

### 3.1 Publishable summary

#### 1. Project objectives

The socio-economic burden of diseases affecting the human central nervous system (CNS) is estimated to constitute 35% of all EU disease burden. Demographic changes in ageing societies of the EU will increase this rate considerably and this will represent a crucial challenge to forthcoming generations. Curative therapies still do not exist for most CNS diseases but gene therapy is a promising new approach. The NEUGENE consortium has been founded to improve CNS gene transfer technology substantially in order to enable advanced therapeutic applications in patients. NEUGENE's major objectives are to develop and to functionally validate the tools necessary for safe, efficient, durable, targeted and regulated expression of therapeutic molecules within the CNS. Complementary strategies are used to optimize adeno-associated virus (AAV) and lentivirus (LV) based gene transfer tools for cell type-specific delivery of transgenes, for their ability to regulate transgene expression levels, and for enhanced safety. These tasks are reflected by the respective work packages (WP1 = Targeting; WP2 = Regulation; WP3 = Safety / Immunology). Work package 4 (Functional validation) proved the enhanced therapeutic options of the developed gene transfer tools in an established animal model of a major neurodegenerative disorder, Parkinson's disease (PD). Thus, vector tools are primarily optimized for use in the nigro-striatal system, degeneration of which is a hallmark of PD. However, advanced, safe, targeted, and regulated gene transfer vectors will have major impacts for gene therapy of other brain diseases as well.

#### 2. Description of work performed since beginning of the project

Several new techniques could be implemented in the partner's laboratories, such as 2Photon live brain imaging to follow regulated and cell-type specific transgene expression in living animals, novel behavioural tests to monitor animal performance under lesion and / or therapeutic conditions, advanced reporter systems for selection of functional drug/aptamer pairs, or online *in vivo* microdialysis of dopamine and metabolites.

During the first project period the consortium assembled a tremendous number of prototype vector tools. As deduced from presentations at the NEUGENE project meetings and the more detailed reports (see below) almost 100 different vector genomes were constructed, propagated into recombinant viruses and functionally evaluated.

During the 2<sup>nd</sup> project period functional evaluations of novel vector tools were performed in a standardized animal model of PD, and optimized delivery methods were developed. The majority of proposed approaches proved to be successful, and the most suitable therapeutic concept available today is entering into clinical development already.

#### 3. Main results achieved so far

##### 3.1 *Targeting of AAV and LV vectors*

Functionality of the brain by no means depends on neurons only, but glial cells and especially astrocytes serve essential roles in maintenance of the CNS. Thus, making brain astrocytes available for CNS gene transfer strategies was one major focus of

NEUGENE, which has been fully achieved. Using different strategies for AAV and LV vectors the consortium was successful in developing astrocytes-specific vectors, which are already used for functional studies including gene therapy in animal models. For example, Mokola pseudotyping was used to shift the tropism of lentiviral vectors toward astrocytes in association with a detargeting strategy based on the incorporation of target sequences for the neuron-specific miR124 to effectively eliminate residual expression in neurons post-transcriptionally. To achieve astrocyte-specific RNAi-mediated gene knock-down three methodologies had to be combined, tissue-specific promoters, tetracycline expression system and miR-embedded siRNA expression and the efficacy of the approach was demonstrated in vivo.

Transcriptional control was also used to direct transgene expression of mosaic AAV-1/2 and AAV-5 serotypes exclusively to astrocytes. These vector prototypes now offer novel venues for both gene therapy and studies of basic brain functions, and astrocyte-specific regulated vectors have proven excellent functionality in pre-clinical animal models (see 3.4). Moreover, we optimized gene transfer into the main target cell population affected by Parkinson's disease, nigral dopaminergic neurons. Retrograde transduction of dopaminergic neurons in the substantia nigra after viral injections to the striatum proved most efficient for AAV6, followed by AAV9 and AAV8, and the combination of AAV serotype 6 with AAV9 opened up the possibility for repeated delivery of the same or sequential delivery of two therapeutic transgenes to the Substantia nigra.

Evaluation of several neuronal subtype-specific promoters allowed for targeting of transgene expression to neuronal subtypes or specific disease-affected neurons by LV vectors: these vectors provide safe tools, that are more selective than others available, for the administration of therapeutic molecules in the CNS.

Our studies did also prove that non-integrating LV can be generated with different pseudotypes and promoters and that these vectors show the same transduction properties as integrating LV. Thus, superior tools which lack the risk of insertional mutagenesis are now available for CNS gene therapy approaches.

### *3.2 Regulation of transgene expression*

We follow two different strategies for development of systems capable of controlling transgene expression levels in the brain: regulatory protein-based and RNA aptamer-based. The RNA-aptamer-based strategy is completely novel and represents a typical high risk / high gain project. Considerable progress has been made in assembling the assay system necessary for identification of suitable regulatory RNA aptamers, and the principle to generate specific aptamers was proven. However, the generated libraries were not sufficiently complex to allow for further in vivo testing yet. This work is on-going, but for the time being we have not met this objective.

In contrast, two different regulatory protein-based strategies have been tested successfully in vivo. Both regulated systems under development towards clinical applicability have demonstrated excellent manageability. They are based on two different principles: i) fusion of destabilizing domains (DD) to proteins involved in DOPA synthesis (TH and GCH1), expression levels of which are regulated by binding of the ligand trimethoprim (TMP) to the DD. This vector system aims at restoring DOPA in striatal neurons in late PD patients. ii) Expression of the neurotrophin GDNF in either neurons or glial cells, where the expression level is regulated by binding of the clinically approved drug mifepristone (Mfp) to the regulatory pSwitch protein, which controls activity of the minimal promoter driving GDNF expression. Both systems needed a large body of work (and indeed more work than expected) for fine

tuning of their respective components to achieve optimal results in pre-clinical animal models. As outlined below, both systems have been functionally tested in relevant pre-clinical animal models of PD.

### 3.3 Safety / Immunology

Interference of the human immune system with viral gene transfer vectors is only poorly understood, as exemplified by recent failures in visceral gene therapy trials. However, especially for AAV based gene therapies its understanding is crucial, since most humans become naturally immunized against AAV-2 during childhood. In order to mimic the human situation as closely as possible, we developed an immunization protocol for rats making use of the antigen which humans encounter, i.e. the wild-type AAV-2 virus. We found that in response to single subcutaneous immunization with wt AAV-2 rats developed neutralizing antibodies against AAV-2 vectors. Furthermore, preimmunization with wt AAV-2 significantly decreased transgene expression in the brain following intracerebral administration of AAV-2 vector encoding EGFP (75 % reduction in EGFP positive cells in preimmunized versus immune-naïve animals). These results clearly indicate that preexisting immunity to AAV-2 may compromise the efficiency of AAV-2-based vector systems even in immune privileged organs such as brain. Additional induction of a lesion which causes up-regulation of inflammatory markers did not further reduce transduction efficacy, indicating that it was only the specific anti-capsid directed immune reaction precluding successful transgene expression.

Furthermore, we investigated immune issues related to EIAV lentiviral vectors, to which humans are not naturally immuno-competent. Our results demonstrated that CNS application of these vectors is not substantially hindered by peripheral immunity, arguing for a superior safety profile.

During the course of our studies, novel safety aspects arose and were investigated: recently introduced serotypes of AAV (like AAV-6, -8, -9) demonstrate superior transduction efficacy as compared to e.g. the prototype AAV-2, but evidently these vectors also are processed quite differently within transduced cells. To address potential safety issues associated with the use of AAV vectors, we explored virus persistence following injection in the nigrostriatal system. It was shown that AAV vector genomes are detectable extra-nuclearly for long time in CNS neurons and that intact AAV particles persist within the nucleus without disassembly, which might impose an additional safety issue. However, our studies also demonstrated that these particles are unlikely to spread to undesired location upon release from degenerating neurons, suggesting that the novel serotypes of AAV are not *per se* introducing uncontrollable safety risks.

### 3.4 Functional validation

The major goal of the NEUGENE consortium was to evaluate the developed advanced genetic treatment options in a pre-clinical animal of PD. The major results obtained during these studies are:

- An AAV vector was generated to constitutively express optimized levels of DOPA synthesizing TH/GCH1, which successfully restored motor performance

in a model of late PD, without induction of any dyskinesia. This tool is in a clinical development program already.

- An AAV vector was generated to constitutively express GDNF in astrocytes, thereby preventing off-target delivery of the neurotrophin without any restriction in therapeutic efficacy. This tool offers novel delivery options for neurotrophic factor-based gene therapy.
- An AAV vector system was generated to express GDNF in a tightly regulated manner, and short-term intermittent induction of GDNF expression provided substantial improvements in motor performance and neuroprotection in a model of early PD. This tool is currently developed further towards clinical applicability.
- A non-integrating LV vector expressing GDNF was generated and provided the same level of neuroprotection as conventional integrating LV vectors. These tools will provide added safety for forthcoming therapeutic strategies using LV vectors.
- Neuronal subpopulation-specific and glia-specific AAV and LV vectors were generated, which are currently exploited to generate advanced animal models of neurodegenerative diseases.

#### 4. Expected final results and potential impact

Optimized AAV vectors to constitutively express DOPA restoring TH/GCH1 proteins in striatal neurons have entered clinical development programs at ULUND. The respective regulated versions are expected to reach clinical applicability within 2-3 years. The regulated GDNF expression vector system is currently being further optimized to fit into a single vector design together with a leading European gene therapy SME (uniQure, formerly AMT) in cooperation with UMG-GOE. The same company is interested in RNAi-based strategies developed by CEA for further pre-clinical research. Thus, tools developed through NEUGENE are very close to clinical applications already.

Further benefit is delivered by tools developed for advanced basic research on genetic therapies for CNS disorders, i.e. optimized and safe non-integrating LV vectors, LV and AAV vectors targeted to specific neuronal or glial sub-populations, protocols for re-delivery of AAV vectors into the CNS etc. In addition, optimized vector tools are currently used to generate advanced animal models of neurodegenerative disorders, targeting key components like the synuclein and huntingtin genes.

In conclusion, we anticipate that tools developed by the NEUGENE consortium will substantially promote applicability of gene medicines for the brain, thereby offering treatment opportunities for large numbers of patients.

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More contact details and links to participating institutions can be found on the project web page: **[www.neugene.eu](http://www.neugene.eu)**