Laboratory techniques in multidisciplinary research of epilepsy

Tehnici de laborator în cercetarea multidisciplinară a epilepsiei

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Abstract

This review focuses on a number of laboratory techniques which can be used complementary to electrophysiology in animal models of temporal lobe epilepsy in order to assess the structural and molecular bases of epileptic syndromes. Immunohistochemistry is used for identifying changes in certain molecules, receptors and neuron subpopulations. Advantages and drawbacks of this method are discussed, mentioning also the limitations of quantitative measurements. The γ-aminobutyric acid (GABA)ergic inhibitory interneurons control the excitability of cortical networks, therefore emphasis is given to the methods used for identification of these cells and the expression of GABA receptor subtypes. The use of quantitative real time polymerase chain reaction (RT-QPCR) in studying GABA(A)-receptor subunit expression is presented in detail. It is underlined the necessity to devise experiments where the behavioral and electrophysiological modifications are studied in parallel with the neuroanatomic, cellular and molecular changes in order to investigate the causal relationship between the altered morphological/laboratory parameters and the clinical symptoms.

Keywords: *immunohistochemistry, polymerase chain reaction, epilepsy, animal model.*

Rezumat

În acest referat sunt prezentate metode de laborator care pot fi folosite în mod complementar electrofiziologiei cu scopul studierii bazelor structurale şi moleculare ale sindroamelor epileptice utilizând modele experimentale pe animale ale epilepsiei de lob temporal. Imunohistochimia este utilizată pentru identificarea unor modificări moleculare, a unor receptori sau subpopulaţii de neuroni. Sunt prezentate avantajele şi dezavantajele acestei metode, menţionând şi limitele măsurătorilor cantitative. Interneuronii inhibitori GABAergici (γ-aminobutyric acid) controlează excitabilitatea reţelelor corticale. Din acest motiv, s-a pus accent pe metodele folosite pentru identificarea acestor celule şi a expresiei subtipurilor de receptori pentru GABA. Este prezentata detaliat

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utilizarea reacţiei de polimerizare în lanţ cantitativă în timp real (RT-QPCR) pentru studierea expresiei subunităţilor receptorilor GABA(A). Este subliniată necesitatea introducerii unor experimente în care modificările electrofiziologice şi de comportament sunt studiate în paralel cu schimbările neuroanatomice, celulare şi moleculare, cu scopul investigării relaţiilor cauzale dintre parametrii morfologici sau de laborator şi simptomatologia clinică.

Cuvinte cheie: *imunohistochimie, reacţie de polimerizare în lanţ, epilepsie, modele pe animale*

Epileptic syndromes are one of the most frequent neurological diseases affecting 0.5-3% of the globe's population. The number of people affected indirectly by this disease (i.e. family members, etc) is actually much higher. (1) Although usually the disease is not lethal the loss of consciousness can lead do dangerous situations (i.e. when driving, etc). Almost indifferently of the type of epilepsy the disease will lead to psychopathological complications (hiposexuality, memory loss, depression) as well as the stigmatization of the patients.(2) Thus epilepsy causes an important socio-economic burden as well as affects the quality of life for both patients and relatives.

Epileptic seizures are caused by an array of complex neurochemical, pathophysiological and anatomical alterations of the brain; therefore epilepsy cannot be considered and treated as a homogenous disease. That's why both in fundamental research as well as the clinical practice instead „epilepsy" is better to use „epileptic syndromes". By this term we refer to a series of diseases different from many points of view but sharing two criteria: (1) the presence of epileptic modifications on electroencephalographic recordings and (2) the recurrence of fairly similar clinical symptoms, which start and end abruptly – called convulsions or ictus. At the very foundation of these symptoms lies the hyperexcitability of a population of neurons with transient abnormally synchronized activity.

Cellular and molecular changes in certain neurons and glial cells have been proposed as determinants for the development of spontaneous seizures. A morphologic change found in association with an epileptic syndrome may be causally related to the abnormal discharge or may be only a consequence of abnormal activity. In order to understand the mechanisms involved, it is necessary to study the time course of such changes. Experiments that could address the causal relation between structure and function are difficult (or impossible) to carry out in human patients. Only animal models can assess the causal relationship between structural abnormalities and epileptogenesis.(3)

This review focuses on a number of laboratory techniques which can be used complementary to electrophysiology in animal models of temporal lobe epilepsy (TLE) in order to assess the structural bases of epileptic syndromes.

In rodents, large dosages of pilocarpine(4) or kainic acid(5) introduced systemically or intracerebrally induce severe acute seizures with subsequent status epilepticus, which is followed by a quiescent period of usually several weeks. This latent period is followed by the development of spontaneous recurrent seizures. An other induction method is kindling, whereby repetitive, focal application of initially subconvulsive electrical stimulation ultimately results in intense partial and generalized convulsive seizures.(6)

Neuronal cell loss is one of the most frequently described modification in human TLE.(7) Nissl staining (e.g. with cresyl violet) offers a detailed qualitative overview of light microscopic features of the brain tissue. However, because Nissl staining is not specific to cell types, quantitative counts of neurons or glial cells are better carried out on tissue that has been processed for a cell specific marker (e.g. NeuN immunohistochemistry for neurons, glial fibrillary acidic protein (GFAP) for glia and glutamic acid decarboxylase (GAD) for in-

hibitory γ-aminobutyric acid (GABA)ergic interneurons.

The most obvious modifications are the cell death in hilus, CA3 and CA1 pyramidal layer of hippocampus and sprouting of mossy fibers in the dentate gyrus of epileptic hippocampi.(8) In the cortex principal cells are intermingled with inhibitory interneurons, therefore the information regarding the cell loss does not indicate whether all cell types are affected or just a certain type of cell. .Interneurons control the excitability of principal cell networks. In the kainate model of epilepsy it has been shown that the GABAergic interneurons are relatively resistant to excitotoxic damage.(9) Inhibitory interneurons, however, do not form a homogenous population. Some studies show that subpopulations of GABAergic interneurons are selectively lost following prolonged seizures or status epilepticus.(10) Interneurons differ in their morphological, electrophysiological and neurochemical features and these differences are functionally relevant. Identifying classes and subclasses of interneurons is very difficult task and a universally acceptable classification scheme is just emerging.(11)

Immunohistochemistry have been particularly valuable in epilepsy research by identifying changes in receptors, interneuron subpopulations and certain molecules. Distinct populations of interneurons can be identified by immunostaining against two calcium binding proteins, calbindin (CB) and parvalbumin (PV), the neuropeptide somatostatine (SOM) and the α1 subunit of GABAA receptors (α1-GABAAR). Using these markers besides positive identification of interneurons, the differentiation of the most important interneuronal subpopulations (basket, bistratified, axo-axonic cells) is possible. Calbindin immunoreactivity is observed in interneurons that make synaptic connections with a variety of cells, including smooth non-pyramidal (GAD positive) neurons, and pyramidal and spiny non-pyramidal cells in the hippocampus and/or cortex. PV immunoreactive interneurons, in contrast, primarily innervate principal cell soma and their axon initial segment. (12) Especially in the CA1 area SOM is expressed in bistratified cells co-expressing PV. The α1-GABAAR is expressed both in PV expressing principal cells and interneurons in the hippocampus with a strong immunoreactivity of cell bodies and dendrites which probably represents extrasynaptic receptors contributing to the strong tonic inhibition of hippocampal neurons.(13)

Although immunohistochemical methods are very useful for identification of neuronal subpopulations and their expression of functionally important molecules the results should be interpreted with caution. Loss of staining for a certain marker in a subpopulation of neurons may not necessarily mean the loss of those cells, because the cells may survive, but no longer produce the protein of interest. Or they can even make the protein, but its structure has been modified, so that the antibody no longer recognizes the epitope against it was produced. Immunohistochemistry focusing entirely on cell bodies may give negative results for proteins that have exclusive dendritic or axonal localization. Even if proteins are produced in the cell body and are transported to the appropriate target sites, slow turnover and low somatic concentration may make the protein undetectable at the cell body. These limitations can be partially avoided using multiple antibodies that provide complementary information.

When immunohistochemistry is used for identification of neuron subpopulations, especially when rare cell types have to be marked, relatively thick sections (60-80 μm) must be used. In this case the penetration of the reagents in the depth of slices can be incomplete. In order to overcome this, usually longer incubation periods are used, the vials are put on rocking table and the primary antibody is often left overnight on specimens. Quantitative measurements are difficult on immunostained specimens due to the variable staining intensity seen

with different primary antibody concentration and exposure time, variable visibility associated with choice of secondary antibody and several uncontrolled factors (degree of fixation, sectioning method, etc). Furthermore, sometimes it is difficult to decide what level of staining should be considered immunopositive. Subjective determinations are acceptable in cases of clear positivity-negativity dichotomy. Computer programs can provide a more objective basis for quantitative densitometric measure of immunostained tissue.

In models of epilepsy not only cell death is observed but also permanent changes in excitatory and inhibitory transmission. It has been suggested that hypersynchrony may arise from altered inhibitory synaptic transmission owing to inappropriate GABAA receptor expression.(14) On the other hand, in other studies altered expression of GABAA receptor subunits was proposed to be compensatory to interneuronal loss and not causative.(15) Differences in GABAA receptor expression on interneurons may have a significant impact on the control of synchronous behavior and as such could be an important pathophysiological mechanism for seizure induction.(16)

In situ hybridization can clearly identify the cells which are making the mRNA for a given protein but provides no information where the protein is localized for appropriate function. The results are easily quantifiable and provide data regarding which cell are able to make a certain protein and in what quantities. If the cellular localization of the protein (and mRNA) is not important for the given scientific question, a more precise method, quantitative real time polymerase chain reaction (RT-QPCR) can be used.

Several experimental models of TLE suggest that expression of GABAA receptors in the hippocampus, amygdala and adjacent cortical areas is abnormal. In genetically seizure-prone rats an overexpression of α2, α3 and α5 subunits is observed, which normally predominate the immature brain. Conversely in seizure-resistant strains the major α subunit found in adult, the α1 is overexpressed.(17) For an exact comparison of mRNA transcription in different samples it is crucial to choose the appropriate reference gene. Endogenous controls can normalize the expression levels of target genes by correcting differences in the amount of cDNA that is loaded into PCR reaction wells. The ideal endogenous control must have a constant RNA transcription level under different experimental conditions, therefore usually constitutively expressed housekeeping genes, or ribosomal RNAs such as 18S are used. In experiments using rats, the commercially available pre-designed probe and primer sets for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an enzyme involved in the glycolytic pathway, and beta actin (ACTB), component of the cytoskeleton are tempting choices. Although GAPDH and ACTB are commonly used as endogenous controls, some studies suggest that they are not appropriate for all experimental conditions. (18)

It was demonstrated that GAPDH besides being a glycolytic enzyme, it functions as a kinase too, which is involved in the phosphorylation of the GABAA-receptor α1-subunit, a mechanism necessary to maintain normal GABAergic neurotransmission.(19) This glycolysis-dependent phosphorylation of the α1-subunit was found to be deficient in human epileptogenic cortex obtained during curative surgery, (20) therefore GAPDH seems to be an inappropriate control gene for RT-QPCR used in animal models of epilepsy.

It was shown by immunoblot analysis that in kainate-induced seizures the ACTB content of the postsynaptic terminals is decreased; (21) acute dendritic injury and actin depolymerization was observed in vivo after status epilepticus.(22) These results suggest that ACTB expression may be affected by epileptogenesis, so it is safer to avoid as endogenous control.

Synaptophysin has been shown to be

stably expressed under conditions of status epilepticus,(23, 24) therefore even if it is not available as a pre-designed commercial kit, we consider that synaptophysin is a more reliable endogenous control.

As changes in GABAA-receptor subunit expression plays an important role in the etiology of TLE, it is important to clarify the intracellular signalling pathways leading to these modifications. For this purpose primary neuronal cell cultures can be used as a model system. Signalling inhibitors and/or activators can be applied and subsequent Western blot analysis can be performed on the whole cell lysates.(25) Using identically treated plates, cells can be used for protein extraction and Western blot in parallel with DNA/RNA extraction and PCR or RT-QPCR.

Most of the presented laboratory methods provide a static picture of the mechanisms involved, therefore they show a one-point-in-time snapshot of the system of interest. In order to investigate the causal relationship between the altered morphological/laboratory parameters and the clinical symptoms, it is necessary to devise experiments where the behavioral and electrophysiological modifications are studied in parallel with the neuroanatomic, cellular and molecular changes. In this sort of experiments the analysis can be performed at several points in time during epileptogenesis. Modifications that appear before the animal reaches a given stage of epileptogenesis can not be a consequence of abnormal function of the system.

In future, a well designed kindling protocol (which assures a progressive development of epileptic symptoms) combined with the above presented immunohistochemical and molecular biological methods will be necessary to investigate the pathologic modification causally involved in epileptogenesis. The results of such studies may open the way for developing truly antiepileptic drugs instead of symptomatic anticonvulsive ones.

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