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Table of Contents

	<u>Page</u>
1. Introduction.....	1
2. Keywords.....	2
3. Accomplishments.....	2
4. Impact.....	13
5. Changes/Problems.....	13
6. Products.....	14
7. Participants & Other Collaborating Organizations.....	14
8. References.....	17
8. Appendices.....	19

Abstract

Amyotrophic lateral sclerosis (ALS) is characterized by the progressive degeneration of motor neurons leading to skeletal muscle atrophy, paralysis, and the death of patients within 2 to 5 years of disease onset. Currently, ALS cannot be prevented and disease progression can be only minimally delayed. Many previous reports have shown that glial cell line-derived neurotrophic factor (GDNF) ameliorates certain aspects of the disease in a number of different animal models of ALS. Despite many encouraging results, a strategy aimed at delivering GDNF has yet to be used in a clinical trial for ALS. Recent research and phase I/II clinical trial successes using adeno-associated virus (AAV) as a therapeutic tool have led to a renewed interest in a gene therapy approach for various disorders of the nervous system. To date, no clinical trial for ALS has yet exploited a gene therapeutic strategy, which prompted us to investigate this approach for ALS. Here, we have chosen to use an AAV based gene therapy approach as a straightforward strategy to promote GDNF production in muscles. **Hypothesis:** Intramuscular AAV5-GDNF injection will ameliorate motor neuron function in the SOD1G93A rat model of ALS. **Objectives:** To perform crucial and extensive pre-clinical studies to enable an investigational new drug (IND) application with the Food and Drug Administration (FDA) for the approval to move the use of intramuscular GDNF delivery by AAV5 into humans affected by ALS. **Findings:** Using a combination of DOD, ALS Association and institutional funding we have investigated the potential of intramuscular AAV1, AAV5, AAV2/6 and AAV9 encoding GDNF as a therapeutic approach to ALS. In all cases intramuscularly administered AAV encoding GDNF did not have an over beneficial effect on motor neuron function. **Alternative treatment:** We propose to pursue the project using an ex-vivo gene therapeutic approach based on the intramuscular transplantation of mesenchymal stem cells (MSC) secreting GDNF. MSC-GDNF based cell therapy has reproducibly improved motor function, motor neuron survival and neuromuscular innervation in ALS rats [1]; [2].

Introduction

Amyotrophic lateral sclerosis (ALS) is the most common adult-onset motor neuron disease and leads to death of patients within 2 to 5 years from diagnosis. Approximately 20,000-30,000 Americans are affected by ALS and an estimated 5,000 new patients are diagnosed every year. There is no cure and Riluzole, the only FDA approved drug for the treatment of ALS, has minimal effects on disease progression. The incidence of ALS has been reported to be higher among US military veterans but further studies are needed to confirm these findings [3]. Numerous studies have demonstrated a direct beneficial effect of growth factors, such as insulin-like growth factor-1 (IGF-1), ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) on motor neuron survival in vitro, and on motor neuron function in experimental models of ALS [4]; [5]. GDNF is a potent trophic factor and several studies have shown the benefits of the beneficial effect of muscle-derived GDNF on motor neuron survival and function has been shown in acute models of motor neuron injury and in transgenic mouse models of ALS using various delivery strategies by a number of investigators [6]; [7]; [8]; [9]; [10]

Although our group specializes in stem-cell based therapeutics, gene therapy using an adeno-associated viral vector encoding GDNF appeared to be the most direct and rapid path to the clinic. This approach was supported by our preliminary data using an AAV2/6-GDNF and AAV5-GDNF and was endorsed by an industry partner, UniQure, a world leader in the development of human gene based therapies.

Keywords

Glial cell line-derived neurotrophic factor, motor neuron, amyotrophic lateral sclerosis, adeno associated virus, gene therapy, muscle

Accomplishments

What were the major goals of the project?

The overall goal of the research was to perform pre-clinical studies enabling an investigational new drug (IND) submission for the intramuscular delivery of an AAV5 encoding GDNF as a therapeutic approach to ALS. As such, we proposed in year one, the following aims:

Specific Aim 1A: To determine the optimal dosage of AAV5-GDNF necessary to maximize muscle innervation and maintain motor neuron function in a single hindlimb of pre-symptomatic and symptomatic SOD1G93A transgenic rats.

Specific Aim 1B: To determine the optimal dosage of AAV5-GDNF necessary to maintain muscle innervation and respiratory motor neuron function in the diaphragm of pre-symptomatic SOD1G93A transgenic rats.

What was accomplished under these goals?

This is a slightly extended progress report due to problems reaching specific milestones. Please note that the data presented in the section is unpublished.

Preliminary pilot data submitted in the grant application indicated that AAV5-GDNF (2.5E11 viral particles/muscle) bilaterally targeted to hind and forelimbs could enhance motor function and extended lifespan compared to AAV5-GFP in male SOD1G93A rats (n = 4). The same approach was not successful in female rats. We first repeated the study in a large cohort of animals (Table 1) but we were not able to reproduce the effect on lifespan observed in male SOD1G93A rats (Figure 1A). Data was analyzed with a generalized linear mixed model (using Asreml 3.0 in R 3.14 x64) generated using the restricted maximum likelihood (REML) method to model the effect of multiple independent variables from behavioral assays over time. In contrast to our pilot data, analysis of BBB forelimb function indicated a significant (p<0.05) worsening of motor function in the AAV5-GDNF cohort compared to controls (Figure 1B). No significant

effect on hindlimb function was observed (See appendix for complete statistical analysis, Svendsen laboratory experiment 33-Bilateral IM injection of 2.5E11 viral particles/muscle of AAV5-GDNF).

Rat	Treatment	Male	Female	Total
SOD1G93A	AAV5-GFP	6	9	15
SOD1G93A	AAV5-GDNF	8	7	15

Table 1 – AAV5 treatment matrix. AAV5-GDNF or AAV5-GFP was bi-laterally administered intramuscularly into the hindlimb (gastrocnemius, tibialis anterior, quadriceps) and forelimb (triceps) of the SOD1G93A rat model of ALS.

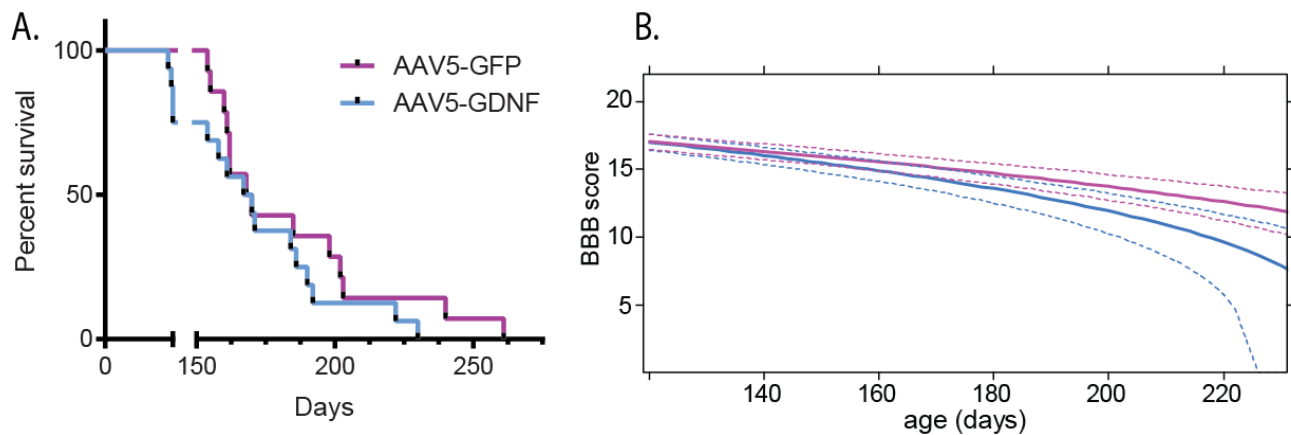


Figure 1 - No beneficial effect of bilateral intramuscular injection (IM) of AAV5-GDNF in the SOD1G93A rats model of ALS. (A) Kaplan-Meier survival curves. No effect on lifespan in males or females following IM AAV5-GDNF treatment was observed compared to control AAV5-GFP animals. (B) Treatment with AAV5-GDNF caused an exacerbation in forelimb motor function ($p < 0.05$) based on the BBB locomotor rating scale (data represented as an average of BBB forelimb values (full lines: mean BBB scores overtime; dashed lines: Upper and lower confidence intervals

Following lifespan and behavioral function analysis, we confirmed GDNF expression via enzyme-linked immunosorbent assay (ELISA) from homogenates of the gastrocnemius muscle of AAV5-GDNF compared to AAV5-GFP injected animals (Figure 2). GDNF expression was variable but was found to be higher than in control tissue in a majority of muscles assayed (Figure 2A) but was not statistically significant due to high variance (GDNF pg/ml, mean \pm SEM, AAV5-GFP: 78.02 ± 5.830 , AAV5-GDNF: 13218 ± 5836). Due to the variability of GDNF expression, we investigated if the degree of expression of the growth factor in hindlimb muscles could be correlated to improved motor function or lack of effect. No overt relationship was observed (Figure 2B). As lack of effect on motor function may have been caused by low distribution of GDNF across the entire muscle, the distribution of GDNF expression was verified by dividing muscle into segments and assaying for GDNF. Although uniform expression across the gastrocnemius was not observed in all muscles investigated, in most cases, expression was found to cover more than 50% of the muscle area (Figure 2C). Interestingly, GDNF expression in the serum of these animals was not detected via

ELISA (Data not shown). This is surprising as several studies report the detection of therapeutic molecules in serum of treated animals following IM injection. This could indicate that although GDNF was present in the targeted muscle that it was not properly secreted which would negate the therapeutic effect of GDNF at the muscle. Although muscle transduction have been used in multiple gene therapeutic approaches, some studies have shown low levels of secretion of gene product following muscle transduction in comparison to other tissue types [11]. We are currently investigating the possibility that GDNF secretion was not efficient following AAV5-GDNF transduction as well as pursuing further characterization of the samples in this including quantification of motor neuron survival. Indeed, we have shown in other studies that therapeutic approaches in SOD1G93A rats do not always have an overt effect on motor function but can preserve the motor neuron cell bodies in the spinal cord [12].

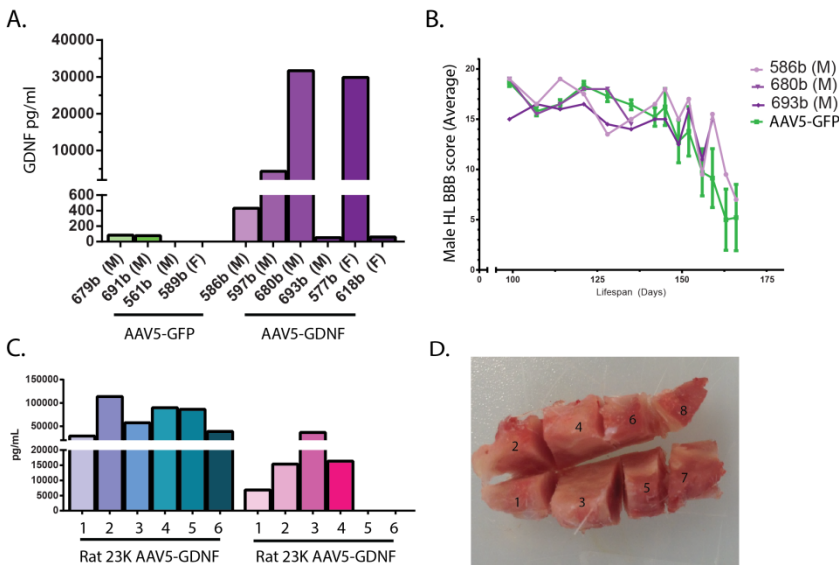


Figure 2 – GDNF expression in the gastrocnemius muscle following AAV5-GDNF injection. (A) Variable expression GDNF expression was observed in the left gastrocnemius of AAV5-GDNF injected animals sampled (x-axis represents rat identity and sex of a subset of animals sampled). In some cases (rat 693b and 618b) GDNF level were similar to AAV5-GFP injected animals. (B) No overt correlation between the degree of GDNF expression or respective limb function was observed in animals receiving AAV-GDNF (representative rats shown in individual traces) compared to average hindlimb BBB scores of controls. (C) Gastrocnemius muscle were divided into segments and assayed for GDNF expression. (D) Example of a divided muscle, note that muscle may have been divided into fewer segments due to overall size of the muscle due to muscle atrophy associated with ALS.

The lack of effect of AAV5-GDNF on motor function was unexpected based on the pilot data performed in the Svendsen laboratory as well as the previous data obtained by Dr. Masatoshi Suzuki (University of Wisconsin-Madison) with AAV2/6-GDNF. As methods used in the current study were identical to the pilot work (age of administration of treatment, IM injection technique etc.) it is possible that the low number of animals in the pilot resulted in unrepresentative data. Although unfortunate, the study has clearly emphasized the need for sufficiently powered cohorts even in preliminary pilot work. The study summarized above with AAV5-GDNF is well powered and has undergone thorough statistical analysis.

In light of the obtained data in the study evaluating IM administration of AAV5-GDNF in SOD1G93A, our collaborators at UniQure recommended pursuing the aims of the application using the AAV1 serotype rather than AAV5. Their AAV1-based product Glybera is approved for commercialization in the European Union for intramuscular AAV-1-based gene therapy of familial lipoprotein lipase deficiency (LPLD). AAV1-follistatin was also successful in a phase 1/2a clinical trial for becker muscular dystrophy [13] and has been used in mouse models for the intramuscular delivery of therapeutic

molecules [14]; [15]). As such, we pursued our work using AAV1-GDNF produced by UniQure.

Specific Aim1A, Major Task 1: Characterize the effect of AAV1-GDNF (*adjusted from AAV5-GDNF based on the above*) intramuscular injections, at various doses, on the motor function of SOD1G93A rats (see appendix II for the statement of work).

Upon funding notification, an animal protocol was generated, reviewed by Cedars-Sinai IACUC (protocol 5375) and approved by ACURO (*Aim1A, Milestone 1*). Please note that AAV serotype was not specified in the approved IACUC protocol and no other changes were performed. As such, we did not seek a secondary ACURO approval due to use of AAV1 rather than AAV5.

SOD1G93A rats were then mated to generate animals for the study (*Aim1A, Subtask 1*). Animals allocated to the protocol were divided into treatment cohorts with increasing dose of AAV1-GDNF (Table 2).

Table 2 - Experimental matrix for unilateral dosing of AAV1-GDNF in SOD1G93A rats.

Rat	Rat/group	Treatment		[Viral particles/muscle]	Euthanasia
		Hindlimb 1	Hindlimb 2		
SOD1G93A	5F/3M	AAV1-GDNF	Vehicle	2.50E+10	Disease end point
SOD1G93A	4F/5M	AAV1-GDNF	Vehicle	2.50E+11	Disease end point
SOD1G93A	4F/5M	AAV1-GDNF	Vehicle	2.50E+12	Disease end point
WT	5F/3M	vehicle	Vehicle	Vehicle	Disease end point

Measurements of BBB and grip strength began shortly prior to intramuscular injections of AAV1-GDNF or vehicle and were maintained until disease end point at which time tissue was collected (*Aim1A, Subtask 2*). Note that other disease monitoring strategies (Beam walking, Electrical impedance myography (EIM) and MRI) were not performed. We considered that the multiple anesthesia sessions and/or manipulations required to perform these procedures could adversely affect disease progression. Note that in other protocols testing alternative therapeutic approaches, BBB has been shown to be sufficiently sensitive between treated and non-treated limb to determine efficacy a therapeutic approach [1]; [16].

At the time of surgery, treatment side for vehicle or AAV1-GDNF injection was randomized by the surgeon and documented in a surgical log. All behavioral monitoring was performed blinded. Using generalized linear mixed modeling to assess the effects of the treatment groups (see appendix 1, experiment 43 (DOD) - Unilateral injection of AAV1-GDNF statistical report), no overt benefit of AAV1-GDNF was observed. In fact, in the group of animals treated with 2.5E11 (dose 2) viral particles of AAV1-GDNF, the ipsilateral treated hindlimb showed a slight worsening ($p < 0.05$) compared to the contralateral side (Figure 3B). This effect was not observed at other doses (Figure 3A, C). However, an overall acceleration in BBB decline in the hindlimb of rats treated with 2.5E12 (dose 3) AAV1-GDNF viral particles was observed ($p < 0.01$) in comparison with animals treated with 2.5E10 (dose 1) viral particles. Interestingly, this cohort (dose 3) of

animals showed a slowed decline in hindlimb grip strength compared to animals treated with $2.5E10$ (dose 2) or $2.5E9$ (dose 1) viral particles of AAV1-GDNF (Figure 3E). No difference in ipsilateral (AAV1-GDNF) versus contralateral (vehicle) grip strength was found at any dose investigated. As BBB is an overall assessment of gait/locomotion and grip strength is specifically designed at assessing grasping and hand function, it is possible that these parameters are differentially affected by the treatment. However, Kaplan-Meier survival analysis revealed an overall shorter lifespan of animals (combination of males and females, $p < 0.05$) injected with $2.5E12$ (dose 3) compared to animals treated with $2.5E10$ (dose 1) AAV1-GDNF viral particles (Figure 3F). A significant effect on lifespan was not reached when males and females were separately analyzed. As such, despite the benefit observed in hindlimb grip strength test, the treatment of ALS rats with AAV1-GDNF $2.5E12$ (dose 3) viral particles had an overall detrimental effect on ALS pathology and also indicates that BBB scoring rather than grip strength may be the more relevant behavioral assay to determine treatment outcome in this animal model.

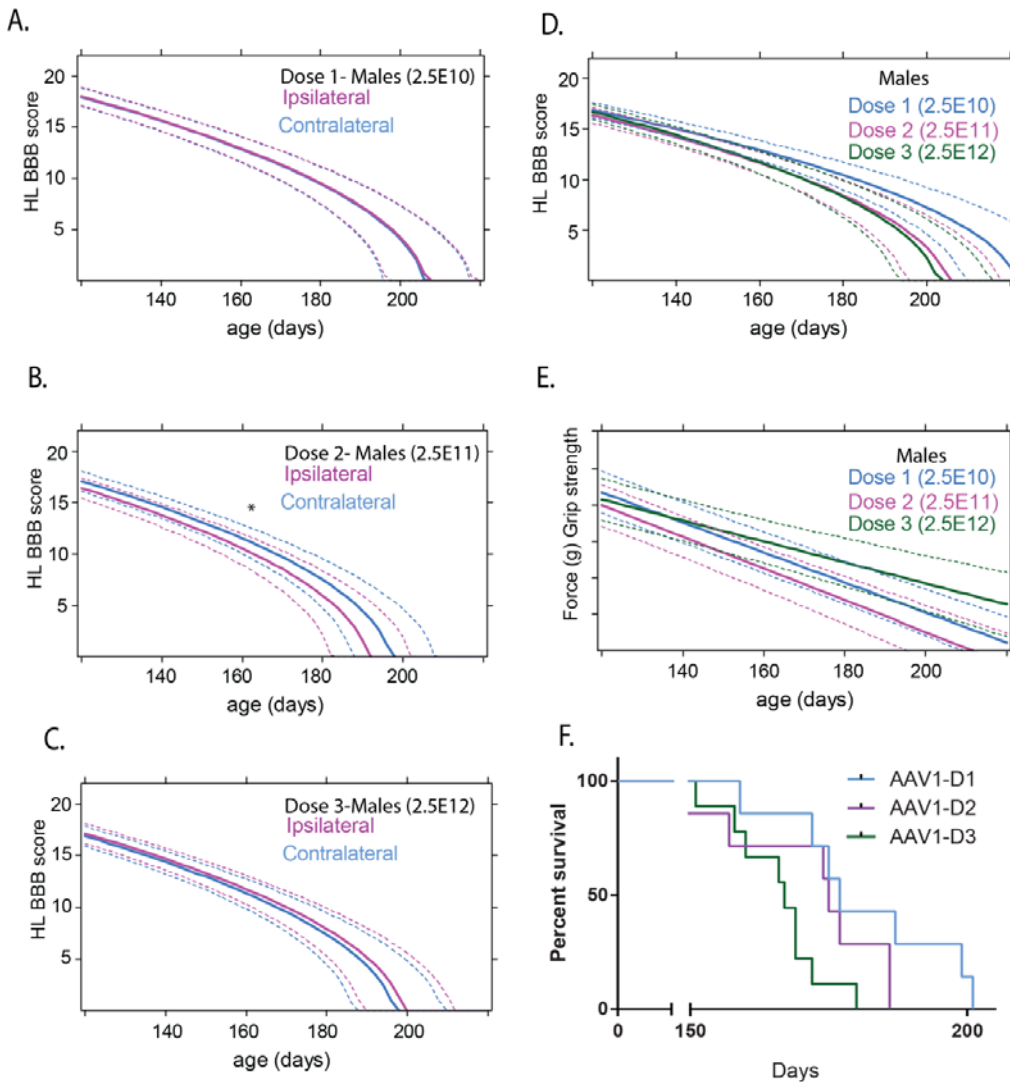


Figure 3. No overt beneficial effect of AAV1-GDNF on motor function or lifespan in SOD1G93A rats. (A-E) Male data is presented and is representative of female data. (A-C) Hindlimb treated with $2.5E11$ viral particles of AAV1-GDNF has significantly lower BBB scores compared to vehicle treated contralateral control. (D) An acceleration of BBB decline was observed with increasing dose of AAV1-GDNF.

Using non-biased stereological techniques, we have performed quantification of choline acetyltransferase (ChAT; (Figure 4A)) and neurofilament heavy (NF-H; (Figure 4B)) motor neurons in the spinal cord of animals treated with the highest dose of AAV1-GDNF (2.5E12 viral particles, dose 3). Motor neuron counts did not indicate any significant difference between the ipsilateral AAV1-GDNF treated limb compared to vehicle treated contralateral limb. Motor neuron counts for the other study cohorts are currently underway.

As in the previous experiment, a subset of injected muscles were verified to confirm the expression of GDNF (Figure 4A, B). Interestingly, no significant GDNF was detected in muscles from AAV1-GDNF treated with 2.5E10 viral particles (Dose 1) compared to contralateral vehicle treated muscles. Similarly, the muscle treated with 2.5E11 (Dose 2) viral particles showed high variance and a significant difference with contralateral vehicle treated muscle was not obtained. However, in muscles treated with 2.5E12 (Dose 3) viral particles a significant increase in GDNF was observed ($P < 0.01$). Moreover, ELISA for GDNF performed on serum of animals treated with 2/5E12 viral particles (dose 3) of AAV5-GDNF indicated a low concentration ($< 51\text{pg/ml}$) of GDNF in 50% of the animal tested. The presence of GDNF in the serum of lower dose cohorts remains to be confirmed. However, due to the minute amounts detected in the serum of dose 3 AAV1-GDNF, detection of GDNF in serum of dose 2 and 1 cohorts is not expected. The presence of GDNF in the serum of dose 3 animals suggests systemic distribution of GDNF and could potentially explain the bilateral effects observed on grip strength, BBB and lifespan of the highest dosed cohort.

Again, due to the variance of GDNF expression in individual muscles of the various study animal, we investigated if any correlation could be observed between the level of GDNF expression and BBB score in the individual the limbs of study animals (figure 4C), no overt relationship was observed.

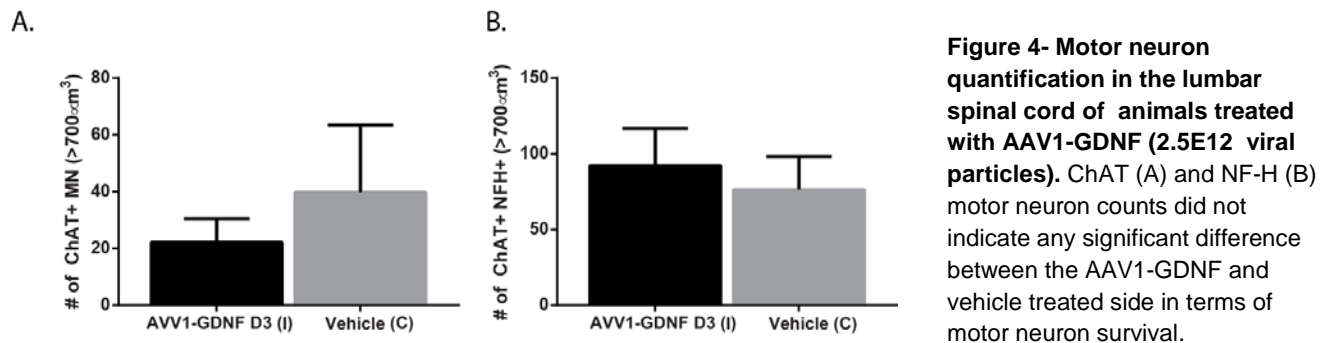


Figure 4- Motor neuron quantification in the lumbar spinal cord of animals treated with AAV1-GDNF (2.5E12 viral particles). ChAT (A) and NF-H (B) motor neuron counts did not indicate any significant difference between the AAV1-GDNF and vehicle treated side in terms of motor neuron survival.

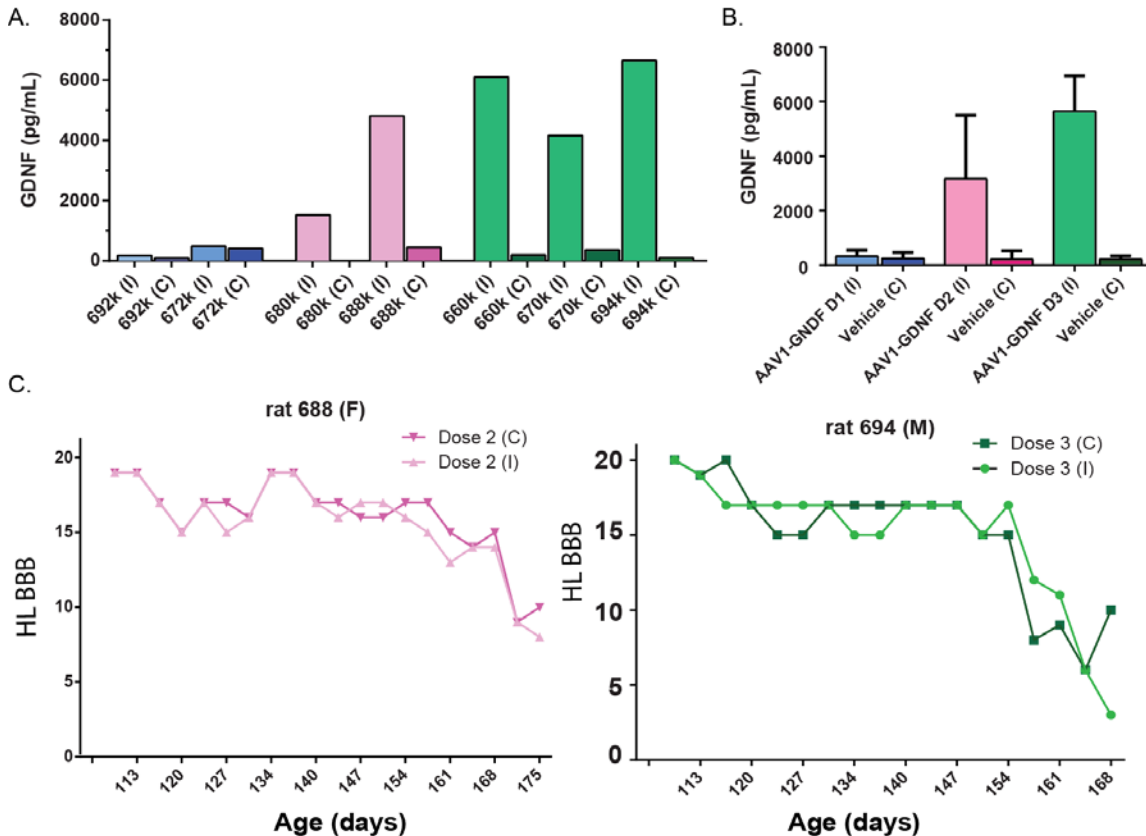


Figure 5. GDNF expression in tibialis anterior muscles of AAV1-GDNF (I; ipsilateral) and vehicle (C; contralateral) treated muscles. (A) Note the variable expression of GDNF at all doses (D1: dose 1, 2.5E10 viral particles; D2: dose 2, 2.5E11 viral particles; D3: dose 3, 2.5E12 viral particles). (B) GDNF expression is significantly enhanced in the muscle of dose 3 treated muscle. (C-D) Representative BBB scores over time for dose 2 and 3 treated animals. Note that despite high GDNF expression in T.A. muscle, no effect on ipsilateral (I) AAV1-GDNF treated limb is observed compared to vehicle treated contralateral limb (C).

In a final effort, we returned to our original data obtained via the intramuscular injection of AAV2/6-GDNF. This particular virus was generated by the Svendsen laboratory and differs from UniQure provided viruses (AAV1 and AAV5) by serotype but also as constitutive GDNF expression is obtained via the use of the mouse phosphoglycerate kinase 1 promoter (mPGK) rather than the combination of the cytomegalovirus (CMV) early enhancer element and portions of the chicken beta-actin gene and the rabbit beta-globin gene (CAG). The mPGK-GDNF construct has been extensively validated by the Svendsen laboratory. Unfortunately, the unilateral treatment of SOD1G93A rats with 1.25E8 (identical viral concentration as pilot data provided in the TDA award application) or 1.25E9 viral particles had no effect on forelimb or hindlimb motor function as assessed by BBB locomotor score (data not shown; see appendix 1, experiment 46- Unilateral injection of AAV2/6-GDNF statistical report)

In a separate set of experiments funded by the ALS association, an enhancement in forelimb BBB score ($P < 0.01$) was observed following the intravenous (IV) injection of AAV9-GDNF (GDNF under the control of the mPGK promoter). This approach was associated with only a slight increase in GDNF expression at the muscle. However, this treatment also resulted in adverse effects such as decreased overall activity in the open field and a decrease in exploratory behavior in Y-maze. Direct intramuscular injection of AAV9-GDNF had no effect on forelimb function. The enhancement in forelimb function following IV injection of GDNF but not subsequent to direct muscle injection indicates

that injection of the virus itself may lead to adverse events such as inflammation or an immune response in the muscle tissue resulting in the negation of the GDNF effect.

Subaward Masatoshi Suzuki, PhD (University of Wisconsin-Madison):

The main objective of the entire proposal (PI. Prof. Clive Svendsen, Cedars-Sinai Regenerative Medicine Institute) is to perform pre-clinical studies enabling an IND submission for the intramuscular delivery of an AAV1 encoding GDNF as a therapeutic approach to ALS. As a sub-contract, our specific role in the proposal was to contribute the experiments described in Specific Aim 1B. The project is to determine the optimal dosage of AAV1-GDNF necessary to maximize muscle innervation and maintain respiratory motor neuron function in ALS model rats (SOD1G93A transgenic).

Our subtasks included: 1) Generate pre-symptomatic SOD1G93A rats for diaphragm injections; 2) Perform AAV injections into the diaphragm of SOD1G93A rats; 3) Tissue collection and histological analysis; and 4) Data analysis and review. As Subtask 1, we obtained four SOD1G93A male breeders from Svendsen lab and expanded our colony size to prepare animal studies. We also worked with UniQure and Cedars-Sinai to set up the material transfer agreement for using AAV at UW-Madison. Although we prepared our animal colony for diaphragm injections of AAV-GDNF, we decided to postpone the experiments based on the latest results done by Svendsen lab.

What opportunities for training and professional development has the project provided?

The significant amount of data associated with this project resulted in the application of advanced statistical modelling to multiple data sets. It has also prompted more collaboration and discussions regarding proper statistical data analysis techniques within the laboratory. The statistical methods learned in the course of the project are now being applied to all in-life data obtained in the laboratory.

We have also adjusted the number of animals used in pilot studies based on the results of the data obtained in the AAV5 studies.

How were the results disseminated to the communities of interest?

Once analysis is complete (motor neuron counts, ELISA etc.) the data from the AAV studies will be submitted for publication.

What do you plan to do during the next reporting period to accomplish the goals?

This section contains unpublished data.

The studies performed in year 1 have enabled us to determine that targeting GDNF to the muscle using AAV is not an effective therapeutic approach. Interestingly, a similar problem was encountered when AAV-IGF1 delivery to the muscle was moved from mice

to rats and monkeys. Following amazing pre clinical data in the mouse [17] the following studies in rats and monkeys did not show retrograde transport of the IGF or functional effects and the path to the clinic was stopped (Kaspar personal communication).

In contrast to the direct GDNF delivery by AAV, our group has shown in two separate publications with large cohorts of SOD1 G93A transgenic rats [2]; [1]) that mesenchymal stem cells (MSCs) secreting GDNF (MSC-GDNF) can sustain motor function and enhance lifespan when transplanted at the muscle. This strategy was initially not favored by our group as direct injection of AAV-GDNF appeared a far simpler protocol and more rapidly clinically viable. However, as we have conclusively shown this approach does not work in this model we will switch to the dual approach of mesenchymal cells releasing GDNF. It is very likely that the reason this has worked so well in the past is the fact that the mesenchymal cells are a good type of cell for releasing GDNF (vs the muscle that showed very variable release of GDNF – see Figure 2 and 5) and that MSCs release a large set of other factors that may contribute to the support the motor neuron connections and function.

In our continuing collaboration with Dr. Suzuki a passage 2 master cell bank (MCB) of hMSC has been produced under good manufacturing practice (GMP) by the Waisman Biomanufacturing at the University of Wisconsin. Dr Masatoshi Suzuki (University of Wisconsin-Madison and current collaborator on this grant). Dr Derek Hei (Director, Waisman Biomanufacturing, US-Madison) intends to develop a clinical grade MSC-GDNF product that can be used for both pre-clinical animal studies and future human clinical trials. Therefore, we would like to support this program in the new year of studies for this proposal and switch from using GDNF viral approach to a dual stem cell and GDNF approach through the use of mesenchymal cells.

As such, in the course of the next reporting period, we would like to propose 2 major objectives:

Objective 1 (3 months): Finalization of data associated with the AAV-GDNF based therapeutic approaches and submission of manuscripts.

Objective 2 (6 months): Confirm the therapeutic potential of GDNF delivery at the muscle using mesenchymal stem cells

Objective 3 (12 months): Complete dosing studies for MSC-GDNF cells and get back on track for pre clinical testing in year 3

As a preliminary study, we obtained a research-grade, bone marrow derived hMSCs from Waisman Biomanufacturing and transduced them with GDNF-expressing lentivirus. These research grade hMSCs are similar to the hMSC line already established as a cGMP bank at Waisman Biomanufacturing. hMSC were transduced with lentivirus encoding GDNF used previously by our group for growth factor

production in human stem cells [18]; [19]; [20]; [21]; [12]; [22]; [23]; [24] and showed over 90% infection efficiency in total cells. A clinical grade lentivirus has already been produced by the Svendsen laboratory for their current preclinical work targeting stem cells to the spinal cord of ALS patients and is available for the production of hMSC-GDNF cells at Waisman Biomanufacturing. As shown in Fig. 6, GDNF protein was efficiently produced by the modified hMSCs and detected in the conditioned medium by ELISA. We are now ready to use these cells in transplant proof-of-concept studies.

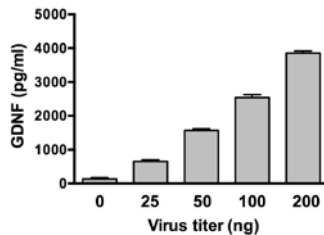


Figure 6- Preparation human mesenchymal stem cells expressing GDNF. Bone marrow-derived hMSCs were plated in a 24 well plate, incubated for 72 hours in medium containing various lentivirus titers (0-200 ng/p24/million cells). GDNF concentration in the conditioned medium was determined by ELISA.

The mission of the Svendsen laboratory ALS team is to translate potent therapeutic approaches to the clinic. Although direct intramuscular administration of AAV encoding GDNF will not be pursued, we have made several advancements in our translational program this year. Our published and current studies have repeatedly shown that the transplantation of human neural progenitor cells (hNPC) secreting GDNF to the spinal of SOD1G93A rats protects motor neurons from degeneration. Supported by the California Institute for Regenerative Medicine (CIRM), we are currently finalizing small and large animal safety studies for delivery of hNPC-GDNF to the spinal cord of ALS patients. An investigational new drug (IND) application is currently under preparation for this therapeutic approach with targeted submission in January of 2016. Moreover, our recent work has indicated that the brain may be a potent therapeutic target in ALS [16] and exciting new data from our group has indicated that hNPC-GDNF can have beneficial effect on motor function and lifespan in SO1G93A rats following transplantation into the motor cortex (Figure 7; unpublished data). We are currently in the process of repeating this study to ensure reproducibility. In the unlikely event that the mesnchymal-GDNF data this year fails to reach significance, we would like to switch to a cortical approach upon approval from DOD. In the

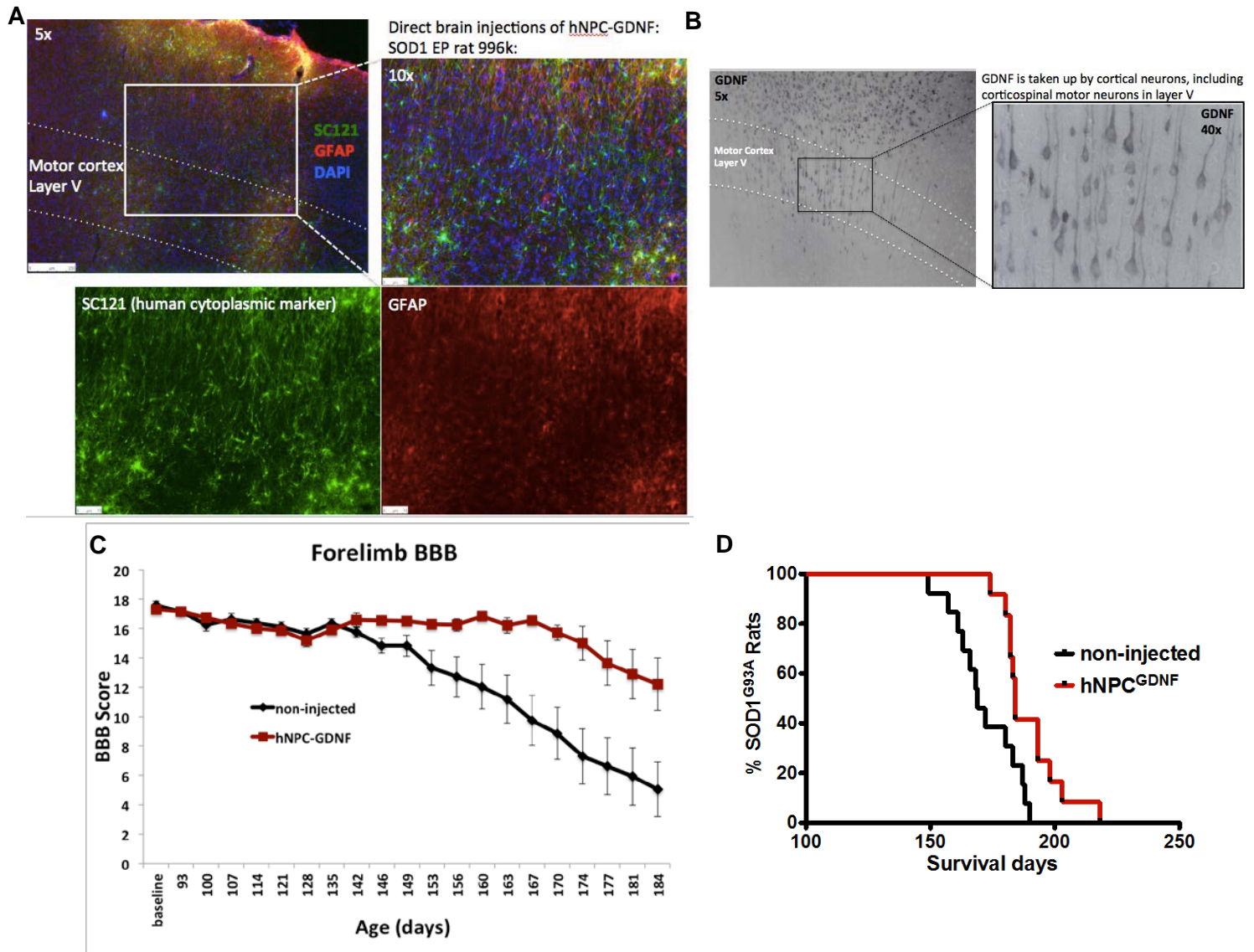


Figure 3 (Above): Brain transplants of hNPC^{GDNF}. (A) Immunostaining for human cytoplasmic marker SC121 (green) and astrocyte marker GFAP (red) in coronal brain sections of SOD1G93A rats revealed that after transplantation of hNPC-GDNF directly into the motor cortex, these cells survive and migrate throughout the cortex. (B) Images of a SOD1G93A rat brain section at endpoint after injections of hNPC-GDNF show cells, including corticospinal motor neurons, in layer V of the motor cortex expressing GDNF. Following brain injections of hNPC-GDNF, (C) forelimb motor function (BBB) was significantly improved as shown using a generalized linear mixed model of behavioral data, which generated predicted values, +/- 95% confidence intervals. (D) Survival was significantly extended, relative to sham rats. n=14 non-injected controls, n=11 hNPC-GDNF, 4 rats were excluded from the hNPC-GDNF group after no sign of engraftment was observed.

Impact

What was the impact on the development of the principal discipline of the project?

The main impact of the study is that we have conclusively shown that direct intramuscular injection on AAV-GDNF is not an appropriate therapeutic approach to ALS. A secondary impact was the development of a standardized and sensitive method for the statistical analysis of longitudinal behavioral data.

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer

Nothing to report

What was the impact on society beyond science and technology

Nothing to report

Changes/Problems

Changes in approach and reason for change

As discussed above, the serotype of the AAV used in the study was changed from AAV5 to AAV1 based on previously obtained data as well as the literature supporting the use of AAV1 rather than AAV5 for our study.

Based on the data generated in the study, the use of a direct gene therapeutic approach for GDNF to the muscle is not suitable for translation as a treatment for ALS. As such, we have proposed to pursue our DOD funded studies to perform the necessary pre-clinical studies to translate the use of MSC-GDNF to the clinic.

Actual or anticipated problems or delays and actions or plans to resolve them

Changes that had a significant impact on expenditures

Items reducing expenditures:

- MRI procedure were not performed
- Biobehavioral core: Beam walking and data analysis performed by Svendsen lab staff
- Reagents and supplies were not all purchased
- Reduced experimental load resulted in reduced percent effort of staff on the project

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.

AAV1-GDNF was used in our DOD funded studies rather than AAV5-GDNF. Both viruses were generated under the same manufacturing conditions by UniQure.

Products

Nothing to report

Participants & other collaborating organizations

What individuals have worked on the project?

Clive Svendsen, PhD

Role: Principal Investigator

Nearest person month worked: 1

Contribution: During the entire course of this project, Dr Svendsen will supervise staff and coordinate the efforts for the accomplishment of the aims of this proposal. He will also be responsible for the generation of progress reports and expense management.

Genevieve Gowing, PhD

Role: Project Scientist

Nearest person month worked: 7

Contribution: Dr. Gowing generated IACUC protocol associated with the studies, coordinated the generation and allocation of animals to the project, supervised experimental procedures, interpreted data, and generated figure/progress report. Dr Gowing will also be responsible for the generation of any manuscripts associated with the study.

Gretchen Miller, PhD

Role: Post-doctoral fellow

Nearest person month worked: 2

Contribution: Dr. Miller has assisted with general duties associated with animal care and euthanasia and tissue processing.

Pablo Avalos, MD

Role: Post-doctoral fellow

Nearest person month worked: 1

Contribution: Dr Avalos performed all the surgical procedures on the animals and participated in monitoring on study animals.

David Rushton, PhD

Role: Post-doctoral fellow

Nearest person month worked: 1

Funding: Institutional Support

Contribution: Dr Rushton performed all statistical analyses.

Brandon Shelley

Role: Research Associate III, Promoted to Research Associate IV

Nearest person month worked: 1

Contribution: Mr Shelley is the laboratory manager and ensured the availability of reagents and supplies required for the study, performed equipment maintenance and was consulted for the optimization of ELISA on muscle tissue.

Roksana Elder, MS

Role: Research Associate II

Nearest person month worked: 5

Contribution: Ms Elder was responsible for supervising mating, weaning, tagging, the daily monitoring of experimental animals and overall animal welfare. She also performed the majority of the euthanasias and tissue collection associated with the study.

Annie Ma

Role: Research Laboratory Associate I

Nearest person month worked: 1

Contribution: Ms Ma assists with stocking supplies associated with animal work and histological processing.

Leslie Garcia, ASHT

Role: Histologist

Nearest person month worked: 5

Contribution: Ms Garcia maintained the sample inventory, supervised the histological processing of samples, performed histological processing, microscopy and stereological quantifications of samples. She also assisted in processing orders for laboratory supplies and reagents.

Christine Chiu

Role: Research Laboratory Associate I

Nearest person month worked: 2

Contribution: Ms Chiu was responsible tissue section and staining of samples and maintenance of histological reagents.

Marlesa Godoy

Role: Research Associate I, promoted to Research Associate II

Nearest person month worked: 5

Contribution: Ms Godoy assisted in surgical procedure, data entry for behavioral assessments, performed ELISA on muscle tissue and is proceeding to stereological quantification of motor neuron counts.

Has there been a change in the active other support of the PI or senior/key personnel since the last reporting period?

New Active support:

U54NS091046-01 Thompson (MPI), Svendsen (MPI) 07/01/2014– 06/30/2020

NIH/LINCS /NINDS

Neuron and Glial Cellular Signatures From Normal and Diseases iPSC Cells

We will use existing iPSC lines from control patients and patients with SMA, fALS and sALS. We will then differentiate them into neural phenotypes and perform a series of assays on the cells including time lapse microscopy, cell death assays, high content screening and a series of omics – transcriptomics, proteomics and genomics in addition to epigenetic analysis.

W81XWH-14-1-0189 Svendsen (PI) 08/01/14 – 05/31/17

Department of Defense (DOD)

Muscle-derived GDNF: A gene therapeutic approach for preserving motor neuron function in ALS

Glial cell line-derived neurotrophic factor (GDNF) is a potent trophic molecule and can promote motor neuron survival in vitro and in vivo. This study will use a gene therapy approach to deliver GDNF to the muscle of rats. We aim to file an IND with the FDA by the end of this proposal.

University of Technology Sydney Svendsen (PI) 11/01/2014 – 10/31/2016

AHDS Patient-derived Induced Pluripotent Stem Cells

Provide a Disease in a Dish Model to Elucidate the Role of Mct8 in the Human Brain

We propose 4 specific aims in order to further understand of the mechanisms that underlie Mct8-deficiency, develop these iPSC-based platforms and establish molecule screens for the treatment of AHDS.

ALS Association Svendsen (PI) 07/01/2015 – 03/30/2016

Application of MultiOmyx to iPSC Models of ALS

Studies on ALS in collaboration with GE.

ALS Association

Svendsen (PI)

01/01/2015 – 06/30/2016

Using Novel Imaging Agents as a Biomarker for ALS

Progression in the fALS Rat

We will assess whether degeneration in both the motor cortex and spinal cord can be detected in the G93A preclinical animal model using novel MR and/or optical imaging agents developed at GE.

ALS Association

Svendsen (PI)

11/01/2014 – 12/31/2016

ENROLL ALS: DNA, Inflammatory and IPSC

Markers and Model of ALS

Our goal is to identify biomarkers in people with ALS to expand our understating of ALS pathology, treatment targets, disease progression, and anatomical differences between different disease phenotypes. This pilot project will allow us to conduct future efficient ALS clinical trials and learn more about the causes of ALS.

What other organizations were involved as partners?

UniQure provided AAV1-GDNF used in the DOD funded study as well as AAV5-GDNF used

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Appendix I: Statistical report

Methods

Rat behavioural unilateral analysis

Animals were injected unilaterally with a treatment, the injected side was referred to as ipsilateral and the uninjected side was considered a negative control, and referred to as contralateral. BBB score and grip strength were assessed independently on both sides of the animal over time following their recovery from post-surgical affects. A generalised linear mixed model (using Asreml 3.0 in R 3.14 x64) was generated using the restricted maximum likelihood (REML) method to model the effect of multiple independent variables behavioural assays over time (BBB score and grip strength). The effectors of experimental significance, including the type of injection (eg. Dose), side of the animal (ipsilateral or contralateral), days since surgery, and sex of the rat, and all 2nd order interactions, and plausible 3rd order interactions were included in the fixed model. The individual rat identification was implemented in the random model to model rat variability, being equivalent to a compound symmetry covariance structure this allowed for the modelling of trends within repeated measures from individual animals. In accordance with the assumptions of a generalised linear model, the model's standardised residuals were checked for a Gaussian distribution visually by normal quantile-quantile plot, and for homoscedasticity by plotting standardised residuals against fitted values. Where necessary, transformation of the Y-variable (behavioural assay) was used to improve the model's adherence with the previously mentioned assumptions. It was not possible to entirely avoid restrictions on the spread of standardised residuals against fitted values in all cases due to ceiling/floor effects inherent to the data. The model was then refined iteratively by removing the least significant, highest-order related term in the fixed model determined by the Wald statistical test until all terms in the fixed model were at least meeting the $P < 0.05$ significance level. The generalised linear mixed model was then used to generate predictions with 95% confidence intervals, to show generally the trends which occurred as a result of each treatment within a standardised rat. Post-hoc statistics were used to estimate the statistical significance between treatment levels at specific time points assessed by the model.

Rat behavioural bilateral analysis

Rats were injected bilaterally with treatments, and the behavioural score (both BBB or grip strength) was assessed over multiple time points following injection and compared to untreated (sham) animals. A generalised linear mixed model (using Asreml 3.0 in R 3.14 x64) was generated using the restricted maximum likelihood (REML) method to model the effect of multiple independent variables behavioural assays over time (BBB score and grip strength). The effectors of experimental significance, including the type of injection (eg. Dose), days since surgery, and sex of the rat, and all 2nd order interactions, and plausible 3rd order interactions were included in the fixed model. The

individual rat identification was implemented in the random model to model rat variability, being equivalent to a compound symmetry covariance structure this allowed for the modelling of trends within repeated measures from individual animals. In accordance with the assumptions of a generalised linear model, the model's standardised residuals were checked for a Gaussian distribution visually by normal quantile-quantile plot, and for homoscedasticity by plotting standardised residuals against fitted values. Where necessary, transformation of the Y-variable (behavioural assay) was used to improve the model's adherence with the previously mentioned assumptions. It was not possible to entirely avoid restrictions on the spread of standardised residuals against fitted values in all cases due to ceiling/floor effects inherent to the data. The model was then refined iteratively by removing the least significant, highest-order related term in the fixed model determined by the Wald statistical test until all terms in the fixed model were at least meeting the $P < 0.05$ significance level. The generalised linear mixed model was then used to generate predictions with 95% confidence intervals, to show generally the trends which occurred as a result of each treatment within a standardised rat. Post-hoc statistics were used to estimate the statistical significance between treatment levels at specific time points assessed by the model.

Results:

Experiment 33: Bilateral IM treatment with AAV5-GDNF or AAV5- GFP

Hindlimb BBB score

A generalised linear mixed model was generated to assess the effects of treatment groups in the fixed model and the random model used to modify the covariance structure to account for rat variability. A y-variable transformation $((y+1)^2)$ was found to result in a model which best fitted the assumptions of a generalised linear model.

The model was then refined by stepwise removal of insignificant factors determined by a $P > 0.05$ by the Wald statistical test. The final significant factors table can be found below (table 1).

Factor	Wald statistic	P value
Age (continuous)	387.8	$P < 0.0001$
Sex	81.9	$P < 0.0001$
Group	1.47	NS
Age:Sex	4.88	$P < 0.05$
Rat ID (random model)	NA	NA

Age was unsurprisingly the most significant factor, indicating the expected decline due to ALS-progression in the rats. The significance of the age:sex factor suggests that the progression of the diseases is different across males and females. However, group was eliminated from the model, suggesting the treatment had no effect.

Forelimb BBB score

A generalised linear mixed model was generated to assess the effects of treatment groups in the fixed model and the random model used to modify the covariance structure to account for rat variability. A y-variable transformation $((y+1)^3)$ was found to result in a model which best fitted the assumptions of a generalised linear model.

The model was then refined by stepwise removal of insignificant factors determined by a $P > 0.05$ by the Wald statistical test. The final significant factors table can be found below.

Factor	Wald statistic	P value
Age (continuous)	335.6	$P < 0.0001$
Group	0.64	$P < 0.0001$
Age:Group	6.6	$P < 0.05$
Rat ID (random model)	NA	NA

Age was unsurprisingly the most significant factor, indicating the expected decline due to ALS-progression in the rats. In contrast to the hind limb data sex was found to be insignificant but age:group significant. This implies that there was no difference between males and females, but the treatments may have had an effect on disease progression.

Interestingly, the GDNF treatment group rats showed an increased rate of progression compared to the GFP treatment group ($\Delta \Delta_{bbb}/\Delta_{age} = -2.2 \pm 1.5$, $T = 2.6$, $P < 0.05$, $df = 717$).

Experiment 43 (DoD): Unilateral injection of AAV1-GDNF (dose ranging)

Hindlimb BBB score

A generalised linear mixed model was generated to assess the effects of treatment groups in the fixed model and the random model used to modify the covariance structure to account for rat variability. A y-variable transformation $((y+1)^2)$ was found to result in a model which best fitted the assumptions of a generalised linear model.

The model was then refined by stepwise removal of insignificant factors determined by a $P > 0.05$ by the Wald statistical test. The final significant factors table can be found below.

Factor	Wald statistic	P value
Age (continuous)	1553.4	$P < 0.0001$
Sex	2.1	NS
Group	9	$P < 0.05$
Side	0.64	NS
Group:Side	8.36	$P < 0.05$
Rat ID (random model)	NA	NA

Age alone was unsurprisingly the most significant factor, indicating the expected decline due to ALS-progression in the rats. More interestingly, the significance of the group:side interaction, suggests that one or more treatments might have had an effect and resulted in an ipsilateral vs contralateral difference.

The refined model was then used to generate predictions with 95% confidence intervals comparing the ipsilateral and contralateral sides for each condition and gender.

comparison	$\Delta bbb \pm SE$	T-value, df	P-value
Dose 1 vs Dose 2	-5.89 ± 4.17	2.0, 24.7	NS
Dose 1 vs Dose 3	-6.30 ± 4.17	2.3, 24.7	$P < 0.05$
Dose 2 vs Dose 3	-2.22 ± 4.16	0.3, 25.7	NS
comparison	$\Delta bbb \pm SE$	T-value, df	P-value
Dose 1: Ipsi vs Contra	-1.28 ± 2.78	0.21, 1266.7	NS
Dose 2: Ipsi vs Contra	-4.83 ± 2.93	2.73, 1266.7	$P < 0.01$
Dose 3: Ipsi vs Contra	-3.10 ± 2.94	1.09, 1266.7	NS

- Dose 3 was found to have a significantly faster rate of progression compared to dose 1.
- Dose 2 showed a faster progression on the ipsilateral side vs contralateral.

Forelimb BBB score

A generalised linear mixed model was generated to assess the effects of treatment groups in the fixed model and the random model used to modify the covariance structure to account for rat variability. A y-variable transformation $((y+1)^2)$ was found to result in a model which best fitted the assumptions of a generalised linear model.

The model was then refined by stepwise removal of insignificant factors determined by a $P > 0.05$ by the Wald statistical test. The final significant factors table can be found below.

Factor	Wald statistic	P value
Age (continuous)	1324.9	$P < 0.0001$
Sex	1.2	NS
Group	3.75	NS
Age:Sex	18.9	$P < 0.0001$
Age:Group	4.6	NS
Rat ID (random model)	NA	NA

In contrast to the hind-limb data the groups were not found to have a significant effect on forelimb BBB score.

Hindlimb grip strength

A generalised linear mixed model was generated to assess the effects of treatment groups in the fixed model and the random model used to modify the covariance structure to account for rat variability.

The model was then refined by stepwise removal of insignificant factors determined by a $P > 0.05$ by the Wald statistical test. The final significant factors table can be found below (table 1).

Factor	Wald statistic	P value
Age	445.3	$P < 0.0001$
Sex	8.2	$P < 0.01$
Group	3.8	NS
Age:Sex	4.5	$P < 0.05$
Age:Group	10.4	$P < 0.01$
Rat ID (random model)	NA	NA

Age alone was unsurprisingly the most significant factor, indicating the expected decline due to ALS-progression in the rats. The significance of the sex suggests a difference between males and females in grip strength, including the interaction with age, which may indicate a difference in disease progression. More interestingly, the significance of the age:group suggests that one or more group is having a significantly different effect.

However, since side was eliminated from the model there is no ipsilateral vs contralateral difference within any group.

The refined model was then used to generate predictions with 95% confidence intervals comparing the ipsilateral and contralateral sides for each condition and gender, this was plotted using matrix plot.

comparison	Δ (Δ bbb/ Δ age) \pm SE	T-value, df	P-value
Dose 1 vs Dose 2	-0.23 \pm 0.48	0.47, 685.8	NS
Dose 1 vs Dose 3	1.24 \pm 0.50	2.48, 680.8	P<0.05
Dose 2 vs Dose 3	1.47 \pm 0.55	2.65, 683.8	P<0.01

- Dose3 was found to have significantly slower progression to both dose 1 and 2.

Experiment 46: Unilateral injection of AAV2/6-GDNF (dose ranging)

Hindlimb BBB score

A generalised linear mixed model was generated to assess the effects of treatment groups in the fixed model and the random model used to modify the covariance structure to account for rat variability. A y-variable transformation $((y+1)^{2.5})$ was found to result in a model which best fitted the assumptions of a generalised linear model.

Factor	Wald statistic	P value
Age	2709.2	P<0.0001
Group	0.11	NS
Sex	3.8	NS
Age:Sex	11.5	P<0.0001
Age:Group	1.4	NS
Rat ID (random model)	NA	NA

The interaction of age:group and group were found to be insignificant, implying no effect as a result of treatment.

Forelimb BBB score

A generalised linear mixed model was generated to assess the effects of treatment groups in the fixed model and the random model used to modify the covariance structure to account for rat variability. A y-variable transformation $((y+1)^{1.8})$ was found to result in a model which best fitted the assumptions of a generalised linear model.

Factor	Wald statistic	P value
Age	1619.8	P<0.0001
Group	0.34	NS
Age:Group	1.65	NS
Rat ID (random model)	NA	NA

Consistent with hind limb data, the interaction of age:group and group were found to be insignificant, implying no effect as a result of treatment. In contrast with hind limb, sex was also insignificant in the fore limb data.