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Functional aspects of early brain development are preserved in tuberous sclerosis complex (TSC) epileptogenic lesions

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ABSTRACT

Tuberous sclerosis complex (TSC) is a rare multi-system genetic disease characterized by several neurological disorders, the most common of which is the refractory epilepsy caused by highly epileptogenic cortical lesions. Previous studies suggest an alteration of GABAergic and glutamatergic transmission in TSC brain indicating an unbalance of excitation/inhibition that can explain, at least in part, the high incidence of epilepsy in these patients. Here we investigate whether TSC cortical tissues could retain $GABA_A$ and AMPA receptors at early stages of human brain development thus contributing to the generation and recurrence of seizures. Given the limited availability of pediatric human brain specimens, we used the microtransplantation method of injecting *Xenopus* oocytes with membranes from TSC cortical tubers and control brain tissues. Moreover, qPCR was performed to investigate the expression of $GABA_A$ and AMPA receptor subunits ($GABA_A$ α1–5, β3, γ2, δ; GluA1, GluA2) and cation chloride co-transporters NKCC1 and KCC2. The evaluation of nine human cortical brain samples, from 15 gestation weeks to 15 years old, showed a progressive shift towards more hyperpolarized $GABA_A$ reversal potential (E_{GABA}). This shift was associated with a differential expression of the chloride cotransporters NKCC1 and KCC2. Furthermore, the GluA1/GluA2 mRNA ratio of expression paralleled the development process. On the contrary, in oocytes micro-transplanted with epileptic TSC tuber tissue from seven patients, neither the $GABA_A$ reversal potential nor the GluA1/GluA2 expression showed similar developmental changes. Our data indicate for the first time, that in the same cohort of TSC patients, the pattern of both $GABA_A$R and GluA1/GluA2 functions retains features that are typical of an immature brain. These observations support the potential contribution of altered receptor function to the epileptic disorder of TSC and may suggest novel therapeutic approaches. Furthermore, our findings strengthen the novel hypothesis that other developmental brain diseases can share the same hallmarks of immaturity leading to intractable seizures.

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid; AMPAR, AMPA receptor; CCCs, cation chloride cotransporters; FCD, focal cortical dysplasia; GABA, gamma-aminobutyric acid; $GABA_A$R, GABA type A receptor; E_{GABA}, $GABA_A$ reversal potential; TSC, tuberous sclerosis complex.

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1. Introduction

Tuberous sclerosis complex (TSC) is an autosomal dominant, multisystem disorder that results from mutations in the TSC1 or TSC2 genes leading to a constitutive activation of mammalian target of rapamycin (mTOR) pathway (ECTS C., 1993). The neurological manifestations of TSC include developmental delay, neurobehavioral dysfunctions (such as autism) and severe epilepsy and are believed to reflect complex structural and functional brain abnormalities (Bolton, 2004; Curatolo et al., 2015). One of the TSC structural brain lesions is cortical tubers, which are considered the major neuropathological substrate for epilepsy in TSC patients (Aronica et al., 2012), although increasing evidence supports the importance of more subtle structural abnormalities present throughout the brain (Marcotte et al., 2012). Both experimental and human data suggest that increased neuronal excitability may reflect the persistence in TSC and focal cortical dysplasia (FCD) brain of immature features, involving both GABAergic and glutamatergic neurotransmitter receptors (Abdijadid et al., 2015; Talos et al., 2008, 2012; Wang et al., 2007). Recent data (Abdijadid et al., 2015; Cepeda et al., 2014) based on immunocytochemical and electrophysiological evidence of abnormal neuronal maturation observed in tissue samples from pediatric epilepsy surgery patients, put forward the notion of cortical "dysmaturity" as unifying hypothesis of epileptogenesis in development brain diseases. Interestingly, immature $GABA_AR$ phenotypes, with altered expression of $GABA_AR$ subunits and cation chloride cotransporters (CCCs) NKCC1 and KCC2 have been reported in cortical tubers (Abdijadid et al., 2015; Cepeda et al., 2012; Talos et al., 2012). While electrophysiological analysis of $GABA_AR$ responses in TSC has been performed in one single case in comparison to an epileptic control without dysplasia (Talos et al., 2012), and patch-clamp recordings, using cortical slices from TSC versus FCD patients have been previously reported (Cepeda et al., 2012), a functional analysis of $GABA_AR$ responses during human cortical development and in TSC, in comparison to controls "without epilepsy", is still lacking. Furthermore, AMPA glutamate receptors undergo a change in their subunit composition during development, being the GluA2 subunit upregulated in mature brain (Kumar et al., 2002; Livesey et al., 2014). However, the functional aspects of this issue are not fully investigated in TSC. Here, we hypothesized that the neuronal excitability in epileptogenic TSC cortical tubers could be caused by an imbalance of glutamatergic/GABAergic neurotransmission due to $GABA_AR$ and AMPAR that retain immature properties resembling the early stages of human brain development. Therefore, given the limited availability of fresh human material, we performed voltage-clamp recordings on *Xenopus* oocytes "micro-transplanted" with membranes from cortical samples at different pre- and post-natal ages. Using this cell expression system, we also studied the $GABA_AR$ and AMPAR properties in TSC samples in comparison to age-matched control tissue. The electrophysiological experiments were supported by parallel qPCR analysis on the same human samples.

2. Subjects/materials and methods

2.1. Subjects

The control cases included in this study were selected from the databases of the Department of Neuropathology of the Academic Medical Center, University of Amsterdam. We used 9 autopsy specimens ranging from 15 weeks of gestation (GW) to 15 years old (Table 1; #1–9, Table 1). Fetal brain was obtained from spontaneous or medically induced abortions with appropriate maternal written consent for brain autopsy. Gestational ages were based on obstetric data, fetal and brain weights and standard fetal anthropometric measurements. We performed a careful histological and immunohistochemical analysis and evaluation of clinical data. We excluded cases with chromosomopathies, major central nervous system (CNS) malformations, brains with postmortem autolysis, severe hypoxic/ischemic encephalopathy, intraventricular hemorrhages, severe hydrocephalus, meningitis or ventriculitis and known history of epilepsy. We only included cases as controls if the specimens displayed a normal cortical structure for the corresponding age and without any significant brain pathology. All autopsies were performed within 10 to 24 h after death.

The TSC cases included in this study were obtained from the archives of the Departments of Neuropathology of the Academic Medical Center (AMC, University of Amsterdam), the University Medical Center in Utrecht (UMCU), Motol University Hospital (Prague, Czech Republic) and the Medical University Vienna. We evaluated one postmortem specimen and 6 surgical specimens from patients who underwent surgery for medically intractable epilepsy (#10–16, Table 1). Three of the seven TSC patients (#11, 14, 16, Table 1) showed symptoms of autism. Perituberal tissue was available from two TSC patients. For one of these, the amount of tissue was not enough (#15; Table 1), while the second has been used to perform electrophysiology experiments (#10; Table 1). In some experiments, we used i) cortical tissues from two children with refractory epilepsy due to focal cortical dysplasia (FCD): one 3 months old (FCDIIA) and another, 2 years old (FCDIIB) and ii) cortical tissue from two adult female controls: one 47 years old and the other 52 years old.

In all surgical cases extensive presurgical evaluation, including at least 24 h to 5 days video-EEG monitoring, high-resolution MRI and neuropsychological testing, was performed in order to characterize the epileptogenic zone (EZ), and to define the optimal surgical strategy. The predominant seizure types were medically intractable complex partial seizures, and all patients had seizures which were resistant to maximal doses of different anti-epileptic drugs (summarized in Table 1). Epilepsy duration was calculated as the interval in years from age at seizure onset to age at surgery; no patients included in our series had seizures in 24 h before surgery.

Informed consent was obtained for the use of brain tissue for research purposes. Tissue was obtained and used in accordance with the Declaration of Helsinki and the AMC Research Code provided by the Medical Ethics Committee and approved by the science committee of the UMC Utrecht Biobank. The local ethical committees of all participating centres gave permission to undertake the study.

2.2. Tissue preparation

Brain tissue from control, TSC and FCD patients was snap frozen in liquid nitrogen and stored at −80 °C until further use (RNA isolation for qPCR and membrane preparation). Additional tissue was fixed in 10% buffered formalin and embedded in paraffin. Representative sections of all specimens were processed for haematoxylin and eosin and for immunocytochemical stainings used for the routine analysis of TSC cortical specimens.

2.3. Membrane preparation and injection

The preparation of human membranes, their injection in *Xenopus laevis*, GABA and AMPA current recordings in oocytes expressing human functional receptors was carried out as previously described (Eusebi et al., 2009). The use of female *Xenopus laevis* frogs conformed to institutional policies and guidelines of the Italian Ministry of Health (no. authorization 78/2015-PR).

2.4. Electrophysiology

Twelve to 48 h after injection, membrane currents were recorded from voltage clamped oocytes by using two microelectrodes filled with 3 M KCl. The oocytes were placed in a recording chamber (volume, 0.1 ml) and perfused continuously, 9–10 ml/min, with oocyte Ringer's solution (OR) at room temperature (20–22 °C).

Current–voltage (I–V) relationships were constructed holding the oocytes at −60 mV and stepping the membrane potential for 2–4 min