Neuron-Schwann cell cultures as platforms to study new therapeutic targets in the peripheral nervous system

**Background:**

The membrane glycoprotein M6a (gpm6a) -together with proteolipid protein (PLP), DM20 and M6b- belongs to the tetraspan PLP family. M6a is a neuronal surface protein that promotes neuronal stem cell differentiation, migration, neurite outgrowth, filopodia/spine induction and synapse formation in primary neuronal cultures and (non) neuronal cell lines\(^{(1-11)}\). In humans, alterations in M6a levels or single nucleotide polymorphisms (SNPs) in GPM6A, are associated with depression, schizophrenia, claustrophobia, bipolar disorders and learning disabilities\(^{(12-16)}\). In mice, variations in gpm6a expression are linked to chronic stress/depression, claustrophobia and Alzheimer's disease\(^{(13,17,18)}\). Likewise, M6a cooperates with PLP and M6b in regulating membrane growth of oligodendrocytes and neurons, respectively\(^{(19,20)}\). Even though the peripheral dorsal root ganglion neurons express M6a, the role of M6a outside the central nervous system remains uncharacterized\(^{(21,22)}\).

According to topology predictions, M6a and PLP family members share structural similarity with tetraspanin protein family members containing four transmembrane domains (TMs), two extracellular loops (EC1 and EC2), and their N- and C-terminus facing the cell cytoplasm\(^{(23)}\). Tetraspanins are ubiquitous molecules involved in cell adhesion, migration, proliferation and differentiation via cell–cell, matrix–cell and lateral associations. Mutagenesis studies of the extracellular loops of tetraspanins demonstrated that they are crucial for the specificity of protein-protein interactions. Regarding M6a, blocking M6a-loops with a monoclonal structural antibody (M6a-mAb) arrests neurite extension and synapse formation in neurons\(^{(3, 23)}\). Moreover, certain cysteine residues within the EC2 of M6a are functionally crucial sites for M6a folding and filopodia formation\(^{(24)}\). In summary, we and other groups proved the functional value of M6a-loops in neuronal plasticity\(^{(3, 7, 23-27)}\). However, the complete mechanisms of action of M6a and the proteins associated with it are unknown.
Based on the experimental evidence and our previous work, our hypothesis is that a **broad range** of M6a’s functions depend on its specific interactions through its extracellular loops in each particular cell and in a particular stage of development. In this regard, the main goal of my PhD thesis is to seek for endogenous proteins that could functionally interact with M6a’s extracellular loops. For that purpose, we performed a coIP assay- between recombinant M6a-loops and cellular extracts from hippocampus- followed by quantitative TMT-MS, (Proteomics Core Facility-EMBL). A list of proteins highlighted by our proteomics approach followed by manual curation and enrichment analysis (Toppgene, Panther, GeneOntology and KEGG) allowed us to identify proteins that are candidates to interact with M6a-loops. In fact, a great number of proteins identified such as: PLP1, MOG, MBP, VNP, and CNTN1 are glial-specific not only for central myelinating glia but also for Schwann cells (SCs), the peripheral nervous system (PNS) glial cells, Figure 1.

**Figure 1: Identification and quantification of M6a-loops interactors.** A) Heatmap representation of peak intensity-based quantification of the three independent coIP coupled to TMT-MS identification. Heatmap of significantly changed proteins with fold change > 2.5 and the average top3 more than 7 equivalent to P < 0.01. B) and C) PLP and piccolo (PCLO) proteins were assessed in a co-localization assay. Representative immunofluorescences performed in B) M6a-RFP and PLP1-GFP co-expressing N2a cells and C) hippocampal culture neurons at 15 DIV showing endogenous levels of M6a (red) and piccolo PCLO (green). Cells were observed with a confocal microscope with a 60x oil-immersion objective. The yellow color in the magnifications demonstrates the co-localization of M6a with PLP and dots of colocalization between M6a and PCLO.
As mentioned previously, there is no evidence supporting a role of M6a in the PNS; however, our preliminary data suggest that M6a could play a role in the PNS as key regulator of sensory neuron and Schwann cell interactions. Therefore, it is possible that M6a interacts with membrane proteins expressed on the surface of Schwann cells. Hence, to validate in M6a-loop interacting proteins, we aimed to perform a PNS co-culture model to study neuron-glia interactions. These co-cultures between dorsal root ganglion (DRG) neurons and Schwann cells recapitulate many of the sequential features of peripheral myelination observed in vivo including the alignment of Schwann cells to axons, the ensheathment of axons by Schwann cell processes, the formation of a basal lamina and finally the wrapping of myelin membranes\(^{(28-30)}\). Hence, to perform co-cultures of DRG neurons with Schwann cells is a good platform to study interactions between proteins. Therefore, the main goal of this project was to establish and characterize a model for the study of M6a-protein interactions in a co-culture of DRG neurons and Schwann cells.

**Methodology:**

DRG explant cultures were established from embryonic 15 days Sprague Dawley rats and maintained for up to 3 weeks in culture to allow the extension of neuronal axons and Schwann cells migration and proliferation. We used heregulin supplemented culture medium to enhance the proliferation of SCs as heregulin is a potent mitogenic factor. Once the co-culture was settle we characterized our system by immunofluorescence and confocal microscopy to detect axons from the neurons and proteins within the Schwann cells surface (figure 2)
Summary of Results:

The first step was to learn how to prepare and maintain primary cultures of DRG neurons and Schwann cells (SCs). For that purpose, Dr Monje, who has plenty experience in co-cultures, trained me to set up all experiments. This means, she showed me how to carry out the dissections of the animals, prepare the tissue and culture media for maintaining the DRG explants in culture for up to 3 weeks before doing the immunofluorescence experiments. Figure 3 shows how this DRG explants cultures grow during this period of time. The image shows a DRG explant from a week in culture. In the panels A and C we can observe the centre of the ganglia and how the axons from the DRG neurons emerge from there. The DRG neurons somas remain at the ganglia (fig. 3 A and C). In panels B and D we can observe how the Schwann cells are attached to the axons and also attached to the plate surface; giving the support to the axons to continue to grow.
Once I developed my skills to established and maintain the DRG explant cultures at Dr. Monje’s lab, we characterized the DRG explant culture by doing immunofluorescence and microscopy analysis. For that purpose, we cultured DRG explants for the mentioned period of time and immunolabelled them with antibodies that recognize proteins within the DRG neurons axons, and proteins that are express at the cell surface of Schwann cells. Figure 4 shows a 21 days in culture DRG explant labelled for Schwann cells processes (p75, green) and DRG neurons axons (NF, neurofilament, magenta). The image shows a region of the explant far from the DRG ganglia, where we can observe how the axons and Schwann cells are in close contact between each other. In this sense, figure 5 (upper panel) shows a DRG explant at low magnification, were the DRG ganglia can be observed. The image shows the DRG axons emerging from the ganglia and Schwann cells attached.

**Figure 3:** Develop of the DRG explants after 1 week in culture. The images were taken with a 10X objective from a phase contrast microscope, under sterile conditions.
to the axons. Schwann cells were labelled with a specific marker for these cells, S100β (green) and DRG axons were labelled with neurofilament (magenta). The DRG neurons and Schwann cells nuclei were labelled with Dapi (magenta). The bottom panel shows a magnification with the 60X objective were Schwann cells are attached to the DRG axons. These experiments allowed us to establish a co-culture model to study proteins that are expressed within the membrane of the DRG neurons axons.

**Figure 4: DRG explants after 3 weeks in culture.** The images were taken with a 10X objective fluorescent microscope. The upper panel shows Schwann cells labelled with the surface protein p75 and DRG axons labelled with neurofilament (magenta).
Figure 5: DRG explants after 3 weeks in culture. The images were taken with a 20X and 60X objectives with an Olympus FV1000 confocal microscope. The upper panel shows Schwann cells labelled with anti-S100β antibodies (green) and DRG axons labelled with anti-neurofilament antibodies (magenta). The DRG neurons and Schwann cell nuclei were labelled with Dapi (magenta). The bottom panel shows a magnification with the 60X objective were Schwann cells are attached and in close contact with the DRG axons.
Summary of Results:

During this project, I developed a co-culture approach method to study protein-protein interactions between DRG neurons and Schwann cells. First, we developed the protocols for the maintenance of the co-culture for long periods of times and finally, we characterized by immunofluorescence our co-culture system.

My training at Indiana University (IUPUI) under Dr. Monje’s supervision gave me the opportunity to learn how to master new culture systems of stepwise complexities that were not established in my lab in Argentina at the time this project began. In Dr. Monje’s lab, I developed the protocols for studying protein-protein interactions in a PNS model of DRG and Schwann cells co-culture by immunofluorescence and confocal microscopy. At the present moment I am developing these same techniques in my home Institution, IIBio-UNSAM-CONICET under the supervision of my PhD advisor, Dr. Camila Scorticati. We found good preliminary data about the protein we particularly studied in this co-culture system and we hope to have our first results by the end of the year.

Also, during my stay in Dr. Monje’s lab, I also collaborated with on-going projects at the lab and I participated in the writing of a research article that were recently published in Biological Procedures on line in which we acknowledged the ISN Society for the support given to me to perform this stay at Dr. Monje’s lab. You will find some pictures of me with Dr. Monje and other members of the lab at IUPUI at the bottom of this report and also attached in the email.


Finally, I want thank the ISN and CAEN committee for your support. This funding not only contributed to my field of work but also to my personal development as a young scientist. Not only I developed new lab skills but also, I performed research in a new lab, with new and friendly colleagues and in another country. So, for me it was a great experience from both work and personal aspects. I will encourage my lab partners in Argentina to apply for these opportunities.
References

Dr. Monje and myself at the lab.