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CONTACT DETAILS OF THE APPLICANT

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PROJECT: "Perturbation of plasticity-related genes and corticospinal tract integrity in a conditional mouse model of TDP-43 proteinopathies"

BACKGROUND

Mislocalization and aggregation of the nuclear protein TDP-43 are hallmark features of the neurodegenerative diseases amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD), two incurable pathologies now classified under the term "TDP-43 proteinopathies" (*1, 2*). Today it is widely recognized that ALS and FTD represent two ends of one spectrum disorder, termed ALS/FTD. The pathophysiological roles of TDP-43 are just beginning to be understood. Although the best described function of the protein is the regulation of RNA splicing, it has been reported to be involved in additional nuclear and cytoplasmic processes (transcriptional repression, microRNA biogenesis, mRNA localization and translation) (*3*).
Cellular and animal models have been recently developed to investigate the impact of TDP-43 dysfunction in disease. We have shown in mice that inducible overexpression of a cytoplasmically-localized form of TDP-43 (TDP-43-ΔNLS) in forebrain neurons evokes neuropathological changes that recapitulate several features of TDP-43 proteinopathies (4). Moreover, as a further validation, we recently reported in *The Journal of Neuroscience* profound behavioral deficits in these mice, including motor, cognitive and social phenotypes. Some of these alterations were reversed upon transgene repression, indicating that, at early stages of disease, TDP-43-mediated behavioral dysfunction is sustained by functional/morphological changes in populations of affected neurons independently of extensive neurodegeneration (5).

HYPOTHESIS

The general hypothesis under investigation is that through aberrant activity of TDP-43, dysregulation of gene expression is a key mechanism in TDP-43 proteinopathies, which leads to biochemical, morphological and behavioral changes. In this context, the overall goals of this project are to identify changes in candidate plasticity-related genes (PRGs) and to study the degree of corticospinal tract (CST) degeneration upon TDP-43-ΔNLS expression. Thus, these effects elicited by TDP-43 dysregulation might underlie the behavioral abnormalities observed in this model, and understanding them will increase our knowledge of the etiology of ALS/FTD spectrum disorders.

SPECIFIC AIMS

AIM 1:

To analyze *in vivo* the basal and behaviourally induced levels of plasticity-related genes in TDP-43-ΔNLS mice.

AIM 2:

To study the anatomo-pathological correlates of the behavioral changes observed in TDP-43-ΔNLS mice, using clearing, tracing and 3D-reconstruction techniques.

METHODS

AIM 1

1.1. Animal care and breeding. Mice are housed at the IFIBIO breeding facility under controlled photoperiod (12-hour light/ 12-hour dark cycle, lights on from 7 a.m. to 7 p.m.), with tap water containing Doxycycline hyclate (Dox, 0.2 mg/ml) to repress transgene expression during development, and standard laboratory chow available ad libitum. CamkIIα/tTA mice are crossed with tetO/hTDP-ΔNLS mice as previously described (*4, 5*) to generate control (non-TDP-43 expressing) and bigenic mice. Genotypes are confirmed by standard PCR protocols.

1.2. Experimental design. Transgene expression was activated at weaning (P28) by removing Dox from drinking water as in (4,5). Mice were analyzed at 1 month post-induction. For the basal condition, animals were sacrificed after removal from home cage. For analysis after behavioral challenge, mice were subjected to a novel environment (a 40x40x40 cm open field) for 20 min (*4*), and sacrificed 1 hr later.

1.3. **Quantification of plasticity-related proteins.** Microtome frozen sections (50 µm) of paraformaldehyde-perfused brains were subjected to IF and the number of positive cells or signal intensity using c-fos, Arc or Zif268 primary antibodies (Santa Cruz) was quantified in cortical (SSC, MC, mPFC) and hippocampal subregions (CA1, dentate gyrus) as in (*4*).

AIM 2

2.1. Experimental design. TDP-43 mice were raised as in **1.1**, and analyzed at 0.5 or 1 month post-induction (at weaning), time points already showing behavioral changes (*4*). For the suppression protocol, mice raised on Dox until P28 were treated again with Dox starting at 0.5 months [1 mo-(sup) mice] after weaning to suppress transgene expression for 2 weeks, and analyzed at 1 month after weaning (*4*).

2.2. Tracing, clearing and 3D-reconstruction. Fluoro-dextran tracer was delivered into motor cortex with a microinjection system to label the pyramidal neurons 5 days prior to sacrifice. At terminal time points described in **2.1**, mice were PFA-perfused, cleared with THF-dichloromethane-BABB treatment and Fluoro-Ruby signal was collected by serial confocal microscopy (Olympus FluoView FV1000/IX81) and 3D-reconstruction and quantification of the CST (Imaris software) performed as in (*6, 7*).

RESULTS

AIM 1:

To analyze if IEGs protein levels were altered in response to hTDP-43-ΔNLS expression, we decided to study the basal expression pattern of Zif268, cFos and Arc. We found a profound decrease in the basal levels of these three IEGs in multiple brain regions (**Figs 1-3,** Naïve condition) involved in different functions that were described to be altered in hTDP-43-ΔNLS mice, including social and cognitive domains (*4*).

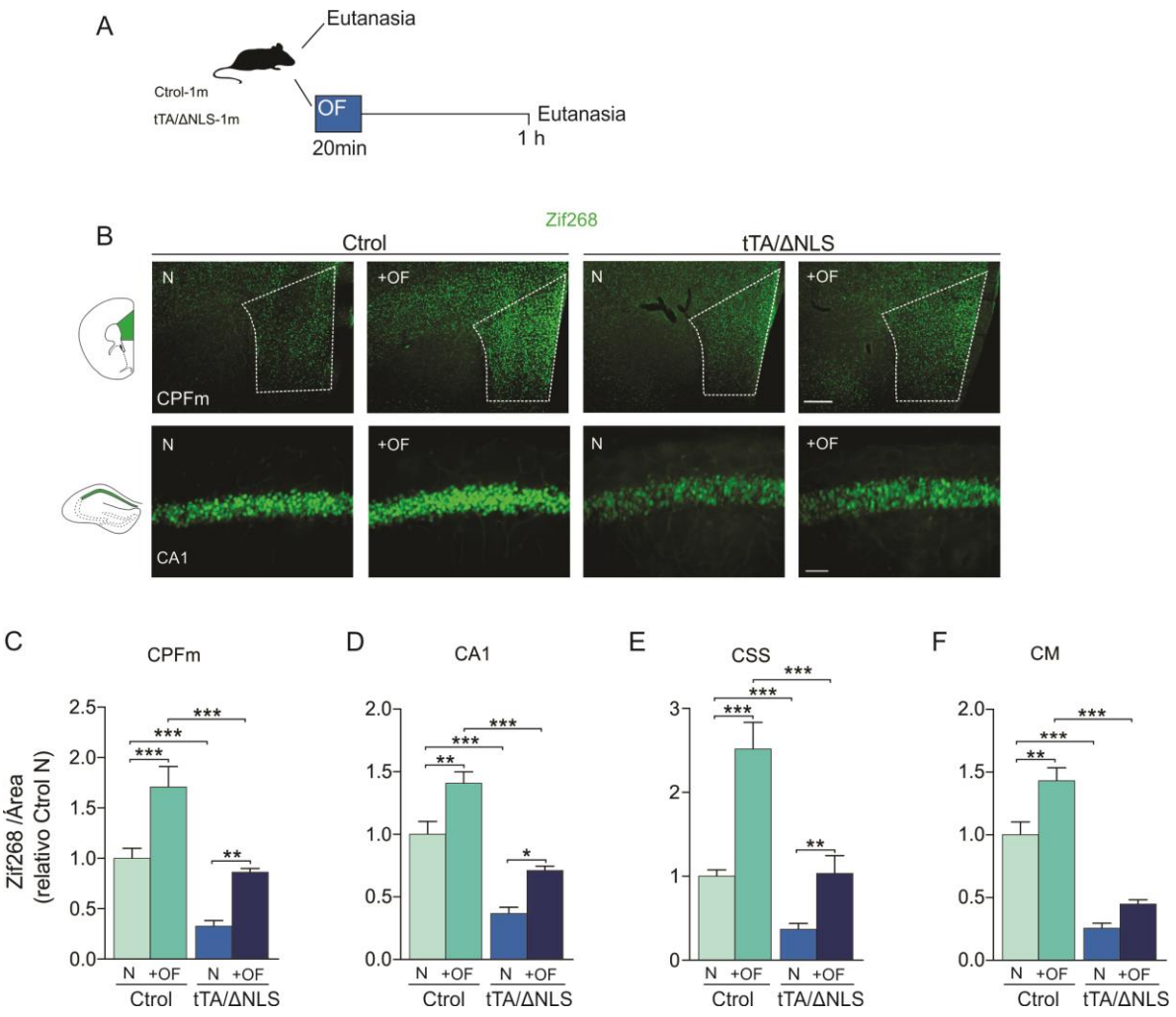

Figure 1. Abnormal basal and behaviorally-induced levels of Zif268 protein in transgenic hTDP-43-ΔNLS mice. (A) Experimental design: Ctrol-1m and tTA/ΔNLS-1m animals were exposed to the open field test as a novel context (+OF) and sacrificed (euthanasia) 1 hour after. These animals were compared with naive (N) mice, which were not exposed to the novelty. **(B)** Immunofluorescence for Zif268 protein in coronal brain slices. Representative photographs of medial Prefrontal Cortex (CPFm) and CA1 region of the hippocampus from N or +OF conditions for both Ctrol and bigenic mice are shown. Scale bar: 250 µm (CPFm) and 50 µm (CA1). **(C-F)** Quantification of the Zif268 signal (intensity/area, relative to Ctrol N) for CPFm (C), CA1 (D), Somatosensory Cortex (CSS) (E) and Motor Cortex (CM) (F). ** p <0.01, *** p <0.001, one-way ANOVA / Newman-Keuls post hoc test, n = 6-8 per group. Data are expressed as mean ± SEM.

After we showed that the levels of expression of zif268, c-fos and arc are decreased in basal conditions in hTDP-43-ΔNLS mice, we decided to study the expression of these IEGs in animals subjected to a behavioral task that is known to physiologically induce these genes, to determine if, in addition to their basal levels, the mechanisms of regulation of expression by activity and / or plasticity was also affected. In effect, IEG response as a consequence of the behavioral challenge (open field exposure) was either significantly lower or completely absent, compared to OF-exposed control littermates (**Figs 1-3,** +OF condition).

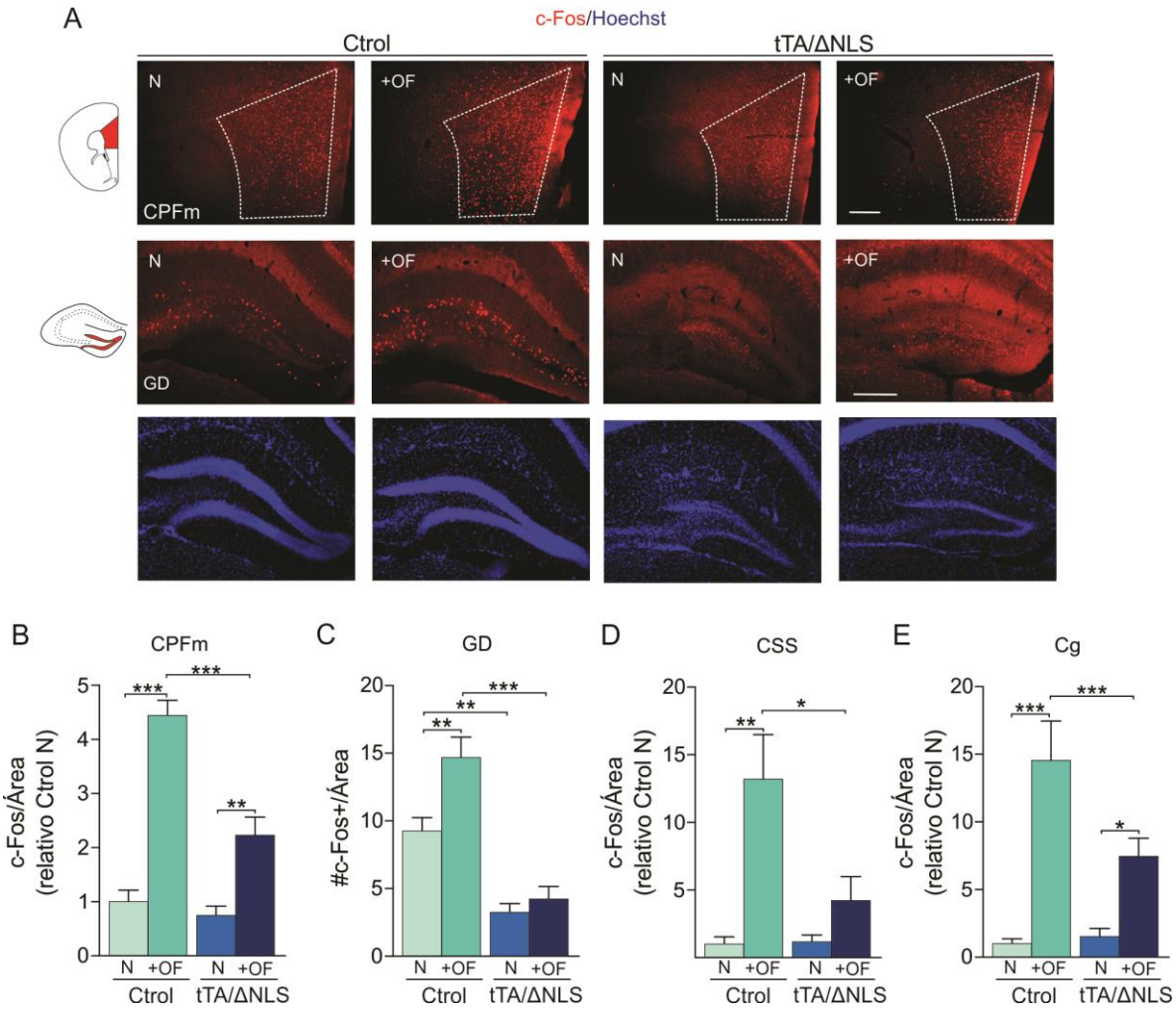

Figure 2. hTDP-43-ΔNLS expression modifies the induction pattern of c-Fos.
Control (Ctrol-1m) and bigenic (tTA/ΔNLS-1m) animals were exposed to the open field test as a novel context (+OF) and sacrificed 1 hour after. These animals were compared with naive (N) mice, which were not exposed to the novelty as described in **Fig 1A**. (**A)** c-Fos immunofluorescence in coronal brain slices. Representative photographs of medial Prefrontal Cortex (CPFm) and dentate gyrus (GD) region of the hippocampus from N or +OF conditions for both Ctrol and bigenic mice are shown. Hoechst staining for nuclei is provided below as anatomical reference. Scale bar: 250 µm. **(B-E)** Quantification of the cFos signal (intensity/area, relative to Ctrol N) for CPFm (B), GD (C), Somatosensory Cortex (CSS) (D) and Cingulate Cortex (Cg) (E). * p <0.05, ** p <0.01, *** p <0.001, one-way ANOVA / Newman-Keuls post hoc test, n = 6-8 per group. Data are expressed as mean ± SEM.

The overexpression of the cytoplasmic variant of TDP-43 leads to remarkable changes in gene expression in the brain of our animal model. In particular, there is a decrease in the basal expression of IEGs like zif268, c-fos and arc, and a deficient or absence of induction after exposure to a behavioral novelty. Neuronal activation, evidenced by the induction of IEGs in this model, seems to be compromised and, therefore, we hypothesize that some of the behavioral changes observed in these animals (*4*) might be related to a general decrease in activity levels and/or neuronal activation caused by the presence of cytoplasmically localized hTDP-43-ΔNLS.

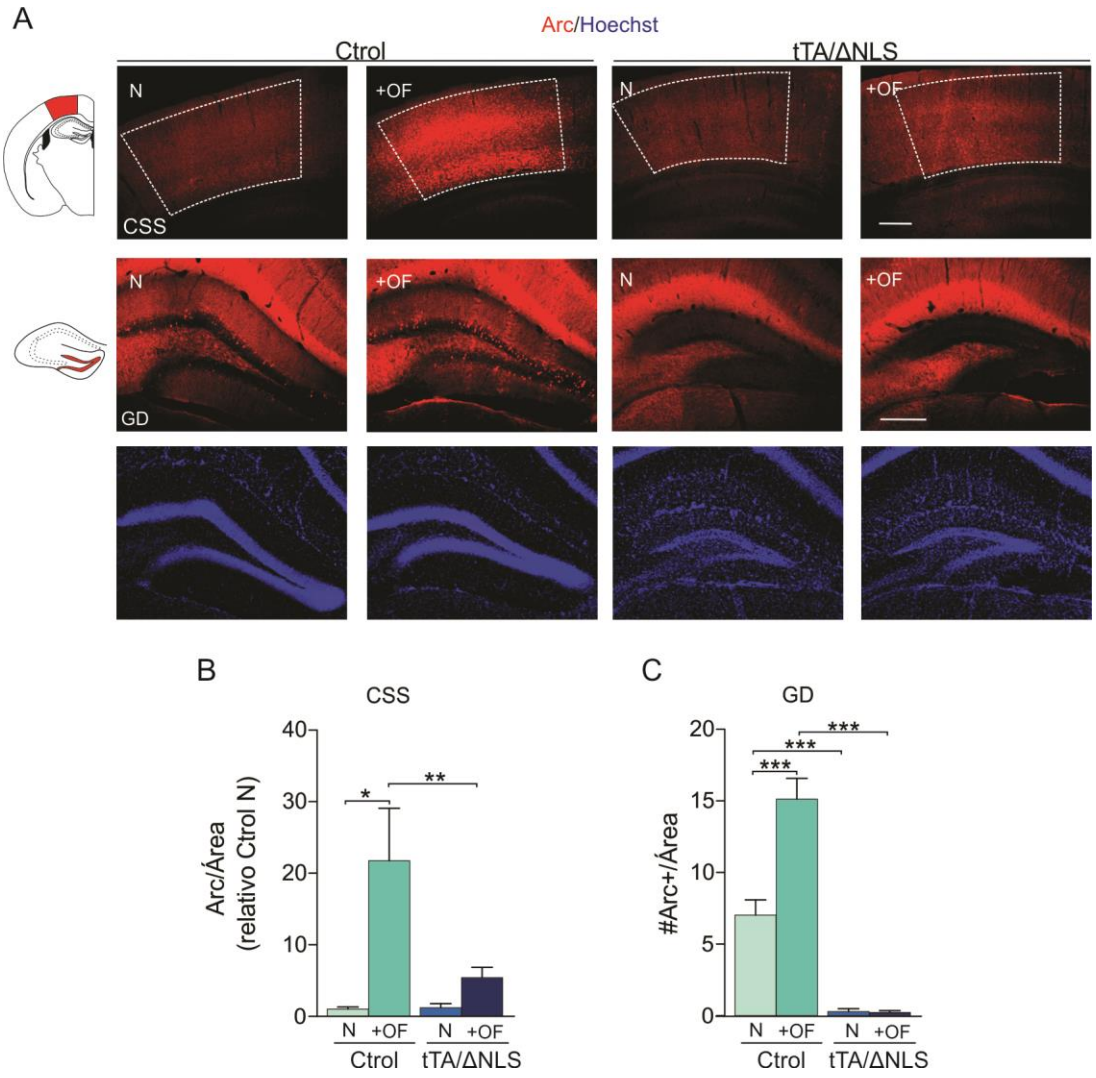

Figure 3. Arc protein levels are dramatically decreased in hTDP-43-ΔNLS transgenic mice.
Experimental design as in **Fig. 1A**. **(A)** Immunofluorescence for the Arc protein in coronal brain slices. Representative photographs of Somatosensory Cortex (CSS) and dentate gyrus (GD) of the hippocampus from N or +OF conditions for both Ctrol and bigenic mice are shown. Hoechst staining for nuclei is provided below as anatomical reference. Scale bar: 250 µm. **(B-C)** Quantification of the Arc signal (intensity/area or number of ARC positive cells, relative to Ctrol N) for CSS (B) and GD (C). * p <0.05, ** p <0.01, *** p <0.001, one-way ANOVA / Newman-Keuls post hoc test, n = 5 per group. Data are expressed as mean ± SEM.

AIM 2:

We proposed the use of a novel approach to study the anatomo-pathological correlates of the behavioral changes observed in our TDP-43-ΔNLS mouse model, combining a cost-effective unsectioned brain/spinal cord clearing technique, fluoroRuby staining, one-photon confocal microscopy and three dimensional reconstruction to study the morphological changes in motor cortex (MC) and corticospinal tract (CST) of TDP-43 transgenic mice.

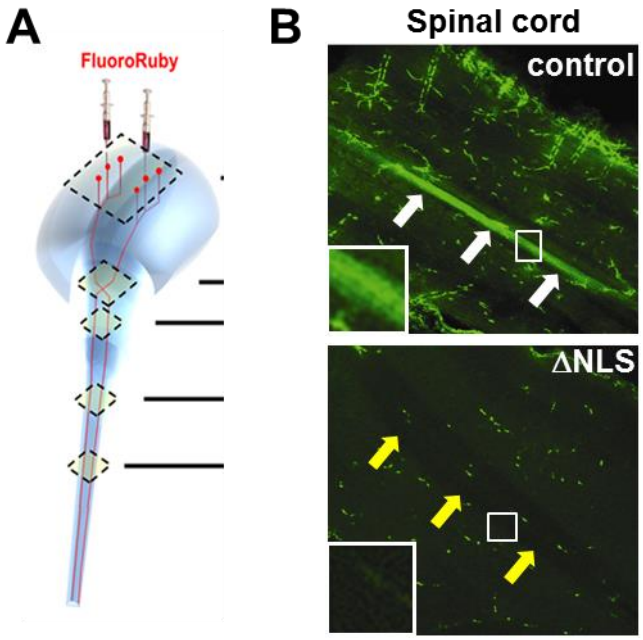

Figure 4. (A) Schematic drawing of anterograde Fluoro-dextran CST labeling. B) CST pathway from spinal cord segments labelled with FluoroRuby. The tracer was delivered into motor cortex with a microinjection system to label the pyramidal neurons. Modified from (*6*). **(B)** Representative image of CST from spinal cord at thoracic level 1 month post transgene induction. The orthogonal views show the pathway and size of CST (white arrows). Notice almost complete loss of CST fibers in TDP-43 transgenic mice 4 weeks after induction (yellow arrows). Insets show higher magnification of boxed area.

We were able to successfully adapt this methodology in transgenic TDP-43-ΔNLS mice, and demonstrated that these animals show an almost complete loss of CST fibers in the spinal cord after 1 month of transgene expression (**Fig. 4**). Currently, we are performing efforts to quantitatively analyze this loss with the use of Imaris 3D Software (Bitplane Sci Software, Switzerland) as in (*6*).

Using this approach, we were also able to determine the time course of this dramatic CST degeneration at the level of brainstem, where a remarkable decrease in CST fibers can be appreciated as soon as 0.5 after transgene expression and a subsequent near complete loss of fibers by 1 month of hTDP-43-ΔNLS (**Fig. 5B**). Interestingly, through 3D reconstruction we identified a region of interest in the brainstem, surrounding the CST decussation, where most axons seems to be lost during this process (**Fig. 5C**).

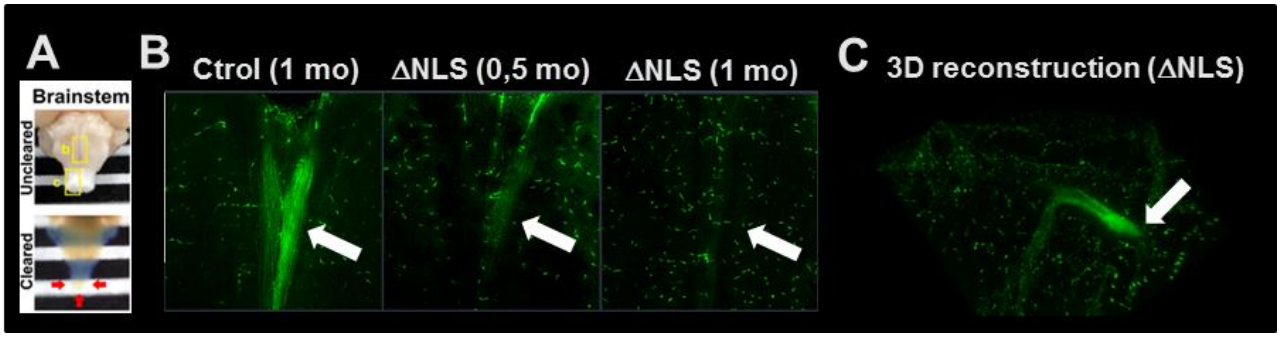

Figure 5. Corticospinal tract (CST) fibers from brainstem of TDP-43-ΔNLS mice labeled with FluoroRuby. (A). Representative images of brainstem (uncleared and cleared) 5 days after tracer injection. **(B).** Representative images of CST segment above decussation. Note time-dependent loss of CST fibers in TDP-43 mice rapidly after transgene induction. **(C).** 3D reconstruction of CST decussation in TDP-43-ΔNLS mice, 1 month post-transgene induction. Note that the area where most axons are lost is within this region (arrow).

DISCUSSION AND CONCLUSIONS

AIM 1

We assessed changes in well-known plasticity-related gene products (Arc, c-fos, Zif268) in a novel mouse model of TDP-43 proteinopathies. IF staining of several brain areas (involved in processing of the behavioral tasks impaired in these mice) revealed a profound decrease of all three genes in transgenic versus control mice. Since these genes are necessary for cognitive processing, we evaluated the response upon a behavioral challenge, and these mice display a reduced/absent induction of all three genes in cortices and hippocampus. These results suggest a novel TDP-43 driven mechanism underlying the behavioral abnormalities displayed by TDP-43 mice and potentially in human TDP-43 proteinopathies.

AIM 2

We performed exploratory, proof-of-concept studies that confirm that these techniques can be successfully applied to a novel neurodegenerative disease model, specifically to study TDP-43 proteinopathies. The application of the combined approach (clearing + 3D reconstruction) allows for evaluation of the damage caused by TDP-43 manipulation along the entire affected pathway, and to help elucidating the mechanisms underlying the motor phenotype recovery after transgene suppression, with implications for ALS/FTD. We are currently performing experiments analyzing the anatomical changes (macroscopic and microscopic) that underlie the remarkable recovery of motor function after transgene suppression.

The results from this project, taking advantage of a unique inducible mouse model recapitulating multiple features of the ALS/FTD spectrum, are allowing us to shed light onto the pathophysiological roles of TDP-43 in the nervous system. We firmly believe that addressing the pathogenic mechanisms underlying TDP-43 proteinopathies will be vital to develop new and more effective therapies for these disorders.

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SCIENTIFIC COMMUNICATIONS

1. "*Biochemical evidence for altered protein levels of plasticity-related genes in inducible TDP-43-ΔNLS transgenic mice*"
de Landeta Ana Belén, Alfieri Julio, Katche Cynthia and Muller Igaz Lionel.
XXXII Argentine Society for Neuroscience Research (SAN) Annual Meeting. Mar del Plata, Argentina, September 25-27, 2017.

2. "C*onditional mouse models of TDP-43 proteinopathies*"
Lionel Muller Igaz.
Invited speaker at the IBRO Alumni Symposium "Basic and translational research in Neurodegenerative disease: from molecules to animal models". 2nd FALAN (Federation of Latin American and Caribbean Neurosciences) Congress. Buenos Aires, Argentina, October 17-20th, 2016.

3. *"Evaluation of a new rodent model of TDP-43 related neurodegenerative disease".*
Lionel Muller Igaz.
Seminar at the Instituto Multidisciplinario de Biología Celular (IMBICE), September 9th 2017, La Plata, Buenos Aires, Argentina.

4. *"New transgenic models to study TDP-43 associated neurodegenerative disease: implications for amyotrophic lateral sclerosis and frontotemporal dementia research"*
Lionel Muller Igaz.
Seminar at the Instituto Multidisciplinario de Biología Celular (ININFA), August 8th 2017, Buenos Aires, Argentina.

Manuscripts in preparation:

- Decreased protein levels of plasticity-related genes Zif268, arc and c-fos in TDP-43-ΔNLS transgenic mice. *de Landeta, Alfieri et al.*

- Time-dependent degeneration of corticospinal tracts in TDP-43 transgenic mouse models of ALS/FTD: application of 3D reconstruction in cleared tissue. *Quintá et al.*

GRANT EXPENSES

The funds from this support were used to acquire the following:

Immunofluorescence supplies and antibodies: $ 1611
Genotyping/PCR reagents: $ 926
Chemicals and drugs (including Doxycycline): $ 928
Animal housing and care costs: $ 1158
Other laboratory consumables: $ 377

TOTAL EXPENSES: 5000 $