Escola Superior De Tecnologia Da Saúde Do Porto
Instituto Politécnico do Porto

Manuella Mendes Martins

"Identification of neuronal alterations induced by SHANK3 mutations using iPS cells from Phelan-McDermid Patients' Fibroblasts"

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Dissertação submetida à Escola Superior de Tecnologia da Saúde do Porto para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Tecnologia Bioquímica em Saúde, realizada sob a orientação do Professor Doutor Carlo Sala, MD do Instituto de Neurociências, CNR Milano.

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"When you have problems like an experiment doesn't work, which often happens, it's nice to remind yourself that perhaps after all you are not so good at this job and the schoolmaster may have been right"

by John Gurdon

Nobel Prize for Physiology or Medicine 2012

"Discovery that mature cells can be reprogrammed to become pluripotent""

Dedico aos meus pais porque sem eles, eu nada seria e, mais ainda,
dedico a Deus porque sem Ele, nada seriamos.

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Abstract

Shank proteins family are the major scaffold proteins that organize postsynaptic density (PSD) at excitatory synapses. The three genes, SHANK1, SHANK2, SHANK3 encode large scaffold proteins containing an ankyrin repeat near the N terminus followed by Src homology 3 and PDZ domains, a long proline-rich region and a sterile α motif domain at the C terminus.

These proteins molecularly link two glutamate receptors subtypes, NMDA receptors and type-I metabotropic GluR (mGluRs). The Shank PDZ domain binds to C terminus of GKAP, which binds to PSD-95-NMDA receptor complex. Homer interaction with proline-rich domain ensures the association of Shank with type I mGluRs.

SHANK3 gene haploinsufficiency is likely to cause the majority neurological features associated with 22q13 deletion/Phelan-McDermid syndrome such as, intellectual impairment, absent or delayed speech and autistic-like behaviour.

In this study, we investigated Shank3 role in synaptic function to understand why alterations in this protein can cause the neurological features presented by Phelan-McDermid Syndrome patients.

We used two different models, shank3 KO mice and hiPS cells derived from Phelan-McDermid patients. Genetically modified mice are useful tools for investigating gene function and comprehending better mechanisms that *in vitro* experiments cannot reproduce, but to understand better human pathologies, we work also with human cells.

We reprogrammed Phelan-McDermid Patients' fibroblasts into hiPS cells, differentiated them into neurons and compare with neurons obtained from healthy age-matched donors. The patients' fibroblasts were reprogrammed into iPS cells, by lentivirus infection with four reprogramming genes OCT4, c-MYC, SOX2 and KFL4, to posteriorly be differentiated into neuron with every step successfully confirmed by precise neuronal markers.

Through differentiated neurons characterization we analysed Shank3 protein expression as well as other synaptic proteins. Patients' neurons with Shank3 haploinsufficiency showed increased mGluR5 protein levels and decreased Homer protein levels suggesting that haploinsufficiency lead to deregulation of the mGlur5-Homer-Shank3 complex and reduced levels of Shank3 protein cause defects in synapses maturation.

Therefore, mGluR5 expression is altered in PMS patients and this should be correlated with defects that we found in neuronal differentiation and synapses maturation observed in neurons derived from PMS patients.

Concluding iPS cells represent a useful model to study the role of Shank3 in the pathogenesis of PMS.

Keywords: Shank3, induced-pluripotent stem (iPS) cells, Autism Spectrum Disorder (ASD), Phelan-McDermid Sindrome (PMS), mGluR5

Resumo

A família de proteínas Shank é o principal conjunto de proteinas de suporte e está localizada na densidade pós-sináptica das sinapses excitatórias. Existem 3 genes na família Shank, Shank1, Shank2 e Shank3 e são caracterizados por múltiplos domínios repetidos de anquirina próximo ao N-terminal seguido pelos domínios Src homologo 3 e PDZ, uma região longa rica em prolina e um domínio de motivo α estéril próximo ao C-terminal.

Shank proteínas conectam duas subunidades de receptors glutamatérgicos, recetores NMDA e recetores metabotrópicos de glutamato do tipo-I (mGluRs). O domínio PDZ da Shank conecta-se ao C-terminal do GKAP e este, liga-se, ao complexo recetor PSD-95-NMDA. Por outro lado, a proteína Homer interage com o domínio rico em prolina para confirmar a associação entre a proteína Shank com o mGluR tipo-I.

A proteína específica em estudo, Shank3, é haploinsuficiente em pacientes com sindrome Phelan-McDermid devido à deleções no braço comprido do cromossoma 22 levando à danos intelectuais, ausência ou atraso no discurso, comportamentos semelhantes ao autismo, hipotonia e características dismórficas.

Neste trabalho, investigamos o papel da Shank3 na função sináptica para compreender a relação entre alterações nesta proteína e as características neurológicas presente em Pacientes com síndrome Phelan-McDermid.

Foram utilizados dois modelos diferentes, ratinhos knockout Shank3 e hiPSC de pacientes com PMS. Ratinhos geneticamente modificados são ferramentas uteis no estudo de genes e na compreensão dos mecanismos que experiências *in vitro* não são capazes de reproduzir, mas de maneira a compreender melhor as patologias humanas, decidimos trabalhar também com células humanas.

Os fibroblastos dos pacientes com síndrome Phelan-McDermid fora reprogramados em hiPS cells, diferenciados em neurónios e comparados com os neurónios obtidos a partir de doadores saudavéis e da mesma idade. A reprogramação em iPSC foi realizada por infecção de lentivirus com quatro genes de reprogramação OCT4, c-MYC, SOX2 e KFL4 para posteriormente serem diferenciados em neurónios, com cada passo sendo positivamente confirmado através de marcadores neuronais.

Através dos neurónios diferenciados, analisamos a expressão de proteínas sinápticas. Pacientes com haploinsuficiencia na proteína Shank3 apresentam níveis elevados de proteína mGluR5 e decrescidos de proteína Homer sugerindo que a haploinsuficiencia leva a desregulação do complexo mGluR5-Homer-Shank3 conduzindo também, a defeitos na maturação sináptica. Assim, a expressão da proteína mGluR5 está alterada nos pacientes com PMS podendo estar relacionada com defeitos encontrados na diferenciação neuronal e maturação sináptica observados nos neurónios de pacientes.

Conclusivamente, iPS cells representam um modelo fundamental no estudo da proteína Shank3 e a sua influência no sindrome de Phelan-McDermid.

Palavras-chave: Shank3, células estaminais pluripotente induzidas (iPS), Autism Spectrum Disorder (ASD), Phelan-McDermid Sindrome (PMS), mGluR5

Riassunto

La famiglia di proteine Shank sono proteine scaffold codificate da tre geni *shank1*, *shank2* e *shank3*.

Queste proteine hanno un peso molecolare maggiore di 200 kDa e la loro struttura comprende un dominio ankyrin repeats vicino dall'estremità N-terminale, un dominio SH3, un dominio PDZ, una lunga regione ricca di proline e un dominio SAM all'estremità C-terminale.

Le proteine Shank sono in grado di legare due tipi di recettori glutamatergici, i recettori NMDA e i recettori metabotropi per il glutammato di tipo I (mGluR1, mGluR5).L'interazione tra Shank e i recettori mGluR avviene attraverso il legame di Homer alla regione ricca in proline di Shank. Mentre l'interazione tra Shank e i recettori NMDA avviene grazie al legame tra il dominio PDZ di Shank e la sequenza C-terminale della proteina GKAP.

L'aploinsufficienza del gene *shank3* è la causa "strutturale" del quadro clinico neurologico associato alla sindrome da delezione 22q13o sindrome di Phelan-McDermid. Gli individui affetti presentano ipotonia neonatale, ritardo globale dello sviluppo, assenza o ritardo di linguaggio e lievi segni dismorfici.

In questo lavoro è stato studiato il ruolo della proteina shank3 nella sinapsi per capire come le alterazioni di questa proteina causino alterazioni neurologiche associate alla sindrome di Phelan McDermid.
Per questo studio sono stati utilizzati due modelli di patologia, topi trangenisci KO per il gene shank3 e cellule ottenute da pazienti affetti dalla sindrome di Phelan McDermid.

Gli animali transgenici rappresentano sicuramente un buon modello per studiare i difetti genetici, ma per capire meglio le alterazioni presenti nei pazienti abbiamo deciso di utilizzare anche cellule umane ottenute direttamenti da pazienti affetti da questa sindrome.

In particolare abbiamo utilizzato fibroblasti che sono stati riprogrammati in cellule iPS, ossia cellule staminali pluripotenti indotte, attraverso l'uso di un vettore lentivirale esprimente 4 geni di riprogrammazione OCT4, c-MYC, SOX2 and KFL4. Queste cellule sono state differenziate in neuroni, che sono stati studiati per trovare eventuali alterazioni nella composizione e nella maturazione sinaptica.

I dati ottenuti mostrano un aumento dei livelli di espressione del recettore metabotropico per il glutammato mGluR5 e una diminuzione dell'espressione della proteina Homer nei pazienti affetti da aploinsufficienza per il gene shank3, suggerendo che l'assenza di shank3 può causare una disregolzione del compleso proteico formato da mGluR5-Homer-Shank3. L'alterzione dell'espressione proteica del mGluR5, potrebbe essere correlato ai difetti nella maturazione sinaptica e nel differenziamento neuronale che sono stati riscontrati nei neuroni ottenuti da questi pazienti.

In conclusione dunque le cellule staminali umane pluripotenti indotte (iPs) rappresentano un buon modello per lo studio del ruolo di shank3 nella patogenesi della sindrome di Phelan McDermid.

Parole-chiave: Shank3, cellule staminali pluripotente indotte (iPS), Autism Spectrum Disorder (ASD), Phelan-McDermid Sindrome (PMS), mGluR5

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Abbreviation List

AD - Alzheimer's disease

AMPc – Cyclic adenosine monophosphate

AMPA - 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid

ASD – Autism syndrome disorder

ATD – Amino N-terminal domain

ATP – Adenine triphosphate

CAM – Cell-adhesion molecules

CaMKII - Ca^{2+}/calmodulin-dependent protein kinases II

CAZ – Cytomatrix of active zone

CDPPB - 3-cyano-*N*-(1,3-diphenyl-1*H*-pyrazol- 5-yl)-benzamide

CNS – Central Nervous System

DAG – diacylglycerol

DMEM - Dulbecco's Minimum Essential Medium

DHPG - 3,5-dihydroxyphenylglycine

DIV – Days *in vitro*

EAAT – Excitatory amino acid transporter

EM – Electron microscopy

ER – Endoplasmic reticulum

ERK - Extracellular signal-regulated kinase

ESC – Embryonic Stem Cell

FBS - fetal bovine serum

FISH - fluorescent in situ hybridization

GABA – γ-aminobutyric acid

GDP - guanosine diphosphate

GLAST – Glutamate aspartate transporter

GLT – Glutamate transporter

GPCR – G-protein coupled receptor

GTP – Guanosine Triphosphate

GluAs – AMPA Receptor subunits

GluKs – Kainate Receptor subunits

HBSS - Hank's Balanced Salt Solution

HEK - Human Embryonic Kidney

iGluR – Ionotropic Glutamate Receptor

IP3 - 1,4,5-triphosphate

iPSC – induced pluripotent stem cell

KAR – Kainate Receptors

LBD – Ligand Binding Domain

LTD – Long-Term Depression

LTP – Long-Term Potentiation

MAGUK - Membrane-associated guanylate kinase

MAP2 – Microtubule associated protein-2

MEF - Mouse Embryonic Fibroblast

mGluRs – metabotropic glutamate receptors

MOI - multiplicity of infection

NLRR - Neuronal leucine-rich repeat

NMDA - N-Methyl-D-aspartate

NMP - Nucleoside Monophosphate

NSC - Neuronal Stem Cells

NSF - N-ethylmaleimide sensitive fusion

NT – Neurotransmitter

PBS - Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PDZ - **P**SD-95/**D**iscs large/**Z**onula occludens (ZO)-1

PKC - Protein Kinase C

PMS – Phelan-McDermid Syndrome

PSD – Post-synaptic density

ProSAP - **Pro**line-rich **s**ynapse-**a**ssociated **p**rotein

RIM - Rab3-interacting molecule

RT-PCR – Real-Time PCR

RT-PCR - Reverse Transcription PCR

SAM - sterile alpha motif

SCZ - schizophrenia

SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis

SH3 – SRC homology 3 domain

shRNA – small hairpin RNA

siRNA – small interfering RNA

SNAP - Soluble NSF Attachment Proteins

SNARE – Soluble NSF attachment receptors

SSTR - somatostatin receptor type 2

TUJ1 - Neuron-specific class III beta-tubulin

TMD – Transmembrane domain

VGLUT – Vesicular glutamate transporte

Introduction

An integral synaptic homeostasis is essential for a healthy brain. This homeostasis depends on the functional properties of synaptic proteins because these molecules control the synaptic assembly, stability, plasticity and maturation. Therefore, any dysfunctions in the synaptic morphology will lead to pathologies called synaptopathies. In this group are included Schizophrenia, Alzheimer's disease and Autism Spectrum Disorders (ASD) (Durand CM, 2007).

ProSAP/Shank proteins are the main scaffolding molecules at postsynaptic density (PSD) and act harboring multiple protein-protein interactions due to their SH3 and PDZ domains. The Shank family is constituted by Shank1, Shank2 and Shank3 and splicing variants have been described to all the three shank genes. However, six intragenic promoters were identified in Shank3 gene, causing a large number of Shank3 splicing variants. The splicing consequences are yet to be defined but it is possible to say, based on where the mutation is localized, that the truncated protein obtained can have diverse functional consequences (Boeckers T et al., 2002).

Phelan-McDermid Syndrome (PMS) is a pathology included in the autism spectrum disorders in which, mutations/deletions in the long arm of chromosome 22 provoke Shank3 gene haploinsufficiency leading to global development delay, moderate to severe intellectual impairment, absent or severely delay speech and neonatal hypotonia (Phelan K and McDermid HF, 2011).

In this specific work, we are going to concentrate our study in Shank3 protein and its haploinsufficiency.

In order to a better comprehension about Phelan-McDermid Syndrome, as well as Shank3 alterations in this pathology, we came up with two different models to study: Patients' fibroblasts that were reprogrammed into induced-pluripotent stem cells and posteriorly, these cells were differentiated into neurons and at the same time, we start to work with mice knockout for Shank3 gene.

The main goal of this work is characterize Shank3 proteins by fibroblasts, neuronal stem cells and differentiated neurons analyzes. Along the study, we are going to evaluate alterations in proteins that could be implicated in Phelan-McDermid syndrome due to correlation with Shank3 protein such as Homer, mGluR5 and PSD-95. Besides, we are

going to try to understand how the communication among neurons is performed and how different are Phelan-McDermid synapses comparatively to normal synapses' functions by analyzing neurons' synaptic formation and synaptic maturation.

From all the results obtained with this work, we hope to have a better understanding about this syndrome and all its mechanisms in way to find a method capable of reestablish Shank3 functions and in this manner, finding a cure to Phelan-McDermid syndrome.

State of the Art

I. The Brain

The brain is the central and most complex organ in the human body. It is able to control and command everything we do. All thoughts, feelings, memories, our capabilities to move, breathe, speak, think, communicate with others, make decisions and even our hopes and dreams are related to cerebral functions. In this way, is easy to understand that the brain regulates all the basic elements to a perfect and healthy body.

The human brain only represents 2% of body weight but needs about 20% of the body's oxygen supply and blood flow otherwise within 3 to 5 minutes begins to die. Its constitution is based on billions of nerve cells, called neurons, connected to each other through a very complex system responsible for carrying out the information from and to the brain. Each neuron is constituted by three elements, which are:

- **Cellular body**, also known as, soma that possesses all the contents essential to the cell as nucleus, mitochondrias, endoplasmastic reticulum and ribosomes.
- **Axon**, is responsible for the transmission of electric impulses from the cell body until a farther place as a muscle or another neuron.
- **Dendrites**, are the branched projections of neurons that act receiving the synaptic signaling from other neuron's axon and passing to the cell body (Gundelfinger et al., 2000).

1.1 Synapses

In the nervous system, a synapse is a structure that allows a neuron or a nerve cell to transmit an electrical or chemical signal to another cell (neuronal or not neuronal) (Schacter DL, 2011).

The synapses are responsible for all the connections in the brain, which are constantly changing, being able to store memories, skills and shaping personalities by strengthening certain patterns of brain activity and losing others.

Based on how the mechanism of transmission is, the synapses can be electrical or chemical.

1.1.1. Electrical synapses

Electrical synapses are a much smaller group when compared to chemical synapses. The electrical synapses act connecting the membranes of the two neurons in communication and put them very close at the synapse, linked through an intercellular specialization called a gap junction. The gap junction contains accurately aligned paired channels forming pores in the membrane of the pre- and postsynaptic neurons, allowing ions, ATP and other important intracellular metabolites to be transferred by simple diffusion through an ionic current that flows passively between the cytoplasm of the pre- and postsynaptic neurons. The ionic current depends on the potential difference generated locally by the action potential. In electrical synapses the transmission can be bidirectional so, the current will flow across the gap junction according to the member of the coupled pair that has received the action potential. The electrical synapse is extremely fast because the passive current flow across the gap junction is almost instantaneous so there is no delay in the communication (Purves D et al., 2001) (Barry W Connors, 2004).

1.1.2. Chemical Synapses

Chemical synapses are complex cell-cell contact sites formed by the axon terminal membrane of one neuron, that contains the presynaptic machinery for neurotransmitter release and the postsynaptic membrane of another neuron responsible for the reception of neurotransmitter signals (Gundelfinger et al., 2000). The space between the pre- and postsynaptic neurons is much bigger when compared to the gap junctions in the electrical synapses and is called synaptic cleft. In the presynaptic membrane, there are small, membrane-bounded organelles called synaptic vesicles filled with one or more neurotransmitters. These neurotransmitters are chemical signals secreted from the presynaptic neuron acting as messengers between the communicated neurons. In this kind of synapse, the signal transmission is unidirectional and starts when an action potential invades the neuron's presynaptic terminal active zone. The active zone is a specialized region of the presynaptic plasma membrane located exactly opposite to the postsynaptic reception apparatus. At the ultra-structural level, both sides of the synapse are characterized by electron-dense projections. When these projections are located in the presynaptic side, are called presynaptic dense projection or cytomatrix assembled at the active zone (CAZ) of neurotransmitter release (Hirokawa et al., 1989) and at the postsynaptic side, they are called postsynaptic density (PSD) (Peters A et al., 1991).

In this way, when the active zone is hit by an action potential, the presynaptic membrane potential changes and the voltage-gated calcium channels open, causing a fast Ca^{2+} influx into the presynaptic terminal, elevating the concentration of Ca^{2+} in the terminal. Consequently, the synaptic vesicles merge to plasma membrane of the presynaptic neuron and this fusion leads to the neurotransmitters release into the synaptic cleft. On the postsynaptic side, neurotransmitter receptors and signaling molecules align with sites of presynaptic vesicle release in an electron-dense specialization, the postsynaptic density (PSD). These bindings lead to the opening of the channels in the postsynaptic membrane and therefore changing the ions capability to flow into the postsynaptic cells. The current flow induced by neurotransmitters alters the conductance and frequently the membrane potential of the postsynaptic neuron, modifying the neuron's probability of firing an action potential, transmitting the information from one neuron to another (Purves D, 2001).

The synapses can, also, be classified according to the neurotransmitters that are released during the synapse and the corresponding postsynaptic receptors' apparatus. In this way, there are inhibitory and excitatory synapses.

1.1.3. Inhibitory Synapses

The inhibitory synapses are those, which the nerve impulse in a presynaptic cell results in the release of inhibitory neurotransmitters leading the opening of multiple ion channels in the post-synaptic cell membrane. This opening facilitates the entrance of negative ions or the exit of positive ions, in either way stabilizing the resting potential of the cell and consequently decreasing the probability for the postsynaptic cell to fire an action potential. The main inhibitory neurotransmitter is γ-aminobutyric acid (GABA). The inhibitory synapses, although forming 10% to 20% of all synapses in the brain, are indispensable for proper and stable functioning of the brain because they modulate the nervous system activity (Chavas J, 2003).

1.2. Excitatory Synapses

The excitatory synapses represent the main positions of communication between neurons in the Central Nervous System (CNS) of Mammalians. This type of synapse is situated on dendritic spines or shafts and is characterized by a prominent PSD. The amino acid L-Glutamate is the main excitatory neurotransmitter in the Mammalians Central Nervous System (CNS) (Erecinska M, 1990) (Figure 1).

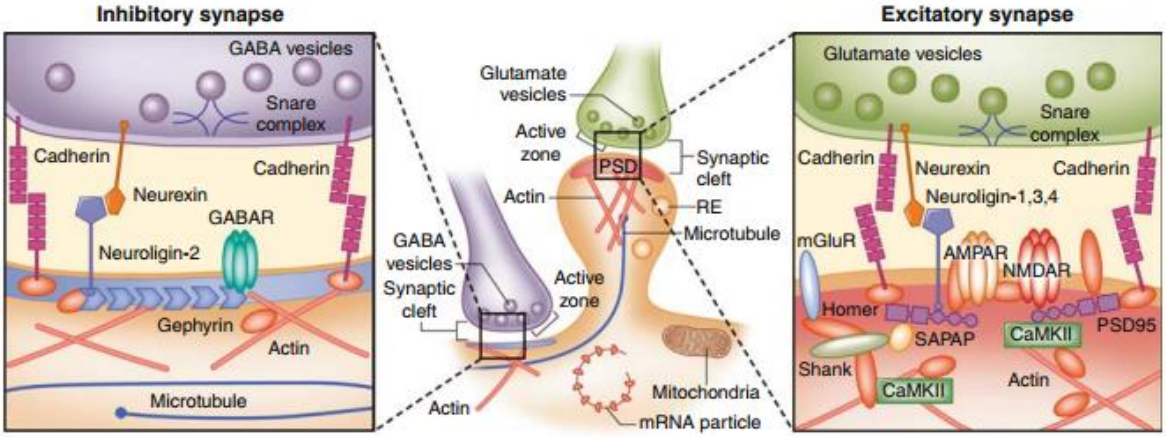


Figure 1 - Molecular architecture of inhibitory and excitatory synapses
(Spronsen MV and Hoogenraad CC, 2010)

Glutamate is synthetized, stored in high concentration in the glutamatergic neurons, and is released from vesicles into extracellular space by a Ca^{2+} dependent mechanism that involves N- and P/Q type voltage-dependent Ca^{2+} channels that appear to be intimate linked to vesicle docking sites. The Ca^{2+} dependent mechanism is developed through particular stimuli, for example, when an action potential is fired.

1.2.2. Pre synaptic machinery

The presynaptic structure is the neuron's region responsible for producing neurotransmitters that are accumulated into synaptic vesicles and after, are released in the synaptic cleft. This releasing is initiated through the docking, which allows the vesicle filled with neurotransmitters and the pre-synaptic membrane to line up in a fusion-ready state at the active zone. The active zone is constituted by the presynaptic membrane and a dense collection of proteins called the cytomatrix at the active zone (CAZ) (Zhai and Bellen, 2004). Once at the active zone, neurotransmitter-filled synaptic vesicles and plasma membrane proteins, with the help of cytoplasmic proteins, recognize each other and

docking occurs (Chua JJE et al., 2010). The cytoplasmic proteins, N-ethylmaleimide Sensitive Fusion (NSF) proteins and Soluble NSF Attachment Proteins (SNAPs) are responsible in assuring proper vesicle targeting. Both of these proteins together with SNAP receptors called SNAREs are able to build a complex between the vesicle and presynaptic membrane (Marz EK et al., 2003). Synaptobrevin is the vesicle membrane protein and syntaxin is the pre-synaptic membrane that act as SNAREs and allow these two structures to recognize each other (Broadie K et al., 1995).

After docking, the priming occurs. This procedure prepares the docked synaptic vesicles so they are able to fuse with the plasma membrane rapidly in response to a calcium influx creating a small opening, which grows larger until the vesicle membrane collapses into the pre-synaptic membrane. The fusion is completed and the exocytosis occurs. This step is thought to involve the formation of partially assembled SNARE complexes (Chua JJE et al., 2010).

In the presynaptic compartment are involved several classes of proteins, such as mammalian Unc-13 Homolog (Munc13), Rab3 and Rab3-interacting molecule (RIM) that had been postulated to recruit synaptic vesicles to the presynaptic membrane (Dulubova I et al., 2005). Besides these proteins, a presynaptic scaffold is arranged near the docked vesicles modulating neurotransmitter (NT) release (Schoch and Gundelfinger, 2006). The presynaptic supramolecular assembly is constituted by large multidomain proteins responsible for forming the building blocks of the active zone, for instance Bassoon (Dieck T et al., 1998), Piccolo (Fenster SD, 2000), ELKS/Rab6-interacting/CAST family members (ERCs), liprin-α, MINT1 (Rogelj et al., 2006), MALS (Olsen O et al., 2005) and CASK (Hsueh YP, 2006). In the presynaptic compartment are also found the cell adhesion molecules (CAMs) for example cadherins such as CDH2 (N-cadherin) found in neurons, neuroxins and integrins. The CAMs, although found in nerve cells are not specifically located here and can be found in others cell's compartments. In the group of synaptic cytoskeleton proteins are included microtubule-associated proteins such as MAP1A, MAP2 and MAP5, neurofilament proteins and actin-associated proteins such as fodrin, drebrin A and α-adducin. This last protein, α-adducin, is responsible for promoting the assembly of the actin-spectrin cytoskeleton, being found both in pre- and post-synaptic compartments (Seidel B et al., 1995) (Gundelfinger ED, 2000).

1.2.1.1 *Glutamate Transporters*

According to the structure and site of action, glutamate transporters can be divided in two different superfamilies, the plasma membrane excitatory amino acid transporters (EAATs) that are dependent on an electrochemical gradient of sodium and the ones that are not dependent and are called the vesicular glutamate transporters (VGLUTs).

The cytoplasmic glutamate is transported inside vesicles by VGLUTs and then released in the synaptic cleft. Studies revealed that the quantity of VGLUT protein in the synaptic vesicle regulates the amount of glutamate released, but the VGLUT quantity is not enough to calculate the release probability (Wojcik C, Yano M, DeMartino GN, 2004).

The accumulation of glutamate in synapse vesicles is not dependent on a Na^+ electrochemical gradient. Instead, the vesicular glutamate transporters are dependent on a proton gradient that they create by hydrolyzing ATP with V-type H^+-ATPase, enabling H^+ flow into the synaptic vesicle becoming more positive, generating a membrane potential and then, finally originating electrochemical proton gradient (Monika Liguz-Lecznar et al., 2007).

Three highly homologous proteins compose the VGLUT family: VGLUT1, VGLUT2 and VGLUT3. Both VGLUT1 and VGLUT2 are expressed mainly in glutamatergic neurons and VGLUT3 is localized in a limited number of glutamatergic neurons in multiple brain regions, such as, neocortex, hippocampus and hypothalamus and has also been found in hippocampal and cortical GABAergic neurons (Hergoz E, 2004), cholinergic neurons in the striatum and serotonergic neurons in the raphe nuclei (Christelle Gras et al., 2002).

Studies have demonstrated that VGLUT1 and VGLUT2 are able to control the synaptic response to the release of neurotransmitter from a single vesicle (quantal size) as well as the efficacy of transmission (Moechars D et al., 2006). Based on these facts, is possible to modulate glutamatergic transmission, causing influence on the synaptic plasticity, specifically homeostatic scaling enabling neurons to keep on firing within optimal range (Monika Liguz-Lecznar et al., 2007).

In the glutamate transporters' superfamily is also included the plasma membrane excitatory amino acid transporters (EAATs). After the VGLUTs transport the glutamate

inside the synaptic vesicles until the presynaptic terminal and release it in the synaptic cleft, the glutamate is transported, with the help of the EAATs, to the astrocytes where is converted into glutamine.

The first studies on the expression patterns and localization of glutamate transporters were done by cloning the genes that code for excitatory amino acid transporters (EAATs) allowing, in this way, glutamate transporters studies in neurons and glia to be carried out (Anastassios V Tzingounis and Jacques I Wadiche, 2007). These transporters are named EAAT1 (GLAST), EAAT2 (GLT-1), EAAT3 (EAAC1), EAAT4 and EAAT5.

The most abundant EAATs, EAAT1 (GLAST) and EAAT2 (GLT-1) (Shashidharan et al., 1994) are found in astrocyte membranes (Lehre KP and Danbolt NC, 1998) (Rothstein JD et al., 1994) (Lehre KP et al., 1995).

The other members of this glutamate transporter gene family, not expressed by astrocytes, are EAAC1 (Kanai Y and Hediger MA 1992) that is present on neuronal cell bodies, EAAT4 (Fairman WA et al., 1995) recognized in cerebellar Purkinje cells and EAAT5 (Arriza JL et al., 1997) expressed in the retina (Anderson CM and Swanson RA, 2000). EAAT1 and EAAT2 are predominantly present in glial cells while EAAT3, EAAT4 and EAAT5 are expressed by neurons throughout the brain.

Any problem in glutamate's transporter can provoke an accumulation of this neurotransmitter in the extracellular space leading to cell damages and consequently, cell death. In this way, to decrease neurotoxicity and because glutamate is not able to cross the blood-brain barrier, glutamate is converted in another compound, glutamine. This process is called glutamate-glutamine cycle and glutamate is recycled in glial cells (astrocytes) because presynaptic nerve terminals are largely devoid of transporters. In this way, glutamate is transported into astrocytes, converted to glutamine with glutamine synthetase's help and recycled back to neurons in the form of glutamine where is reconverted in glutamate through phosphate-activated glutaminase (Danbolt CN, 2001). In this way, glutamate transporters play a fundamental role in preventing the accumulation of extracellular glutamate because they are able to remove glutamate to maintain low levels in the synaptic cleft, they limit glutamate diffusion between synapses to make sure that the

fidelity of transmission is guaranteed and they recycle glutamate for sustained release (Hediger MA and Welbourne TC, 1999).

1.2.3. Postsynaptic compartment

As demonstrated previously, the synapse is the communication between an axon from the presynaptic neuron and a dendrite from the postsynaptic neuron.

When compared to axons, dendrites are often shorter and less uniform because they are thicker near the origin at the cell body and then taper off the farther away they are from the soma (Jan YN, Jan LY, 2001). As far as the molecular composition concerns, the dendrites contain mRNA and basically all the same organelles that are present in the cell body, such as ribosomes, endoplasmic reticulum (ER) and Golgi Complex (Guardiol A, 1999) (Steward O and Schuman EM, 2001). In opposite of axons that do not have mRNA and ribosomes, dendrites have and so, they are able to synthetize proteins (Weiler IJ and Greenough WT, 1999).

The postsynaptic element of major part of excitatory synaptic transmissions in the Central Nervous System occurs at mushroom-like protrusions distributed with semi-regular spacing along the dendrites known as dendritic spines (Swulius M et al., 2010) (Newpher TM and Ehlers MD, 2008). Palay in 1956 and Gray in 1959 first described the spines through electronic microscopy while studying cytoarchitecture of the CNS and had given the name, *spine*, because the electron opaque appearance in negative stained micrographs of thinly sectioned neuronal tissue (Lee KFH et al., 2012). The spines are shaped like mushrooms, each with a resembling spherical head connected to the dendritic shaft by a cylindrical neck and in these spines occurs more than 90% of all excitatory synapses in the mammalian central nervous system.

These dendritic protrusions constitute a powerful structural scaffold for the majority of excitatory synapses in the brain, sheltering a balance of biochemical signalling machinery as well as a postsynaptic density (PSD) composed by, amongst others, ionotropic glutamate receptors of the AMPA and NMDA subtypes (Lee KFH et al., 2012).

The dendritic spine structure reflects a dynamic arrangement that may undergo several changes in shape, slowly and rapidly, over its lifetime (Sorra KE and Harris KM, 2000).

All these changes in spine number and/or structure occur because brain uses long-lasting modifications of synaptic strength in neuronal circuits (Malenka RC, 1994). These modifications regenerate a strengthening or weakening of synapses between CA3 and CA1 neurons in the hippocampus. One of these mechanisms is a long-lasting enhancement of synaptic transmission, the Long-Term Potentiation (LTP) that leads to a sustained increase in CA1 neurons' synaptic strength. This strength is provoked by momentary high frequency stimulation of Schaffer collateral axons projected by CA3 neuron. The other apparatus that is capable of shaping the dendrite structure is the Long-Term Depression (LTD) that, on the other hand, is related to a low-frequency stimulation leading to long-lasting weakening of the same synaptic population (Ito M.,1989) (Dudek SM and Bear MF, 1992). The LTD is generating by prolonged low frequency stimulation, about 1Hz (Bramham CR and Srebro B, 1987). Studies affirm that these two machineries combined are thought to be related with synaptic plasticity, being able to modulate learning and memory mechanisms (Malenka and Nicoll, 1999) (Lynch MA, 2004).

1.2.2.1 *Postsynaptic density*

In the postsynaptic membrane of excitatory synapses is placed a region defined as containing thickening and increased density called postsynaptic density (PSD) (Gold MG, 2012).

The PSD dimensions are proportional to total spine volume, number of presynaptic vesicles and quantity of organelles within the spine (Spacek J and Harris KM, 1997). The PSD was first measured at ~50nm by electron microscopy (EM) of isolated PSDs (Carlin RK et al., 1980). Electron microscopy has been a valuable method to characterize, at the ultra-structural level, the formation, size and shape of dendritic spines along diverse phases of development of both postsynaptic density and actin cytoskeleton (Swulius M et al., 2010).

The postsynaptic density is situated on the opposite side of the transmitter-releasing site on the presynaptic membrane most precisely, the active zone (Feng W and Zhang M., 2009). A highly organized neurotransmitter reception apparatus clustered to the postsynaptic membrane attached to the submembranous cytoskeleton and physically connected to components of intracellular signaling pathways constitute the postsynaptic density protein complex (Gundelfinger ED and Dieck ST, 2000).

Over the past few years, the postsynaptic density has been received a lot of attention and consequently, a very large group of proteins have been identified such as, membrane receptors and ion channels, proteins involved in signaling, cell adhesion proteins, scaffold proteins, cytoskeleton proteins constitute the PSD proteome (Sheng M and Hoogenraad CC, 2007).

1.3. Glutamatergic Receptors

The glutamate is involved in a wide range of physiological processes in the CNS that are associated with emotion, cognition and motor functions. After released from the presynaptic membrane, glutamate diffuses through the synaptic cleft and binds to postsynaptic neurotransmitter receptors that modify the membrane potential and activate signal transduction cascades (Jin Y and Garner CC, 2008).

Many types of glutamate receptors (GluRs) are co-clustered at postsynaptic membrane of excitatory synapses. In the glutamate receptors' group is included metabotropic receptors (mGluRs) and ionotropic receptors (iGluRs). The metabotropic receptors are responsible for mediate transmembrane signal transduction via trimeric G proteins while the ionotropic receptors harbor an intrinsic neurotransmitter-gated cation channel (Hollmann M and Heinemann S, 1994).

1.3.1. Ionotropic Receptors

The Ionotropic Glutamate Receptors (iGluR) are ligand-gated cation channels that couple the binding of agonists to a soluble ligand-binding core to the opening and desensitization of a transmembrane ion channel and are fundamental for fast synaptic transmissions between nerve cells (Madden DR, 2002).

Three classes of ionotropic glutamate receptors mediate these fast excitatory transmissions and are divided according to their sensibility to diverse agonists. The ionotropic receptors are α-amino-3- hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA), (2S- 3S,4S)-3-(carboxymethyl)-4-prop-1-en-2-ylpyrrolidine-2-carboxylic acid (Kainate) and N-methyl-D-aspartate (NMDA) (Lodge D, 2009) (Collingridge GL et al., 2009).

Architecturally, iGlu receptors are normally tetrameric or pentameric assemblies of multiple subunits. Each subunit is related exclusively with a receptor class, and so, there is

no assembly between different ionotropic receptors (Rojas A and Dingledine R, 2013). Besides, each subunit has an extracellular N-terminal domain (ATD), not less than one extracellular ligand binding domain (LBD), a channel-forming transmembrane domain (TMD) and cytoplasmic C-terminal domain involved in signaling. (Wolter T et al., 2013).

1.3.1.1. *NMDA Receptors*

The N-methyl-D-aspartate receptor, also known as NMDA receptor is responsible for fundamental brain functions from excitatory neurotransmission to learning and memory mechanisms and so, NMDA is considered one of the most fundamental receptor in the brain.

NMDAR is constituted by seven subunits, GluN1, GluN2A, GluN2B, GluN2C, GluN2D, GluN3A and GluN3B. In this way, the subunits are able to form NR1/NR2/NR3 that has reduced calcium permeability. They can also form the complex NR1/NR3 and because glutamate binds to NR2 subunit, in this case, the NMDA receptor will not reply to glutamate, only to glycine or D-serine, both co-agonists, which bind to NR1 and NR3 subunits (Henson MA et al., 2010).

NMDARs are located typically within the postsynaptic density (PSD) bound into it at the synapses but they are also found in many other locations on neurons' surface and in numerous other types of cells both in the nervous system and in other structures of the body (Petralia RS et al., 2010) (Gladding CM and Raymond LA, 2011). Evidences have shown that NR1 subunit is well distributed all over the brain, while NR2A is present in synapses (neocortex and hippocampus) and NR2B is extrasynaptic and so, could have influence in synaptic plasticity as well as neuronal cell death (Hardingham EG et al., 2002) and long-term potentiation (Massey PV et al., 2004). On the other side, NR2C and NR2D are very expressed in the cerebellum (Nakanishi S, 1992) and NR3 has been proved to be present in the cortex. A deficient regulation of this NR3 during development is related to schizophrenia (Das S et al., 1998).

NMDA receptor's role in learning and memory is related to the blocking by magnesium ions in a voltage-dependent way. When the membrane is resting, polarized, the Mg^{2+} ions are blocking the NMDAR's but when these receptors receive a synaptic input, the neuron becomes depolarized, the Mg^{2+} is removed and so, there is an influx of Na^{+} and Ca^{2+} ions and efflux of K^{+} ions. This Ca^{2+} influx mechanism through NMDA receptor

allows long-term modifications in synaptic strength, synaptic structure and connectivity (Zito K and Scheuss V, 2009). In this way, is possible to affirm that NMDAR's act both on presynaptic side because they are required to glutamate release and on postsynaptic side becoming depolarized and allowing Ca2+ influx.

1.3.1.2. *AMPA Receptors*

The α-amino-3- hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors, together with NMDA receptors, are involved in the major part of fast excitatory neurotransmission in the central nervous system (CNS) and have fundamental roles in all aspects of brain function, such as learning, memory and cognition *(*Henley JM and Wilkinson KA, 2013).

Long-Term Depression (LTD) and Long-Term Potential (LTP) studies are initiated by activation of post-synaptic NMDA receptors and expressed by modifications in the number as well as in the composition and/or properties of postsynaptic AMPA receptors, causing alterations in synaptic plasticity (Morris RGM et al., 1986).

The AMPA receptors are constituted by four subunits, GluA1, GluA2, GluA3 and GluA4. All the four subunits share a mutual membrane topology with each other and with NMDA and Kainate receptors, meaning, extracellular N-terminus and intracellular C-terminus, although this tail is able to interact with a numerous variety of cytoplasmic proteins. AMPARs accumulate in the endoplasmic reticulum (ER) primarily as dimers and then, combined dimers with dimers to form tetramers. In adult rat hippocampal neurons, AMPARs include arrangements mostly of GluA1/2 and GluA2/3 subunits while synaptic AMPARs comprise combinations of GluA1 and GluA2 (Lu W et al., 2009).

Studies have classified GluA1 as a fundamental AMPAR subunit related to adaptation processes, because this molecule could be involved in the initial steps that could eventually lead to compulsive drug use and a morphine state-dependency. Impairment in this subunit leads to damages in stimulus reward learning by demonstrating an abnormal second-order answering (Aitta-aho T et al., 2012).

GluA2 subunit has a RNA editing site able to substitute the Q607 glutamine residue to an arginine residue, Q/R editing and so, the major part of GluA2 is modified in adult neurons. When this subunit changes the glutamine residue to an arginine residue it

becomes able to act as an ER retention motif and reduces GluA2-containing AMPARs that are calcium impermeable. On the other side, GluA1 does not have this residue change capability and so it is calcium permeable and does not remain in the ER, being quickly exported from this structure and transferred to the plasma membrane (Henley JM and Wilkinson KA 2013) (Sommer B et al., 1991) (Greger IH et al., 2007) (Wright A and Vissel B, 2012).

Both GluA3 and GluA4 have fundamental functions in synaptic AMPAR currents in thalamic neurons; once studies have demonstrated that deletion in one of these two subunits abolish synaptic AMPAR responses in the thalamic neurons indicating that GluA3 and GluA4 represent a big part of these neurons' configuration. GluA4 is expressed majorly during early development and present low levels in the adult brain (Wang H et al., 2011).

1.3.1.3. *Kainate Receptors*

The (2S- 3S,4S)-3-(carboxymethyl)-4-prop-1-en-2-ylpyrrolidine-2-carboxylic acid receptors, mostly known as Kainate Receptors (KARs) is one of the three ionotropic glutamate receptors subgroups for the excitatory transmitter L-Glutamate. This group has GluK1, GluK2, GluK3, GluK4 and GluK5 as Kainate receptors' subunits (Wolter T et al., 2013). The GluK1, GluK2 and GluK3 have low glutamate affinity and are skilled of originating functional homomeric channels. On the other hand, GluK4 and GluK5 have high glutamate affinity but need to associate with one or more GluK1, GluK2 and GluK3 to assembly functional channels (Huettner JE, 2003)

This type of receptors is the least understood and all the physiological and synaptic transmission properties known have been discovered only recently. They have their own category because the selective depolarization of isolated dorsal root fibers by kainate was distinct from the binding sites activated by NMDA and AMPA (Huettner JE, 2003).

Kainate receptors have been involved in functions related to synaptic transmission and plasticity and are present in the most various locations throughout the nervous system, such as, hippocampus, hypothalamus, cerebellum, amygdala, striatum, among others. (Rojas A and Dingledine R, 2013)

1.3.2. Metabotropic Receptors

Metabotropic Glutamate Receptors (mGluRs) cluster at the outer edges of the PSD and are present in half of the hippocampal synapses (Sorra KE and Harris KM, 2000). They are responsible for neuronal excitability regulation in the CNS through modulation of numerous types of ion channels such as voltage-dependent potassium channels, voltage-dependent calcium channels, non-selective cation channels and ligand-gated ion channels (Wheal H and Thomson A, 1992) (Saugstad JA et al., 1996).

First, it was thought that glutamate used its neurotransmitter actions entirely via ionotropic glutamate receptors (iGluR) such as NMDA (Nmethyl-d-aspartate), AMPA (α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid) and Kainate receptors but studies in the late 1980's had demonstrated that glutamate was able to stimulate also receptors mediated by a G-protein and they are named G-protein coupled receptor (GPCR) (Conn PJ and Pin JP, 1997)

All the G-protein coupled receptors (GPCR) contain seven membrane-spanning regions with their N-terminal section on the exoplasmic region and their C-terminal region on the cytosolic part of the plasma membrane (Lodish H et al., 2004). The signal-transducing G protein is constituted by three different polypeptide chains, called α, β and γ. The G_s α subunit, α_s, is a GTPase switch protein that alters between an active (on) state bound to GTP and an inactive (off) state bound to GDP while the subunits β and γ remain bound together and are typically referred to as the $G_{\beta\gamma}$ subunit.

In its inactivated form, G-protein appears as a trimer with GDP bound to α_s but when this protein binds to a ligand-activated receptor, α_s transforms the GDP into GTP, its activated form. Following this activation, α_s dissociates from the βγ complex, being alone and so, is possible for it to bind to an adenylyl cyclase molecule leading to its activation and consequently, cyclic AMP production (Lodish H et al., 2004) (Azevedo C, 1999).

Once the mGluRs are associated with heterotrimeric G protein, this molecule can open the ion channels directly or using second messengers in the cytoplasm such as intracellular Ca^{2+} or cAMP. The Ca^{2+} acts directly on the ion channels or through a protein kinase while the cyclic-AMP depends on the adenyl cyclase activation and can also regulates the ion channels' opening directly or mediated by protein kinase A which

phosphorylate the ion channels, permeabilizing them (Azevedo, C 1999). In this way, mGluRs are able to modify neuronal and glial excitability.

The mGluR family contains eight receptors divided in three groups according to amino acid sequence homology, agonist profile and signal transduction pathway (Nakanishi S, 1994) (Saugstad JA et al., 1996).

Table I - Classification and Characteristics of mGlu Receptors (Yasuhara A and Chaki S, 2010)

	Group I		Group II		Group III			
	mGlu1	mGlu5	mGlu2	mGlu3	mGlu4	mGlu6	mGlu7	mGlu8
Structure	Class C GPCR		Class C GPCR		Class C GPCR			
Signaling	Gq/11 Activation of phospholipase C		Gi/o Inhibition of adenylyl cyclase		Gi/o Inhibition of adenylyl cyclase			
Agonists	DHPG	CHPG	LY404039, LY354740, MGS0028, MGS0008	LY404039, LY354740, MGS0028, MGS0008	L-AP4, PHCCC	HomoAMPA	L-AP4, AMN082	L-AP4, RS-PPG
Positive allosteric modulators		DFB, CDPPB, CPPHA, ADX47273	LY487379, BINA				AMN082	
Antagonists	JNJ16567083, LY367385	MPEP, MTEP	LY341495, MGS0039	LY341495, MGS0039	CPPG	CPPG	CPPG	CPPG

The Group I receptors includes mGlu1 and mGlu5 and couple preferentially to Gq/11-proteins, leading to activation of phospholipase C and consequent mobilization of intracellular Ca^{2+}. This enzyme is able to hydrolyze phosphatidylinositol 4-5 biphosphate originating 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 is an intracellular messenger that interacts with its correspondent in ER (endoplasmic reticulum) receptor leading to Ca^{2+} liberation and increasing its concentration in the intracellular level. DAG is located in the membrane and activates protein kinase C (PKC), a Serine/Threonine kinase responsible for a multiplicity of cell functions from enzyme to biological functions (Newton CA, 1995).

Group I metabotropic receptors is able to activate a numerous signaling pathways, such as MAP/ERK signaling pathway. This via is constituted by MAP (Mitogen activated protein) or, also known as, ERK (Extracellular signal regulated kinase) that are Serine/Threonine kinases, which activation implies a threonine residue and a tyrosine residue phosphorylation. When the MAP kinase is activated, it moves from the cytoplasm to the nucleus where is able to phosphorylate various types of molecules (Sweatt JD, 2001). Besides, the receptors belonging to Group I are selectively activated by 3,5-dihydroxyphenylglycine (DHPG).

The Group II receptors include mGlu2 and mGlu3, and Group III receptors embrace mGlu4, mGlu6, mGlu7 and mGlu8. Both group II and group III, in contrast to what happens to group I, are negatively coupled to adenylyl cyclase and so, when activated they inhibit forskolin-stimulated cyclic AMP formation. The receptors belonging to group II are specifically activated by (1*S*,2*S*,5*R*,6*S*)-(1)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid (LY354740) or (2)-2-oxa-4- aminobicyclo[3.1.0]hexane-4,6-dicarboxylic acid (LY379268), and Group III receptors are stimulated by L-(1)-2-amino-4-phosphonobutyric acid (L-AP4). (Yasuhara A and Chakib S, 2010)

As far as function is concerned while Group I receptors are located mostly in the postsynaptic membrane and responsible for increasing NMDA receptor activity leading to a bigger risk of excitotoxicity, Group II and Group III are located mainly in presynaptic membrane and perform doing the opposite action, that means, decreasing NMDA receptor activity and so, diminishing excitotoxicity probability. In this way, is possible to affirm that they act in order to establish the homeostasis. Specifically, mGlu3 receptors are highly expressed in glial cells (Mineff E and Valtschanoff J, 1999) and even though the function of these receptors is not very well understood, because of the large contribution given by glia cells to the glutamate uptake and synthesis, this receptor activation probably result in important functional effects (Cartmell J and Schoepp DD, 2000). Finally, mGluR6 is expressed exclusively in the retina.

A large number of ligands such as agonists, antagonists, positive/negative allosteric modulators have been used to define physiological and pharmacological significance and possible therapeutic application in some of the metabotropic glutamate receptors such as, mGlu2, mGlu3 and mGlu5 (Yasuhara A and Chakib S, 2010). CDPPB, 3-cyano-*N*-(1,3-diphenyl-1*H*-pyrazol- 5-yl)-benzamide, is the most studied positive allosteric modulator potentiating mGluR5 and through this molecule' studies is possible to conclude and define pharmacological answers for diseases as Autism Disorder Spectrum. (Stauffer SR, 2011).

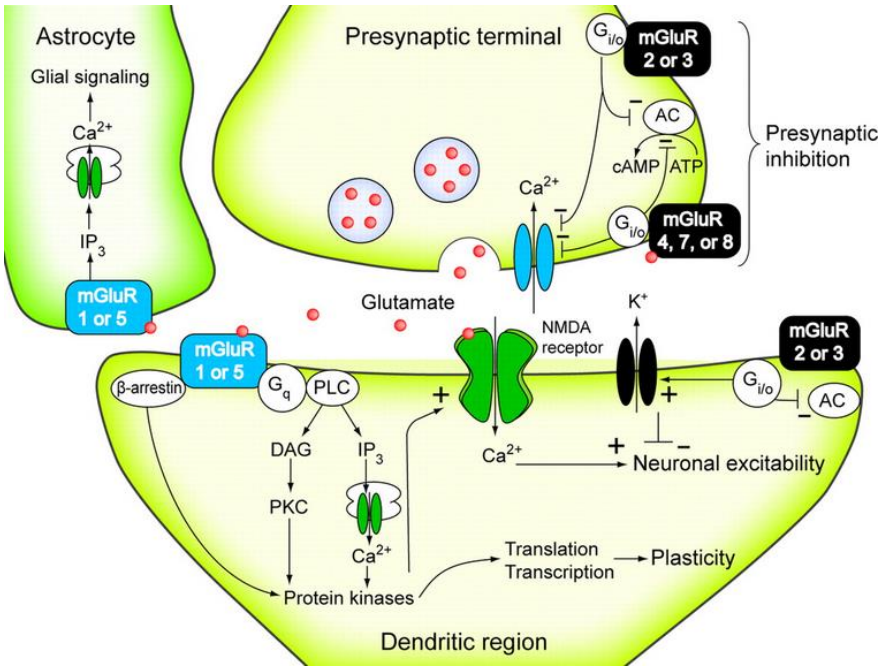


Figure 2 - Metabotropic Glutamate Receptors (Benarroch EE, 2008)

1.4. Scaffold proteins

The scaffold proteins are fundamental to a perfect and healthy neuronal function and are located in a prominent thickening region at the cytoplasmic surface of the postsynaptic density (PSD). The PSD machinery is constituted by receptors with associated signaling and scaffolding proteins that organize signal transduction pathways near the postsynaptic membrane (Walikonis RS et al., 2000).

Together with scaffolding proteins, PSD is able to control postsynaptic glutamate receptors clustering and functions, clustering cell-adhesion molecules and regulating adhesion between presynaptic and postsynaptic membranes (Siekevitz P, 1985), recruiting and controlling signaling proteins in response to receptor activation and anchoring all the PSD components to the microfilament cytoskeleton of the spine (Chua JJE et al., 2010).

Initially, the first proteins found in the PSD were identified by biochemical characterization of the most abundant proteins, followed by SDS-PAGE of purified PSDs, finding proteins such as CaMKII and PSD-95 protein (Kennedy MB et al., 1983) (Cho KO et al., 1992).

After this first procedure, different proteomic studies had recognized more than 400 proteins present in the postsynaptic density through MS fingerprinting.

Over the past twenty years, has been identified a superfamily of proteins called Membrane-associated guanylate kinase (MAGUK) classified as ubiquitous scaffolding molecules concentrated at sites of cell-cell contact such as synapses. They are constituted by several protein-protein domains, which improve MAGUK capability to recruit large protein assemblies. The three protein-protein domains are PDZ (PSD-95/Discs large (zonula occludens (ZO)-1)-domains, an SH3-(Src homology-3)-domain and a guanylate kinase (GUK)-domain. Some members are constituted by one or two L27 (Lin-2/Lin-7)-domain belonging in their N-terminal. (Funke L et al., 2005). All the proteins belonging to this family have exactly this same structure (except MAGI that will be mentioned). Curiously, studies have discovered that the association between SH3 and GUK domain, originating SH3-GUK integral structural unit, result in different functions from those SH3 and GUK domains that are isolated (Zhu J et al., 2011).

MAGUK family proteins, interact with membrane proteins through their PDZ domain and this superfamily is fundamental for the structural integrity of the PSD (Chen X et al., 2011).

In the MAGUK's family is included 10 subfamilies classified phylogenetically according to genomic sequence of the central PDZ-SH3-GUK region and they are: CASK, membrane protein palmitoylated 1 (MPP1), MPP2-7, MPP5, zona occludens (ZO), caspase recruitment domain containing MAGUK protein (CARMA), DLG, discs large 5 (DLG5), calcium channel b subunit (CACNB), and MAGUK with an inverted repeat (MAGI) (Mendoza A et al., 2010).

The PDZ domain is a modular protein-protein interaction domain and like many other protein-protein interaction domains, PDZ is relatively small with a length about 100 amino acids. The PDZ fold is constituted by six β-strands (βA, βB, βC, βD, βE, βF) and two α-helices into a compact globular fold and by a N- and a C-terminal close to one another in the folded structure. In this way, the domains are highly modular and are able to integrate in proteins without relevant structural changes. The ligation to its target proteins is done through the C-terminal peptide motif of the ligand proteins (Oliva C et al., 2012) (Harris BZ and Lim WA, 2001) (Funke L et al., 2005).

The best-known MAGUK is PSD-95 and is highly abundant in the postsynaptic density of excitatory neurons. This protein has three PDZ domains and can bind to a large variety of proteins found in the postsynaptic density. The first two PSD-95 PDZ domains are able to bind to the NMDA glutamate receptor subunit, NR2B C-terminal suggesting a role in receptor clustering at synapse (Niethammer et al., 1996).

The SH3 domain was one of the first modular protein interaction domains identified. From the beginning was characterized as a large homology sequence with about 60 amino acids and present in many proteins. The SH3 domain usually binds proline-rich sequences (PXXP) in target proteins and identifies PXXP as an essential conserved binding motif. Although SH3 domain is able to bind proline-rich sequences, binding partners for MAGUK's SH3 domain have not been found. Studies indicate that GUK and SH3 domain establish an interaction that blocks PXXP motif and so, it is not recognize and consequently, SH3 domain is not capable of interacting with these proline-rich sequences in other proteins (McGee AW and Bredt DS, 1999) (McGee AW et al., 2001).

Protein-protein interactions are facilitated by proteins with a SH2 or a PTB domain because they are able to recognize phosphotyrosine and consequently, leading to tyrosine phosphorylation. However, in opposite, the tyrosine phosphorylation of SH3 domain blocks or at least, decreases the protein-protein interactions. This can lead to a switch in cell performance as happens in chronic myeloid leukemia cells, phosphorylation of SH3 domain of c-Abl gene leads to transformation potential (Tatarova Z et al., 2012) (Mayer BJ, 2001) (Oliva C et al., 2012).

GK domain has originated from the enzyme guanylate kinase that is a member of nucleoside monophosphate (NMP) kinases catalyzing phosphoryl transfer from ATP to GMP and GK domain is related to tissue development, cell polarity control, synaptic formation and plasticity (Zhu J et al., 2012) (Li Y et al., 1996). The GK domain is catalytically inactive; however, it has always a preceding SH3 domain or tracked closely by a WW (two conserved Trp residues) motif (Verpelli C et al., 2012). Each guanylate kinase is constituted by three domains known as Core, LID and GMP binding domains. Although the molecular bases leading the mechanisms underlying the bindings of MAGUK domain to its targets are not very clear, the functional roles are well defined. GUK domain is functionally fundamental once, for instance, a truncation mutation of

CASK originates the loss of its entire GK domain, has been associated to mental retardation a human patient with microcephaly (Najm J et al., 2008).

1.4.2. SHANK Protein

The SHANK proteins are fundamental to a well-organized postsynaptic density. These proteins are believed to work as the main coordinators of the PSD, once they are able to form complexes with postsynaptic receptors, signaling molecules and cytoskeletal proteins present in dendritic spines and PSDs (Durand CM, 2007) (Naisbitt S, 1999) (Figure 3).

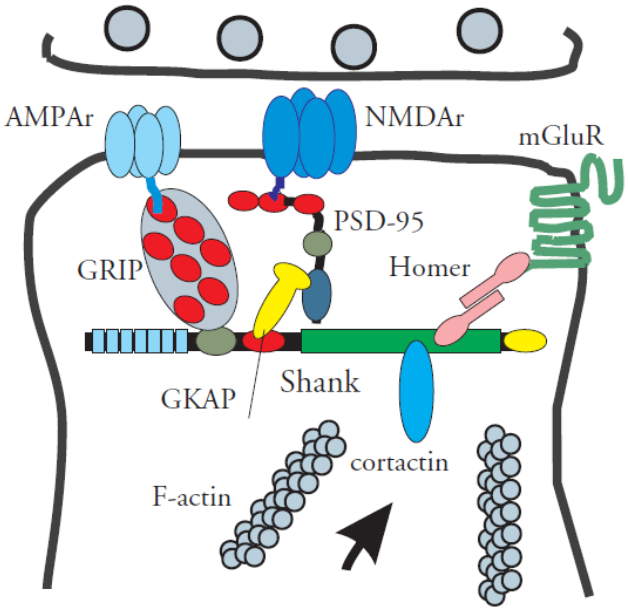


Figure 3 - A postsynaptic protein assembly organized by Shank protein. (Sheng M and Kim E, 2000)

The SHANK protein family is constituted by SHANK1 (also known as Synamon or SSTRIP), ProSAP1/SHANK2 (also named CortBP1) and ProSAP2/SHANK3. The name ProSAP, **pro**line-rich **s**ynapse-**a**ssociated **p**rotein has origin in the common proline-rich clusters present in all the three Shank proteins (Boeckers TM, 1999a) (Boeckers TM, 1999b) and the Shank denomination is related to SH3 domain and multiple ankyrin repeats (Naisbitt S, 1999).

ProSAP/Shanks are relatively large proteins: the long splice variants are around 2000 residues in length and Molecular Mass is more than 180kDa. They are constituted by a N-terminal, followed by ankyrin repeat domain, SH3 (Src homology 3) domain, a PDZ

domain, a proline-rich region, a SAM (sterile α-motif) domain in the C-terminal at the end of the protein.

The SH3, PDZ and SAM domain are highly conserved in all three proteins and among them, they share 63% to 78% homology. In spite of this, is not very clear the Shank2 constitution because is not known if this subfamily possesses ankyrin repeats, since as far as this gene is concerned the splice variants of this gene do not have the N-terminal region connected to SH3 and PDZ domains (Lim S et al., 1999).

All the three family members are highly expressed in the hippocampus and cortex but each one of them has a particular expression. Shank1 is only expressed in the brain and specifically in the cerebellum, primarily appears in Purkinje cells (Yao I et al., 1999). ProSAP1/Shank2 is expressed in cerebellum granular cell layers and likewise in non-neuronal tissue such as pituitary gland, lung, kidney and testis and (Dobrinskikh, E et al., 2010) and ProSAP2/Shank3 seems to be expressed in nearly all observed tissues (Lim S et al., 1999).

Although all the members of Shank family are much more expressed in the cortical and hippocampal areas, they are present also in the glial cells, in the postsynaptic specialization of the retina and the synapse surrounding area.

This superfamily forms a large multimeric scaffold proteins complex at the base of the post-synaptic density (PSD) and co-assembles group-I mGluR1 with NMDA receptors through dimeric adaptor proteins, Homer family, here Homer c-c stands for Homer containing a coiled-coil domain. The Homer family is considered a part of a mechanism of homeostatic plasticity that depresses the neuronal responsiveness when input activity is too high and the GKAP (guanylate kinase-associated protein), which is one of the major components of the PSD, functions as a scaffold protein for various ion channels and associated signaling molecules. GKAP binds to the PSD-95 NMDA receptor complex forming the GKAP-PSD95 complex (Bertaso F, 2010).

Shank and GKAP combine to form aggresomes that are degraded by proteasomes when PSD-95 is lacking. On the other hand, Shank association to the GKAP-PSD95 complex seems to be needed to ensure correct Shank1 targeting to synapses in developing and mature neurons (Romorini, S. et al., 2004). Besides, Shank1 relies also on the PDZ domain to assure the transport in the synapses (Sala C et al., 2001). On the other hand, the

synapse targeting for ProSAP1/Shank2 occurs in the very early stages of synaptogenesis even before the PSD95-GKAP complex formation. Finally, ProSAP2/Shank3 has an independent synaptic targeting from PDZ domain.

Even though studies have improved the knowledge about the Shank superfamily, there is still much more to study and to know about the mechanisms and all the proteins interactions. As it is described previously, Shank proteins are constituted by a large number of domains and is fundamental comprehending each one so, in the end becomes easier the understanding as a whole (Figure 4).

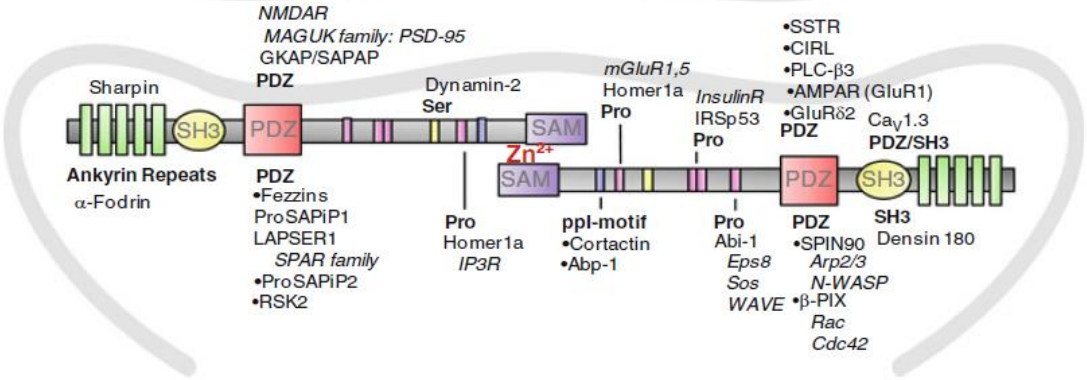


Figure 4 - ProSAP/Shank protein composition at the PSD (Verpelli C et al., 2012)

- Ankyrin repeat domain

The ankyrin repeat is a 33-residue sequence motif discovered in the yeast cell cycle regulator Swi6/Cdc10 and the Drosophila signaling protein Notch. The name ankyrin has origin in the cytoskeleton ankyrin protein, which has 24 copies of this repeat domain and is present in super kingdoms such as, *bacteria*, *archaea*, in a variety of viral genome but mostly, in *eukarya*. The fact that this domain is present in so many different types of proteins, can suggest its fundamental function for many cellular processes such as cell fate determination, endocytosis, transcription regulation, cell cycle control or any other help the cell might need such as recruit a substrate to a catalytic domain (Mosavi LK et al., 2004). These types of mechanisms are essential for signal diversity and integration.

The α-Fodrin, also known as brain α-spectrin, is a multidomain protein constituted by 22 spectrin repeats, one SH3 domain and two EF-hand calcium binding motifs and is a very important part of the PSD (Carlin et al., 1983). This protein is an actin-regulating protein and connecting directly to F-actin of dendritic spines through its spectrin repeat 21 is able to interact with N-terminal ankyrin repeat of Shank1/SSTRIP and ProSAP2/Shank3 (Bockers et al., 2001). This protein is processed by calpain proteases and so, in a calmodulin-calcium manner, acts in response to calcium level elevation, synaptic activity or both (Vanderklish et al., 2000). Consequently, any changes in calcium levels will interfere to the interaction between ProSAP/Shank with α-fodrin, leading to alterations in the postsynaptic density (Boeckers, 2001).

Besides, Shank also interacts through ankyrin domain with Sharpin (Shank-associated RH domain interactor), also known as SILP1. Studies have demonstrated a communication between this protein C-terminal and the ankyrin repeats of Shank1 and so, probably thanks to this interaction, Shank is capable of forming protein filaments (Naisbitt S et al., 1999).

- SH3 (Src homology 3) domain

The most known SH3 domain protein that interacts with Shank family is densin-180. This interaction antagonizes dendritic branching to improve the growth of functional spines and synapses.

Densin-180 is very concentrated at synapses and co-localizes with others proteins located in the postsynaptic density in the hippocampal neurons. This protein is included in the scaffold proteins group and belongs to the LRR glycoproteins family together with trk, trkB, oligodendrocytes myelin glycoprotein and NLRR-3 (Ohtakara K et al., 2002).

Studies have demonstrated that Densin-180 seems to bind directly to CAMKII, α-actinin, α-catenin and shank (Walikonis RS et al., 2001). Although is not known what Densin-180 functions are, is known when this protein is deleted the α-actinin level decrease in the brain, consequently reducing metabotropic glutamate receptor mGluR5 and DISC1 in the PSD fraction and a misregulation of a third, CAMKII. If mGluR and NMDA are modified, there are alterations in the CAMKII regulation and changes in spine morphology. Therefore, is possible to affirm, that deletion in Densin-180 leads to disturbs

in the postsynaptic signaling causing alterations in the brain with mental illness phenotype such as, autism (Carlisle HJ et al., 2011).

- PDZ domain

The Shank family PDZ domains are high homologous among Shank1, Shank2 and Shank3 with about 80% homology. However, when Shank's PDZ domain is compared to other proteins' PDZ domains, the homology is much lower (Lim et al., 1999).

The best known interaction through the PDZ domain of Shank3 is with GKAP (guanylate kinase associated-protein) (Boeckers et al., 1999b) (Naisbitt et al., 1999). This protein includes a group of PSD proteins that bind directly to postsynaptic scaffold proteins, such as PSD-95, S-SCAM and nArgBP2. The GKAP C-terminal (QTRL) is common to all four GKAP family members (GKAP/SAPAP1, GKAP/SAPAP2, GKAP/SAPAP3, GKAP/SAPAP4) and so, probably they are all capable of interact with Shank. However, some splice variants of GKAP, for instance, GKAP1b, has a different C-terminal and consequently, when this subunit is overexpressed inhibits the synaptic localization of endogenous Shank. In this way, is possible to affirm that it is fundamental for the recruitment of Shank to postsynaptic sites the correct interaction between C-terminal PDZ domain of GKAP and Shank protein. (Sheng M and Kim E, 2000).

The consensus C-terminal sequence (X-T/S-X-L) is recognized by Shank's PDZ domain, in which leucine is the last amino acid residue (Naisbitt et al., 1999). Through two-hybrid assay, mGluRs terminating with a different cytoplasmic tails, sequence SSSL (mGluR1α) or SSTL (mGluR5) interact with Shank PDZ domain directly but there is not yet a meaning for this interaction when practiced *in vivo* assays (Tu et al., 1999).

The somatostatin receptor type 2 (SSTR2) is another G-protein-coupled receptor, which tail end with QTSI sequence. SSTR2 also interacts with PDZ domain of Shank1 and Shank2, but, again, is not possible to confirm this interaction *in vivo*. (Zitzer et al., 1999a,b).

One PDZ domain can only interact with one of these partners and is not yet very well understood if the interaction is just with a specific group of cells, specific subdomains in the cell or if it is a competition where the binding partners in the PSD compete for

ProSAP/Shank's PDZ domain according to binding affinity or abundance (Boeckers TM et al., 2002).

- Proline-rich region

The Proline-rich region is placed between the PDZ and SAM domains of Shank and has more than 1000 residues, mostly proline and serine residues. This region acts usually as binding sites for SH3, EVH1 and WW domains and there are two proteins that bind to the proline-rich region, Homer and Cortactin, both of these interactions were discovered through two-hybrid screens using parts of these proteins as baits **(**Du Y et al., 1998) **(**Tu JC et al., 1998).

Homer is a scaffolding molecule that links mGluR1 with inositol 1,4,5-triphosphate (IP3) receptors. The EVH1 Homer domain interacts directly with one of the proline-rich clusters of ProSAP/Shank proteins. **(**Tu JC et al., 1998). The two EVH1 domains present in Homer protein are able to connect mGluRs to the consensus sequence PPXXF found in all ProSAP/Shank family members, allowing a cross-link in the postsynaptic density between Homer and SAP90/PSD-95. The PPXXF sequence is also found in mGluR1 and IP3 receptor. While EVH1 Homer domain binds, directly, to Shank1 and Shank3, the binding to Shank2 remains to show if it is directly (Xiao B et al., 1998). Homer links physically and functionally the phospholipase-C-coupled mGluR with IP3, its downstream effector. The receptor is stimulated and consequently, the intracellular calcium release is more efficient, indicating that perhaps Homer could have contribution in accumulating signaling complexes implicated in excitation-calcium connection. Since Shank binds directly to Homer and interacts with G-protein-coupled receptors, for instance, mGluR5, it is possible to think that maybe Shank helps Homer in its function **(**Tu JC et al., 1999).

The Shank family proteins also interact with cortactin. In fact, cortactin-binding protein (Du Y et al., 1998) seems to be a splice variant of Shank2, which lacks the ankyrin repeat and SH3 domains (Lim S et al., 1999). This protein was identified as a substrate of Src tyrosine kinase and is a F-actin binding-protein enriched in cell-matrix contact sites, lamellipodia and in growth cones of neurons (Du Y et al., 1998). Cortactin plays a fundamental role in the reorganization of cytoskeletal structures when it receives extracellular and intracellular stimuli (Wu H and Parsons JT, 1993). The SH3 domain of

cortactin interacts exactly with a SH3 binding motif known as ppl (PPΨPXKP) domain (Du Y et al., 1998) present in ProSAP1/Shank2 and ProSAP2/Shank3 but apparently not present in Shank1. The interaction between the Shank family and cortactin is fundamental to the morphological plasticity contributing to dynamic changes of spine and PSDs (Boeckers TM et al., 2002).

Mapping experiments with the yeast two-hybrid system, another protein seems to have a direct interaction with ProSAP/Shank proteins. This protein is called dynamin, more exactly, the isoform dynamin-2, (Okamoto PM et al., 2001) which C-terminal proline-rich domain is able to interact with a small serine-rich sequence conserved in all ProSAP/Shank family members. This interaction could be related to the membrane turnover regulation and glutamate receptor recycling because this protein is located underneath the postsynaptic membrane (Boeckers TM et al., 2002).

- SAM (sterile alpha motif) domain

The sterile alpha motif domain, also known as SAM domain is present in a diverse group of organisms such as fungi, protozoa and animals thus, are found in a variety of proteins, including Eph receptors.

The Shank C-terminal is constituted by a SAM domain, which is able to bind to other SAM domains in a homomeric and heteromeric manner (Naisbitt S et al., 1999), leading to the conclusion that ProSAP/Shank proteins can connect in a tail-to-tail way through its own SAM domains (Boeckers TM et al., 2002).

Shank family proteins are capable of oligomerization allowing the cross-linking of many groups of proteins complexes such as PSD-95 and Homer-based complex at postsynaptic sites. (Sheng M and Kim E (2000). This oligomerization ability of Shank family is fundamental because helps the SAM domains of ProSAP1/Shank2 and ProSAP2/Shank3 in the postsynaptic targeting and facilitates the interaction of large ProSAP/Shank pieces through zinc ions consequently originating the PSP (postsynaptic platforms) (Gundelfinger, ED, Kreutz MR et al., 2006).

II. Autism Spectrum Disorders

As described previously, synaptic homeostasis is a fundamental prerequisite for a healthy brain. A single neuron usually has hundreds and thousands of excitatory and inhibitory synapses at its dendrites and cell bodies. The neuron capability of firing an action potential rests on the total input of all these synapses (Zhai RG and Bellen HJ, 2004). Consequently, any kind of alteration in the synaptic balance, affecting brain morphology and function can lead to molecular pathogenesis of so-called synaptopathies such as autism, schizophrenia (SCZ) and Alzheimer's disease (AD).

Intellectual disability is one of the most common neurodevelopmental disorders and it is diagnosed by an intelligence ratio of 70 or below. Damage in communication and social interaction skills and limited and repetitive patterns of activities and interests are some of the characteristics of autism spectrum disorders (ASD) (Kelleher III RJ et al., 2012) (Verpelli C and Sala C, 2011). As the occurrence of autism endures to raise, with one out of every 110 children receiving an ASD diagnosis within the first 3 years of life, period with a big synaptogenesis activity, information on behavioral treatment continues to be in high demand (Mayville EA and Mulick JA Eds, 2011) (Moessner R et al., 2007).

Although the genes responsible for Autism remain mostly unknown, through familial and twin studies it has been proved that ASD is majorly related to genetic neuropsychiatric disorders (Durand CM, 2007). Autism is characterized mostly by impairments in reciprocal interaction and communication restriction and stereotyped patterns of interests and behaviors (Uchino S and Waga C, 2013). Some of these phenotypical characteristics including developmental delay, childhood hypotonia, absence of speech and some minor dysmorphisms such as dolichocephaly, dysplastic ears and epicanthal folds (Punzo C et al., 2009) have been described for further than a decade ago in more than 100 cases.

Mutations that have been associated with autism syndrome are mostly located in two neuroligin synaptic genes (*NLGN3* and *NLGN4*) on the X chromosome, *RPL10* at Xq28 and *SHANK3*/*ProSAP2* on chromosome 22q13. These neuroligins are adhesion molecules responsible for synapses formation and function because these molecules act in the direct connection between synaptic proteins, cognition and autism. Autism has been related to other mutation families but these represent remarkably rare events, typically of

unconfirmed pathogenicity or they have not yet been validated by other studies (Moessner R et al., 2007).

Mutations associated with a documented cause represent currently about 10% of cases and are related with fragile X syndrome, tuberous sclerosis and cytogenetically detectable chromosome abnormalities. In what concerns cytogenetic abnormalities the most frequent one is maternally derived duplication of chromosome 15q11-q13. However, a various number of other chromosomal regions are described with a higher frequency of events observed in syndromic forms of ASD (Moessner R et al., 2007). Evidences for autism loci were found on 20 different chromosomes (Sebat J. et al., 2007) but the most common being deletions and duplications on chromosomes 7q, 15q and 22q. In this last chromosome, the chromosome 22, were detected specifically rare structural changes in a long arm of this chromosome (Uchino S and Waga C, 2013).

2.1 Phelan-McDermid Syndrome

The most important mutation in the chromosome 22 is a telomeric deletion of variable length of the chromosomal region 22q13. The loss of 22q13.3 could result from simple deletion, translocation, ring chromosome formation and rare structural changes in a long arm of chromosome 22 (Uchino S and Waga C, 2013).

Watt et al., initially described this pathology in 1985. In 1992, Katy Phelan and Heather McDermid elaborated the clinical picture of the 22q13.3 deletion so this syndrome has also been called Phelan-McDermid syndrome (Verhoeven W et al., 2012) and in 2001, Bonaglia et al. confirmed a direct genetic relation between a defect of ProSAP2/Shank3 gene and this syndrome (Boeckers TM et al., 2002).

Among the genes nominated as mutated in autism, *SH3* and *SHANK3/ProSAP2* gene, which is located inside the deleted region, are the ones with more associations to the disease (Verhoeven W et al., 2012) because through genetic analysis was discovered a balanced translocation between chromosome 12 and 22 leading an interruption of the ProSAP2/Shank3 gene at exon 21. This mutation was registered in a child with all the features of the 22q13.3 deletion syndrome. Shank3 gene is mostly present in the cerebral cortex and cerebellum and encodes a multidomain scaffolding proteins, ProSAP2 or Shank3 in the postsynaptic density. This protein interacts with the cytoskeleton, attaches and clusters glutamate receptors in the exactly opposite site to the presynaptic

neurotransmitter release site (Denayer A et al., 2012) and so, the ProSAP2/Shank3 haploinsufficiency (Wilson HL et al., 2003) leads to an abnormal organization and function of excitatory synapses (Romorini S et al., 2004).

The Phelan-McDermid syndrome includes physical and behavioral abnormalities, such as absent to severely delayed speech in 99% of the cases, hypotonia in 97% of the cases, normal to accelerated growth in 95% of the cases and some less present like thin and flaky toenails in 78% of the cases and prominent or poorly formed ears in 65% of the cases. Inside the behavioral alterations are included chewing on toys or clothes, hair pulling, tongue pushing and teeth crushing (Manning MA et al., 2004).

The diagnosis of Phelan-McDermid syndrome is established throughout deletion/disruption findings of 22q13.3 using cytogenetic banding and fluorescent in situ hybridization (FISH) or bacterial artificial chromosome comparative genomic hybridization (CGH array) techniques. Using FISH technique, a 310kb probe (ARSA probe) mapped the 22q13.3 and a 80 kb subtelomeric probe (D22S176) which mapped the least 300 kb of chromosome 22. The probability of these two probes together find the 22q13.3 deletion is almost 100%, but as usually is a very expensive technique and is not a common practiced method in the clinics (Anderlid BM et al., 2002). An even newer and better technique to detect smaller deletions or accurately measure deletion size or breakpoints is oligo-array CGH that permits much better resolution of the chromosomal breakpoints performing genome-wide survery and molecular profiling of genome aberrations with a resolution of 75 kb (Sarasua SM et al., 2011) (Bonaglia MC et al., 2006).

Recently, in 2011 Sarasua et al., studied the relation between chromosome 22 size deletion and phenotype in 71 individuals from 0,4 to 40 years old with Phelan-McDermid syndrome using a total of 84 clinical phenotypic characteristics and Shank3 deletion size ranged from 0,22 to 9,22Mb. The results strongly indicated a direct relation between increased deletion size and physical features such as facial asymmetry, fleshly hands, and dysplastic toenails and neonatal features for instance hypotonia, feeding problems and developmental delays including speech delay, later age to crawl and to walk. On the other hand, Behavioral features were not related to deletion size (Sarasua SM et al., 2011).

Uchino S and Waga C, 2013 studied the SHANK3 gene in 134 autistic patients and alterations in this gene were found in about 10% of the ASD patients.

Almost 80% of the patients with Phelan-McDermid syndrome have a terminal or an interstitial deletion involving 22q13.3. However, there are other kinds of mutations in ProSAP2/Shank3 that lead to PMS. In 2001, Bonaglia et at described an apparently balanced chromosome translocation t(12;22)(q24.1;q13.3) without loss of genetic material in a male with Phelan-McDermid syndrome. The breakpoints were localized in the chromosome 22 within the exon21 of Shank3 and in the chromosome 12 within an intron of APPL2. This disruption of SHANK3 result from a *de novo* mutation (Bonaglia MC et al., 2001).

Later, in 2010, Phelan et al. identified a deletion which resulted from an unbalanced chromosome translocation, which is characterized by 22q13.3 deletion and partial trisomy of a second unrelated chromosomal segment. This type of mutation represents around 20% of all Phelan-McDermid syndrome mutation (Phelan MC et al., 2010). Misceo et al., in 2011 identified a very rare abnormality in a 20-year-old woman with physical and development characteristics of PMS where an intragenic deletion of SHANK3 was associated with a translocation between 22q13.3 and Xq21.33 leading to truncation of SHANK3 in the last two exons, 22 and 23 (Misceo D et al., 2011).

Trying to find the best treatment to cure autistic patients, studies have proven that intranasal treatment with insulin is a very promising way to achieve this goal. Lately, a restricted group of patients has improved the developmental delay with this application. Six children with 22q13 deletion syndrome have received intranasal insulin for more than 1 year and have been detected improvements in speech and communication, emotional state, cognitive capabilities, motor skills from 6 weeks of treatment until the end of the clinical trial (Schmidt H et al., 2009).

Previous studies have demonstrated that knock down of Shank3 in hippocampal and cortical mouse and rat cultures induce a reduction of the mGluR5 expression and the absence of DHPG that functions as a group I mGluR agonist induced Erk1/2 (extracellular signal-regulated kinase 1/2) and CREB phosphorylation that leads to the activation of their kinases activity. Besides, reduces the mGluR5-dependent synaptic plasticity and modulation of neuronal network activity (Verpelli C et al., 2011). In this way, a treatment

that has been proved to be effective as therapy to Shank3 reestablishment functions in mice neurons knock down for Shank3 is the 3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)-benzamide (CDPPB). This drug acts as a positive allosteric modulator potentiating mGluR5-dependent signaling and thus, is capable of restore synaptic physiology in neurons with Shank3 mutations.

The best way to study the SHANK3 mutations in Phelan-McDermid patients and others mental disorders, is using genetically modified mice. This method is based on animals, which had its genome altered through genetic engineering techniques making it possible to reproduce the same gene functions as an autistic patient. The approach consists in modifying embryonic stem cells with a DNA construct containing DNA sequences homologous to the target gene. All the embryonic stem cells that recombine with genomic DNA are chosen and injected into mice's blastocysts. Through this technique is possible to analyze all mutations related to a single gene (Bonaglia M et al., 2009) (Peça J et al., 2011).

Using Shank3 mice, we were able to characterize Shank3 protein mutations as well as phenotype abnormalities found in 22q13 Patients' deletion.

Animal models studies have been a great achievement but is fundamental to understand what happens in Human neurons with Shank3 protein mutations because as we know, although animal models are fundamental to the overall comprehension, they do not express exactly what is going on in Human neurons.

To analyze brain is not that simple. Every single part is fundamental to a healthy and complete function so, it is essential to discover a method that allows us to do brain studies without causing any injury.

Shinya Yamanaka in 2006 published an article that explained how to reprogram *in vitro* mouse embryonic and adult fibroblasts into induced pluripotent stem cells (iPS cells). This method was accomplished by retroviral transduction of four defined transcription factor Oct4, Sox2, c-Myc and Klf4 and the markers Nanog and Lin-28 among others help in the maintenance of pluripotency in both early embryos and ES cells (Takahashi K. and Yamanaka S., 2006).

Embryonic stem (ES) cells originated from inner cell mammalian blastocysts are capable of growing indefinitely while preserving pluripotency characteristics (Evans and Kaufman, 1981) being able to differentiate into all derivatives of the three primary germinative layers: ectoderm, endoderm and mesoderm while adult stem cells are multipotent and are able to differentiate into only a limited number of cell types. The fibroblasts are now induced pluripotent stem (iPS) cells.

This iPS cells have emerge a whole new Medicine Era because they have the potential to cure diseases or damages using derivatives of patient specific stem cells, in other words, now we have the opportunity to create *in vitro* model human for diseases which the etiology is unknown. All of this by producing functional cell types relevant for therapy such as neurons, cardiomyocytes, pancreatic β-cells, hematopoietic cells among others (Wernig M et al., 2008).

Specifically in this work, the iPS cells will be differentiated *in vitro* into Embryoid Bodies, after in Rosettes and consequently in Neuronal Stem Cells (NSC) to posteriorly become neurons (Figure 5).

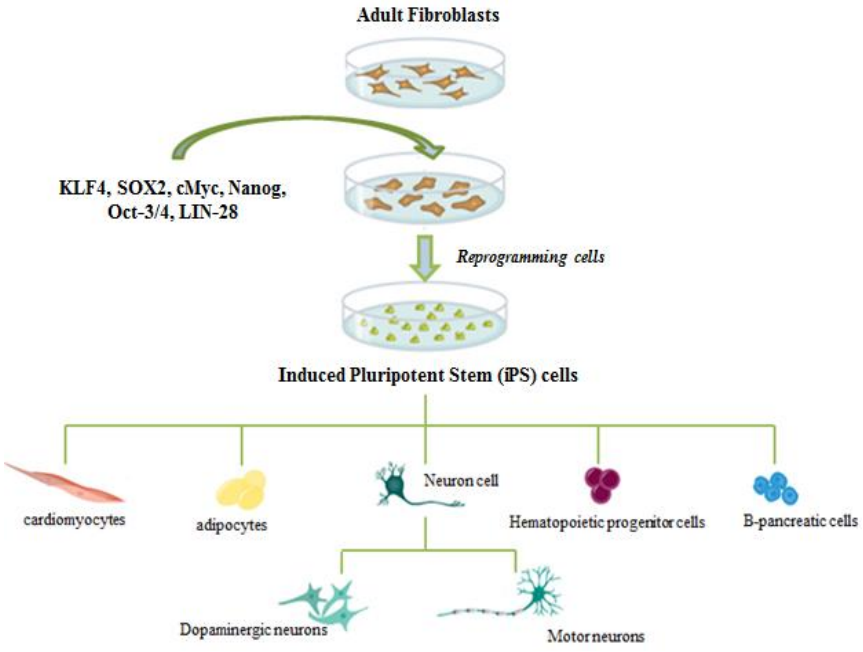


Figure 5- Reprogramming fibroblasts into neurons. The adult fibroblasts are reprogrammed into iPS cells by transcriptional factor. The iPScells can differentiate into a diverse group of cell types. In our specific case, these cells will be differentiated into neurons

In this particular work, we are going to analyze Shank3 functions throughout biochemical and morphological techniques in way to characterize Shank3 role in synaptic signaling since this protein haploinsufficiency is mostly related to Phelan-McDermid Syndrome with mental retardation and autism disorder phenotypes. To achieve this goal we are going to use patients and control fibroblasts and reprogramming them into iPS cells and posteriorly differentiation into neuronal stem cells becoming finally neurons.

Material and Methods

Cell cultures

Mammalian Fibroblasts (line COS- Monkey kidney fibroblast) cells

Cultivated in monolayer using IWAKI plates (10cm diameter) with 10mL DMEM (Dulbecco's Minimum Essential Medium) culture medium containing 2mM Glutammina, antibiotic (gentamicin 0.1%) and enriched with fetal bovine serum supplemented (FBS) in 10%. Each 5 days the cell cultures were treated with tripsin-EDTA solution. The cell suspensions were diluted in ratio 1:10, plated and incubated at 37°C in humidified atmosphere with 5% CO_2.

HEK (Human Embryonic Kidney) cells

Cultivated in monolayer in IWAKI plates (10cm diameter) in 10mL DMEM (GIBCO) containing 10% fetal bovine serum non supplemented (GIBCO origin: united states), 1% Glutammina, 0.1% gentamicin and 1% geneticin (GIBCO). Each 5 days the cell cultures were treated with tripsin-EDTA solution. The cell suspensions were diluted in ratio 1:10, plated and incubated at 37°C in humidified atmosphere with 5% CO_2.

Primary culture preparation of rat embryos hippocampal neurons

Hippocampal neuronal primary cultures have been grown in plates treated on previous days.

Plates for immunofluorescence experiments preparation: 16mm diameter slides (Borosilicate Glass) were incubated at room temperature in 65% nitric acid for 48 hours followed by 2 washes for 2 hours each with distillated and sterile H_2O. After, the slides were sterilized in the stove at 180ºC for, at least, 5 hours and the day before the neuron culture preparation the slides were placed in individual wells in 12-well plates (IWAKI, Bibby).

In each individual well was added 1 mL poly-L-lisin solution (0.5mg/mL, SIGMA) in borato buffer 0.1M.

For Biochemical experiments were used 6-well plates, without slides, treated with the same solution.

On the neuron preparation day, the plates with slides were washed 3 times with distillated and sterile H_2O and then incubated with culture medium (98% Gibco Neurobasal medium 1X, 2% Gibco B-27 supplement 50X, 1% Gibco Pen/Strep (10000U/ml Penicillina G Sodio e 10000 µg/mL Streptomicina sulphate in 0.85% salin solution), Glutamine 2mM, Glutamate 100µM). The plates were incubated at 37ºC, 5% CO_2 until the neurons preparation.

A 18-19 days pregnant rat (Charles River) was sacrificed with CO_2 intoxication.

Then, embryos' brains were extracted and with microscope help for dissection, the hippocamps were extracted.

The hippocampus were washed 3 times with HBSS (Hank's Balanced Salt Solution: 10% Gibco HBSS 10X w/o Ca^{2+} and Mg^{2+}, 3.3% HEPES 0.3M Ph 7.3, 1% Gibco Pen/Strep) and after, were treated with a solution with 2.5% tripsin for 10-15 minutes at 37ºC.

The hippocamps were then washed 5 times with HBSS medium and mechanically dissociated.

The concentration of neurons was 75000 cells/well (12-well plate) or 300000 cells/well (6-well plate).

After a week of neuronal culture preparation, the culture medium was changed with feeding medium (98% Gibco Neurobasal medium 1X, 2% Gibco B-27 supplement 50X, 1% Gibco Pen/Strep (10000U/mL Penincilin G Sodio and 10000µg/ml Streptomicin sulphate in 0.85% salin), Glutamin 2mM).

Immunofluorescence

The hippocampal neurons, fibroblasts, neuronal cell precursor and differentiated neurons were fixated with 4% paraphormaldehyde solution, 4% saccharose, 240 mM phosphate buffer for 10 minutes at room temperature or, alternatively, with 100% cold methanol for 10 minutes at -20ºC. After the incubation, the neurons/cells were washed 3 times with PBS.

The cellular monolayers were incubated with primary antibody diluted in GDB buffer (Jackson Immunoresearch Lab; USA) (30mM buffer phosphate with 0.2% jelly, 0.5% Triton X-100, 0.8M NaCl) for 3 hours at room temperature. The incubation was followed by 3 washes with Alto Sale Buffer (500 mM NaCl, 20 mM $NaPO_4^{2-}$) and after, for 1 hour secondary antibody associated with a fluorophores diluted in GDB Buffer incubation.

When the secondary antibody incubation was finished, the slides were washed with Auto Sale Buffer, PBS and distillated water and, at last, mounted on top of microscope slides with the help of Mounting Medium (Vecta Shield).

The images were acquired with LSM 510 Meta Confocal Microscope with LSM 510 Meta Software, mounted on a Zeiss microscope with Nikon 63X lens and immersion oil with sequencing acquisition at maximum resolution (1024x1024 pixel).

The image quantification at confocal was measured with Open Source ImageJ software (Image Processing and analysis in java).

Statistical Analysis was done through Student's t-test. All data were presented with media ± standard error.

SDS-PAGE and Western Blot

The protein samples were diluted in SDS-PAGE sample buffer 2x (Tris HCl 125mM pH 6.8, SDS 4%, 2-mercaptoethanol 10%, Glycerol 20%, Bromophenol Blue 0.004%) boiled at 100ºC for 10 minutes or at 65ºC for 10 minutes. The proteins were separated in a polyacrylamide gel in 6%, 7.5% or 10% of running buffer (distillated H_2O, 20mM Glycine, 250mM Tris, 1% SDS).

The PageRuler Prestained Protein Ladder used to measure the protein bands weight on the electrophoretic migration is provided by Thermo Scientific.

To visualize the proteins of interest was done an analysis based on Western Blot.

After the SDS-PAGE protein separation, the proteins were eletrophoretically transferred to a nitrocellulose membrane (SIGMA) using a buffer with 0.020M Tris-HCl, 0.150M glycine, 20% Methanol pH 8.3 at 80V constantly for 120 minutes. When the transference was finished, the proteins were visualized with Red Ponceau 1X. After removal of excess dye, the membrane was saturated with blocking solution (5% powdered

milk dissolved in TBS Tween (0.1% Tween 20, Tris 20mM, NaCl 150 mM, pH 7.5)) for 1 hour.

The membrane was, then, incubated during 3 hours at room temperature or overnight at 4ºC with primary antibody diluted in 3% milk. Subsequently, were proceeded 3 washes with TBS Tween 0.1% and secondary antibody anti IgG rabbit, mouse, guinea pig and sheep conjugated with peroxidase incubation and visualization through Enhanced ChemiLuminescence (Perkin Elmer) reaction.

The band intensity was measured with ImageJ software.

The proteins signaling were normalized based on actin/tubulin levels as well as MAP2 levels. MAP2 protein is used as normalization protein because indicates the number of cells that is present and so, we can divide the level of each protein for MAP2 level and then, we will have the real level of each protein.

Antibodies: Rabbit anti Shank3 (Santa Cruz Biotechnology, H-160); Guinea Pig anti Shank3 (Beri S et al., 2007); and rabbit anti-GFP (Cell Signaling Technology); Rabbit anti-mGluR1, Rabbit antimGluR5, Rabbit anti-GluR1, Rabbit anti-GluR2/3 (Millipore Bioscience Research Reagents); Mouse anti-GluR2, Mouse anti-Shank1, Mouse anti-Shank2, Mouse anti-PanShank, Mouse anti-PSD95 (Neuromab, UC Davis/NIH neuromab Facility); Rabbit anti-GKAP (Gently given by Dr. Morgan Sheng); Rabbit anti-Homer (Gently given by Dr. Eunjoon Kim Lab, KAIST, South Korea); Mouse anti-MAP2, Mouse anti-sinaptophysin, Mouse anti-beta actina, Mouse anti-alfatubulina (Sigma-Aldrich); Secondary antibody conjugated with FITC-, Cy3- and Cy5- anti-mouse, anti-rabbit, anti-guinea pig or anti-goat (Jackson ImmunoResearch).

DNA Extraction

The mices are split from their parents after 21 days of birth. A little part of the tail was cut off and the DNA is extracted.

All the procedure was done by a kit from SIGMA – REDExtract-N-Amp^{TM} Tissue PCR Kit.

The REDExtract-N-Amp^{TM} Tissue PCR Kit contained all the reagents needed to rapidly extract and amplify genomic DNA from mice tails and other animal tissues. The

DNA was released from the starting material by incubating the sample with a mixture of the Extraction Solution (E7526) and the Tissue Preparation Solution (T3073) at room temperature for 10 minutes. There was no need for mechanical disruption, organic extraction, column purification or precipitation of the DNA. After, adding Neutralization Solution B (N3910), the extract was ready for PCR.

The primers used in the PCR were S10 (cctctaggcctgctagctgtt), S12 (caagttcatcgctgtgaagg) and AS3 (aagaagccccagaagtgaca). The primers S12 and AS3 together were able to recognize the entire SH3 domain, from exon 10 until after exon 11, identifying a 360bp region, which correspond to wild-type genotype. The knockout doesn't have the exon 11 so, the primers S10 and AS3 had to recognize an upstream region of the exon 11 and then the piece is smaller due to the deletion and the primer will identify a 190bp region.

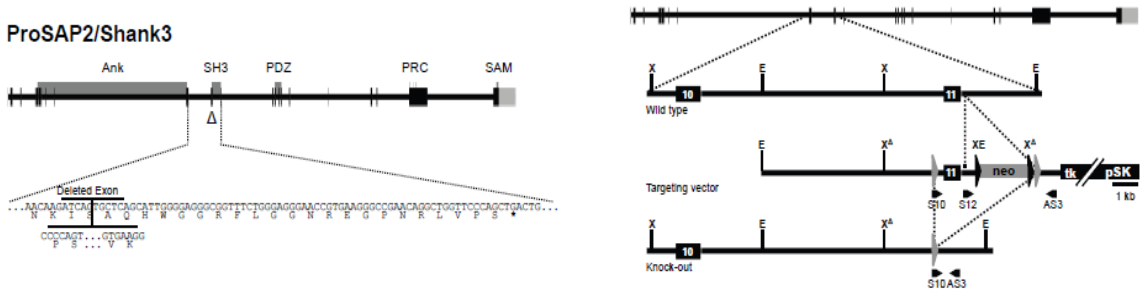


Figure 1 - ProSAP2/Shank3 gene sequence and respective knockout sequence for 22q13.3 deletion of the same gene. Primers S10, S12 and AS3 representing the cut locations for each sequence (Michael J. Schmeisser et al., 2012).

The mastermix contained for each sample: 0.6uL DNA (200ng), 21.5uL Polimerase (invitrogen), 0.5uL Primer S10 (500nM), 0.6uL Primer AS3 (500nM), 0.7uL Primer S12 (500nM), 1.0uL DMSO and H_2O to complete 25uL total.

The PCR program was started with the initialization step at 95ºC for 3 minutes and then, proceeded to denaturation step at 95ºC for 30 seconds. The annealing was carried out at 58ºC for 36 seconds (Initialization, denaturation and annealing are repeated for 36 cycles). After these 36 cycles, the elongation step occured at 72ºC for 30 seconds and the final elongation at the same temperature for 10 minutes. When the PCR was over, the holding temperature is 20ºC that was kept for an indefinite time.

The samples were dyed with loading dye 6x and loaded in a 2% agarose gel.

Reprogramming Human Fibroblasts into Neurons

Human Fibroblasts were extracted from patients bottom and platted at density of 60000 cells per well of a 6-well plate with a final volume of 3mL per each well. Culture medium used was DMEM-F12 20% serum to maintain the target cells in a proliferative state.

To reprogramming fibroblasts, was used a gene cocktail (the same used by Yamanaka) that includes SOX-2, KFL-4 and OCT-4. All these genes were expressed in a viral vector, policistronic lentivirus MILLIPORE® that has all the 3 genes.

Before transduction, the number of cells in one well of the 6-well plate was counted to be determined the volume of virus needed to achieve a target MOI (Multiplicity of infection). To achieve an MOI of 20-50 was used this equation:

$$\text{Virus volume } (\mu L) \text{ required} = \frac{\text{Number of cells seeded}}{\text{Virus Titer (IFU/mL)}} \times \frac{\text{Desired MOI}}{1\ \text{mL}} \times 1000\mu L$$

The medium from each well was replaced with 1mL of new DMEM-F12 20%.

The transfection reagent used was Polybrene at 1:10 dilution, in which 1µL of Polybrene is diluted into 9µL of sterile distilled water. Is added 5µL of the diluted Polybrene transfection reagent to each well to be transduced and the final polybrene concentrarion was 5µg/mL. After the polybrene, was added the required volume of virus directly to the wells containing the attached fibroblasts. Finally, the plate was gently mixed from side to side to thoroughly mix the virus onto the fibroblasts. The plate was incubated overnight in a 37ºC, 5% CO_2 incubator.

At day 2, the medium was replaced from each well with 1mL fresh DMEM-F12 20% and a second virus infection was performed.

After one day, at day 3, the cells were washed 3 times with 3mL PBS 1x per well, aspirating after each wash. After last PBS 1x removal, was applied 3mL fresh media per well.

The two days after, days 4 and 5, the cell morphology was monitored daily, also the medium was replaced with 3mL of fresh medium per well.

The next day, inactivated Mouse Embryonic Fibroblast (MEF) feeder layer was prepared to support the cells being reprogrammed on the day before being used.

A 6well-plate with matrigel (1mL) was prepared and left in the incubator for 30/40minutes. After, the medium was removed and added 10mL of mitomicin for 2/3hours at 37ºC.

MEF has to be inactivated because, other way, interferes with IPS development. Therefore, MEF was treated with mitomicin, which is a fuse mitotic inhibitor that is able to block cellular growth.

Mitomicin comes as 2mg powder. This powder was dissolved in 2mL milliQ water so, the final concentration was 1mg/mL. From this concentration, mitomicin was diluted 1:100 in medium used to culture MEF cells which was DMEM 20% FBS (DMEM High Glucose, FBS 20%, AA no essential 1x, L-glutammina 2mM, Pen-Strep 1x, Na Piruvato 1mM). To this medium was added 500µL mitomicin 1mg/mL. On MEF was used 10mL of this solution.

After 2 hours and a half, mitomicin was removed and the plate was washed with PBS1x and after, added 5mL of tripsin for 10 minutes at 37ºC.

Without remove tripsin, was added 5mL of MEF medium followed by medium resuspension onto the cells to detach very well. All the content was passed to a 15mL falcon and 5 minutes centrifuge at 1000rpm. The supernatant was removed and the pellet was resuspended in 2mL of MEF medium in a 15mL falcon. After, was taken 10µL of this solution and the cells were counted with Burker Camera (The average number of cells is multiplied for 10000 to obtain the number of cells in 1mL and then, the proportion related to an specific volume).

Finally, the matrigel from each well of a 6well-plate was removed, was added 2mL of DMEM 20% and the calculated amount of MEF.

On the 6^{th} day, virus-infected cells were re-plated onto inactivated MEF feeder layer through this process:

The medium from 6-well plate containing MEF was removed and washed once with 2mL PBS1x per well. The PBS was removed and replaced with 3mL Human

Embrionary Stem Cells medium (hESC medium). The medium used to culture Human EScells was composed by DMEM F12, 20% KSR (Knock-out serum replacement), AA non-essential 1x, L-Glutammina 2mM, Pen-Strep 1x, Na Piruvato 1mM, β-mercaptoethanol 2μM and after filtered with 0.22μm filter, was added bFGF 10ng/mL per well.

The medium from 6-well plate containing the virus-infected cells was aspirated and washed once with 3mL PBS 1x and after, replaced for Accutase solution to each well of the plate containing the virus-infected cells. The plate was incubated for 8-10 minutes at 37ºC to dissociate the cells. Subsequently, were added 2mL of Human EScells medium, the cells were resuspended and transferred to a 15mL conical tube. The cells were centrifuged at 800rpm for 5 minutes so could form a pellet and the supernatant was discarded. The pellet was resuspended in 2mL Human EScells medium containing 10ng/mL FGF-2 and the number of cells was counted using a hemocytometer.

Finally, the virus-infected cells were seeded with an approximately concentration of $1x10^4$ to $5x10^4$ onto the 6-well plate containing inactivated MEF.

At the day after, the medium was not changed. The morphology was monitored.

For the next four days, using a 5mL pipette, the medium was removed carefully and replaced with 3mL fresh Human ESC medium containing 10ng/mL FGF-2 to each well. This procedure was done every day, also monitoring cell growth and morphology daily. Small iPS cells colonies started to appear around Day 10-15

When the colonies started to appear, was added fresh inactivated MEF every 7^{th} day to replenish older MEFs during the reprogramming time course.

For the next week, the Human iPS cells colonies growth was monitored daily, always looking for homogeneous colonies that were compact and have defined borders. The iPS cells were ready to be picked when reach approximately 200cells.

On the day before picking the iPS cell colonies, was prepared a fresh 6-well plate with inactivated MEFs (the way MEFs are prepared before). On the day that iPS cell colonies were ready to be picked, the medium was aspirated from 6-well plate containing inactivated MEFs plated from the day before. The plate was washed once with 2mL PBS 1x, removed the PBS, was added 3mL of fresh Human EScells medium containing

10ng/mL FGF-2 to each well of inactivated MEFs. The plate was set in 37ºC, 5%CO_2 incubator until the manually passaged iPS were ready to plated onto it.

On the day that colonies were ready to be picked, the 6-well plate containing iPS cells colonies was transferred to a tissue culture hood containing a dissecting microscope. Using a 21 gauge needle attached to a 3mL syringe, cut each iPS colony into 2 or 3 pieces depending upon the colony size. Using a p200 pipettor that has been set to 30µL volume, all the pieces were transferred from one well into a new well of a pre-equilibrated 6-well plate containing inactivated MEFs. The plates were agitated gently from side to side and forward and backwards to ensure that iPS clumps were evenly distributed over the inactivated MEF feeder layer. The plate was placed in 37ºC, 5%CO_2 incubator for 2 days without any media exchanges. The medium was not changed one day after passaging.

On the second day after manual passaging, the medium was exchanged with 3mL Human ESC medium containing 10ng/mL FGF-2 to each well of a 6-well plate. The medium was replaced daily because for the first 3 to 5 passages, colonies may require a longer length of time to grow to sufficient size to be ready for passaging. By the 3rd to 5th passage, iPS cells can be cultured similarly to human ES cells.

At this point, the cells were ready to differentiate.

The medium was changed to Human Embryoid Bodies (hEB) Medium. This medium was constituted by DMEM F12, 20% KSR (Knock-out serum replacement), AA non-essential 1x, L-glutammina 2mM, Pen-Strep 1x, Na Piruvato 1mM and β-mercaptoethanol 2µM. The hEB medium was used until these Embryoid Bodies become Neuronal Rosettes. At this point, the medium obviously change to other specific medium. The medium used to differentiate Rosettes into Neuronal Stem Cells was the same medium used to IPS and before filtering, was added 5mL N2 1x.

When the cells become Neuronal Stem Cells, the medium was constituted by DMEM/F12 (20%FBS), Glutamine 2mM, B27 (1:1000), N2 (1:100), 1% P/S. This medium was filtered and then, was added 50µL EGF 20ng/mL and 50µL bFGF 20ng/mL.

After cell growth, the medium was changed to differentiation medium that is exactly the same as Neuronal Stem Cells' medium but there is no EGF and bFGF because the grow has stopped.

RNA interference

Shank3 and luciferase are inserted into HindII/BglII site of pLVTHM vector to produce lentivirus vector.

The siRNA used is directly against the 21 exon of the Shank3 gene in rat and mouse: 5' GGAAGTCACCAGAGGACAAGA 3'.

The constructs resistant to siRNA (Shank3r, Shank3R87Cr, and Shank3InsgGr) were obtained by mutating six nucleotides of the binding site for the siRNA, without changing the amino acid sequence of the resultant protein.

Lentiviral vectors production

HEK Cells 293T (Invitrogen) are plated in 100mm Petri plaques (IWAKI).

To produce lentiviral vectors, the cells are used with confluence levels of 60% to 70% and then transfected with three different vectors necessary to lentivirus formations: pLVTHM vector with shShank3 and VsVg and PAX plasmids.

After 48 hours, the medium where the cells have been growing has virus particles. In this way, the medium is centrifugated at 27000g for 2 hours at 4ºC. The pellet obtained is resuspended in PBS, aliquoted and conserved at -80ºC (Lois et al., 2002).

Infection

The hippocampal neurons are infected with lentiviral particles containing pLVTHM at DIV7 with a Multiplicity of infection (MOI) of 5; the cells are lisated with lysis buffer at DIV13-15 for biochemical experiment or fixated for immunofluorescence assays.

RNA Extraction

To perform RNA extraction used posteriorly to RT-PCR is fundamental TRI Reagent (Sigma-Aldrich). This reagent is a mixture of guanidine thiocyanate and phenol in a monophase solution and is able to isolate simultaneously RNA, DNA and protein on homogenization or lysis of tissue sample.

Sample Preparation

The monolayer cells are trypsinized and put on suspension. After, are centrifuged to isolate the cells and then, lisated in TRI Reagent by repeated pipetting. One mL is enough to lysate 5-10x10^6 cells animal, plant or yeast cells or 10^7 bacterial cells.

RNA isolation

RNA isolation execution is done by transferring the aqueous phase to a fresh tube and added 0,5mL of 2-propanol per mL of TRI Reagent used in Sample Preparation and mix. The sample stands for 5 to 10 minutes at room temperature. After, centrifuged at 12000 x g for 10 minutes at 4ºC. The RNA precipitates forming a pellet on the side and bottom of the tube.

The supernatant is slowly discarded and the RNA pellet is washed by adding at least 1mL of 75% ethanol per 1mL of TRI Reagent used in Sample Preparation. The sample is vortexed and centrifuged at 7500 x g for 5 minutes at 4ºC.

The RNA pellet is briefly dried for 5 to 10 minutes by air-drying. Do not let the RNA dry completely because this significantly decreases its solubility. An appropriate volume of formamide, water, or a 0.5% SDS solution is added to the RNA pellet. To facilitate dissolution, mix by repeated pipetting with a micropipette at 55ºC for 10 minutes.

Now, the RT-PCR is proceeded.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The massive majority of cellular RNA molecules are tRNAs and rRNAs. Only 1% to 5% of total cellular RNA is mRNA. To form the construction of cDNA is fundamental the correct split of mRNA from the total RNA pool. The most part of mRNA are constituted by a poly (A) tail but on opposite, the structural RNAs do not have this poly (A) tail and this leads to the conclusion that these tails enrich the mRNA.

To translate mRNA into double-stranded DNA we use ImProm-II Reverse Transcription System (Promega). This experiment makes possible the cDNA production to be consequently, amplified by Polymerase Chain Reaction (PCR) technique.

1μg of RNA is combined with 0,5μg of oligo (dT) primer that connects to poly (A) tails and is added nuclease-free water to 10μL. The primer mix is thermally denatured at 70ºC for 5 minutes so the RNA secondary structures will be denatured and then, cooled on ice. After, the reverse transcription mix is assembled on ice and is constituted by ImPromII 5X Reaction Buffer diluted to 1X, ImPromII Reverse Transcriptase 1uL, magnesium chloride 1,5mM, dNTPs 2mM, RNasin Ribonuclease inhibitor 20U and nuclease-free water to complete 10μL. The reverse transcription reactions of up to 1μg of total RNA are accomplished in 20μL of total reverse transcription mix. Following the initial annealing at 25ºC for 5 minutes so the reverse transcriptase can bind, the reaction is incubated at 42ºC for up to one hour to allow elongation of cDNA. Finally, the temperature is increase to 70ºC for 15 minutes to inactivate RT enzyme and the reaction stop. Once there is no cleanup or dilution after the cDNA synthesis, the product can be directly applied to amplification reactions.

Quantitative Real Time PCR

The quantitative Real Time PCR is a method used to amplify and simultaneously quantify a targeted cDNA molecule. The quantity can be either an absolute number of copies or a relative amount when normalized with an endogenous gene, such as GADPH or β-Actin.

To detect the Real-Time PCR products could be used non-specific fluorescent dyes, such as SYBR Green that intercalate with any-double stranded DNA or can be used sequence-specific DNA probes (TaqMan probes) containing oligonucleotides labeled with a fluorescent correspondent that allows detection only after hybridization with its complementary DNA target.

In our experiments, was used SYBR Green (BioRad) as detection system. An increasing in DNA products during PCR reaction points to an increase in SYBR green fluorescence that is measured at each cycle, permitting the quantification of DNA concentration.

Relative concentration of DNA present during the exponential phase of the reaction is determined by plotting fluorescence against cycle number on a logarithmic scale. A threshold for detection of fluorescence above background is determined. The cycle at which the fluorescence from our sample crosses the threshold is called Cycle Threshold,

Ct. The amount of DNA supposedly duplicated every cycle during the exponential phase and relative amounts of DNA can be calculated with the formula: $2^{-\Delta CT}$, where ΔCT is the difference between the sample Ct and the housekeeping gene Ct.

The reaction occurs in the Thermalcycler iQ5 Real-Time PCR detection system (BioRad) and SYBR Green Supermix (BioRad). This supermix is constituted by 50ng cDNA (retrotranscribed RNA), SYBR Green 2X diluted to 1X, Primer Fw 10μM with final concentration of 0.5μM, Primer Rv 10μM with final concentration of 0.5μM and water to 25μL.

The first cycle is 95ºC for 2 minutes. After, the second cycle occurs at the same temperature for 30 seconds and then 95ºC for 10 seconds. The third cycle is repeated 50 times and the temperature decreases until 59ºC for 20 seconds and then increases for 74ºC for 20 seconds. The fourth cycle occurs at 95ºC during one minute. At the fifth cycle, the temperature goes down until 74ºC for one minute and in the last cycle, the sixth, the temperature is 74ºC for 10 seconds and this cycle is repeated 43 times.

Statistical Analysis

The samples are neuronal preparations from human or rat neurons analyzed and tested from a whole group of different experiments. All experiences were repeated three times for a precise quantification.

The variables are quantitative since all the information extract from each experiment will be measured and then compared and evaluated.

All the samples follow a normal distribution and so, samples were analyzed with parametrical tests statistically studied using student T-test to associate the outcomes.

Results

The Shank family is a group constituted by Shank1, Shank2 and Shank3. This protein, Shank3, is a protein that belongs to the scaffolding protein group in the postsynaptic density. Shank3 is able to connect ion channels, neurotransmitter receptors and other proteins to the actin cytoskeleton and G-protein-coupled signaling pathways. This capacity is related to its PDZ domain that is able of mediate protein-protein interactions. Consequently, any dysfunction of this protein will cause an unbalanced neuronal function affecting the communication among neurons and leading to neurological diseases.

The major part of the genes related to neurological diseases, such as Autism Syndrome Disorders influence glutamatergic excitatory synapses and Shank family genes are no exception.

In the Phelan-McDermid syndrome (PMS), is observed a haploinsufficiency in Shank3 protein caused by a deletion in the long arm of chromosome 22 originating a 22q13.3 deletion and leading to autistic-like phenotypes.

Although the prevalence is not known, PMS is the second most frequent subtelomeric rearrangement where simple deletions represent 75% of the cases and is diagnosed within the first three years of life.

Nowadays, with much more known cases, is fundamental to study it in order to understand and come up with a cure. In this way, we use two different models to study Phelan-McDermid syndrome, mice and humans.

Preliminary Data

As every study, in the beginning not much is known about the subject. In this way, the first step is to figure out how the studies are going to be carried out.

To a better comprehension about Shank3 functions in synapse development and functions, we knocked down Shank3 using RNA interference technique. We infected at DIV7 hippocampal neurons in culture with a lentivirus vector expressing shRNA for Shank3 (shShank3) and a shRNA for control (shControl) with specific siRNA for

luciferase. At DIV14 the neurons were lisated in a specific lysis buffer and then Western-Blot was performed (Figure 1).

We showed that the shRNA for Shank3 is able to reduce specifically Shank3 endogenous levels on both mRNA and protein without interfering with the other two Shank proteins, Shank1 and Shank2.

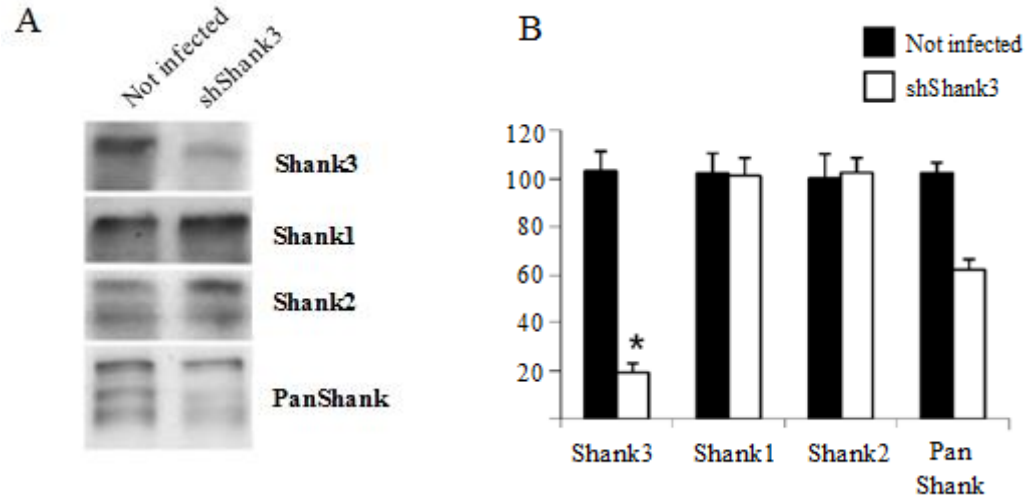


Figure 1 – (A) Western-Blot of lysate Rat hippocampal neuron at DIV7 from shShank3 and non-infected neurons to see levels of Shank1, Shank2,Shank3 and PanShank. Only Shank3 levels are altered in shShank3 neurons indicating that the virus worked. (B) Respective Shank proteins' quantification levels obtained in the lysates. *p< 0.01, Student's t-test.

In this procedure, were used two different antibodies, one against the N-terminal domain and other against the C-terminal domain of Shank3. Western-Blot results demonstrate decreasing in the Shank3 levels in the main bands but the other Shank proteins, do not have significant changes. In this way, we can be sure that lentivirus worked specifically for Shank3 gene.

Western-Blot experiment was followed by Real-Time PCR to check the Shank family mRNA levels in the neurons infected with shShank3 or shControl at DIV7 (Figure 2).

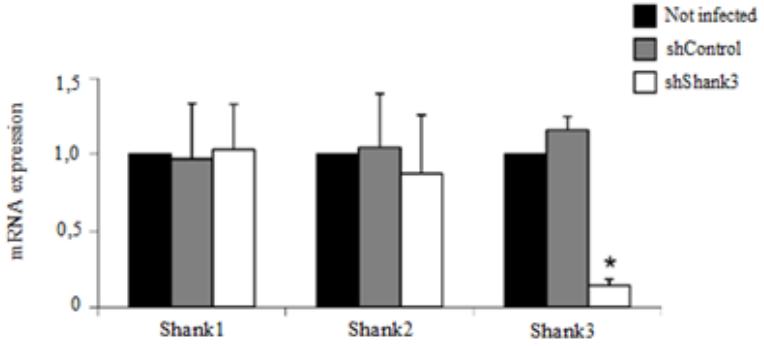
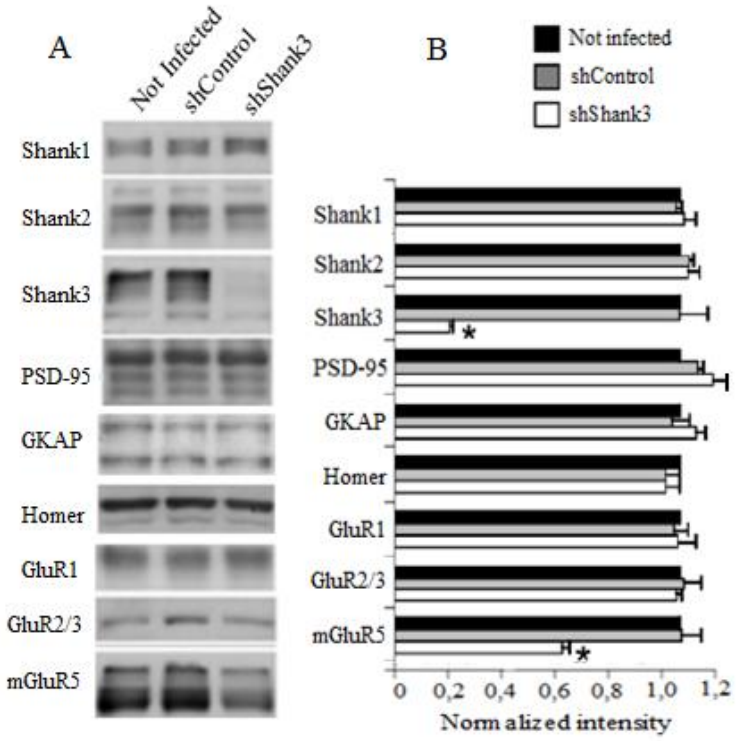


Figure 2 - mRNA levels quantification of Shank protein family *p< 0.01, Student's t-test.

The results indicated lower levels of Shank3 mRNA in the ones infected with shShank3, which means, that shRNA for Shank3 (shShank3) is capable of reduce the levels of endogenous Shank3 mRNA and protein in rat hippocampal culture maintaining the other Shank proteins unaltered. This was observed by Real-Time PCR and Western-Blot assays.

As described previously, Shank3 is fundamental in assembling postsynaptic density because of its protein-protein interactions. In this way, we investigated the consequences of Shank3 knockdown in the protein arrangement of excitatory synapses by analysis of total lysates from hippocampal cultures infected with shShank3 or shControl (Figure 3).



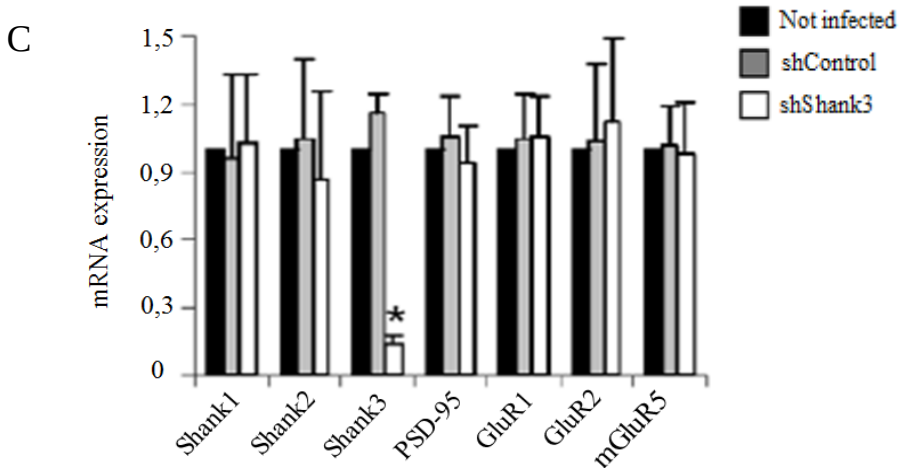


Figure 3 - (A) Hippocampal neurons lysate at DIV7 with shShank3 or shControl and antibodies' analyzes. (B) Respective proteins' level of hippocampal neurons infected with shShank3 or shControl and non-infected. *p< 0.01, Student's t-test (C) Proteins mRNA levels of hippocampal neurons infected with shShank3 or shControl. *p< 0.01, Student's t-test.

Observing the Western-Blot results for the others scaffold proteins, glutamate receptors and signaling molecules we can conclude that mGluR5 levels are decreased in shShank3 infected hippocampal neurons in the total lysate as well as in the synaptosome's fraction (S.E. for normalized intensity in the total lysate is 0.58±0.03 and in the synaptosomal fraction is 0.37±0.05, $p<0.01$, Student's *t* test) (Figure 3A and 3B). However, the decreasing in mGluR5 protein levels are not related to mGluR5 mRNA levels, which are normal (Figure 3C).

Looking to the other analyzed proteins present in synapses and/or postsynaptic density, there is no significant alterations between shShank3 and shControl levels. Even Homer and GKAP scaffold proteins that have direct interaction with Shank3 did not seem to have significantly variations by Shank3 decreased levels (Figure 3A and 3B).

Present Data

To clarify the role of Shank3 in synapses formation and function we decided to use two different models: Shank3 KO mice and hiPS cells derived from Phelan-McDermid patients.

Nowadays, studies using mices are fundamental to comprehend better situations that *in vitro* experiments cannot reproduce. On the other hand, the possibility to work with human cells gives us the opportunity to be closer to the human pathology. In this way we reprogrammed to hiPS cells Phelan-McDermid Patients' fibroblasts and differentiated them into neurons to compare them with neurons obtained from healthy donors same age

The patients studied have all deletion in different sizes on chromosome 22q13.3 and so, are all Phelan-McDermid patients, confirmed using FISH technique.

In this way, the patients were submitted to fibroblast biopsy. After, the Human Fibroblasts were reprogrammed into iPS cells (as described in Material/Methods Chapter). We generated hiPSC lines from fibroblasts obtained from patients and healthy control donors using the hSTEMCCA-loxP virus Pluripotency of each clone was confirmed by alkaline phosphatase expression (Figure 4).

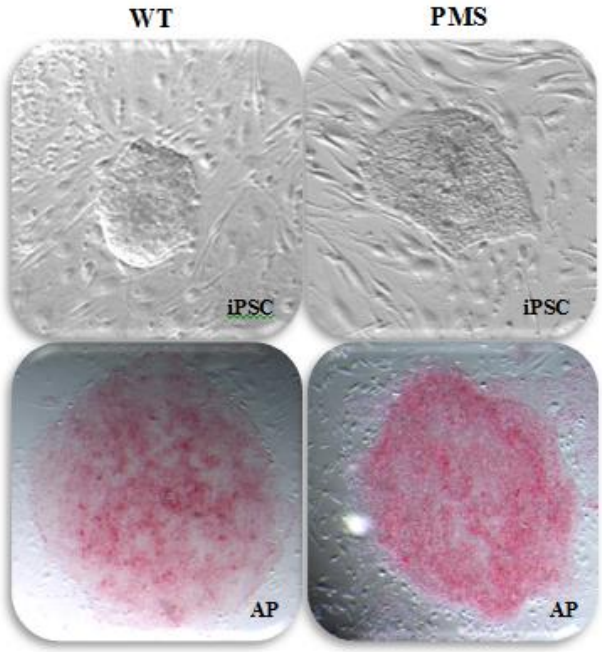


Figure 4 - Description of iPS clones from Control and Phelan-McDermid syndrome and respective alkaline phosphatase test. Both clones are alkaline phosphatase positive, indicating presence of undifferentiated cells with potential to self-renewal

Besides, Real-Time PCR and Immunostaining techniques were used to confirm the presence of Oct4, Nanog and Lin 28 marker genes that are classical markers of pluripotency (Figure 5).

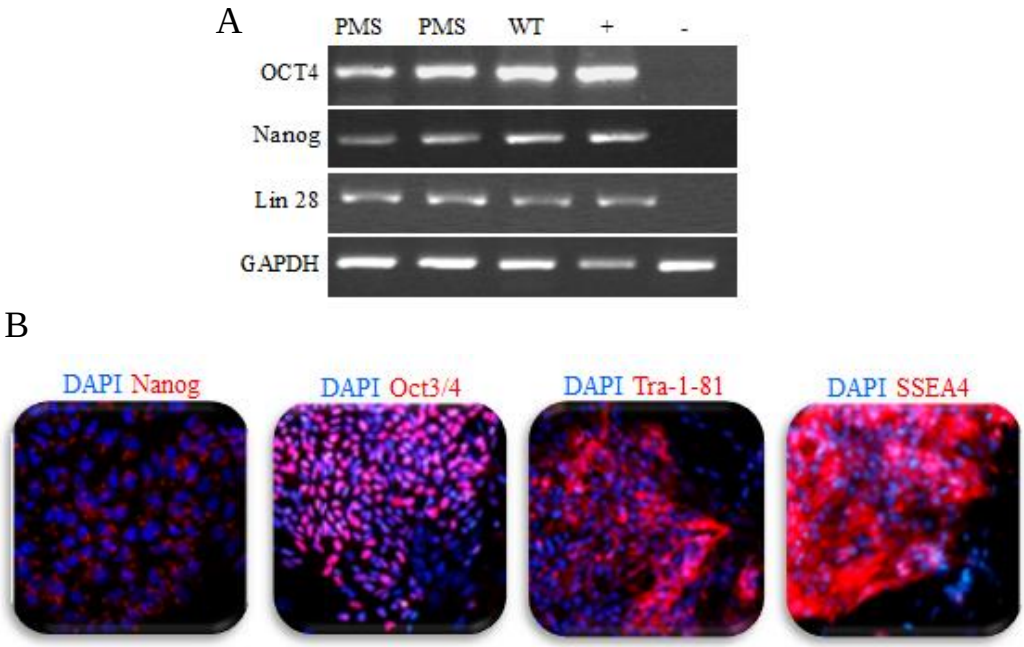


Figure 5 - Marker genes Oct4, Nanog and Lin 28 expression both on Control and PMS confirming the presence of undifferentiated pluripotent human stem cells by Real-Time PCR (A) and Immunostaining (B)

Induced-Pluripotent Stem Cells are able to differentiate into the three germinative layers. We analyzed the ability of our hiPSC clones to spontaneously differentiate into the three germ layers in the absence of mitogenic factors by looking at the expression of βTubulin III, Sox17 and aSMA as markers of ectoderm, endoderm and mesoderm, respectively (Figure 6).

Figure 6 - Immuno staining of Human IPS cells divided in the three germinative layers: βtubIII – ectoderm, Sox17 – endoderm and αSMA – mesoderm

When the iPS cells start its differentiation, in the first step it becomes Embryoid Bodies. The presence of Embryoid bodies is an initial indicator of good differentiation quality in embryonic stem (ES) cells and in induced-pluripotent stem (iPS) cells. To obtain neurons, we differentiated Embryoid bodies into Rosettes that are positive for Nestin a marker of neural stem cells (NSC). We purified a homogeneous population of NSC. All purified hNPs were positive for the neural cell markers Nestin, Pax6 and Sox2. At the contrary, hNPs did not express the pluripotency markers Nanog OCT3/4 and Tra-1-81 (Figure 8I), in addition we did not find cells positive for the neuron specific protein MAP2 and for αSMA a marker of myoepithelial cells (Figure 7).

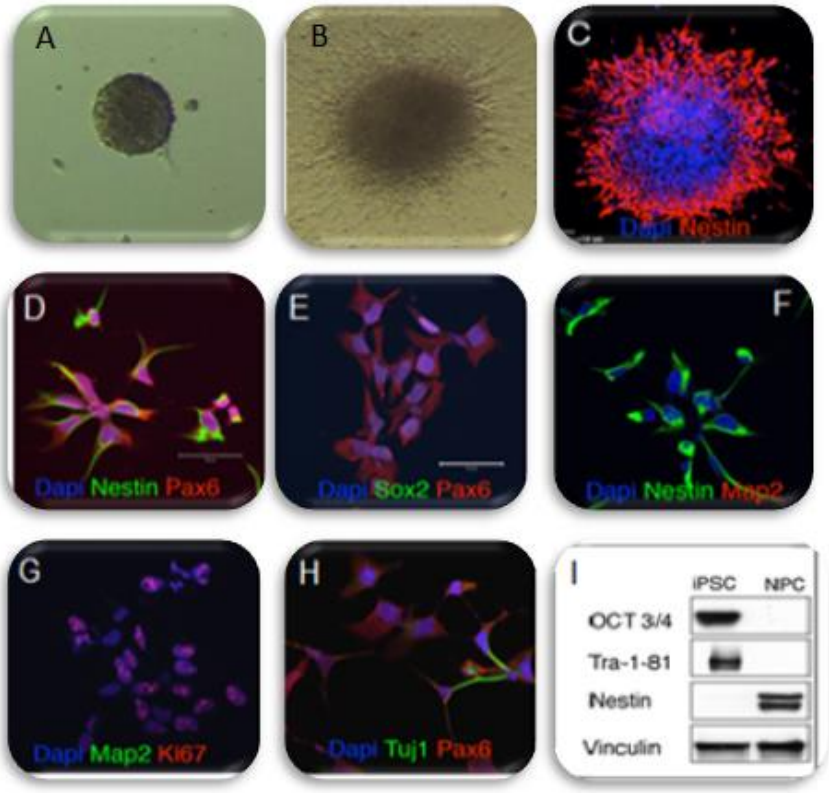


Figure 7 - From Human IPS cells to Neuronal Precursors.
(A) Embryoid Bodied obtained from hIPS, (B) Rossette obtained from Embryoid Bodies, (C) Rossette are tested positive for nestin, a marker for NSC both during embryonic development and in the adult brain, (D and E) Nestin and Pax6 markers to evaluate expression of NPC. (F-G-H) Mature neuronal marker Map2 and Tuj1, an early neuronal marker. (I) NPC, as expected, expressing Nestin however, does not express OCT3/4 and Tra-1-81 iPS cell markers.

Human NSCs were kept under proliferating conditions in the presence of bFGF and EGF and were stable in morphology and for the expression of Nestin for more then 20 passages. (Figure 8).

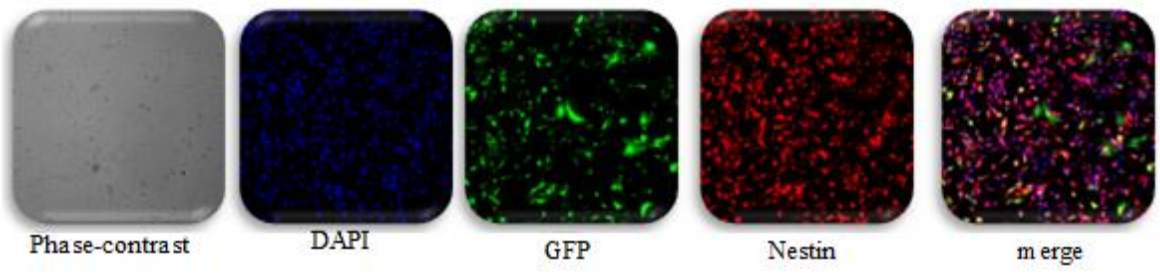


Figure 8 – Immunofluorescence assay using Nestin antibody to confirm NPCs stability after 20 passages

Finally, we demonstrated that NSCs can differentiate into oligodendrocytes, astrocytes or neurons (Figure 9)

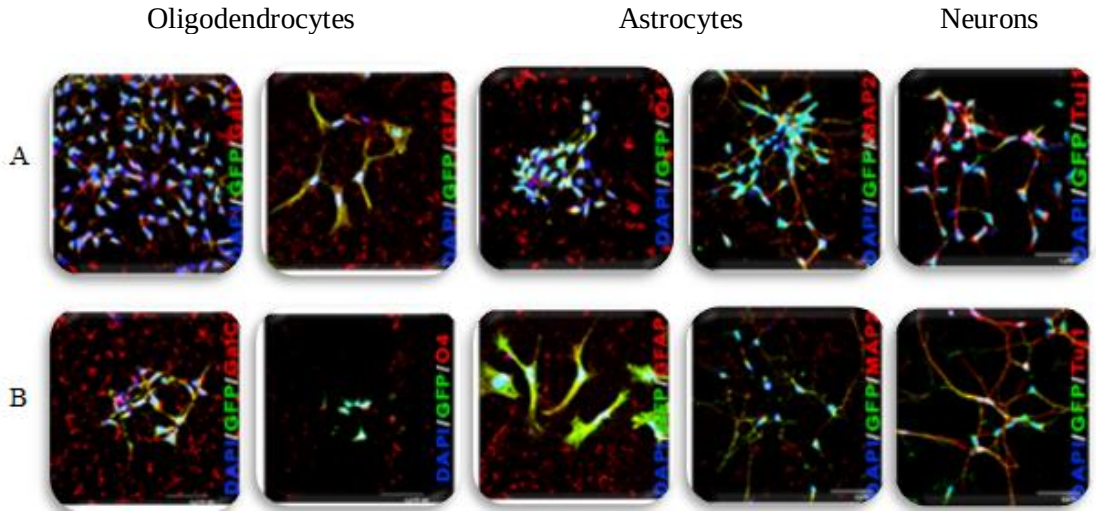


Figure 9 - Immunostaining of NPC from Control (A) and PMS (B) of the three neuronal cells: Olygodendrocytes (GalC and O4 markers), Astrocytes (GFAP marker) and Neurons (Map2 and Tuj2 markers).

NSC Differentiation by Co-Culture with Rats Cortical Neurons

To differentiate NSCs into neuron we co-cultured them with rat primary cortical neurons to help the growth and improve the survival time.

The NSC cells were infected with a lentivirus expressing green fluorescence protein (GFP) so it is possible to recognize and separate the NSCs that have become neurons from the rat neurons. After 60 days in culture, we analyzed the human NSCs-derived neuronal cells using precise markers visualized by immunofluorescence.

After 60 days in culture, we analyzed the human NSCs-derived neuronal cells using specific markers visualized by immunofluorescence. The NSC-derived neurons, green, all expressed neuronal nuclei (NeuN), a classical marker of neuronal cell types; interestingly, immunoreactivity for NeuN is only observed in neurons that have become postmitotic, whereas no staining is observed in proliferative zones. This result clearly indicates that hNPs cocultured onto rat cortical neurons differentiate in a neuronal population. Green cells also expressed the human nuclei protein (hNa), demonstrating that the neuronal cells we obtained derived from human cells. For labeling dendrites, we use MAP2 (Microtubule-associated protein 2). MAP2 is a protein included in the microtubule-associated protein family and is thought to be implicated in microtubule association, a fundamental stage in neurogenesis and is used for tagging dendrites. Finally we used VGLUT1 (Vesicular Glutamate Transporter 1) and synaptophysin to label synapses.

VGLUT1 is a marker for glutamatergic synapses. On the other hand, synaptophysin is a synaptic vesicle glycoprotein present in basically, all neurons and participates in synaptic release (Calhoun ME et al., 1996) (Figure 10).

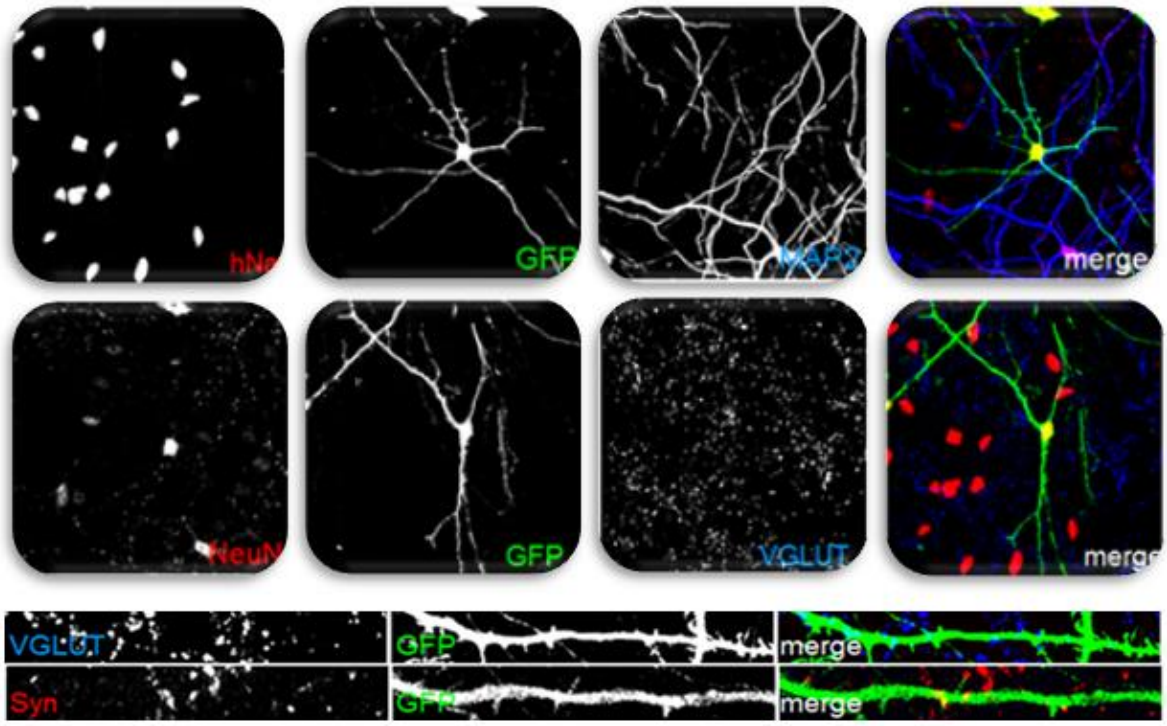


Figure 10 - Human Neuronal Precursor Cells infected with lentivirus expressing GFP and co-culture these NPC with rat primary cortical neurons. The co-culture was evaluated by immunofluorescence using Neuronal nuclei antibody (NeuN), Human Nuclei antibody (hNA), MAP2, VGLUT and synapsin.

Looking at the immunofluorescence assays, the cells are positive for hNA and NeuN indicating the presence of Human neuronal cells. Moreover, the presence of MAP2 validates the existence of dendrites. Therefore, we can confirm that the differentiation is finally over and we have now, human neurons.

Analysis of synaptic formation during neuronal differentiation of Human Neuronal Stem Cells

To evaluate synapse formation during the Neuronal Stem Cell differentiation into neurons, the NSC were infected with lentivirus expressing Homer-GFP. After 30 days of differentiation, at DIV 36, the cells were fixed and immunofluorescence assays were performed (Figure 12).

At this stage, Homer-GFP signal was distributed along the whole neuron leading to the conclusion that the differentiation was not complete yet because Homer was not located specifically at excitatory synapses (Image 11A).

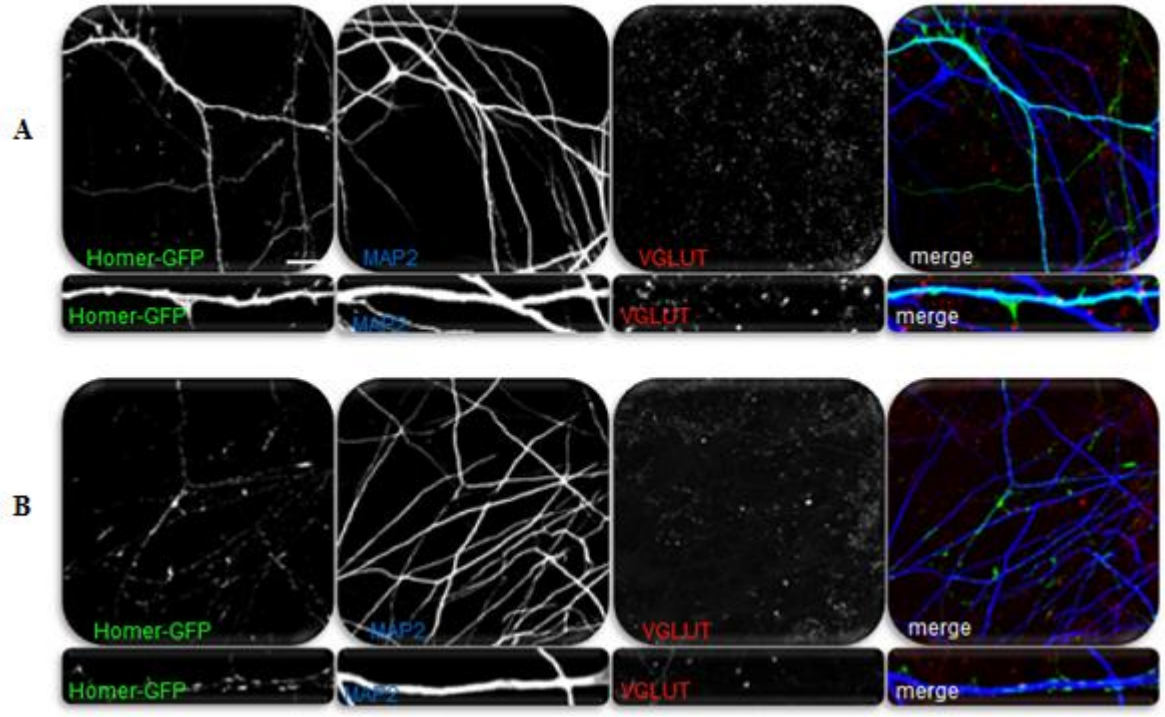


Figure 11 - Analysis of synapse formation during neuronal differentiation in Human Neuronal Precursor infected with lentivirus expressing Homer-GFP under cytomegalovirus (CMV) promoter and plated onto cortical neurons at DIV7. Results analyzed after 30days at DIV 36 (A) and at the end of differentiation, DIV 60 (B)

On the other hand, at DIV60, Homer protein is clustered at excitatory synapses following the classical synapse distribution (Image 11B).

Characterization of Fibroblasts from control and Patients

To a better study of the proteins altered in Phelan-McDermid patients we start by analyzing the patient's fibroblasts, because it is known that Shank3 is not only present in neurons but also seems to be expressed in nearly all observed tissues (Lim S et al., 1999).

In total, we have been studying seven Phelan-McDermid patients with different deletions among them. The major part of the patients presents a macrodeletion in Shank3 protein but there is also one patient with a Shank3 ring mutation, another patient with a 22q ring mutation and finally, a patient with a microdeletion at the C terminus of Shank3 protein.

In the Shank3 fibroblasts' characterization, we analyzed the patients through western-blot, immunofluorescence and RT-PCR.

We also checked the presence of mGluR5 and MAP2 proteins in fibroblasts from control and from patients T, R, H. Besides, as control, we used rat hippocampal neuron and NSC from a healthy control (Figure 12).

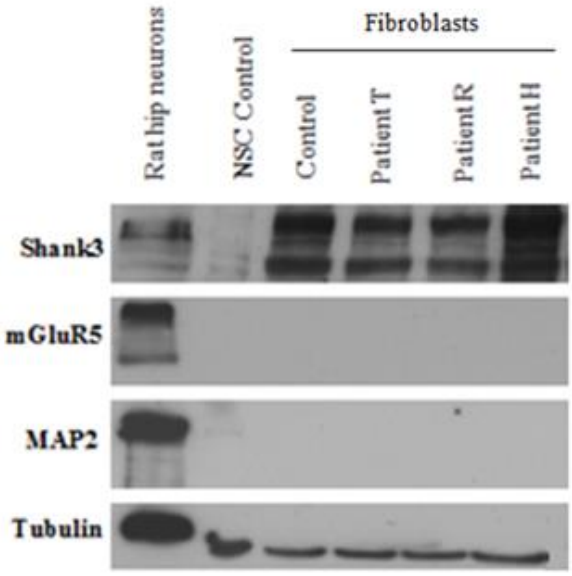


Figure 12 - Protein levels of fibroblasts from patients and control by Western-Blot analysis using antibody against Shank3, mGluR5, MAP2 and Tubulin.

As result, Shank3 protein was observed, although in different quantities, in all samples. As expected, mGluR5 and MAP2 were not present in any of the analyzed samples.

Indeed, we analyzed by immunofluorescence Shank3 levels in control and patients R and H (Figure 13).

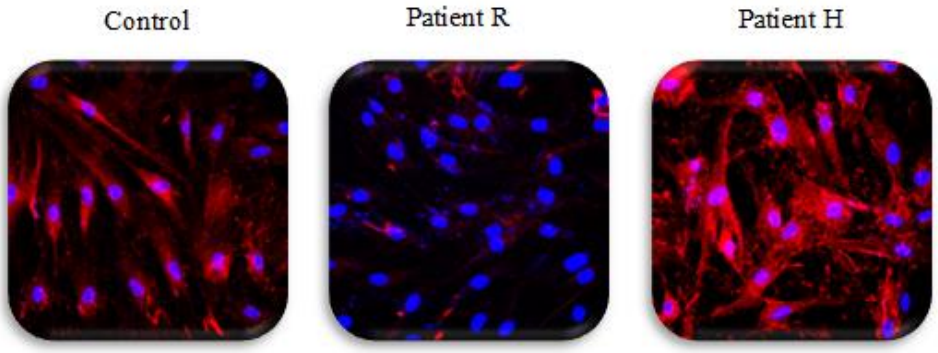
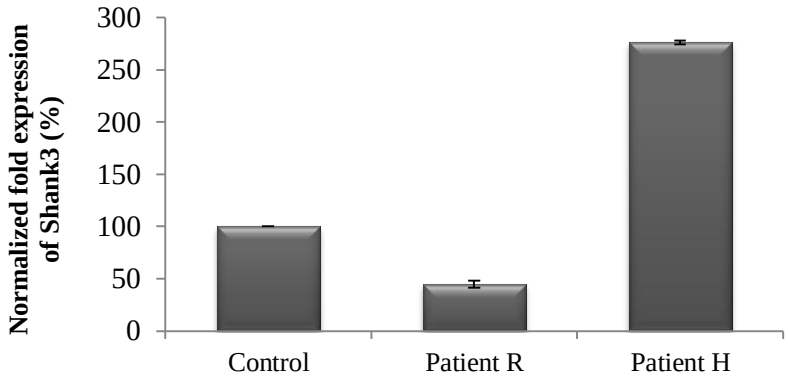


Figure 13 - Confocal microscopy of Fibroblasts from Control and Patients R and H expressing Shank3 protein.

The results confirmed the presence of Shank3 protein in fibroblasts previously obtained by Western-Blot assays.

We also measured Shank3 mRNA levels in fibroblasts using real time PCR (Graphic I).



Graphic I - Normalized fold expression of Shank3 in fibroblasts from Control, Patient R and Patient H, t-Student *p<0.05

Considering that all the patients have mutations or deletions in Shank3 gene, was expected that all the patients have less Shank3 mRNA and Shank3 protein comparatively to the control. Although the majority follows the expected, some patients express more Shank3 mRNA and Shank3 protein comparatively with control fibroblasts. Analyzing these results in fibroblasts, is possible to conclude that there is a large heterogeneity in what concerns Shank3 expression.

Characterization of Neuronal Precursor Cells from Control and Patients

After analyzing fibroblasts from control and patients, the next step was to characterize NSC derived from control and patients. The NSCs are neuronal precursor cells defined by their capacity for self-renewal and multipotency. These cells are originated, as mentioned previously, from fibroblasts and then when achieve this form, are ready to differentiate in any kind of cells present in the CNS, as astrocytes, olygodendrocytes or neurons.

To study the NSCs we started by doing Western-Blot assays on patients and control using antibody against Shank3, PSD-95 and Tubulin. PSD-95 is a scaffolding protein present in the postsynaptic density and tubulin is used as a protein normalization marker (Figure 14).

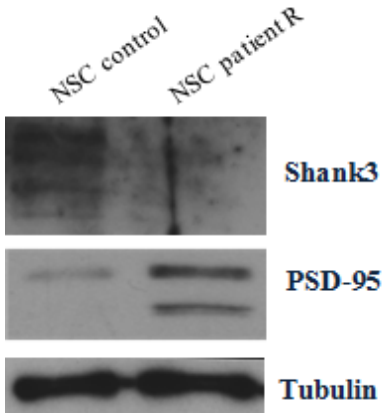
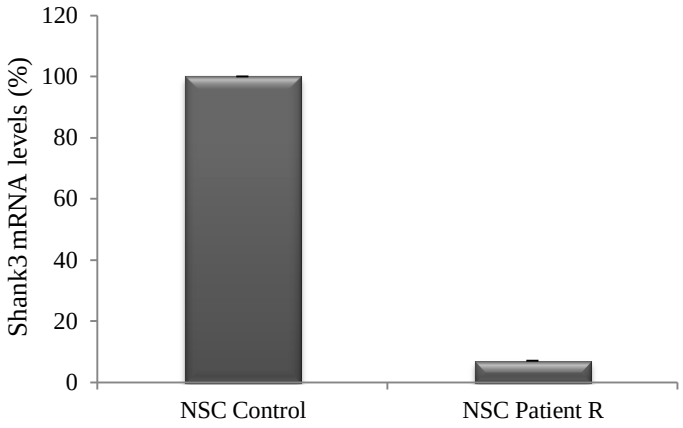


Figure 14 - Western-Blot characterization of Shank3, PSD-95 and tubulin levels in NSC control and NSC from patients R.

As expected, the levels of Shank3 in NSC from patients are much decreased relatively to Shank3 levels in NSC from control. We also checked Shank3 mRNA levels (Graphic II).



Graphic II - Shank3 mRNA levels evaluated by Real-Time PCR in NSC from Control and NSC from Patient R, *p<0.05

The Real-Time results were corresponding to Western-Blot outcomes because we see a strong reduction in Shank3 mRNA levels in the NSC from patients compared to NSC from Control.

Characterization of Differentiated neurons (DIV 60) from Control and Patients

We differentiated NSC into mature neurons and we analyzed through western-blot technique a various number of synaptic proteins.

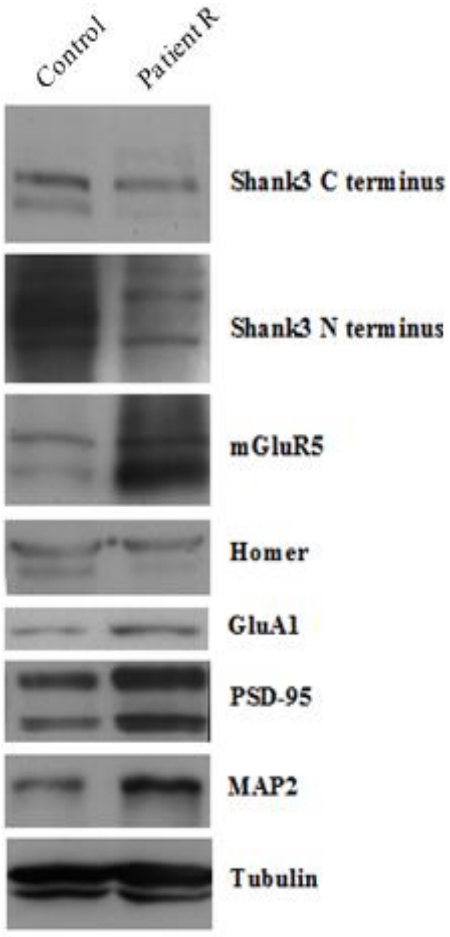
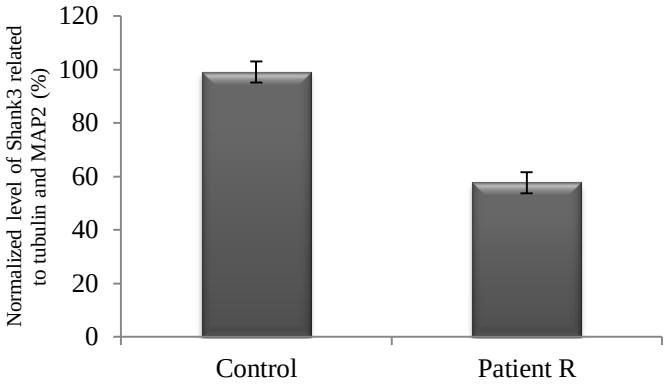
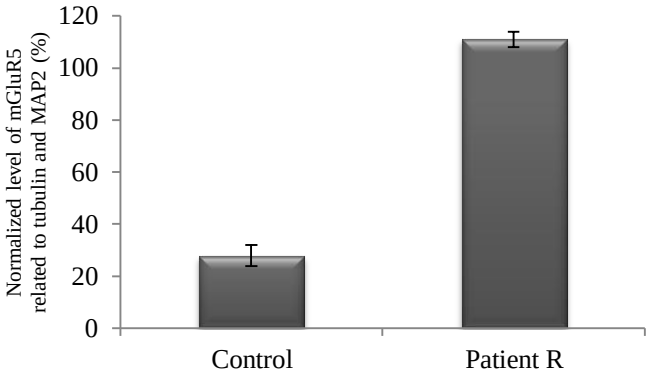


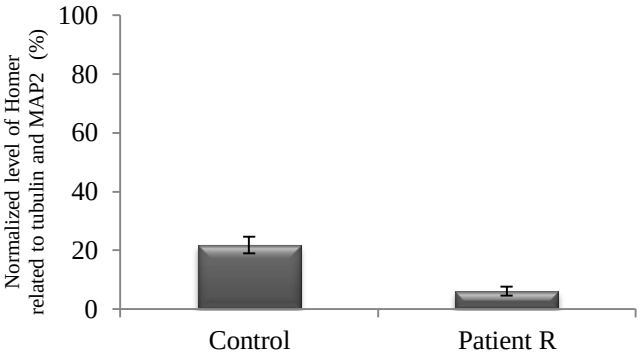
Figure 15 - Characterization of neurons at DIV60 when differentiation is completed in control and Patient R using antibody against Shank3 N and C terminus, mGluR5, Homer, GluA1, PSD-95, MAP2 and Tubulin



Graphic III - Protein levels' quantification of Shank3 in control and patient R both at DIV 60. *p<0.05, t student



Graphic IV - Protein levels' quantification of mGluR5 in control and patient R both at DIV 60. *p<0.05



Graphic V - Protein levels' quantification of Homer in control and patient R both at DIV 60. *p<0.05

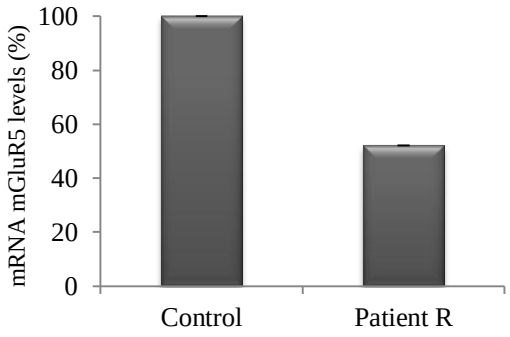
The first result observed is a clear decreasing in Shank3 levels comparatively to control (Graphic III), according to the results obtained previously.

We also found in neurons derived from patient R mGluR5 increased levels compared with neuron derived from healthy control (Graphic IV).

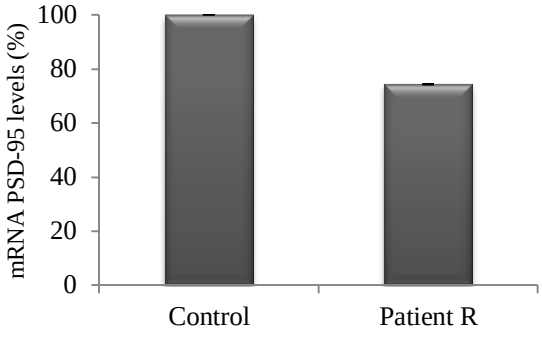
MAP2 was increased in patient R comparatively to control and was used as a neuronal differentiation marker (Figure 15).

Finally, we found in patient R decreased levels of Homer protein relatively to control (Graphic V).

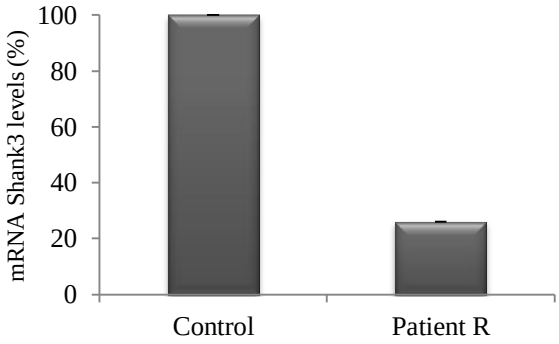
In way to analyze Shank3, PSD-95 and mGluR5 mRNA levels in control and patient, Real-Time PCR assay were executed.



Graphic VI - mRNA mGluR5 levels in control and patient R in differentiated neurons, *p<0.05



Graphic VII - mRNA PSD-95 levels in control and patient R in differentiated neurons, *p<0.05



Graphic VIII - mRNA Shank3 levels in control and patient R in differentiated neurons, *p<0.05

Analysis of mGluR5 levels in differentiated neurons

The result of mGluR5 levels obtained by patient R through western-blot was confirmed by immunofluorescence (Figure 16).

The mGluR5 intensity was calculated using ImageJ software and were analyzed 18 different neurons (Graphic IX).

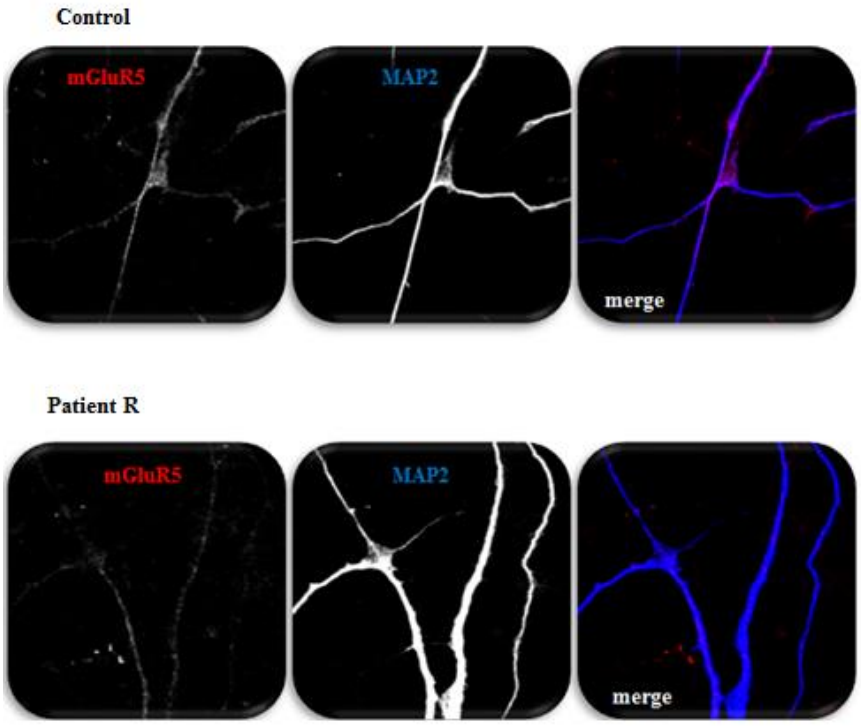
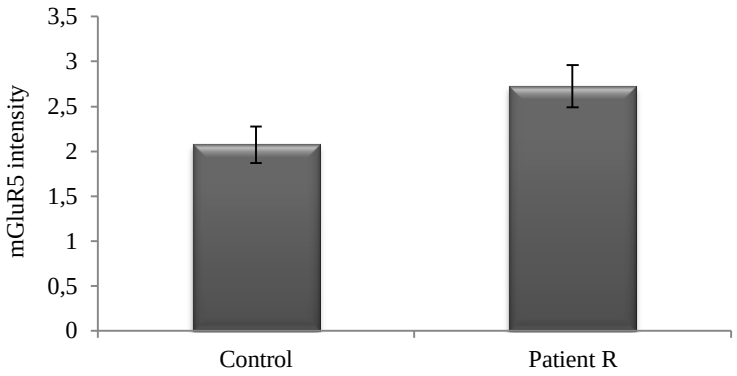


Figure 16 - mGluR5 and MAP2 levels in differentiated neurons of Patient R and Control by immunofluorescence assay



Graphic IX - mGluR5 intensity levels in control and patient R measured by ImageJ software

Analysis of Synaptic Maturation in Phelan-McDermid Patients

To follow up synapses formation in patient R and control we infected NSCs with a lentivirus expressing Homer-GFP and MAP2 antibody was used in an immunofluorescence assay (Figure 17).

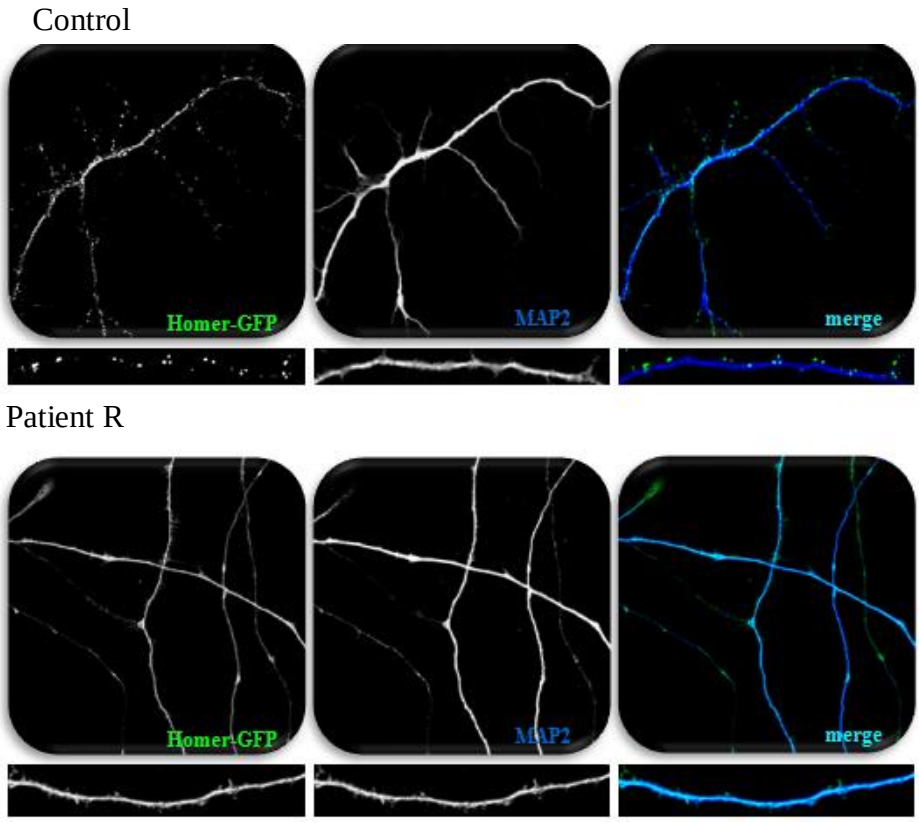
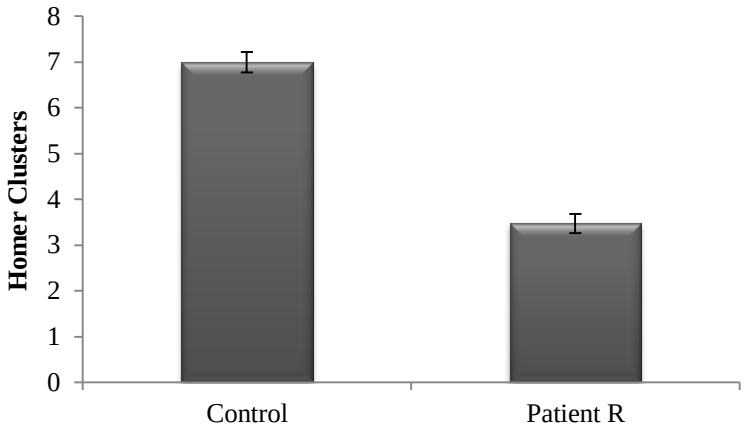


Figure 17 - Evaluation of synaptic clusters in NSC from Control and Patient R infected with lentivirus expressing Homer-GFP.

The immunofluorescence results indicate that Homer is much more spread in patient R comparatively to control because in the control Homer-GFP forms clusters that are located at synapses. These results lead to the conclusion that in patient R there are some defects in synapse maturation.

We also count the number of Homer clusters in neurons, either in patient and control (Graphic X).



Graphic X – Number of Homer Clusters counted obtained by immunofluorescence assay.

Analysing the results, we found a significant reduction in Homer clusters in Patients R neurons in comparison to Control neurons confirming a defect in synapses formation

Mice Genotyping

Along with patients' analysis, we have been also working with mices.

Professor Tobias M. Böckers, PhD MD working at the Institute of Anatomy and Cell Biology in Germany has gently given Shank3 mice to our group at Neuroscience Institute - CNR, Milan.

As described in Material and Methods chapter, we cut a little part of the mice tails and execute the DNA extraction from it. After, the DNA extracted is combined in a master mix and a normal PCR is performed. The DNA amplified runs into a 2% agarose gel with SYBR green and then, the results are analyzed (Figure 18).

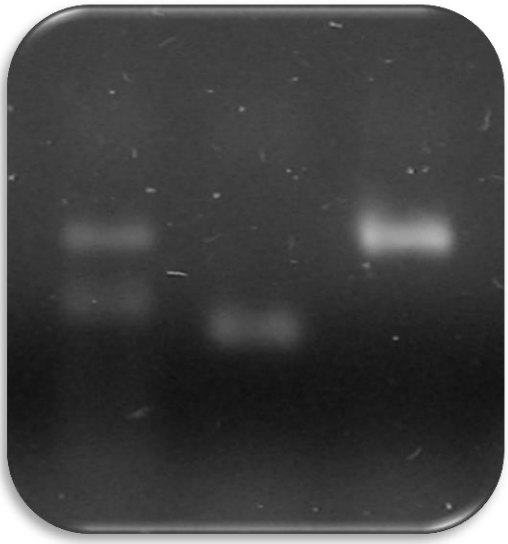


Figure 18 - PCR from mice genotypization to identify heterozygous, knock out and wild-type mice, respectively

After the gel running is finished, the gel is exposed to an UV light and thanks to SYBR green is possible to see the DNA bands. We had better results by doing two different master mixes instead of mixing all the primers together. Therefore, there is one mix to detect wild-type sequence band and other to detect knockout sequence band so, in this manner, we will use two wells in the agarose gel for each mouse DNA.

The gel presented, as an example, demonstrates all the possible results. The two first wells represent the absence of DNA sample, used as control negative to exclude any kind

of possible contamination that could influence the comprehension of the results. The third and fourth bands represent a heterozygous mouse because its DNA has a higher band, the wild-type band (360kb), as well as a lower band, the knockout band (190kb). The fifth band is higher so, is bigger meaning, no deletion is present as explained previously in Material and Methods chapter and the absence of a lower band, indicates that this mouse has wild-type genotype. Finally, and by the same way of thinking, the last two bands represent just one mouse and because there is only the shorter band, the deleted band, this mouse is knockout.

From the moment we have genotyped the mice, we organize them all and select them to perform electroencephalography, electrophysiological assays, behavior procedures and *in vitro* assays.

Discussion

The ProSAP/Shank family proteins are composed by three different proteins, Shank1, Shank2 and Shank3 with distinct expression patterns among the individual members. In our study, we focus the investigation in Shank3 gene. The Shank3 gene is deleted in Phelan-McDermid Syndrome patients leading to Shank3 protein haploinsufficiency. Here we studied the Shank3 role in synaptic function to better understand why alterations in this protein can cause all the neurological features presented by Phelan-McDermid Syndrome patients.

In preliminary data, we used a lentivirus vector expressing shRNA for Shank3 in order to silence all the main Shank3 splicing variants in mice hippocampal neurons and in this manner to characterize what happens when Shank3 gene is silenced. The expression of Shank3 was studied using an antibody against the C-terminal and other against the N-terminal and we demostrated that the shRNA for Shank3 was able to specifically silence all the major Shank3 isoforms without interfering with the expression of the other two Shank genes Suggesting that there is no compensatory mechanisms among the Shank protein family members at least in neuronal cultures.

Although we didn't find any major alterations in pre and postsynaptic compartments after Shank3 silencing we found that mGluR5 levels was significantly decreased when analyzed by Western-Blot assay but not by RT-PCR assay, suggesting that the mGluR5 protein, but not the mGluR5 mRNA, was expressively reduced.

These studies suggest that Shank3 deletion specifically alters synapse function by altering mGluR5 expression. We thus decided to verify this hypothesis by analyzing PMS patients neurons obtained by reprogramming patient fibroblasts into iPS cells and subsequently differentiated into NSC and finally, into neurons.

The Fibroblasts were reprogrammed to induced pluripotent stem (iPS) cells by lentivirus infection with four reprogramming genes OCT4, c-MYC, SOX2 and KFL4. The iPS clones have a typical morphology and as demonstrated in the Results Chapter, are positive for alkaline phosphatase through immunohistochemistry assays and express pluripotent markers such as Oct4, Nanog and Tra-1-81 observed by RT-PCR as well as immunofluorescence techniques. Besides, iPS cells are able to differentiate into the three germinative layers, which are endoderm, ectoderm and mesoderm.

The iPS cells differentiation was achieved by inducing the formation of Embryoid Bodies (EBs) from Human iPSC colonies at passage five. Rosette-like differentiation of attached EBs was obtained after 5-7 days in culture and rosettes expressed on the surface the early neural precursor marker Nestin. All purified hNPs obtained by differentiated rosette were positive for the neural cell markers Nestin, Pax6 and Sox2 and the majority of the cells were positive for Ki67 strongly demonstrating that they are self-renewable. βTubIII positive cells were detected, according with previous reports. On the contrary, hNPs did not express the pluripotency markers Nanog OCT3/4 and Tra-1-81, in addition we did not find cells positive for the neuron specific protein MAP2 and for αSMA, a marker of myoepithelial cells.

Based on these results, we can conclude that we were able to obtain human neuronal precursors that can be used for further neuronal differentiation.

The NSC differentiation was first achieved by co-culturing NSCs, infected with GFP expressing lentivirus, with rat primary cortical neurons. At DIV 60, at the end of differentiation, the NSCs were positive for NeuN and hNA antibodies. Neuronal Nuclei (NeuN) antibody recognized almost exclusively a nuclear protein expressed in the nervous system and its expression is related to neuronal differentiation. On the other hand, hNA antibodies recognized only human nuclei and were used to verify the presence of Human cells, derived by NSCs differentiation, from the co-cultured rat primary cortical neurons.

We also used MAP2 as well as VGLUT1 and synaptophysin antibodies to better characterize terminal neuronal differentiation of NSCs.

After observing the correct NSCs differentiation into neurons, we also analyzed synaptic formation during the differentiation process. In this case, the NSCs were infected with lentivirus that expressed Homer1-GFP under cytomegalovirus promoter. At DIV36 and at DIV60, the cells were fixed to perform immunofluorescence studies using MAP2 and VGLUT1 antibodies.

Homer1 can generate three alternatively spliced mRNA that encode for Homer1a, Homer1b and Homer1c proteins. While Homer1b and 1c are constitutive, Homer 1a is induced by neuronal activity, regulates spine morphogenesis decreasing neuronal responsiveness when the input is too high in order to maintain homeostatic plasticity (Sala et al., 2003). Looking at the immunofluorescence assays we see that at DIV36 Homer-GFP

was still distributed all over the dendritic compartment indicating that synapse differentiation in these neurons was not complete yet although the expression of MAP2 was clearly present also at DIV36. Only at DIV60, Homer-GFP was found clustered at excitatory synapses indicating that the synapse differentiation was achieved.

Since Shank3 is not only expressed in neurons but also in other tissue, we started our study in Phelan-McDermid Patients by investigating Shank3 presence in fibroblasts. Interestingly we found that not only Shank3 is expressed in fibroblast but also the expression level was different among different PMS patients. On opposite, mGluR5 and MAP2 both exclusively neuronal proteins were not expressed in all the fibroblasts obtained from the patients.

After the NSC differentiation was characterized, our next step was to compare differentiated neurons at DIV60 obtained by PMS patients with neurons obtained by healthy individuals (control). We found that, Shank3 protein expression is decreased in patient R relatively to control however, it was possible to see with C-terminal antibody that two of the three Shank3 isoforms did not present significant changes when compared to control levels.

Interestingly these data suggest that PMS patients may differentially express some Shank3 isoforms. However whether different Shank3 isoforms expression in patients may lead to diverse clinical phenotypes remains to be determined (Wang X et al., 2011) (Zhu L, 2011).

Because we previously showed that Shank3 knock down in cultured neurons induces a specific reduction of mGluR5, we looked at the expression of mGluR5 and other synaptic proteins in differentiated neurons derived by iPS cells of patients R. Surprisingly we found that in patient R mGluR5 expression is higher than in control neurons. Increased expression of mGluR5 is also associated to a decreased expression of Homer1 (Homer1a isoform). These results were confirmed by immunofluorescence assay using an antibody against mGluR5 and other against MAP2. The mGluR5 protein intensity was higher in patient R neurons than in control neurons. These data confirmed alteration in mGluR5 protein levels in PMS patients and suggested a deregulation of the mGlur5-Homer-Shank3 protein complex.

Recent evidences indicate that spine density is inversely associated with cognitive function and these results are related to the fact that ASD patients exhibit elongated, dense and twisted spine morphologies (Penzes P et al., 2011). These abnormal spine characteristics lead to deficient circuits that may be the ones responsible for the socio-cognitive impairments typically present in autistic spectrum disorders.

As described previously, Homer is a scaffold protein located at postsynaptic density. Shank promotes maturation of dendritic spines and expansion of spine heads because its capability of enroll Homer into postsynaptic spots (Sala et al., 2001).

To follow up synapses formation in patient R and control we infected NSCs with a lentivirus expressing Homer-GFP and we found that Homer is much more spread in patient R comparatively to control and Homer-GFP clusters are reduced in patient neurons. These results strongly suggest that absence of Shank3 is related with some defects in synapses maturation.

Conclusion

Shank protein (**SH**3 domain and **ank**yrin repeat containing proteins) family is constituted by Shank1, Shank2 and Shank3. These three molecules are scaffolding proteins with multiple domains for protein-protein interactions binding to a variety of membrane and cytoplasmic molecules.

Our specific protein in study, Shank3, is haploinsufficient in Phelan-McDermid syndrome patients due to deletions in the long arm of chromosome 22 so, this syndrome is also called, 22q13 deletion syndrome.

To a better understanding of the mechanisms implicated in this syndrome, we have come up with two different models to study, knockout mice and human patients. Since, the directly study of the brain is impossible in *in vivo* situations, the patients' fibroblasts were reprogrammed into induced-pluripotent stem cells to posteriorly be differentiated into neuron stem cells and finally, neurons with every step successfully confirmation by precise neuronal markers.

The Shank3 protein characterization in patients was done firstly, in fibroblasts and after, in neuronal stem cells and differentiated neurons at DIV60. Patients express different levels of protein Shank3 probably due to differences in mutations leading to possible different phenotypes among them.

Neurons derived from Patient R with Shank3 haploinsufficiency show increased mGluR5 protein levels and decreased Homer protein levels suggesting that Shank3 haploinsufficiency lead to deregulation of the mGlur5-Homer-Shank3 protein complex. Finally, reduced levels of Shank3 protein cause defects in synapses maturation indicating poor neuronal communication that lead to socio-cognitive impairments.

Taken together our data show that iPS cells represent a useful model to study the role of Shank3 in the pathogenesis of PMS. Indeed we have shown that mGluR5 expression is altered in PMS patients and this should be correlated with defects that we found in neuronal differentiation and synapses maturation observed in neurons derived from PMS patients.

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