Al Consiglio Nazionale delle Ricerche
Ufficio Accordi e Relazioni Internazionali
Piazzale Aldo Moro,7
00185
Oggetto: **STM Passafaro N° 0040275 del 05/07/2013**

FINAL REPORT OF THE RESEARCH ACTIVITY

SUMMARY:

Female-restricted Epilepsy and Mental Retardation (EFMR), also known as Early Infantile Epileptic Encephalopathy-9 (EIEE9), is a debilitating neurological condition characterized by early onset seizures followed by mental retardation and autistic features. This condition has been first described in 1971 and mapped more than 20 years later on chromosome X. Only recently PCDH19, which encodes for Protocadherin-19 (PCDH19), has been identified as the causative gene. The Protocadherin family is the largest group within the Cadherin superfamily of calcium-dependent cell adhesion molecules.

We analysed PCDH19 expression: the protein is detectable in the rat brain, with high level in the cortex and in the hippocampus, consistent with a role in cognitive function. At the subcellular level, PCDH19 is present in crude synaptosomes. PCDH19 expression is temporally and spatially regulated even in dissociated cultures of hippocampal neurons. Most of the neurons express PCDH19 at early stages of development, while in mature neurons PCDH19 is more expressed in glutamic acid decarboxylase (GAD) 65-67 positive neurons than in excitatory neurons.

By a yeast two hybrid screening of a human brain cDNA library, we identified the alpha1 subunit of GABA(A) receptor as a direct interactor of PCDH19. We reconfirmed the interaction by CoIP in heterologous cells and in neurons. The silencing of PCDH19 by shRNA reduces GABA(A) receptor alpha1 subunit expression and surface levels in cultured neurons, suggesting that PCDH19 loss of function impairs GABA(A) receptor transmission.

RESEARCH ACTIVITY DESCRIPTION:

The hippocampus is often the focus of epileptic seizures and it may be subjected to damage following seizures, moreover it plays a crucial role in cognitive function, being a crucial structure for learning and memory. For this reason we investigate PCDH19 expression in rat brain and brain regions. PCDH19 is detectable in the rat brain, with high level in the cortex and very high level in the hippocampus, that is consistent with a role of PCDH19 in cognitive function. At the subcellular level, fractionation experiments on rat brain showed that PCDH19 is present in crude synaptosomes (P2 fraction), suggesting that PCDH19 could be directly involved in synapse function.

By doing ICC experiments we observed that PCDH19 expression is temporally and spatially regulated even in dissociated cultures of rat neurons. Most of the neurons express PCDH19 at early stages of development, while mature neurons (DIV18) are characterized by heterogeneous PCDH19 expression: high PCDH19 levels are retained in glutamic acid decarboxylase (GAD) 65-67 positive neurons, while lower levels are detectable in GluA1 positive neurons. This pattern suggests that PCDH19 is required at different developmental stages, in both inhibitory and excitatory neurons .

In order to identify the proteins that interact with PCDH19, we did a yeast two hybrid screening of a human brain cDNA library, by using the intracellular C-terminal tail of PCDH19 as a bait. We identified the alpha1 subunit of GABA(A) receptor as a direct interactor of PCDH19. GABA(A) receptors mediate fast inhibitory neurotransmission in the central nervous system (CNS). Interestingly, GABA(A) receptor alpha1 subunit is associated with CNS excitability throughout the physiological development as well as in animal models of epilepsy. We validated the yeast two hybrid screening result by doing biochemistry and immunocytochemistry assays in heterologous

cells (**Figure 1**) and in neurons (**Figure 2**) and showed that the two proteins colocalize and coimmunoprecipitate.

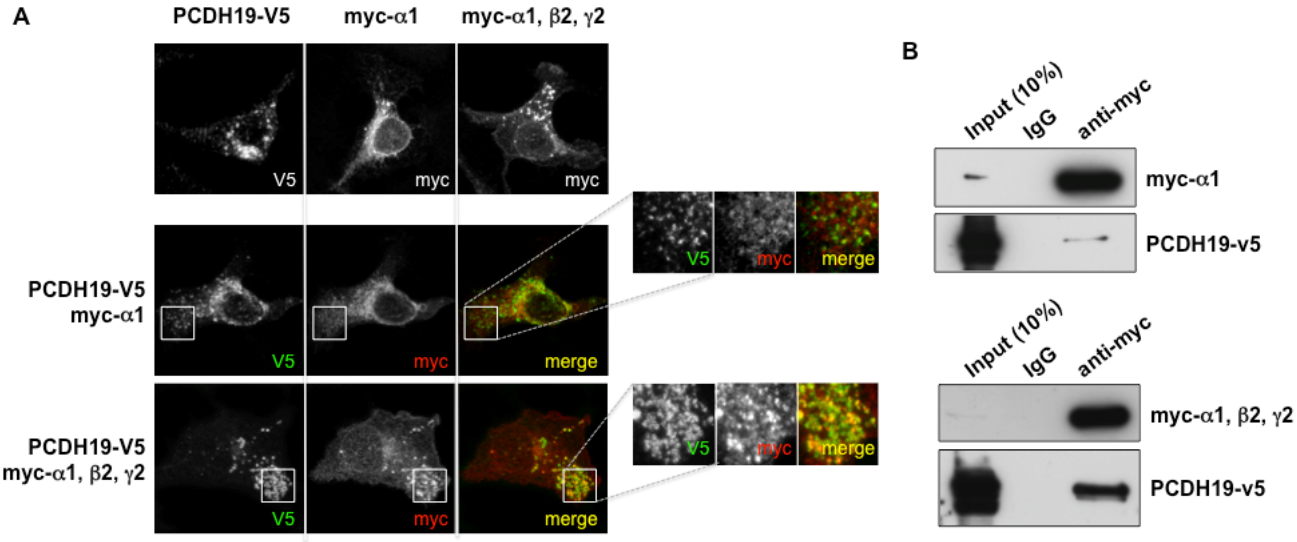

Figure 1. *Interaction of PCDH19 and GABA(A) receptor alpha1 subunit in HEK-293 cells. HEK-293 cells were transfected with PCDH19-V5 and myc-alpha1 subunit or with myc-alpha1 subunit plus beta2 and gamma2 subunits to allow the assembly of the pentameric GABA(A) receptor and immunostained or lysated for CoIP assay. (A) The extent of colocalization between PCDH19 and alpha1 is higher when alpha1 subunit is assembled into a pentameric receptor. (B) PCDH19 coimmunoprecipitates with GABA(A) alpha1 subunit. The amount of PCDH19 immunoprecipitated by anti-myc antibody is bigger when alpha1 is transfected together with beta2 and gamma2 subunits.*

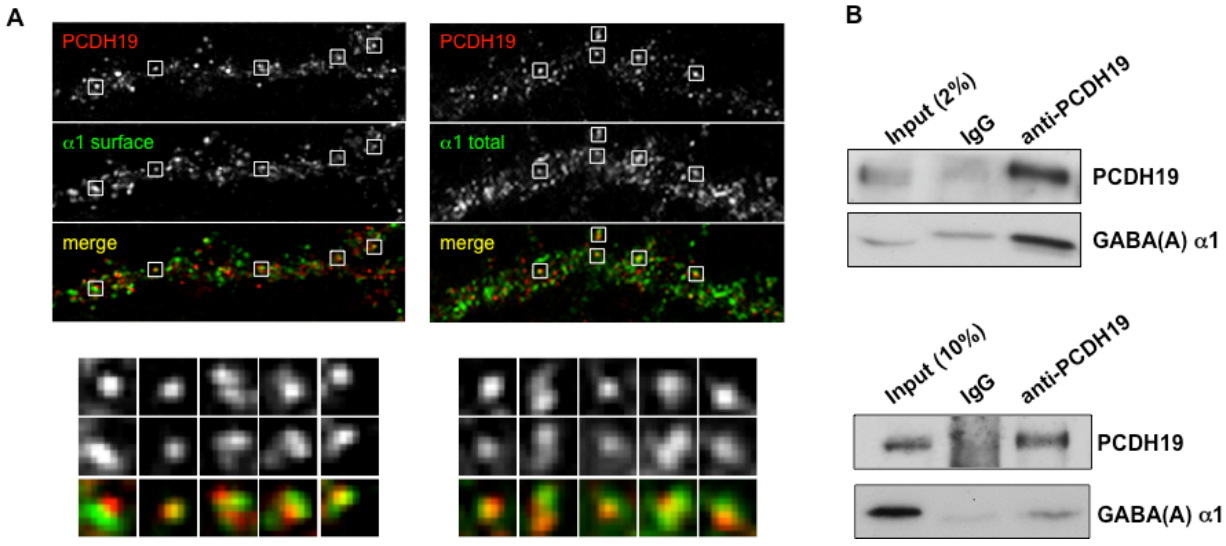

Figure 2. *Interaction of PCDH19 and GABA(A) receptor alpha1 subunit in neurons. (A) Cultured hippocampal neurons (DIV18) were immunostained with anti-PCDH19 and anti-GABA(A) alpha1 subunit antibody. The anti-alpha1 antibody was given either under non-permeabilizing or permeabilizing conditions to stain respectively surface (left panel) and total receptor (right panel). PCDH19 partially colocalizes with GABA(A) alpha1 subunit. (B) GABA(A) alpha1 subunit coimmunoprecipitates with PCDH19 in hippocampal homogenates from adult rats (top panel) and in lysates of cortical neurons at DIV18 (bottom panel).*

In order to mimics the effect of PCDH19 loss of function, we designed a set of three different shRNAs, all specific for PCDH19. In particular sh2120 is able to recognise human mRNA, while sh1240 and 1873 bot human and rat/mouse mRNA. The lentiviral-mediated transfection of these shRNAs significantly reduces the amount of PCDH19 in neurons (**Figure 3**). Interestingly, the knockdown of PCDH19 causes a strong reduction of the GABA(A) alpha1 subunit expression level, while the overexpression of PCDH19 causes an increase of GABA(A) alpha1 subunit expression in neurons (**Figure 4**). Moreover PCDH19 knock-down reduces GABA(A) receptor alpha1 subunit surface level (**Figure 5**), suggesting that PCDH19 loss of function could impair inhibitory transmission in neurons.

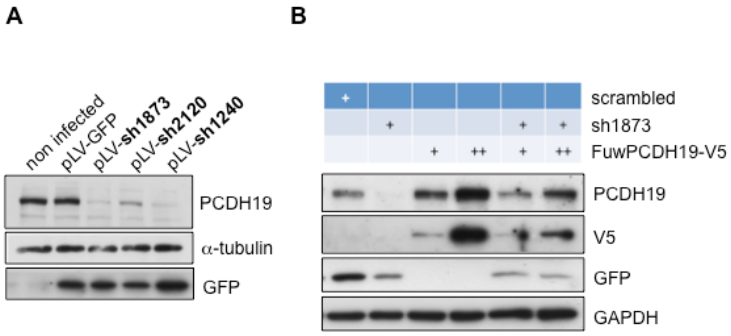

Figure 3. *Validation of PCDH19 shRNAs. Lentiviral-mediated infection of neurons with pLVTHM-GFP, pLVTHM-GFP-shRNA1873, -sh2120, -sh1240 (A) and with pLVTHM-GFP-scrambled shRNA1873, pLVTHM-GFP-shRNA1873, FuwPCDH19-V5 (B). Immunoblots refer to DIV10 cortical neurons infected at DIV3. ShRNAs downregulate PCDH19. FuwPCDH19-V5 infection rescues PCDH19 level.*

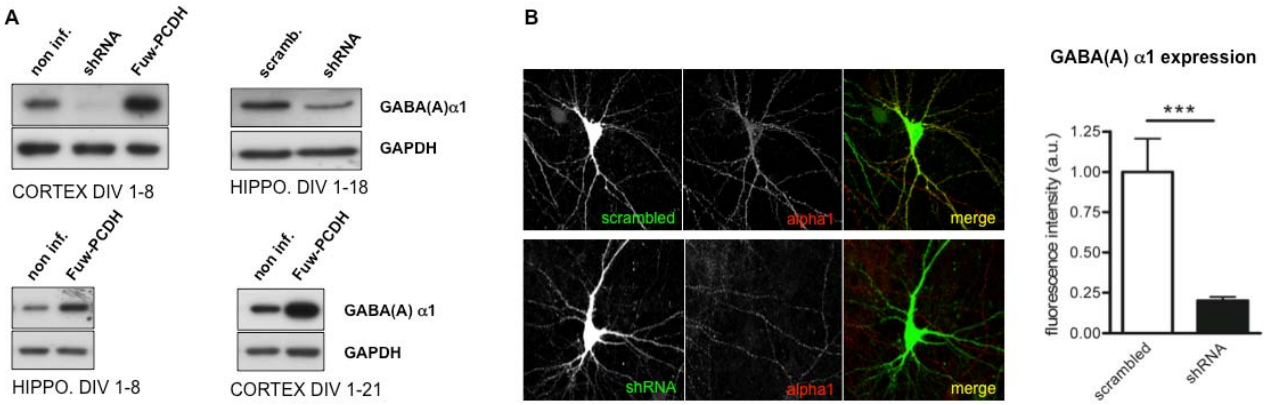

Figure 4. *Modulation of GABA(A) receptor alpha1 subunit expression by PCDH19 downregulation and overexpression. (A) Cultured neurons were infected with PCDH19 shRNA1873 or FuwPCDH19-V5 and immunoblot was performed with anti-GABA(A) alpha1 subunit antibody. The amount of GABA(A) alpha1 varies according to the level of PCDH19 expression. (B) Cultured hippocampal neurons were infected at DIV1 and immunostained at DIV18. The fluorescence intensity of alpha1 subunit is decreased in shRNA1873 expressing neurons compared to control neurons expressing a scrambled shRNA (***p<0,001).*

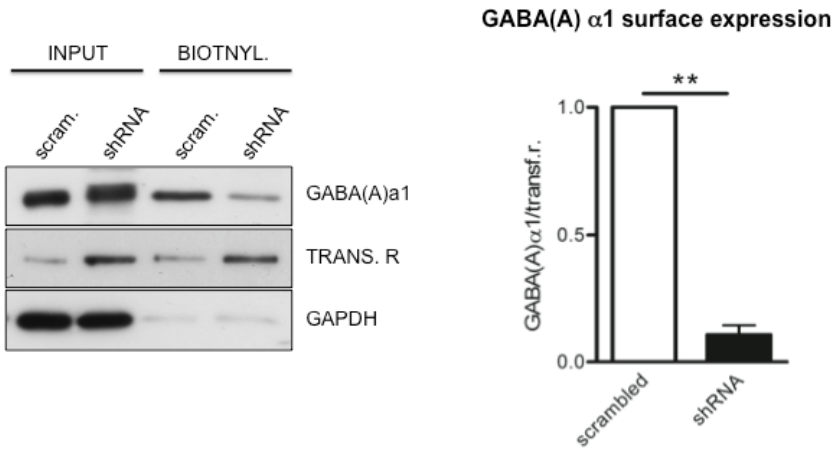

Figure 5. *PCDH19 knock-down reduces GABA(A) receptor alpha1 subunit surface level. Cultured cortical neurons were infected with shRNA1873 or scrambled shRNA at DIV1 and a biotinylation assay was done at DIV12. The amount of surface alpha1 subunit was evaluated and normalized to transferrin receptor (**p<0.01).*

All together the obtained results highlight a role of PCDH19 in GABA(A) receptor expression and possibly inhibitory transmission, as well as a role in neuronal migration during development.

Milano, 11/10/2013

Dott.ssa Maria Passafaro