COMMITTEE FOR AID AND EDUCATION IN NEUROCHEMISTRY (CAEN) CATEGORY 1B: REPORT for Research supplies for use in the applicant's home laboratory - International Society for Neurochemistry ISN CAEN Grants – December 2015 round

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# PROJECT TITLE: FGD6 gene expression in Focal Benign Infantile Epilepsy (FBIE): its potential role in epilepsy pathophysiology.

Epilepsy is the third most common cause of neurological disorders. It encloses a group of syndromes that share the defining feature of spontaneous recurrent seizures. Its aetiology and pathophysiology are largely unknown. Although animal models exist for many of the epilepsies, still after many decades of study they have failed to yield conclusive insight into the mechanisms for any of the syndromes. Current therapy is directed solely at the main symptom: spontaneous recurrent seizures, but about 30%–40% of patients cannot be adequately controlled with antiseizure medications (1, 2). Among the epilepsy syndromes, Focal Benign Infantile Epilepsy (FBIE) is the most prevalent in the paediatric population and belongs to the idiopathic epilepsy group. The symptoms affect patient quality of life and generally revert with age. At present no therapies exist that attacks the cause of the disease, hence understanding its pathogenesis is fundamental for a proper translation in management to improve patients' outcome.

Recently Kauffman et al. (3, unpublished data) performed genetic analysis of two FBIE patients from a family with seven members and determined several genes as potential candidates to be involved in epilepsy pathogenesis. FGD6 (*FYVE, RhoGEF and PH domain-containing protein 6*) was the only gene that displayed perfect segregation with epileptic patients. The affected members presented a p.E276G mutation in homozygosis, suggesting mutated FGD6 as a probable cause of epilepsy in this family.

Although it is expressed in neurons, FGD6 function is not well established. It belongs to a family consisting of six members (FGD1-6) and a related GEF (guanine exchange factor), which possess a similar domain organization. These proteins have a DbI homology (DH) domain that encode a principal GEF catalytic unit and multiple phosphoinositide-binding domains, including two pleckstrin homology (PH) domains and a FYVE domain. The amino acid sequence of these domains is highly homologous but each member has a unique N-terminal region. This domain organization suggests that they may function as cross-linkers between plasma membrane and the actin cytoskeleton (4-6). Among the FGD family members, FGD1 and FGD4 have a known function such as Cdc42 GEF activators and a recent study has described a possible action of FGD6 in regulating Cdc42 activity and cell polarity in osteoclasts (7). Cdc42 is a Rho GTPase and has a key role in CNS regulating cytoskeleton, synaptogenesis, spines and dendrite formation and axon guidance.

Cdc42 is a Rho GTPase and has a key role in CNS, regulating cytoskeleton, synaptogenesis, spines and dendrite formation and axon guidance (8, 9). It has been described a potential relationship between Cdc42 and epilepsy since the deregulated expression of cytoskeletal proteins impairs neuronal excitability in animal models of epilepsy, and Cdc42 expression is altered in patients with epilepsy (10, 11).

#### **HYPOTHESIS:**

The mutation in FGD6 found in FBIE patients affects proper Cdc42 function which in turn hampers neuronal function, contributing to epilepsy pathophysiology.

### **SPECIFIC AIMS:**

To test the functional relevance of FGD6 in neuronal biology *in vitro*.

#### **RESULTS:**

We first developed a neuroepithelium differentiation protocol for hIPS (human induced pluripotent stem) cells from wild type, FGD6 heterozygous and FGD6 mutated homozygous subjects (Figure 1). Our previous reports in this model indicated a decrease in the axonal length and some electrophysiological properties altered in the cells derived from FGD6 mutated homozygous subjects.



We first analysed FGD6 expression in all the groups in a differentiated neuroepithelium model. As can be seen in Figure 2, FGD6 localization differs among the groups. In WT cells, FGD6 was uniformly distributed, while in epileptic subjects with a homozygous mutation in FGD6, the protein was localized in the plasma membrane and aggregated in the cytoplasm.

Figure 2. FGD6 is differentially expressed in FGD6 mutated cells in a neuroepithelial culture model.



FGD6 expression in 10-weeks neuroepithelium culture. Cells were fixed and incubated with anti-FGD6 antibody (green), Hoechst-33258 (nuclei) and the corresponding secondary antibody. Images were acquired with a Confocal - Zeiss- LSM 510 META microscope and analysed using LSM and ImageJ software. Scale bars, 20µm.

In WT cells, FGD6 was uniformly distributed, while in epileptic subjects with a homozygous mutation in FGD6, the protein was localized in the plasma membrane and aggregated in the cytoplasm (arrowhead).

Since FGD6 cellular distribution was altered when the protein is mutated, and FGD6 is thought to be a GEF for Cdc42; we then studied actin cytoskeleton in fibroblasts from wild type, FGD6 heterozygous and FGD6 mutated homozygous subjects. Figure 3 shows disorganization in actin network in cells from epileptic subjects.



Actin cytoskeleton analysis in fibroblasts. Cells were fixed and stained with phalloidin (red) and Hoechst-33258 (nuclei). Images were acquired with a Confocal - Zeiss- LSM 510 META microscope and analysed using LSM and ImageJ software. Scale bars, A=100µm, B=50µm.

Cells from epileptic patients displayed a decrease in actin staining, as well as disorganization of the cytoskeleton.

As mentioned above, FBIE belongs to the idiopathic epilepsy group and the symptoms affect patient quality of life but generally revert with age. To further understand the possible role of FGD6 in this pathology we examined its expression along neuronal differentiation. We used the SK-N-SH neuroblastoma cell line and as shown in Figure 4, during neuronal maturation there is a significant decrease in FGD6 expression.



At early stages of SK-N-SH neuroblastoma cells differentiation, a decrease in FGD6 levels was observed, followed by a raise in FGD6 mRNA as the cells continued to differentiate.

Finally we analysed the effect of FGD6 knockdown in the expression of some actin-related cytoskeleton proteins, downstream Cdc42. We used a specific siRNA for FGD6 and we observed a decrease of 50% in mRNA levels (Figure 5A). We then performed Western Blot analysis of different proteins involved in the cytoskeletal network. Our preliminary results show a decrease in Arp2 expression when FGD6 mRNA is decreased (Figure 5B).



Knockdown protocol for SK-N-SH neuroblastoma cell line. Cells were plated and after 24h, transfected with FGD6 a specific siRNA (siRNA), a scramble sequence (Scr) or non-treated (CST). After 2 days cells were harvested, a fraction of the cellular pellet was used to obtain RNA and FGD6 expression was determined by RT-qPCR analysis. The remaining fraction was used for Western Blot.

A- FGD6 expression levels were determined by RT-qPCR. Values are expressed as mean  $\pm$  SD from 3 independent experiments. mRNA levels were normalized to controls. Statistical analysis was performed using one way ANOVA test. \*p $\leq$ 0,05.

At early stages of SK-N-SH neuroblastoma cells differentiation, a decrease in FGD6 levels was observed, followed by a raise in FGD6 mRNA as the cells continued to differentiate.

B- Expression of Arp2 by Western Blot. Protein extracts were obtained and analysed by SDS-PAGE.

Different proteins involved in actin network were evaluated when FGD6 was partially diminished. Among them, Arp2, a protein that promotes actin filament assembly in dendritic spines, was affected. Preliminary studies suggest that a fall in FGD6 expression results in a decrease of Arp2.

#### **CONCLUSIONS:**

Our results suggest that in neurons FGD6 expression is tightly regulated during neuronal maturation, and that FGD6 mutation affects cytoskeleton dynamics, which in turn hampers neuronal function such as differentiation and neuronal activity, thus possibly contributing to epilepsy pathophysiology.

#### **RESEARCH SUPPLIES:**

CAEN grant funds were used to acquire an anti-FGD6 antibody, cell culture and molecular biology reagents, Lipofectamine 2000 transfectant and mice for fibroblasts cultures as feeder layer for IPS cells.

#### SCIENTIFIC PUBLICATIONS DERIVED FROM THE GRANT:

- Reunión conjunta de Sociedades de Biociencias – SAIC (Meeting), 13-17 november 2017, Buenos Aires, Argentina. Galván D.I., Farías M.I., Casalia M., Casabona J.C., Cavaliere Candedo V., Gonzalez J., Kochen S., Kauffman M., Pitossi F.J., **Garcia C.I.** "FGD6 expression in Focal Benign Infantile Epilepsy (FBIE): its potential role in epilepsy pathophysiology".

- Part of the present results is included in a manuscript currently in preparation.

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