CAEN Awardee full report

This is the CAEN award full report of Tomás Roberto Carden, who has been awarded a category 1A (Visit by the applicant to another laboratory) grant.

Dates

- Starting date: April 1st, 2018
- Finishing date: May 1st, 2018

Background

The maintenance of functional activity in the nervous system (NS) depends on the activity of neurons. These cells are the signaling elements of the NSand, although there are hundredsof different types, all neurons share certain basic morphological characteristics which underlie their capacity to transmit information: they show a round soma from which two different types of processes emerge, axons and dendrites. Dendrites are relatively short processes which receive information from other nerve cells; axons, instead, are typically longer than dendrites and conduct electric signals to other neurons. The differentiation process of neurons *in vitro* consists of a series of quite stereotyped phases which werefirst described by Dottiand colleagues in 1988. In their seminal paper, the authors state that the initial spheric morphology is broken by the emergence of buds which later become neurites; after 24 hours in culture, all neurites look alike and have a similar length. On the second day in culture, one of the processes starts to grow differentially. After 3 days in culture, such process elongates still further while the other neurites remain stable. By the fifth day in culture, the identity of each process has become clear, with the longest one constituting the axon and the shorter ones becoming dendrites. All these phenotypic changes are underlain by alterations in the profile of genetic expression, which has allowed the identification of certain proteins to be used as antigen markers of specific stages of lineage progression.These marker proteins are a useful tool to evaluate the degree of neuronal differentiation. Thus, the expression of doublecortin (DCX) can be used to identify immature neurons, while the expression of NeuN can be used to identify mature ones.

Iron is an essential nutrient for all cell types. It is indispensable to broad biological processes such as mitosis, DNA synthesis and electron transport during cell respiration. Moreover, it is required as a cofactor by many enzymes, thus affecting multiple biological processes. In the NS in particular, iron is involved in the synthesis of neurotransmitters and the formation of myelin. Besides, it has a fundamental role in the development of the NS, as shown by the negative effects its gestational deficit has on synaptogenesis and myelinogenesis.

Neurons incorporate extracellular iron mainly through transferrin (Tf), a glycoprotein of approximately 80kDawhich can bind up to two iron atoms reversibly with great affinity. Once loaded with iron, Tf binds to transferrin receptor 1 (TfR1) and the complex they form is internalized by an endocytic process. The presence of a proton pump within the endosome membrane produces the acidification of its internal medium, which reaches a pH of 5.5. This acidification introduces conformational changes in the Tf molecule that result in the release of iron atoms inside the endosome. Once free, iron must be reduced in order to be exported to the cytoplasm by divalent metal transporter 1 (DMT1). Finally, the Tf-TfR1 complex is recycled to the plasma membrane and Tf is released to the extracellular space, becoming available again to start a new cycle.

While the role of Tf in iron metabolism has been broadly studied, this protein has also attracted the attention of many groups due to the effect it exerts on cell proliferation and differentiation in many tissues. In fact, it has been described many times as a trophic factor. In particular, regarding de NS, work from our lab has shown that Tf treatment accelerates the maturation of the oligodendroglial lineage.However, the effects of Tf treatment on neurons have never been studied, which is why we intend to investigate them in the current project. Moreover, given that Tf and DMT1 work jointly in the process of iron acquisition, we propose there could be a link between the expression of this transporter and neuronal differentiation.

Hypothesis

Treatment of neuronal cultures with aTf, an iron carrier which has been characterized as a trophic factor in the central nervous system, induces maturation. If the effects observed are linked to iron metabolism, the silencing of DMT1 expression can also affect cell differentiation.

Objectives

Objective 1 – Evaluate the effects of aTf treatment on the differentiation of cortical neurons *in vitro*.

It has been reported that Tf acts as a trophic factor on a wide variety of tissues; in particular, our own group has shown that aTf accelerates the process of oligodendroglial differentiation. These previous results suggest that aTf could also act as a pro-maturational factor on the neuronal lineage. Thus, we propose to evaluate whether aTf treatment exerts any effects on the differentiation process of cortical neurons *in vitro*.

The degree of differentiation will be immunocytochemically assessed using primary antibodies which recognize proteins used as antigen markers of specific stages of lineage progression. In this way, immature neurons will be recognized through the expression of DCX and mature neurons will be recognized through the expression of NeuN. The proportion of both populations will be calculated normalizing to total cell number. Moreover, a morphological study will be performed calculating the area occupied by processes stained with neurofilament and microtubule-associated protein 2 (MAP2).

Objective 2 – Evaluate the effects of DMT1 silencing on the differentiation of cortical neurons *in vitro*.

Tf and DMT1 work together in the process of iron internalization and neurons have been previously reported to express DMT1 (Skjorringe et al., 2015). Given that iron is essential for different metabolic processes, we hypothesizethere might be a link between DMT1 expression and neuronal differentiation. To test this hypothesis, we will transiently silence the expression of DMT1 using siRNAs against its mRNA in cultures of cortical neurons and evaluate the degree of neuronal differentiation through immunocytochemistry assays as already described.

Materials and methods

Primary culture of cortical neurons

Cortical neurons were prepared from the brains of 1 to 2-day-old mouse pups using established procedures with minor modifications. Briefly, brains were removed aseptically and placed into DMEM/F12 (Life Technologies). After the brains were dissected, the blood vessels and meninges were carefully removed under a dissecting microscope. Brain cortices were isolated and dissociated by digestion with a solution of 0.05% trypsin (Sigma) containing 0.06%DNase I (Sigma) in Neurobasal medium (Life Technologies) for 10min at 37°C. The digestion reaction was stopped with Neurobasal medium containing 10% fetal bovine serum (Omega Scientific) and triturated by repeated passages (20 times) through a 10ml pipette. The cell suspension was filtered through a sterile cell strainer of 70μm (BD Biosciences) into a 50ml centrifuge tube. The cells were pelleted by centrifugation at 200g for 5min and resuspended in Neurobasal medium plus 2% (v/v) B27 (Life Technologies) supplemented with 0.25mM GlutaMax I (Life Technologies), 0.25mM glutamine (Life Technologies), and 100μg/ml gentamicin (Omega Scientific). Two-thousand cells/mm^2 were plated onto 12mm glass coverslips coated with poly-D-lysine (Sigma). The neurons were kept at 37°C in 95% air 5% CO_2 for 8 and 12 days.

aTf treatments

An aTf sterile 50× stock solution 5 mg/ml was used to treat cultures at a 100 μg/ml final concentration. After treatment, neurons were cultured for 4 and 8 days. Media were replaced every three days during treatment. All the aTf treatments were performed in parallel with control cultures lacking aTf.

siRNA knockdown of DMT1

Neurons were transiently transfected with a combination of four different siRNA duplexes (ON-TARGET plus SMART pool siRNAs, Thermo Scientific) specific for DMT1. Briefly, 6pmol of each siRNA duplex were mixed with Lipofectamine TM RNAiMAX (Life Technologies) and the mixture was added to the coverslips containing neurons 4 days after plating them. After transfection, cells were cultured for 4 and 8 days.

Immunocytochemistry

Cells were stained with antibodies against several neuronal markers and examined by confocal microscopy. Briefly, cells were rinsed in PBS and fixed in 4% buffered paraformaldehyde (Sigma) for 20min at room temperature. After rinsing in PBS, the cells were permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 2min at room temperature. Fixed cells were incubated in a 5% goat serum blocking solutionin PBS followed by overnight incubation at 4°C with primary antibodiesagainst DCX (Santa Cruz), NeuN (Millipore), MAP2 (Millipore), neurofilament M (Millipore), Ki67 (Abcam), activated caspase-3 (Cell Signaling). Cells were then incubated with the appropriate secondary antibodies (Jackson), nuclei were stained with fluorescent dye DAPI (Life Technologies), mounted onto slides with Aquamount (Thermo Scientific), and fluorescent images were obtained using a Olympus spinning disc confocal microscope (Olympus, IX83-DSU). Quantitative analysis of the results was carried out counting the antigen-positive and total DAPI-positive cells in 20 randomly selected fields, which resulted in counts of >2000 cells for each experimental condition. Counts of antigen-positive cells were normalized to the counts of total DAPI-positive cells for each condition. For some antigens, the total area covered by processes in a $1mm^2$ was assessed in 20 randomly selected fields by MetaMorph software (Molecular Devices) using a built-in auto inclusive threshold.

Results

aTf treatment does not affect cell death or proliferation

Ki-67 is a nuclear protein used as a marker of cellular proliferation. aTf treatment did not affect the proportion of Ki-67-positive cells at neither time analyzed (Fig 1. A).
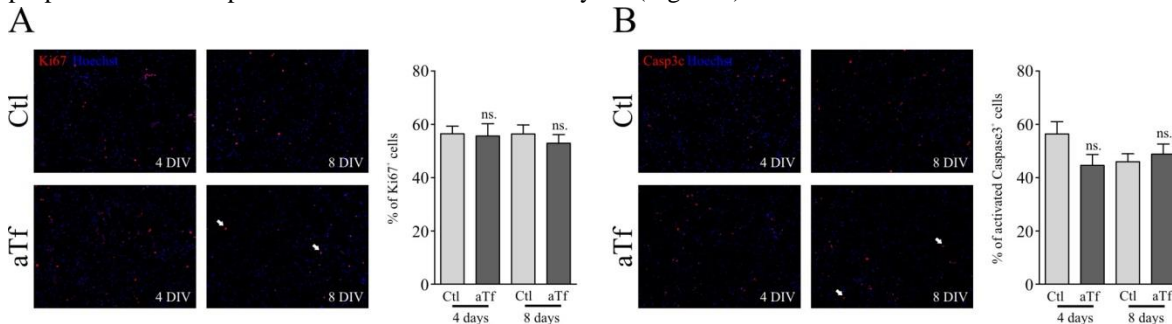

Fig. 1 – (A) Microphotographs showing Ki67+ neurons at 4 and 8 days post-treatment. Cell proliferation was calculated as the percentage of Ki67+ cells in each experimental condition. (E) Microphotographs showing caspase-3+ neurons at 4 and 8 days post-treatment. Cell death was evaluated by calculating the percentage of caspase-3+ cells in each experimental group.

aTf treatment promotes cortical neuron differentiation in vitro

Also a microtubule-associated protein, DCX is expressed by neuronal precursor cells and immature neurons. Downregulation of DCX occurs at the same time that these cells begin to express NeuN, a marker for mature neurons. Thus, the relative expression of both these proteins can be used to assess the degree of neuronal differentiation. As expected,in our cultures the expression of DCX decreased while the expression of NeuN increased over time, as seen when comparing 4 vs. 8 days; this result confirms that neurons are actually differentiating *in vitro*.Regarding our treatment, aTf addition to the culture medium produced a decrease in the proportion of DCX-positive cells at 4 as well as 8 days after treatment. Furthermore, the proportion of NeuN-positive cells was significantly higher in aTf-treated cultures at both time points analyzed (Fig. 2, A and B). These data suggest that aTf treatment further promotes neuronal differentiation *in vitro*.

Besides, the area occupied by MAP2 and neurofilament increased significantly over time, which suggests that neurons are maturing morphologically, but remained unaffected by aTf treatment(Fig. 2, C and D).
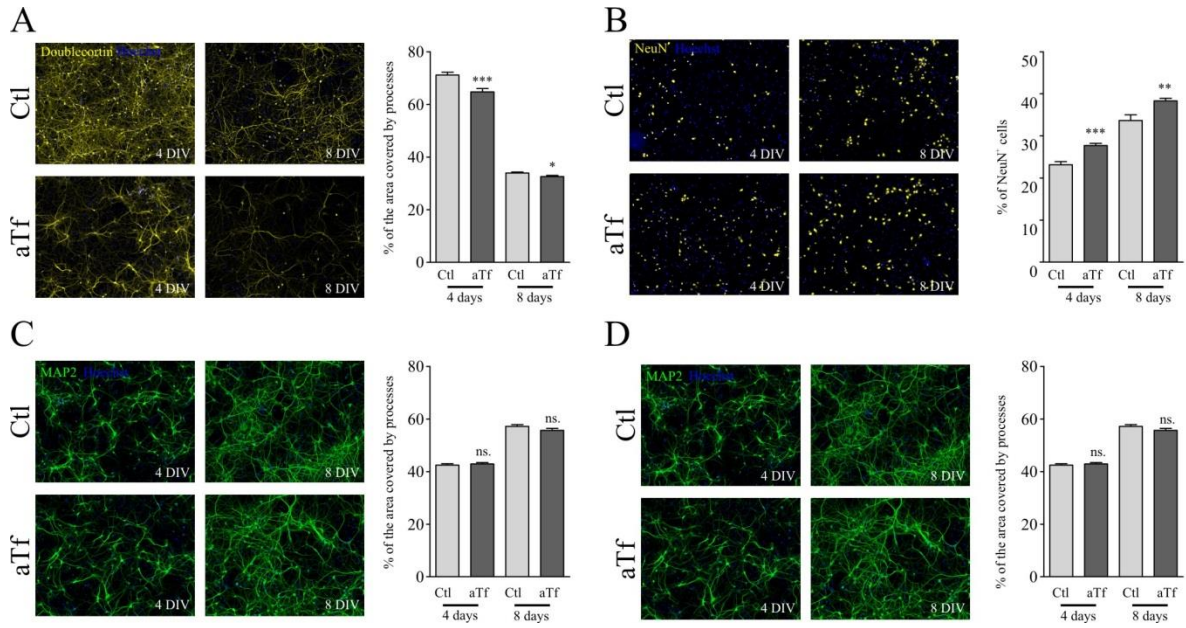
*Fig. 2 – Microgr aphs showing control and treated neurons for 4 and 8 days stained with antibodies against DCX, NeuN, MAP2 and MAP2 (A, B, C and D). For NeuN the proportion of positive cells was calculated for each experimental condition. For DCX, MAP2 and NFs the area occupied by positive cells was determined for each condition. Values are expressed as mean ± SEM of 5 independent experiments. *p<0.05, **p<0.01, ***p<0.001 vs. respective controls.*

DMT1 silencing effects on cortical neurons

Regarding DMT1, all results are presented in Figure 3; however, they are only preliminary and further experiments are currently underway in order to confirm silencing efficiency, given that neither the area of DMT1$^+$ cells nor the IOD DMT1 signal decreased upon treatment suggesting that silencing was ineffective (FigXX).
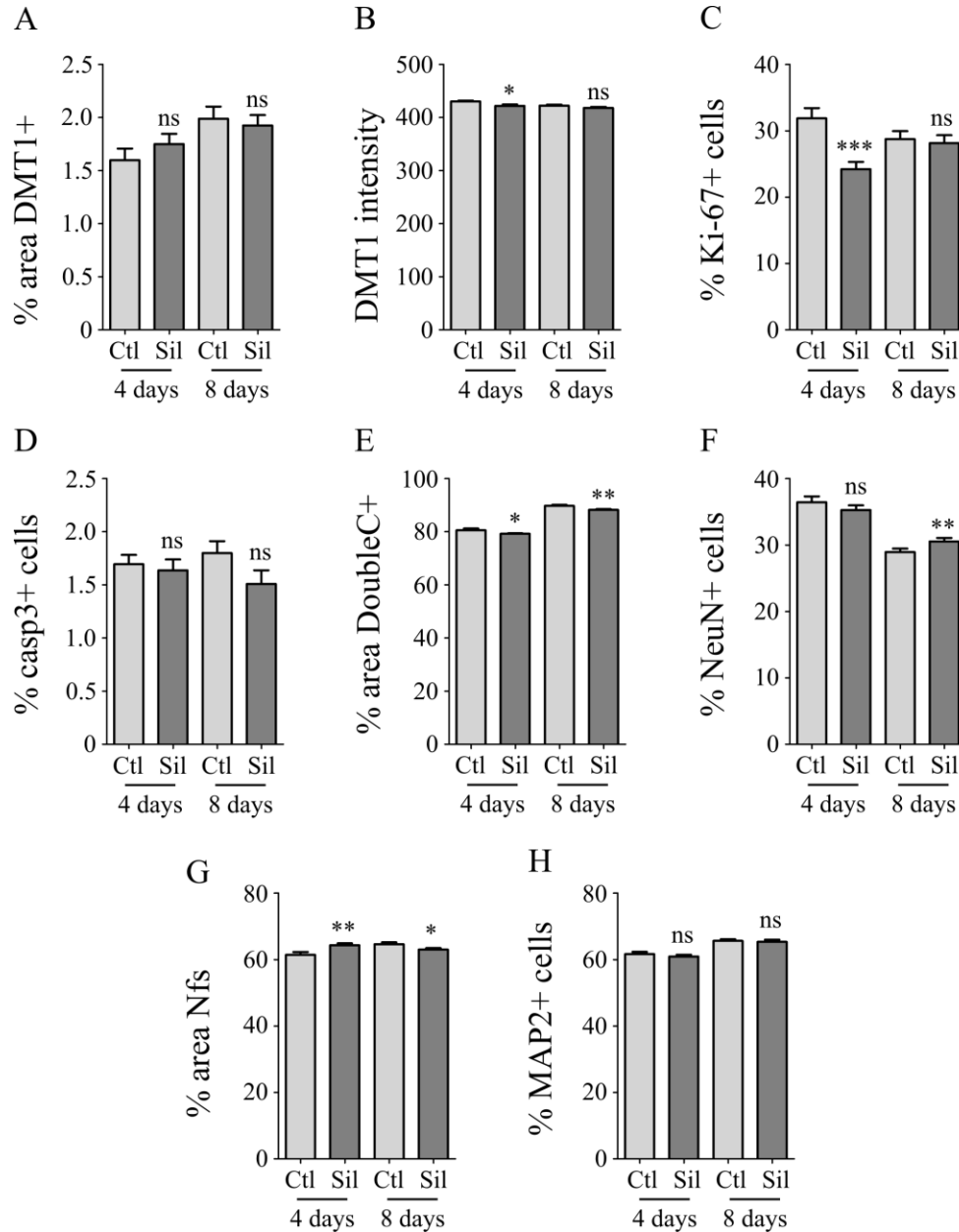

Fig. 3 – Quantifications of mcirographs showing control and DMT1 silenced neurons for 4 and 8 days. The area and intensity of DMT1 were calculated for each condition (A and B), the proportions of Ki67$^+$ and activated caspase 3 (C and D) cells were determined for each condition. Control and silenced neurons for 4 and 8 days were stained with antibodies against DCX, NeuN, MAP2 and MAP2 (E, F, G and H), the proportion of NeuN$^+$ and $^{MAP2+}$ cells were calculated and the area occupied by DCX$^+$ cells and NFs$^+$ cells were determined.

Conclusions

Regarding aTf treatment, our results show that it acts as a pro-differentiation factor, as can be seen by the enhanced expression of NeuN and decreased expression of DCX in the aTf treated condition. However, this enhanced maturation could not be verified by morphological markers such as MAP2 or NFs expression; this could be due to the fact that morphological differences appear later or to the fact that those two particular proteins are not sensible enough to evince differences. Our results also showed that aTf treatment did not affect two classic cell parameters such as proliferation or apoptosis, as can be seen

With regard to DMT1 silencing, we are not yet in a position to conclude anything given that the effectiveness of silencing is not certain.

Outcomes and benefits

Besides the scientific results obtained, I consider that the travel experience has been highly positive for the development of my professional career. The visit to Dr. Paez´ lab gave me the possibility of getting to know how basic research in the field of neuroscience is performed in another country, an experience which will undoubtedly proved rewarding. Moreover the results obtained have a positive effect on the progress of my PhD project and the travel has allowed me to establish networking and scientific interchange.

Financial report

The amount of Argentine pesos (AR$)23,612.56 was spent on plane tickets, as can be checked with the receipt attached below. On that date (February 7th), that was the equivalent of U$D 1,192.

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C.U.I.T.: 30659514628
Ing. Brutos: 901-9907134
INICIO DE ACTIVIDADES: 15/03/1993

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Localidad: CORRIENTE	**Ing. Brutos:**	**Forma de Pago:** CONTADO
Cond. IVA: CONSUMIDOR FINAL	**CUIL:** 23338426704	**Grupo de Pax:** TOMAS ROBERTO CARDENx1

Detalle

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The remainder was spent on local transport (flight to Buffalo, NY),food and accommodation.

Letter from PI of the host laboratory

HJKRI–Hunter James Kelly Research Institute

August 13, 2018

To Whom It May Concern:

This letter is to certify that Tomás Roberto Carden visited our laboratory through the month of April of the current year. During his stay, Tomás performed some experiments in primary cultures of cortical neurons. He has made the interesting finding that transferrin, an iron-binding glycoprotein, promotes the morphological maturation of neurons in vitro. Tomás has found that primary and secondary dendrites from transferrin-treated neurons underwent more extension than controls, leading to a net growth of the dendritic arbor. Furthermore, transferrin treatment induces the expression of mature neuronal markers such as NeuN and decrease the synthesis of immature markers like Doublecortin. These results define the involvement of transferrin on cortical neurons maturation as well as the role played by this iron transporter during the early phases of neuron development. Since iron metabolism is essential for the development of neurons data from this project will be important to advance in new therapeutic strategies for neurodegenerative diseases such as Parkinson and Alzheimer.

To further characterize neuronal iron metabolism, Tomás conducted knock-down experiments using siRNAs for the Divalent Metal Transporter 1 (DMT1),a multi-metal transporter with a primary role in iron transport. Even though DMT1 has been previously described in the central nervous system nothing was known about the role of this metal transporter in neuronal maturation. He is currently analyzing the data and performing some control experiment to establish the role of DMT1 in cortical neurons.

In summary, Tomás visit was very productive and the initiation of an interesting research project and collaboration. Please contact me if you need further information.

Sincerely yours,

Pablo M. Paez
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