPSPs (red trace) recorded in a CA1 pyramidal cell to alveus stimulation (three stimulations, 10 ms apart, time points of stimulation indicated by the middle trace). (Miles et al, partner CR2 Inserm)

The experiments carried out during the first 12 months with electrophysiological, immunohistochemical and biomolecular methods show changes in expression of K^+ channel subunits and in regulation of different K^+ currents. In particular I_h was found to be down regulated in interneurons in Kainic and Pilocarpine experimental models and in human hippocampus resected for therapy of temporal lobe epilepsy. This resulted in reduced resonance of these cells that correlate with reduced capability of inhibitory neurons to generate membrane potential oscillations.

A region-specific up-regulation of somatodendritic I_h in hippocampal DGCs has been found that can be regarded as a result of transcriptional up-regulation of HCN1 and 4 subunits. Such K^+ current plasticity may be an important mechanism in the conversion of a normal to an epileptic brain.

Other important results of SP3 concern the GABAergic postsynaptic compartment and the role of inflammation in epileptogenesis. Two interesting rat models of post-traumatic epilepsy (PTE) and post-status epilepticus (SE) epilepsy have been produced and validated with continuous video-EEG monitoring. Perfused or snap-frozen brains have been processed for immunohistochemistry of calcium binding proteins or in situ hybridization analysis of GABA receptor subunits. Only preliminary results are available, data at least from 2-3 immunohistochemical markers and GABA receptor subunits are expected to be ready by the end of June 2008.

Evidence of activation of inflammatory markers of the IL-1beta system was obtained in rat brain during epileptogenesis and in human tissue from patients with temporal lobe epilepsy. In both cases the activation involved astrocytes as well as some neurons. Exchange of brain tissues has been organized and executed during 2007 in order to collect the samples from TBI models (Dr Pitkanen's lab, CR16) to carry out similar investigations as above reported.

SP4 An important part of the activity was focused on developmental abnormalities also referred to as dysplasia. Genotype-phenotype correlations has been performed in 101 out of 154 patients with epilepsy due to malformations of the developing cerebral cortex recruited from European countries. Twelve different mutations have been identified in LIS1, DCX, FLNA, GPR56 and ARX genes that regulate cortical development. Two mosaic mutations in the LIS1 gene have been identified. Further mutations have been detected in patients with phenotypes not associated with known genes. Animal models of DCX and ARX linked heterotopias have been developed. DCX linked double band heterotopia was suitably modeled, while controls on ARX model are still ongoing. In parallel with biomolecular experiments, electrophysiological methods have been developed to test epileptogenic activities in *in vivo* and *in vitro* models. Preliminary data strongly support the notion that heterotopic cells connect and are connected by superficial layer pyramidal neurons, thus providing a morphological substrate for activity synchronization. To overcome the encountered problems, technical advanced systems are being developed. A novel telemetric EEG recording system has been developed, which allows for the first time electrophysiological recordings from freely moving behaving rats over distances of up to 3 m and under various experimental conditions. Moreover the ability of magnetic nanoparticles (ViroMag) to control, promote, assist and target viral transduction *in vitro* and in embryonic brain are being evaluated. The preliminary results show that high transduction efficiency can be achieved in primary rat hippocampal neurons and in brain of rat embryo with a Lentivirus. Electrophysiological recordings have been obtained from 16 tissue samples from surgically resected specimens from patients between 3 months old and 7 years old. Patch clamp recordings were performed on 49 neurons injected with biocytin and revealed using the ABC system. The large majority of them (37) were pyramidal cells and the remaining were interneurons (10); 2 cells could not be classified. Data analysis is ongoing. Moreover experiments aimed at culturing organotypic explants from human resected cortices have been carried out. Patch-clamp recordings or calcium imaging analysis depicted the presence of synaptic activities. Post-hoc reconstructions of recorded cells, subsequently injected with biocytin, demonstrated the presence of pyramidal cells with normal features. However, the slices no longer preserved the laminar cytoarchitectonic organization. Further studies are required to determine whether these culture conditions allow a reproducible preparation for culturing human cortical neurons and pharmacological analysis.

Results of immunocytochemical and biomolecular analysis of resected human dysplastic tissue from 124 patients are being correlated with clinical outcome. Data analysis of 24 patients with Nodular Heterotopia has already been completed and submitted for publication. Electroclinical, neuropathological and MRI correlations in 100 patients with Taylor's Focal Cortical Dysplasia is in progress.

The analysis of neuropathological data of 291 patients with temporal lobe epilepsy showed a frequent association of hippocampal sclerosis with dysplastic features. An appropriated file with all the available data has been generated and it is now ready for the statistical evaluation that will be performed within the next six months.

SP5 The first 12 months of the project aimed at identifying genetic markers of drug resistance have been devoted to the development of the protocol and a *Case Record Form* for patients' data collection that have now been finalized and to patients enrollment that has also been practically completed. To date, 929 patients with localization-related epilepsy (LRE) have been enrolled, 54.4% of whom (36.1% with cryptogenic and 63.9% with symptomatic LRE) meet criteria for drug resistance, whereas 45.6% (68.5% with cryptogenic and 31.5% with symptomatic LRE) meet criteria for drug responsiveness. In a first stage, a single-locus and multi-locus phased analysis is being used to study associations between common haplotypes and phenotype status (drug resistance vs. drug responsiveness) in 500 patients with pharmacoresistant localization-related epilepsy and 500 pharmacoresponsive controls with localization-related epilepsy. In a second stage, an analysis of the 50 most significant associations identified in the screening phase will be carried out in a separate additional population of 500 pharmacoresistant patients and 500 controls, and a final joint analysis (combined data from both stages) will then be applied for definitive statistics.

A second research line is aimed at assessing new AEDs in severe myoclonic epilepsy of infancy (SMEI) in relation to genotype by a multicentre controlled trial designed to compare topiramate, stiripentol and clobazam. After approval of the EPICURE Project, stiripentol was assessed by the European Medicines Agency (EMA) for the indication "drug refractory SMEI as adjunctive therapy to valproate and clobazam" on condition that the manufacturer (Biocodex, Paris, France) will perform a controlled trial to confirm the efficacy and safety of the compound in this indication. Given the rarity of SMEI it would be unfeasible to have two similarly designed trials (the EPICURE and an independent Biocodex-initiated trial) competing for the same patients' population. Therefore EMA agreed on 21 June 2007, whereby only one single European trial will be conducted under coordination of the EPICURE consortium. EPICURE will retain the property of trial data but, in exchange for Biocodex's support in providing study material and some logistical help, will ensure that the data will be made available to EMA in compliance with the conditional approval requirement.

A detailed study protocol has been prepared, approved by the Ethics Committee of the Italian coordinating Center FMPV on 3 July 2007 and then submitted to the EMA Committee for Human Medicinal Products (CHMP), which requested some amendments. The amended protocol was finally approved by the Ethics Committee of FMPV on 11 September 2007 and by **INSERM**, the French promoter (Ethics Committee Ile de France III (Hospital Tarnier-Cochin) on 8 November 2007. A network of 22 Italian and 14 French epilepsy centers has been created for patient enrolment.

Another research line is on the molecular mechanisms of pharmacoresistance that are investigated by several EPICURE partners on animal models and human tissue by complementary techniques in a synergistic effort. The investigations are focused on HCN channels, responsible for I_h K^+ current and on Na channels responsible for both transient (I_{NaT}) and persistent (I_{NaP}) Na^+ currents. A series of experiments is being carried out in mice lacking either the β_1 or β_2 subunit of the voltage gated Na^+ channel to assess their role in determining the sensitivity of Na channels to AEDs.

The experiments performed during the first year demonstrated that expression and regulation of ion channels are indeed altered in correlation with the development of pharmacoresistance. Interestingly these changes are different in different areas of the brain. Such changes result in a decreased sensitivity to antiepileptic drug effect and are cell-type and AED specific. The results of experiments demonstrating that the mechanism of action of CBZ - use-dependent block of I_{NaT} - is completely lost in CBZ-resistant patients have already been published.

Protein and mRNA expression and function of multidrug transporters have also been found altered and their contribution to the development of drug resistance are object of the ongoing investigations.

Besides the study of established AEDs, innovative therapies are objects of SP5 investigational lines. During the reporting period of the project, it has been shown that neuropeptide Y (NPY) does alter synaptic transmission onto interneurons in the hippocampus, which could be part of its antiepileptic effect. Another neuropeptide, galanin, has been expressed in the cell line clones to use in ex vivo gene therapy based on ECB approach. Also specific BDNF splice variants seem to be transported into the dendrites and might contribute to epileptogenesis, and experiments developing splice-specific siRNAs to interfere with BDNF are ongoing. Moreover, combination of BDNF and FGF in one viral vector, and transduction of the genes into the hippocampus has been shown to protect animals from spontaneous seizures, and novel combination vectors of EGF and GDNF, each alone and in combination with BDNF and FGF-2 are on its way.

Dissemination activities have been undertaken with the design of a detailed dissemination plan; the creation of an EPICURE web site; the release of a bi-annual e-newsletter and the organization of several technical meetings internal to the project on specific research issues. During the reporting period various dissemination tools were produced including a flyer and press release which was issued and distributed during the main congresses and conferences in the field of epileptology. Dissemination has been undertaken through participation of project researchers in several scientific national and international conferences. 13 articles have appeared in peer reviewed journals acknowledging the European Commission support for the EPICURE project. A further 4 articles are in the press, 7 are in preparation and 1 paper has been submitted.

The European Epilepsy Brain Bank (EEBB) is an internet based documentation system for patients operated on for the treatment of focal, chronic epilepsies. Its website is now located at www.epicure-bank.org and linked to the Epicure website. Access is protected and permitted only to members of the consortium. A total of 1999 data sets have already been included at month 12.

Training activities have been implemented in various forms so as to contribute to the training of a new generation of researchers and clinicians and to the transfer of information between laboratories for the development of novel diagnostic/therapeutic strategies and procedures:

- firstly, intensive training courses were planned to guarantee advanced training of the young and senior researchers involved in EPICURE. Two mini-courses to be held during the EPICURE meeting of January 2008 were outlined on phenotyping of epileptic patients and on the characterization of animal models of epilepsy;
- secondly, to achieve staff mobility and exchange among partners, a page was activated on the EPICURE web site to advertise both local training offers and open postdoctoral and PhD positions. Furthermore, exchanges of senior scientists and junior scientists within EPICURE were successful.

Management activities were undertaken via the delivery of documents for the establishment and adoption of common operational procedures; internal monitoring of research activities and auditing of expenses; compliance with external scientific and financial reviews and audits; research guidelines and coordination of activities; logistics and IT; legal and administrative issues; reporting and the definition of the financial and implementation plan.