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Aggregation and hyperphosophorylation of the microtubule protein Tau (MAPT) are mechanisms that are major factors in the development of brain diseases, collectively called Tauopathies. These includes disorders such as Alzheimer´s disease, Pick´s Disease, Progessive Supranuclear Palsy, Corticobasal degeneration and Frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17), which the latter is purely a genetic disorder [1, 2]. The Tau protein exist in six different isoforms, generated from alternative splicing, and are derived from the inclusion / exclusion of a 29 or a 59 amino acid long insert (Exon 2 and 3) and the inclusion of 3 or 4 microtubule binding site (Exon 10) [2]. Although there is extensive knowledge and research around tau and related diseases, until today there is no current treatment for Tauopathies and options for symptomatic treatment is very limited. A target treatment strategy that has been suggested is down-regulation of mutated and malign Tau protein expression, which causes aggregation and hyperphosphorylation. Such a strategy would lead to less protein accumulation and concomitant slower disease progression, or even a halt of the disease. As FTDP-17 is purely a genetic disorder, this disease has been used to understand gene functions and mechanisms of Tau protein. Several transgenic mouse cell lines and mouse models have been developed upon the more than 20 distinct mutations found for FTDP-17.

Small interference RNA (siRNA) has been developed as a powerful tool to silence genes interest and its corresponding protein expression [3]. We have here suggested and experimentally worked on siRNA interference technique to knockdown mutated and malign expression of human tau protein. For this we have characterized a transgenic mouse model expressing the human P301S mutation in the *tau* gene, developed by Prof. Goedert, University of Cambridge [4]. This model shows tau aggregation and following severe behavioural impairment, in particular, in motor behaviours. The symptoms are subtle and non-significant in early adulthood, but can be picked up in test such as spontaneous rearing behaviour (Fig. 1A). This behaviour gradually decline in this model over months reaching significant at 6 months of age, as compared to early adulthood at 3 months. After 5-6 months the animals also show severe signs of paralysis, in particular, in the hindlimbs. These behaviours can be observed visually and readily pick up in behavioural tests measuring distance travelled (in open-field conditions; Fig. 1B) and time spend on a rotating rod (Rotorod test; Fig. 1C). These data provide us with important information about the model, of which test paradigm to use and at which time-points significant behavioural effects, of treatments strategies like siRNA interference of the tau protein, can be evaluated.

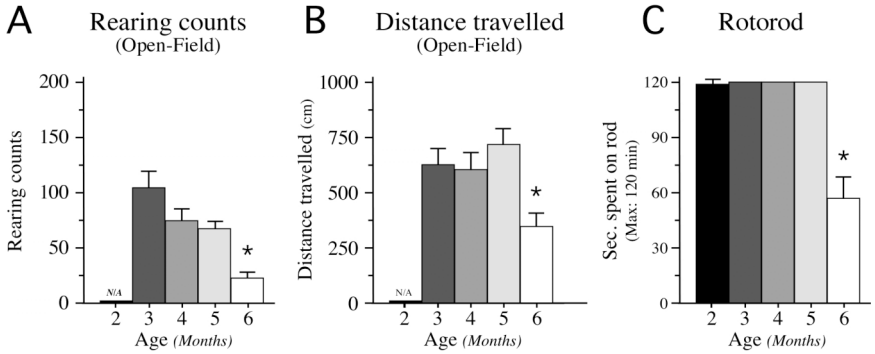

Figure 1. ***Behavioural progression in human P301S Tau transgenic mice****. Mice overexpressing human Tau with the P301S mutation show deficits in rearing behaviour, which significantly progresses over time (**A**). Deficits can also be picked up in ambulation as distanced travelled, in the open-Field (**B**) and the performance in rotorod test (**C**).These severe and significant deficits can be observed at an age of 6 months. * different from all other timepoints. One-way ANOVAs F(3,59)=4.9635, 11.199, and 28.409 followed by each pair student´s t-tests for A, B and C respectively. p<0,004 for all comparisons.*

In order to deliver the siRNA in to cells several techniques has been suggested and tested with various successes. We have here investigated multiple transfection agents *in vitro* and PolyethyleneImine(PEI-)-based and Accell-siRNA™ (Dharmacon) based delivery *in vivo*. First, we evaluated siRNA interference efficacy of the enhanced Green Flourescent Protein (eGFP) and the house-keeping protein Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using “naked” siRNA molecules. We used several transfection agents including Hi-Perfect (Quigen), Dharmafect (Dharmacon), X-treme Gene (Roche), Saint-RED (Synvolux), Trans-TKO (Mirus) and Interferrin (Polyplus). We prepared and used primary cortical cultures from E15-17 Actin- eGFP transgenic and C57/B6 embryos, as well as human mesenchephalic cell culture (LuhMes). We found unfortunately that, in our hands, the transfection agents were not effective or toxic. Further, we established collaboration with Prof. Culmsee at the Department of Pharmacology and Clinical Pharmacy, Philipps-University Marburg, where we started to work with Lipofectamine 2000 (Invitrogen) as transfection agent, in which the Prof. Culmsee had experience. We used here both “naked” and modified

(ON-TARGETplus™, Dharmacon) siRNA against the home-keeping genes GAPDH and Cyclophilin B (CycloB) in primary cortical cell cultures expressing P301S mutation, derived from our transgenic P301S mutant Tau mice as well as LuhMes cells and mouse neuroblastoma cells (HT22). We also predicted good siRNA sequences for the human *MAPT* gene using the Whiteheads siRNA prediction tool (*http://jura.wi.mit.edu/bioc/siRNAext/home.php*). We design siRNAs that targeted human mutant P301S tau, with a less affinity endogenous mouse tau, targeting the heterogene sequences between the human and the mouse *MAPT* genes. Four sequences were selected due to their poor overlap with the mouse tau gene and relative low overlap with any mouse genes. Although, using single or pools of GAPDH or CycloB, or the four Tau sequences alone or in a pool, we were not able to produce any consistent knockdown using Lipofectamine 2000. As the primary goal was to knockdown the protein level, we used western blot as the primary technique, rather than systematically looking at the RNA levels. As the transfection agent did not produce any reliable and consistent protein knock-down, we started to experimentally test the novel Accell-siRNA™ developed by Dharmacon. The Accell-siRNAs have been modified to enter the cell without any transfection agent, which also is more suitable for *in vivo* application. We tested Accell-siRNA™ in both primary neuronal cortical cultures as well as the LuhMes cells. Here, we were able to show that GAPDH and CycloB proteins could significant be knocked down in both cell types, as shown by western blot (Fig. 2A). The more potent protein knockdown, for both tested cells, was observed for CycloB. Furthermore, we also tested pre-designed Accell-siRNA against Tau protein (smartPool of four sequence). Similar, we were here able to significantly knockdown the human and mouse Tau protein both in primary cortical neurons at concentrations low as 250nM siRNA (60-95%; Fig. 2C) and human mesencepahlic (LuhMes) cells at 125nM (75-95%; Fig. 2D). Substantial protein knockdown was observed already at 72h post transfection in the LuhMes cells and at 96 to 144h in the primary neurons.

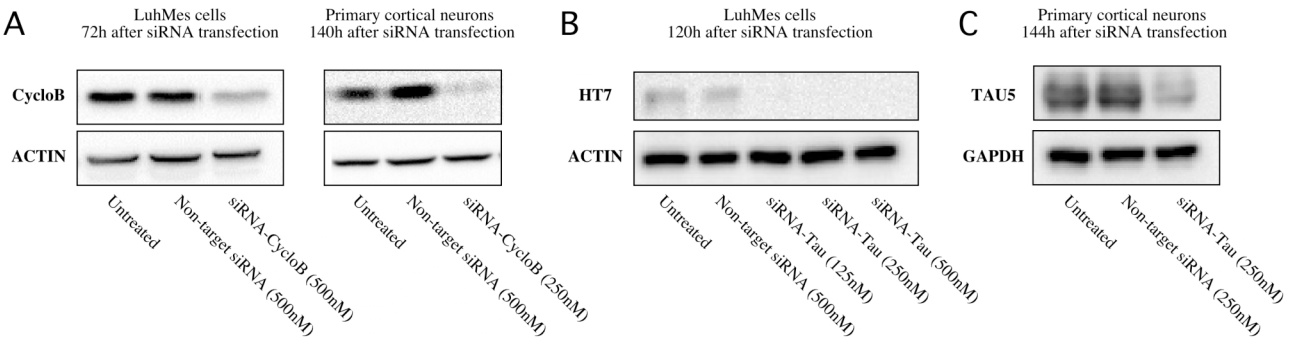

Figure 2. ***Accell-siRNA knockdown of Cyclophilin B and Tau protein in vitro. (A)*** *Cyclophilin B (CycloB) was effectively knocked down in human mesencephalic neurons (LuhMes; 70% at 500nM) and mouse primary cortical neurons (80% at 250nM) at 72 and 140h after transfection, respectively. (**B-C**) Similar, Accell-siRNA against human TAU showed significant knockdown in LuhMes (>95% at 125-500nM; **B**) and primary cortical neurons (75% at 250nM; **C**), derived from P301S mouse model, here shown at 120 and 144h after siRNA transfection.*

In order to deliver the siRNA *in vivo* we investigated PEI-based delivery, in collaboration with Dr. Aigner at Department of Pharmacology, Philipps-University Marburg, as well as the vector free Accell-siRNA transfection in rodent brain. Low molecular weight PEI (Jet-PEI and F25 PEI) relies on non-covalent formation of PEI and RNA molecules, where the RNA is protected and the complex enter the cells via endocytosis. Although, this methods have previously shown very promising data [5], we observed that the PEI used were highly toxic when injected in the brain. We found severe losses of GFP fluorescence and NeuN immunostaining after single PEI injection in the striatum of Actin-eGFP expressing mice (Figs. 3A & B).

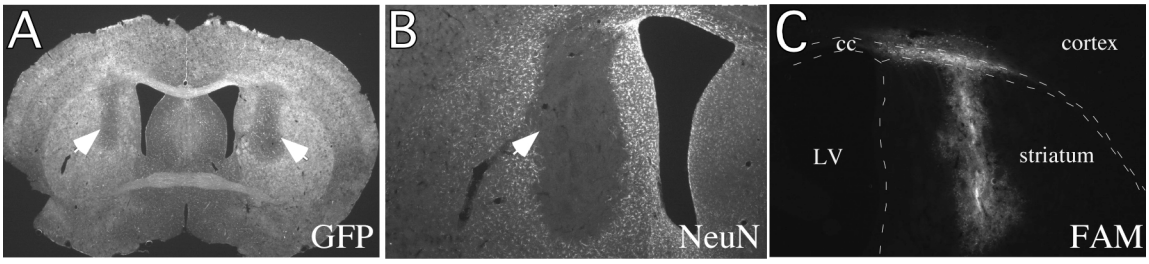

Figure 3. PEI-siRNA delivery to the striatum of Actin-eGFP expressing mice and Accell-siRNA transfection in C57/B6 mice. (A) *Animals received single injections of PEI-siRNA-GFP (white arrow in right striatum) and PEI-Luciferace (white arrow in left striatum), where you can see a absence of GFP staining on both sides. Moreover, in the NeuN staining. **(B)** you can clearly appreciate the*

neuronal cell loss from the PEi-injection in the striatum (white arrow). ***(C)*** *Single injection of Accell-siRNA™ couple with the florescence tag FAM showed robust transfection of the neurons in the striatum and in corpus callosum (cc) and cortex after 72h.*

The PEI delivery system was no longer pursued and we test siRNA transfection *in vivo*, using the Accell-siRNA™ system. In order to evaluate the transfection of cells *in vivo* and distribution of intracerebral injections of the molecules, we used Accell-siRNA™ where the siRNA molecule were labelled with Fluorescein (FAM) dye. Single injections of 2ul (0.05 umol/ul) siRNA lead to intense fluorescent signal in the striatum and corpus callosum (cc) / cortex, seen as a column streching from the ventral part of the cortex through the cc to the striatum after 3 days *in vivo* (Fig. 3C). On-going, we have injected single doses of the pre-designed Accell-siRNA™ against tau (0.06nmol in 3ul), which siRNA showed significant knockdown in primary neuronal cultures, in the hippocampus and cortex of human P301S mutant tau transgenic mice, the areas showing most robust expression and hyperphosphorylation of mutant human tau in the mouse model. The data is, however, not yet available.

Our data represent an important step forward in investigating the effect of siRNA interference in a clinical relevant model of human Tau-related disease. Moreover, applications in an animal's model of the disease (*data in progress*) will further shed light on the effect of siRNA interference and its potential application as a treatment for Tauopathies and related disorders. However, closer collaboration should be established between companies working with siRNA and the academic, and not the least more research funding is required from private and governmental institution to bring new applications in to clinical practice.

The Marie Curie fellowship is an important resource, which has given me personally a great experience and education. It is also of most importance in order to bring translational and basic science forward and to build a good scientific community.

References:

1. Arriagada, P. V., Growdon, J. H., Hedley-Whyte, E. T. & Hyman, B. T. Neurofibrillary tangles but not senile plaques parallel duration and severity of Alzheimer's disease. Neurology 42, 631-9 (1992).
2. Lee, V. M., Goedert, M. & Trojanowski, J. Q. Neurodegenerative tauopathies. Annu Rev Neurosci 24, 1121-59 (2001).
3. Kim, D. H. & Rossi, J. J. Strategies for silencing human disease using RNA interference. Nat Rev Genet 8, 173-84 (2007).
4. Allen, B. et al. Abundant tau filaments and nonapoptotic neurodegeneration in transgenic mice expressing human P301S tau protein. J Neurosci 22, 9340-51 (2002).
5. Grzelinski, M. et al. RNA interference-mediated gene silencing of pleiotrophin through polyethylenimine-complexed small interfering RNAs in vivo exerts antitumoral effects in glioblastoma xenografts. Hum Gene Ther 17, 751-66 (2006).