I N G E B I - C O N I C E T

Instituto de Investigaciones en Ingeniería Genética

y Biología Molecular "Dr. Héctor N. Torres"

Buenos Aires, February 28th 2015

ISN-CAEN Committee

Return Home GRANT 2014

– Progress report-

It is with great enthusiasm that I send this report to inform ISN-CAEN committee about the progress of my work during the year 2014. Despite the current difficulties that we experience in Argentina, thanks to ISN-CAEN support I was able to significantly advance in my research projects upon my return to Buenos Aires.

My team is currently constituted by one PhD student (awarded a doctorate fellowship from National Research Council 2014-2017) and two MSc students (from University of Buenos Aires).

Our group is interested in the study of neurodegeneration mechanisms with the aim to search for novel therapeutical approaches. We are specialized in the production of lentiviral vectors to modulate gene expression in the mouse brain. Technical facilities are now fully established in my lab, including stereotaxic delivery of vectors into the mouse brain and analysis of gene expression by qPCR, as well as behavioural analysis of mice. We are currently breeding three transgenic mice colonies that were shipped from a collaborator lab in the UK. We use these mice as experimental models to analyse behavioural and neurochemical phenotypes related to neurodegeneration. Thanks to the ISN-CAEN funds we were able to afford the purchase of reagents and consumables and the expenses of housing and technical services for mice maintenance. So far, we analyzed the behavioral and neurochemical consequences of Tau depletion and /or re-expression, using the Tau knock-out mice (*mapt* -/-) and the Htau mouse model, which carries a human *MAPT* gene in a *mapt* -/- background. We are currently testing

whether Tau isoforms imbalance influences some phenotypes that mimic cognitive impairments present in human tauopathies. Our ongoing research suggests a direct role of tau isoforms in neuronal function and point towards RNA reprogramming as a promising tool for therapeutical interventions in tauopathies. Part of our results were presented in the Argentina Society for Neurochemistry (SAN) annual conference in October 2014.

However, as our research involves the analysis of aged mice (12-18 months) we were not able to conclude yet all the experiments proposed for this project and we expect to complete this experimental work during the next six months. Hopefully, a first manuscript will be ready to send for publication by mid 2015 and the whole outcome of this project could be presented at the ISN biennal meeting in August 2015.

During this year I have also established and strengthened collaborations with local colleagues and I have been invited to give seminars in different institutes in Argentina.

In summary, I consider that this second year as an independent researcher in Argentina has been very fruitful, both in terms of results, training of human resources and scientific interactions. Although difficulties still exist, I feel myself able to tackle problems to focus on our main objectives, keeping my group motivated and optimistic. Personally, I am satisfied with the lab performance and particularly, I strongly appreciate the commitment and energy of Argentinean young students. I expect we will soon be able to publish our first article to consolidate our group in the Argentinean Scientific community and contribute to enhance the impact of Latin American neuroscience. Once again, I acknowledge ISN-CAEN Return Home Grant for this opportunity that helped me to begin my career as an independent researcher in my home country.

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ISN-CAEN Return Home Grant- Progress report

María Elena Avale

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Date awarded: February 2014

Title: Optimizing molecular strategies with potential therapeutical application in neurodegenerative diseases.

Part I: Modulation of Tau isoforms imbalance: role in Tau aggregation and neurodegeneration

Summary: This project seeks to investigate the relationship between Tau isoforms imbalance and the neurodegenerative processes present in human tauopathies. We use a mouse model lacking the murine Tau and carrying a human Tau transgene (htau mouse). This model develops Tau aggregates from 9 months old and cognitive impairments since 12 months old (Polydoro *et al.,* 2009). Based on previous neurochemical data available about hTau mice (Andorfer *et al.,* 2003) our current hypothesis is that the observed phenotypes are related to the imbalance in Tau isoforms due to alternative splicing of exon 10 in the human transgene in the mouse brain.

Goal: The final goal is to test a therapeutical strategy to restore Tau balance and decrease the neurodegenerative process and the concomitant cognitive deficit in hTau mice. This project has a potential implication for translational neuroscience, to set the grounds for future therapeutical approaches in human tauopathies.

Aims completed:

1- Quantitative analysis of tau isoforms and detection of tau aggregates in specific brain regions in 9 to 12 months-old htau mice.

2- Behavioral analyses of htau mice compared to wild type to assess locomotion and cognitive behaviour.

3- Set up of the *trans-splicing* strategy to rescue the balance of Tau isoforms *in vivo*. Phenotypic recovery in hTau mice rescued by *trans-splicing*.

Results

-**Aim 1**: The content of Tau isoforms containing or not exon 10 was determined by real time quantitive PCR. Primers spanning the junction between exons 9 and 10, or exons 10 and 11 of human Tau were used. Different brain areas were dissected from htau mice at 6, 9 and 12 months of age. Our results show an imbalance between 3R and 4R isoforms significantly detected in the prefrontal cortex from 6 months onwards while other structures such as the hippocampus, the

striatum and the substantia nigra pars compacta did not show a significant difference in the 4R/3R ratio (Figure 1).

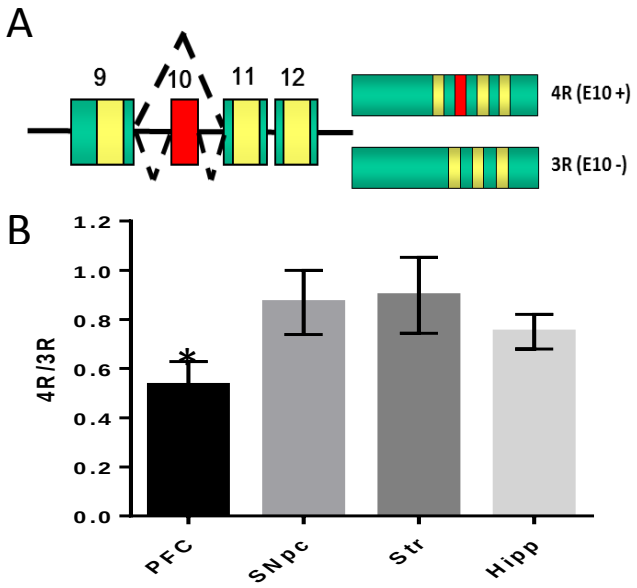

Figure 1. A). Tau isoforms with 3R or 4R were detected based on the presence exon 10 by RT-qPCR from RNA extracted from prefrontal cortex (PFC*), Substantia Nigra pars compacta* (SNpc), striatum (Str) and hippocampus (Hipp) of htau mice. B) The content of each isoform was calculated, related to a reference genes and the ratio between 4R/3R isoforms was determined for each brain structure.
*p < 0.05 (One Way ANOVA followed by Dunnet's test).

These qPCR results are consistent with previous reports showing that in htau mice the human *MAPT* transgene displays a shift towards the exclusion of exon 10 by alternative splicing leading to an increase of 3R Tau isoform over 4R (Andorfer *et al.,* 2003). However, the present results provide the first quantitative analysis performed in different brain areas of this model, which suggests that the imbalance is particularly significant in the PFC of htau mice from 6 months old.

The presence of aggregates of hyperohosphorylated Tau was detected by immunohistochemistry in htau mice brain after 9 months old. Fixed brain slices were stained using an antibody specific to detect both early and late tau aggregates (CP13, Andorfer *et al*, 2003). The presence of aggregates was evident in the prefrontal cortex but not in the other structures analysed (Figure 2).

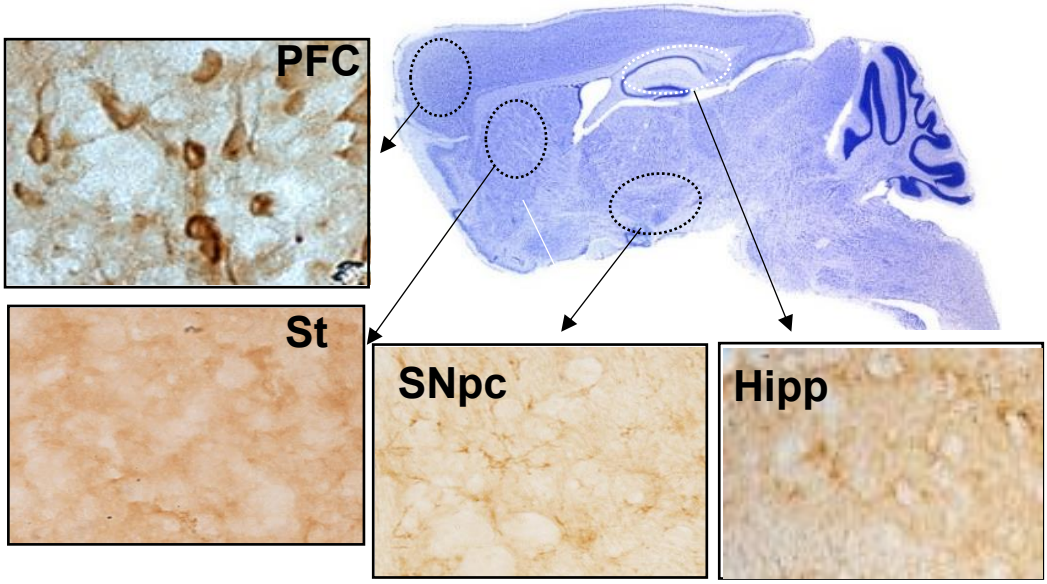

Figure 2. Tau aggregates were detected by immunohistochemistry using an antibody against S202 phosphorylated Tau (CP13) on brain sections htau mice (12 months): prefrontal cortex (PFC*) Substantia Nigra pars compacta* (SNpc), striatum (ST); hippocampus (Hipp). Aggregates of hyperphosphorylated Tau were detected in the PFC.

-Aim 2: To characterize the behavioural phenotypes in the hTau model, mice were subjected to a battery of behavioural test between 9 and 12 months old. In all experiments Htau mice were compared with wild type mice of the same age.To assess locomotion and motor coordination phenotypes mice were analysed in the open field and the rotarod. Cognitive performance was tested either with the Morris Water Maze (to assess hippocampal memory) or Novel Object Recognition Test (NOR) to analyse both cortical and hippocampal memory in response to ovel stimuli.

Htau mice showed normal locomotor behaviour (Figure 3A) and motor coordination in the rotarod (Figure 3B). Memory assessed by Morris Water Maze showed no differences during the training or the testing phase (Figure 3C). However, the NOR test revealed a deficit in the hTau mice performance compared to the WT group (Figure 3D).

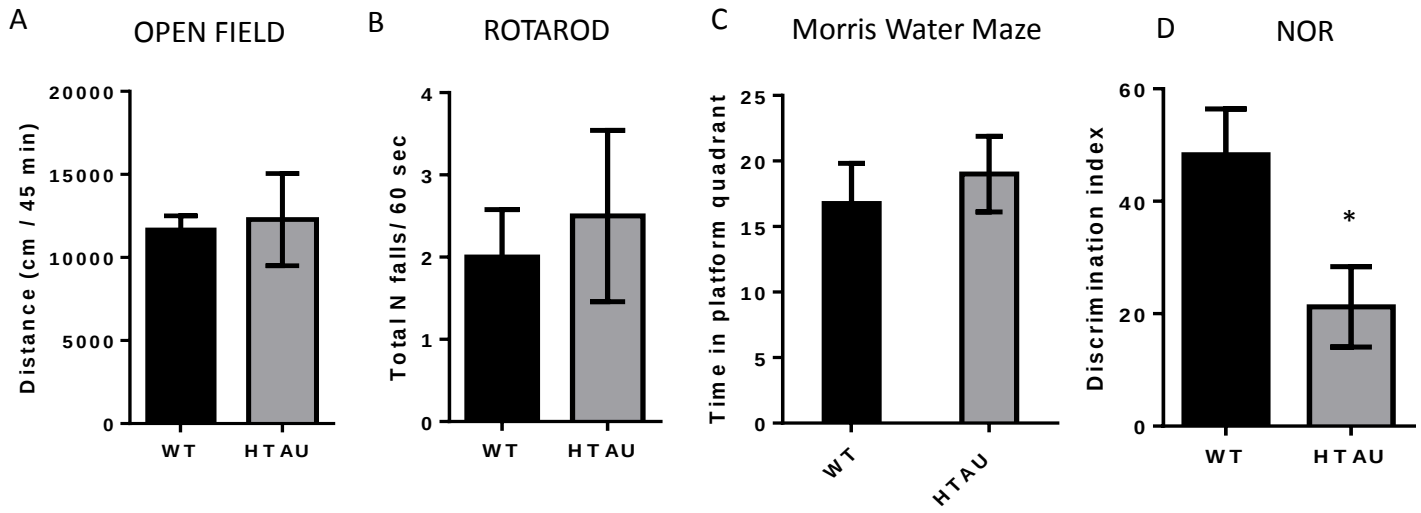

Figure 3. Assessment of Behavioral phenotypes of hTau mice. A) Open field: WT and Htau mice were tested during 45 min in a square open field under dim illumination. B) Rotarod: mice were tested in the rotarod for 60 sec. Maximum time on the rod and latency to first fall were recorded as a measure of motor coordination performance. No significant differences were observed between groups.

C) Mice were trained to swim and find a hidden platform into a 1 m diameter pool, using visual cues around the maze. After 5 days of training the platform was removed and the time spent in the platform quadrant was determined as a measure of memory. Both groups showed similar time spent in the platform area.

D) Mice were tested the Novel Object Recognition (NOR) task. After 10 min habituation per day during 3 days, mice were exposed to two identical objects for 10 min. 5 hours later mice were exposed to one of the familiar objects and a novel one. Time exploring each object was recorded and the discrimination index between familiar and novel objects was determined (DI: (Time in novel object-time in familiar object)/Total exploration of objects). htau mice evidenced a lower discrimination index than WT group (*p= 0,017, t test)

Together the neurochemical and behavioural data obtained strongly suggest that the deficit in the NOR task observed in htau mice is related to cortical dysfunction, which correlates with the presence of Tau aggregates and Tau 4R/3R isoforms imbalance in the prefrontal cortex of Htau mice.

-**Aim 3**: We next sought to to test if restoration of Tau 3R/4R imbalance in the prefrontal cortex of Htau mice could lead to a phenotypic recovery. We used the *trans-splicing* strategy to modulate Tau exon 10 inclusion (Figure 4 A). This strategy was previously validated in neuronal cultures and in a pilot study *in vivo* (Avale *et al*, 2013), showing that pre trans splicing molecules carrying a Tau expression cassette and splicing domain could achieve a *trans-splicing* reaction with the endogenous Tau transcript. Here this strategy is used to induce the conversion of Tau 3R, the prevalent isoform in the PFC of htau mice, into Tau 4R (Figure 4A). Lentiviral vectors carrying the Tau 4R Pre-trans splicing molecule (LV Tau-PTM4R) were injected into the perfrontal cortex of Htau mice at 3 months old. This group is referred as hTau rescued group (htau-RESC).
Behavioral studies were conducted between 9 to 12 months old (e.g. 6 months after Tau4RPTM injection). The control htau group and the wild type control group were injected with a LV carrying 4RPTM with a deleted splicing domain, so it cannot induce the trans-splicing reaction.
A first quantitative analysis comparing the content of Tau isoforms in the PFC between htauResc and htauControl groups showed that the 4R/3R ratio increased due to the injection of the LV Tau-PTM4R.

Cognitive tests were conducted, showing that hTau RESC group display an improvement in their performance in the NOR task (Figure 4C). The performance of htauResc mice in the other behavioural tests (open field, rotarod and Morris water Maze) was not altered and remained equal to wild-type and htau control groups.
This result suggests that restoring the 4R/3R balance in the PFC of htau mice might prevent the development of cortical deficits underlying the NOR test without altering other behaviors.

Work in progress:
Behavioral analyses of the first htau-RESC group were conducted between September 2014 and December 2014. After completing behavioral experiments, brain samples were obtained and the neurochemical analysis of this group is currently under way. Half of mice were transcardiacally perfused to analyze the presence of Tau aggregates, and determine if the restoration of Tau balance has a significant effect over the presence or amount of hyperphosphorylated Tau in the PFC.
The rest of the samples were dissected and frozen to perform RNA and protein extraction and are currently under analysis. qPCR will be performed to further quantify the Tau 4R/3R ratio in htau Resc *vs* hTau control group. In addition, an analysis to determine the level of soluble and insoluble Tau by the sarkosyl extraction tests will be done, as previously described in the working plan. We expect to complete these experiments in the next three months. Our hypothesis is that the behavioural rescue observed in htauResc mice is likely to correlate with a reduction in the amount of hyperphosphorilated and/or insoluble Tau.

In addition, in order to further validate our results, a second experimental group has been already injected with the LV-PTM4R last January 2015. This group will be analysed in the same behavioural tests followed by neurochemical analyses, as was done in the first group. We expect to complete all the analyses of this second experimental group by mid-2015.

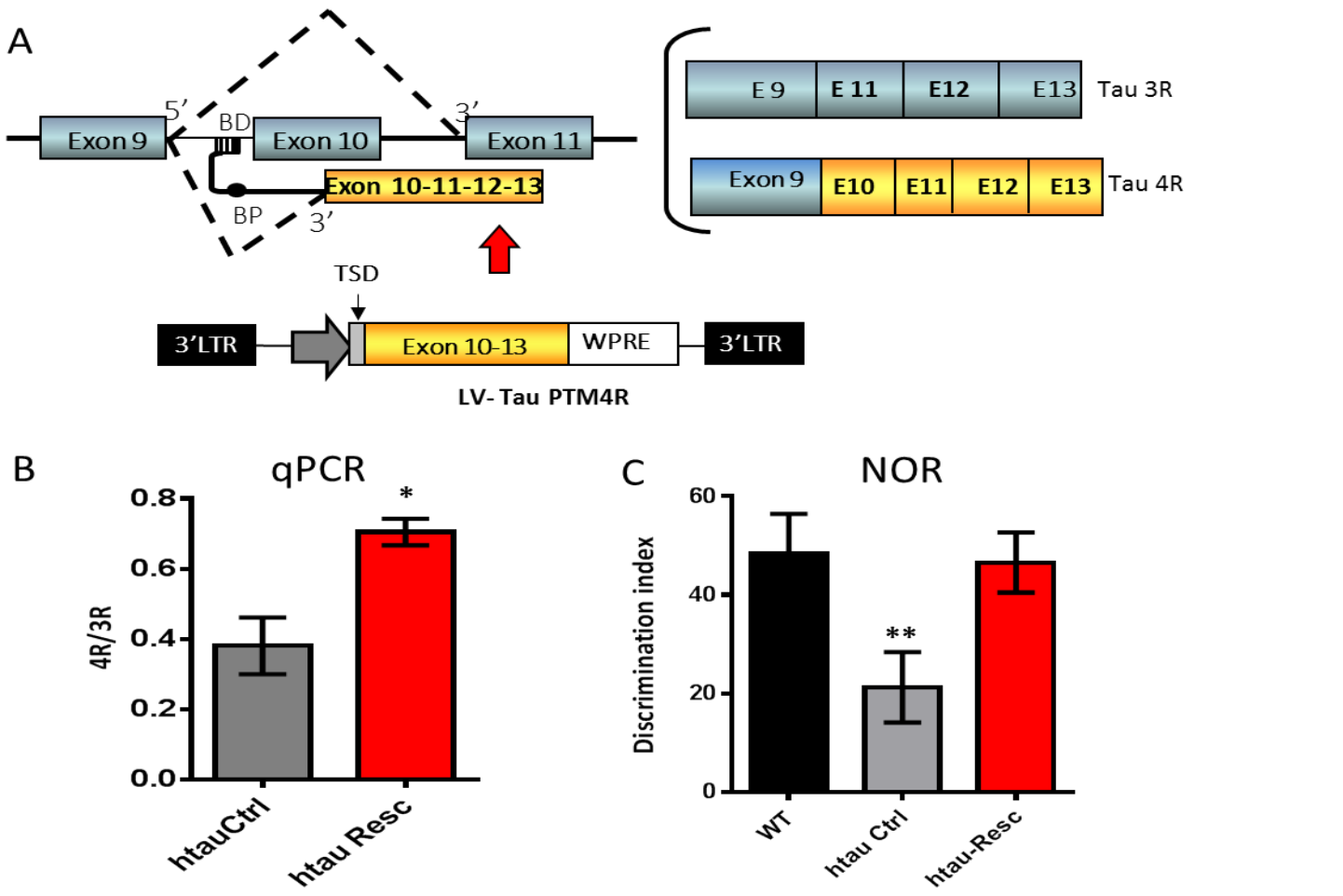

Figure 4 .A) ***Trans*-splicing strategy used to shift Tau isoforms from 3R to 4R in htau mice**. A pre *trans*-splicing molecule containing exons 10 to 13 (Tau-PTM4R) is expressed by a lentiviral vector. The PTM4R generates an RNA fragment that binds to the endogenous tau mRNA transcript (grey) at thelevel of intron 9 through *a trans-splicing* domain (TSD) and undergoes *trans*-splicing generating a chimeric RNA carrying Exon10. B-C) Htau mice were injected with the **LV-TauPTM4R** into the PFC at 3 months old (HtauResc group). B) The relative content of Tau isoforms in the PFC of htauCtrl vs. htauResc groups was determined by qPCR as in Figure1 (**p*<0.05 Student's *t* test). C) HtauResc were tested in the NOR task compared to control groups of htau and WT mice. Htau-RESC group showed preference for the novel object similar to the WT group, indicated by restoration of normal discrimination index (**p<0.05, One way ANOVA followed by Tukey's test; *n*=5 per group).

II: Role of neural activity in neuron survival and the neuroprotective effects of nicotine

Summary: This project is based on the hypothesis that electrical activity may be a neuroprotector of dopaminergic neurons. The aim is to set up an *in vitro* model of spontaneous dopaminergic neuronal death on which we could test neuroprotection exerted by modulators of neuronal activity such as pharmacological agents or heterologous receptors.

Goal: The final goal of this project is to determine whether the increase of neuronal activity could exert a neuroprotective effect over dopaminergic neurons, to develop potential therapeutical strategies for Parkinson's disease.

Aims completed:

1-Set up of the *in vitro* model of dopaminergic neurons degeneration.
2-Testing neuroprotective effects of nicotine and tetraethyl ammonium over DA neurons in culture.

Results

Neuronal cultures were prepared from the mesencephalon of E13.5 mouse embryos, using a protocol previously described (Toulorge *et al.,* 2011). To test optimal conditions, cultures were prepared with embryos either inbred mice (C57/B6) or outbred (CF1) stock, both from University of Buenos Aires Animal Facility. To obtain neurons we tested either mechanical dissociation or a papain containing commercial kit (Worthington, USA). The most reliable results were obtained when using CF1 embryos and mechanical dissociation.

After dissociation, neurons were plated in DMEM/F12 medium supplemented with horse serum (5%) and fetal calf serum (2.5% from day *in vitro* (DIV) 0 and then 0.5% from DIV 3 onwards. In these conditions, it is well described that dopaminergic (DA) neurons, and not any other neuronal type, degenerate progressively. Cultures were fixed at different time points and DA neurons were counted after performing fluorescent immunostaining against tyrosine hydroxylase. Time course of DA neurons counting showed a decrease from DIV 5 onwards, as expected (Figure 5).

We also tested the protective role of nicotine, either alone or combined with a depolarizing agent, the broad-spectrum K+ channel blocker tetraethyl ammonium (TEA). Cultures were treated from DIV 1 with Nicotine (1 µM), or Nicotine (1 µM) plus TEA (100 µM). Results show that nicotine has a neuroprotective effect but when combined with TEA the DA survival increased up to two fold at DIV 7 when compared to control conditions. However, at DIV 14 the DA neuronal death was similar in all treatments (Figure 5).

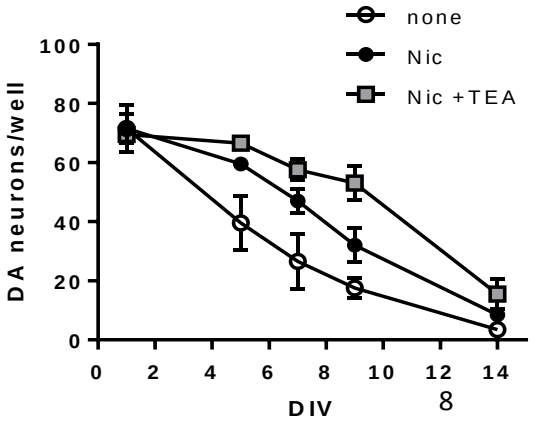

Figure 5. Mesencephalic neuronal cultures prepared from E14.5 mouse embryos. Neuroms were plated and cultured in the different conditions. At the indicated points cells were fixed to perform TH staining and counting (DIV: day in vitro).

These results confirm previous findings showing that nicotine improves the survival rate of DA neurons in culture and that the effect is increased under depolarization conditions (Toulorge *et al.,* 2011), supporting the hypothesis of a role of electrical activity on nicotine neuroprotection.

Work in progress:

After setting up the model and being able to confirm nicotine effect over DA survival we were ready to move forward into the analysis of other potential molecules affecting DA neurons activity as potential neuroprotective agents. Particularly, we are interested on testing whether stimulation of electrical activity through the expression of heterologous receptors could exert a protective effect on cultured DA neurons, similar to the observed with nicotine and TEA. The rationale of this experiment is to develop a strategy to specifically activate DA neurons without altering other neurons present in the mesencephalic culture.

We recently obtained two Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) from Dr. Bryan Roth lab (University of North Carolina, US). These receptors are G-protein coupled transmembrane receptors that are able to either stimulate (hM3D) or inhibit (hM4D) neuronal activity when activated with clozapine-N-oxide (CNO) (Alexander *et al*., 2009).

hM3D and hM4D were cloned into lentiviral expression vectors, LVs particles were prepared and used to transduce DA neurons in culture. Control neurons were transduced with a LV expressing only a reporter gene. All cultures were treated with CNO (10 μM) and DA neurons counted at DIV 10 and 14. Preliminary data shows that hM3D expression and CNO treatment increased survival rate up to 3-fold when compared to control cultures, treated with hM4D + CNO, or CNO alone. These experiments are currently under further analysis to evaluate the time course of survival.

We are also preparing lentiviral vectors carrying hD3M in a conditional "*floxed*" cassette, on which the expression of DREADDs depends on the action of CRE recombinase. These LVs will be used to transduce mesencephalic cultures prepared from DAT-CRE mice, in order to express hD3M exclusively in the DA neurons present in the culture. In addition we will combine the expression of h3MD and nicotine treatment to test a potential synergistic action.

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