Award Number: W81XWH-18-1-0175

TITLE: A GLP-1 Analog for the Treatment of ALS

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REPORT DATE: JULY 2022

TYPE OF REPORT: ANNUAL

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
JULY 2022	ANNUAL	1JUN2021 - 31MAY2022

4. TITLE AND SUBTITLE

NLY01: A GLP-1 Analog for the Treatment of ALS

5a. CONTRACT NUMBER
W81XWH-18-1-0175

5b. GRANT NUMBER
W81XWH-18-1-0175

5c. PROGRAM ELEMENT NUMBER

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The involvement of neuroinflammation manifested by activated microglia and astrocytes in ALS is supported by a wealth of clinical and molecular evidence. In addition to producing neurotoxic cytokines, activated microglia also induce differentiation of astroglial cells into neurotoxic A1 astrocytes - direct mediators of neuronal cell death, including possibly in ALS. Therefore, development of agents that could selectively inhibit the microglial activation and A1 astrocyte formation without off-target toxicity could have profound therapeutic potential since they could be used to treat a variety of neurologic disorders for which there currently are no disease-modifying therapies. NLY01 is a GLP-1R agonist. NLY01 selectively inhibits microglial activation, and blocks induction of cytokines and A1 astrocyte formation; thus, providing neuroprotection. The overall objective is to collect data for a FDA IND application for ALS. We propose to develop NLY01 for ALS its potential as a target using both ALS animal and human ALS iPSC-based models.

15. SUBJECT TERMS

GLP-1R, microglial activation, astrocytes, neuroprotection

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
U	U	U	UU	12	19b. TELEPHONE NUMBER *(include area code)*

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1. INTRODUCTION

The involvement of neuroinflammation manifested by activated microglia and astrocytes in ALS is supported by a wealth of clinical and molecular evidence. Recent studies show that, in addition to producing neurotoxic cytokines, activated microglia also induce differentiation of astroglial cells into neurotoxic A1 astrocytes - direct mediators of neuronal cell death, including possibly in ALS, Parkinson's disease (PD) and Alzheimer's disease (AD). Therefore, development of agents that could selectively inhibit the microglial activation and A1 astrocyte formation without off-target toxicity could have profound therapeutic potential since they could be used to treat a variety of neurologic disorders for which there currently are no disease-modifying therapies. NLY01 is a blood-brain barrier (BBB) penetrant long-acting GLP-1R agonist. NLY01 selectively inhibits microglial activation, and blocks induction of cytokines and A1 astrocyte formation; thus, providing neuroprotection. The overall objective is to collect data for a FDA IND application for ALS. Given the implication of common neuroinflammatory pathways for ALS and PD/AD, we propose to develop NLY01 for ALS by validating its potential as a target using both ALS animal and human ALS iPSC-based models.

2. KEYWORDS

IPS cells, Amyotrophic Lateral Sclerosis, ALS, Astrocyte, Astrocyte Progenitor, Glial Restricted Precursor, Cell Autonomy, Stem Cell

3. OVERALL PROJECT SUMMARY

ACCOMPLISHMENTS

Aim #1: A Dose Ranging Study of Mechanism of Action, Safety, and Therapeutic Efficacy in SOD1^{G93A} and longer-lived SOD1^{G37R} mice (JHU).

a. We have verified that NLY01 accumulates in the brain in SOD1G93A mice—thus confirming its biodistribution.

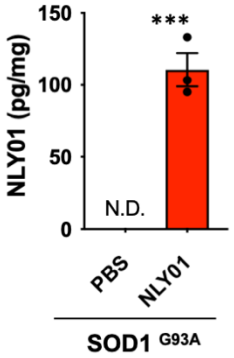


Figure 1. Accumulation of NLY01 in brain. SOD1^{G93A} mice were treated with PBS or NLY01 with a dose of 5 mg/kg twice weekly. The hemisphere regions were prepared from the whole brains and NLY01 concentrations were measured in homogenates by ELISA following manufacturer's recommendations (Phoenix Pharmaceuticals, Inc., CA). Error bars represent the mean ± S.E.M.. (n=3, N.D., not detected. ***P < 0.001).

b. The SOD^{G93A} mouse study (twice weekly injections with 10mg/kg NLY01) is completed. This includes the measurement of survival (no change), forelimb and hindlimb grip strength testing (no change), weight (no change), and onset of disease (no change).

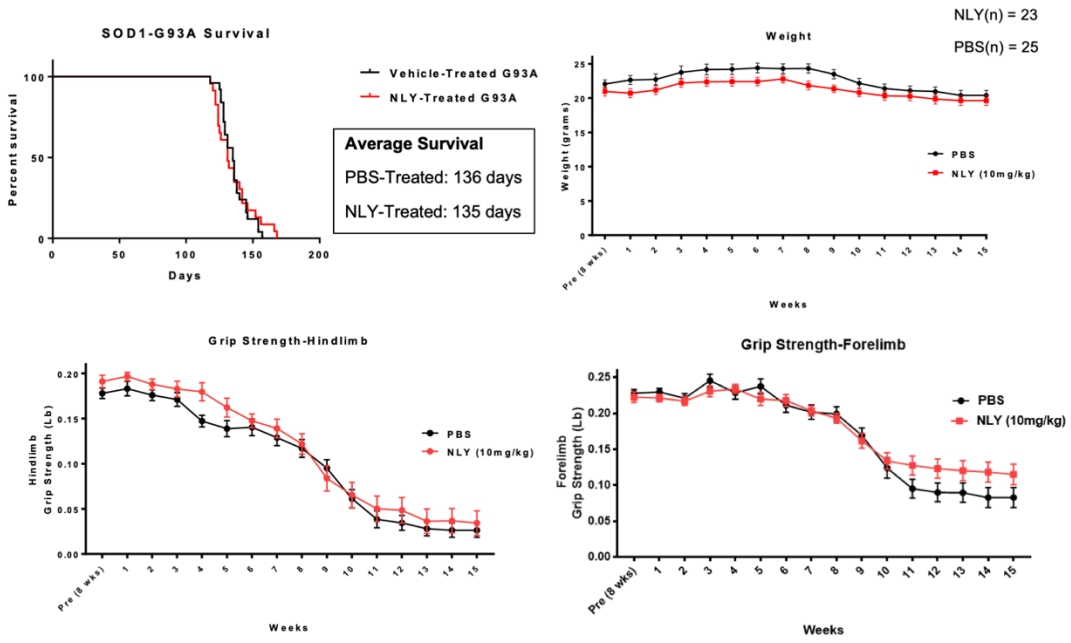


Figure 2. Behavioral analysis of NLY01 treated SOD1G93A mice

c. Dr. Lee's lab has analyzed the spinal cords from SOD1^{G93A} and control mice for the expression of pro-inflammatory neurotoxic cytokines and determined that IL-1α, IL-1β and IL-6 were significantly increased in the cervical region of presymptomatic stage by qRT-PCR. In the symptomatic stage, most of neurotoxic cytokines including C3, TNF-A, IL-1A, IL-1B and IL-6 were highly upregulated in most of the areas of cervical, thoracic and lumbar spinal cords (**Fig. 3**). . The levels of GFAP, C3 and TNF□ were significantly induced at end stage. Protein expression of C3, GFAP from symptomatic/end stage showed a significant increase in the cervical, thoracic, and lumbar regions. The expression of GLP-1R highly increased in the region of cervical and lumbar at the end stage (**Fig. 4**).

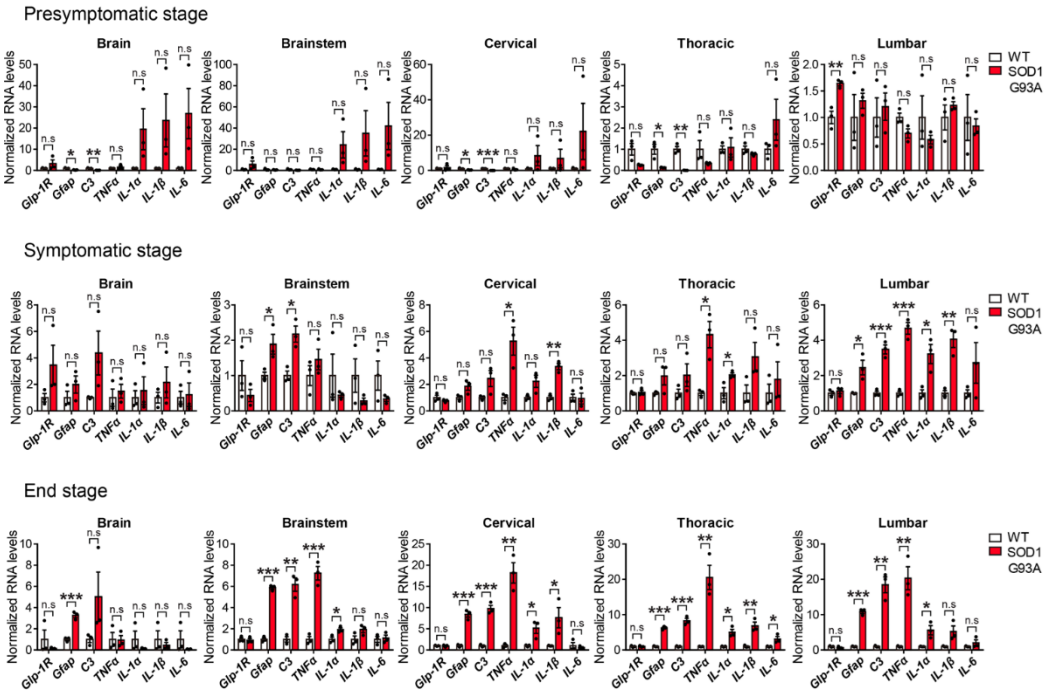


Figure 3. Analysis of spinal cord from SOD1 G93A mice for microglia activation

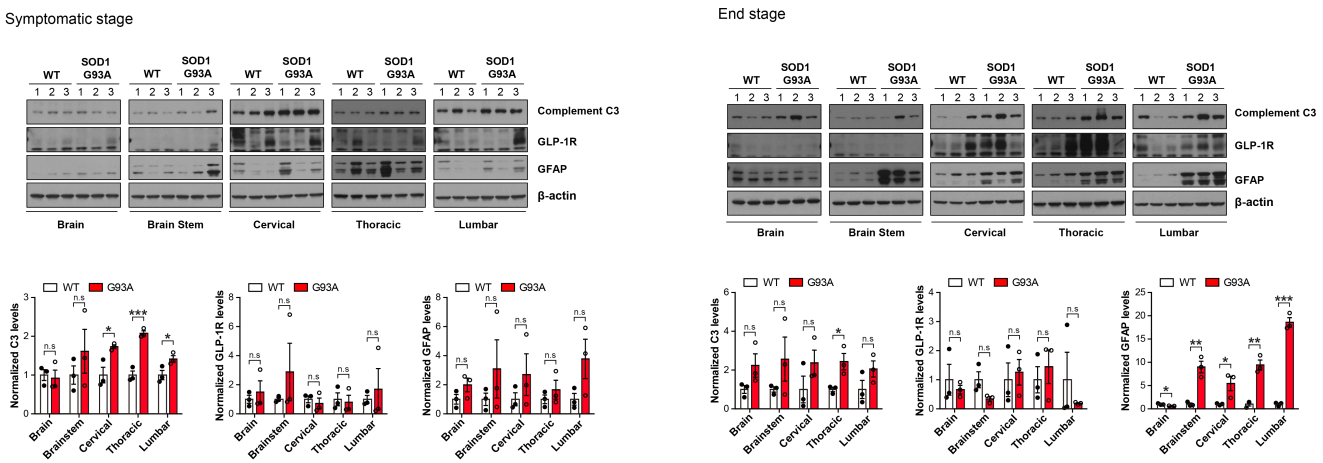


Figure 4. Analysis of spinal cord from symptomatic SOD1 G93A mice for C3 expression by WB.

d. Motor neuron (MN) analysis of these tissues from both the cervical and lumbar spinal cords show that there is no difference in MN survival between the groups (not shown). This is not entirely surprising given the short duration of survival for the SOD1G93A mouse.

e. We have confirmed in a cohort of WT mice treated with NLY01 that there are no adverse events related to weight loss or behavior (**Fig. 5**). This is important since long-term administration of the compound in SOD1G37R ALS mice will take place over the lifespan of that model (approximately 210 days).

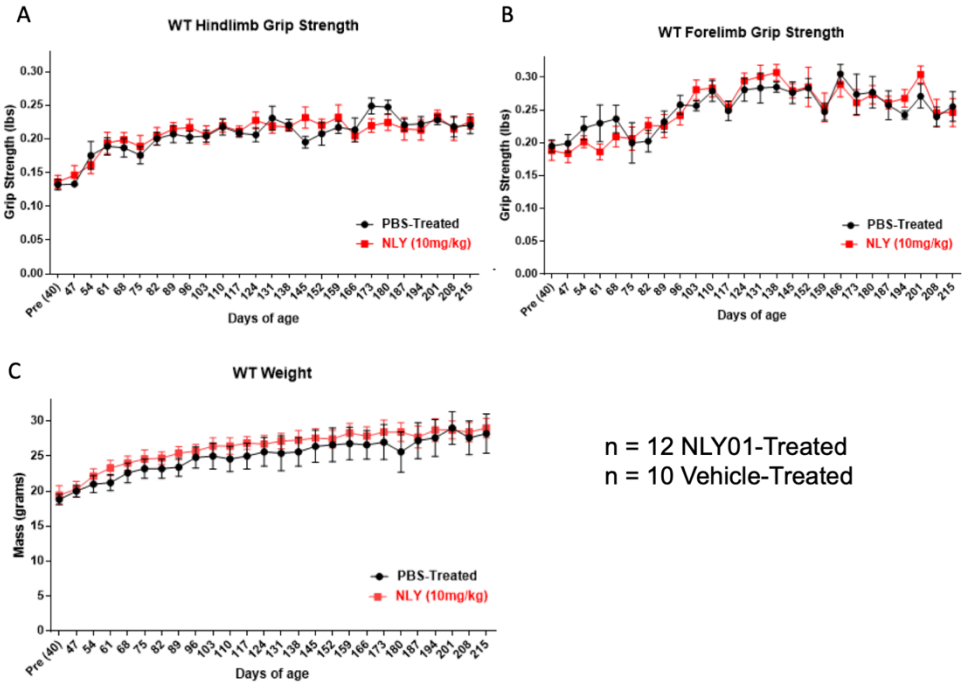
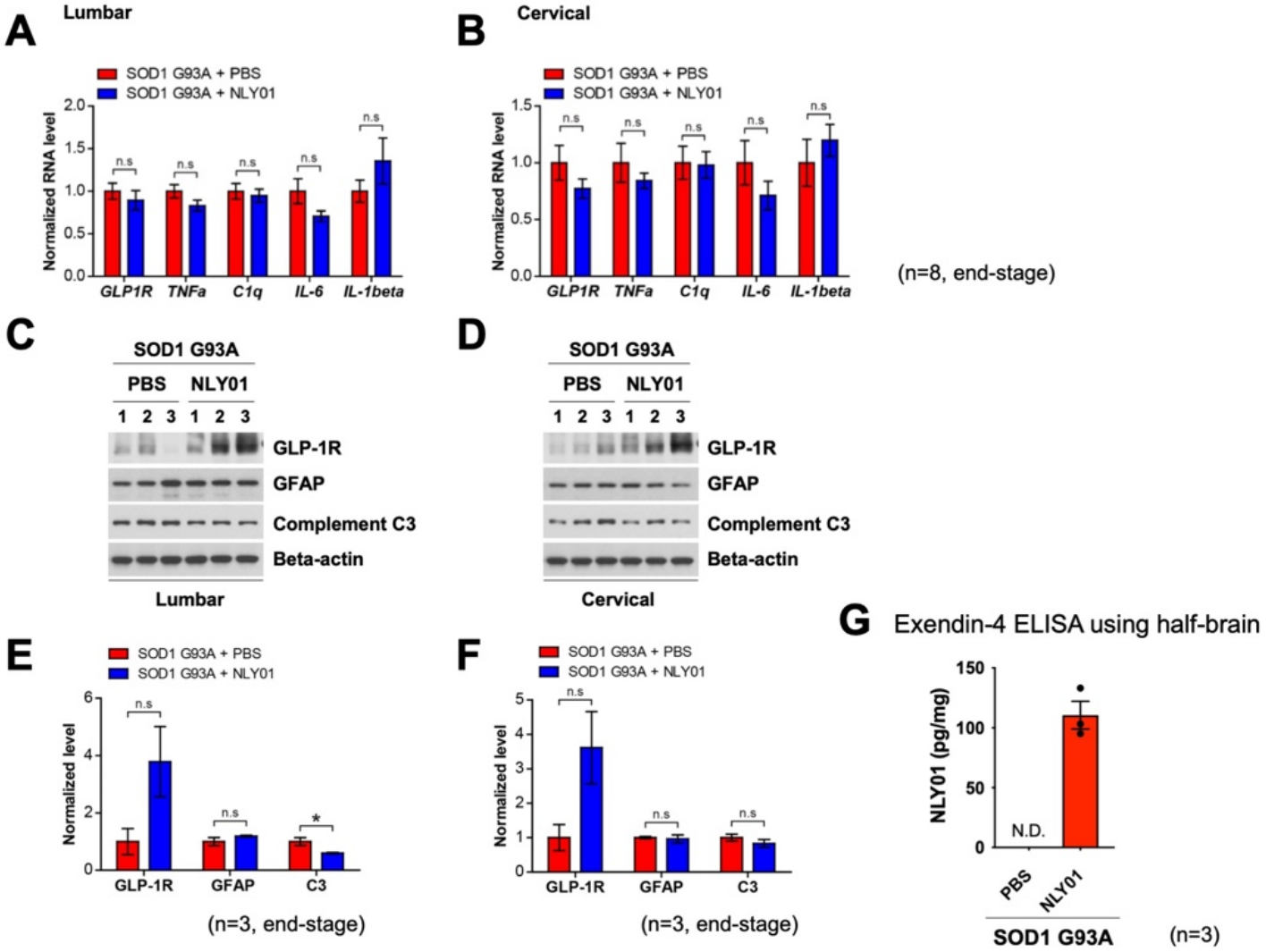
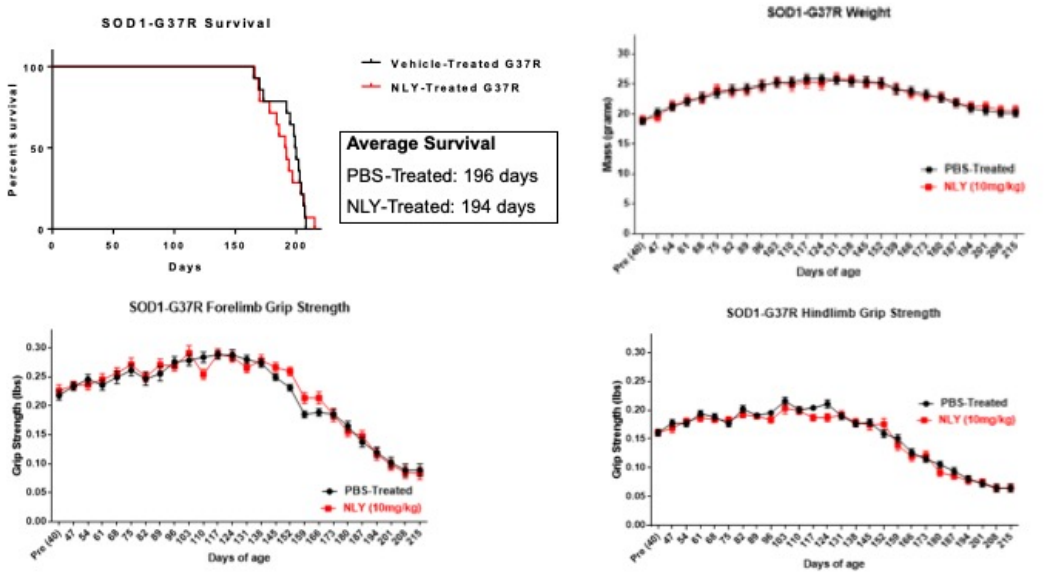


Figure 5. NLY01 has no adverse behavioral effects on WT mice

f. NLY01 treatment inhibits expression of the C3 pro-inflammatory cytokine in the spinal cords of SOD1^{G93A} mice (Figure 6). To examine the potential that NLY01 could reduce expression of selected cytokines, we treated SOD1G93A mice with NLY01 10mg/kg subcutaneously twice weekly beginning at 60 days of age and continuing to the endstages of disease. The gene expression of the GLP-1R and selected cytokines from SOD1^{G93A} mouse lumbar (**Fig. 6A**) and cervical spinal cord (**Fig 6B**) at endstage was examined but we did not appreciate significant differences in these expression profiles in those animals treated with NLY01. Immunoblot of GLP-1R, GFAP, and C3 in lumbar (**Fig. 6C, E**) and cervical spinal cord (**Fig. 6D, F**) shows a reduction in C3 protein expression at endstage in SOD1^{G93A} mice treated with NLY01 (10mg/kg) and a non-significant trend towards an increase in GLP-1R was noted in SOD1^{G93A} mice treated with NLY01. To verify that NLY01 reached the CNS, we examined exendin-4 in the brain via ELISA to demonstrate that NLY01 concentration in treated SOD1^{G93A} mice was over 100-fold greater than vehicle-treated SOD1^{G93A} mice (**Fig. 6G**).



g. NLY01 does not demonstrate any benefit in behavioral measures or survival in SOD1^{G37R} mice (Figure 7). We hypothesized that a longer-lived SOD1 mouse model might allow us to observe a benefit of NLY01 treatment because the duration of NLY01 treatment would be longer than that allowed with the more aggressive SOD1^{G93A} model. Despite high dose 10mg/kg doses of NLY01 twice weekly, we did not observe any differences in SOD1^{G37R} mouse survival, weight, forelimb or hindlimb grip strength.



h. NLY01 does not demonstrate any benefit in electrophysiological measures of motor activity in SOD1^{G37R} mice (Figure 8). We wanted to establish whether NLY01 treated SOD1^{G37R} mice would demonstrate a physiological benefit from treatment. We did not demonstrate any differences in SOD1^{G37R} mice treated with NLY01 at presymptomatic (160 days), symptomatic (180 days), or at endstage (200 days).

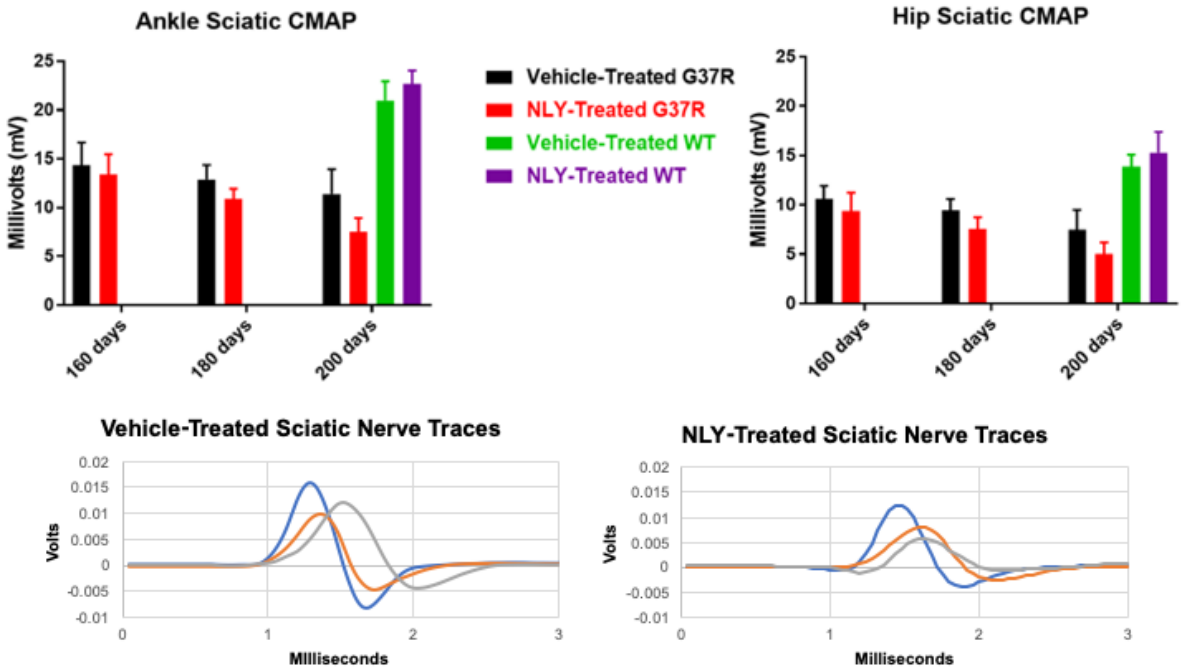


Figure 8. Electrophysiological measures of motor activity in SOD1^{G37R} mice following NLY01 treatment.

i. Analysis of isolated astrocyte in SOD1^{G37R} mice (Figure 9). Next, we isolated the astrocyte from cervical, thoracic and lumbar region to investigate the NLY01 effect in A1 astrocyte at end stage (200 days). The levels of mRNA for Pan and A1 astrocyte increased in the cervical region including LCN2, Serping3n, and H2-T23. However, the change was minimal in the samples of NLY01 treatment.

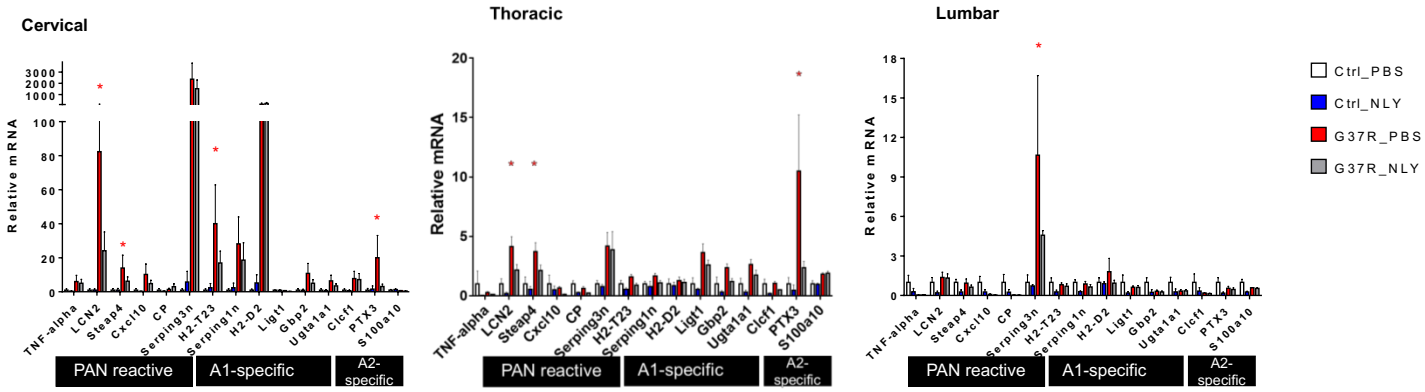


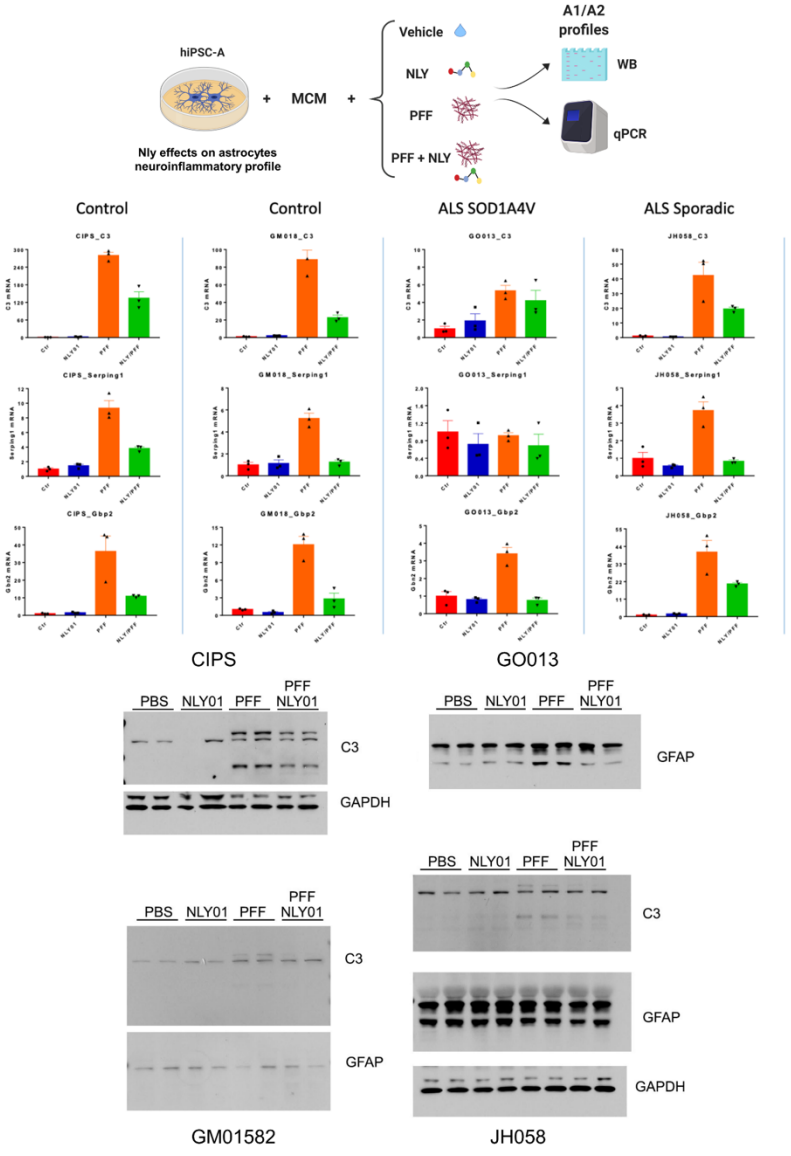
Figure 9. Analysis of isolated astrocyte in SOD1^{G37R} mice following NLY01 treatment.

<u>Aim #2: Establish whether NLY01 can provide neuroprotection via neuroinflammatory pathways in human iPSC-derived neuron/astrocyte cultures (JHU).</u>

a. <u>NLY01 prevents microglia-mediated activation of human astrocytes from control and ALS patients (Figure 10).</u>

We have previously demonstrated *in vivo* and *in vitro* rodent models that α-syn PFF-induced microglial activation promotes astrocytes conversion to an A1 neurotoxic profile through the release of IL-1α, tumor necrosis, factor alpha (TNFα) and C1q. We asked whether this phenomenon could contribute to neuroinflammatory changes in ALS astrocytes with relevance to human pathobiology.

Microglia-conditioned media (MCM) was collected from mouse primary microglial cultures that have been treated with either vehicle, NLY01, PFF or PFF+NLY01 and was then applied to cultures of human induced pluripotent-derived astrocytes (hiPSC-A) from control, sporadic (SALS) and familial ALS (FALS) patients (**Fig. 10**). Untreated human astrocytes from ALS patients and after treatment with vehicle MCM did not display any consistent "A1" neurotoxic profile. However, when ALS hiPSC-A were treated with MCM from PFF- activated microglia their transcriptional profile showed increases



in specific neuroinflammatory markers, including C3, Serping1, Gbp2, though other A1 markers were unaffected. Besides the mRNA levels of chemokine Ccl5 and Cxcl10 were highly increased in the activated astrocyte. These changes were completely prevented by treatment of PFF-activated microglia with NLY01. NLY01treatment of inactive microglia (i.e. non PFF-activated) did not have any effect on astrocyte neuroinflammatory markers. "A2"-specific transcripts were unaffected by MCM independent of treatment conditions. The above-mentioned significant changes in A1 transcripts expression were confirmed at protein levels with Western Blot analysis (CIPS=control, GO013=ALS SOD1^{A4V}).

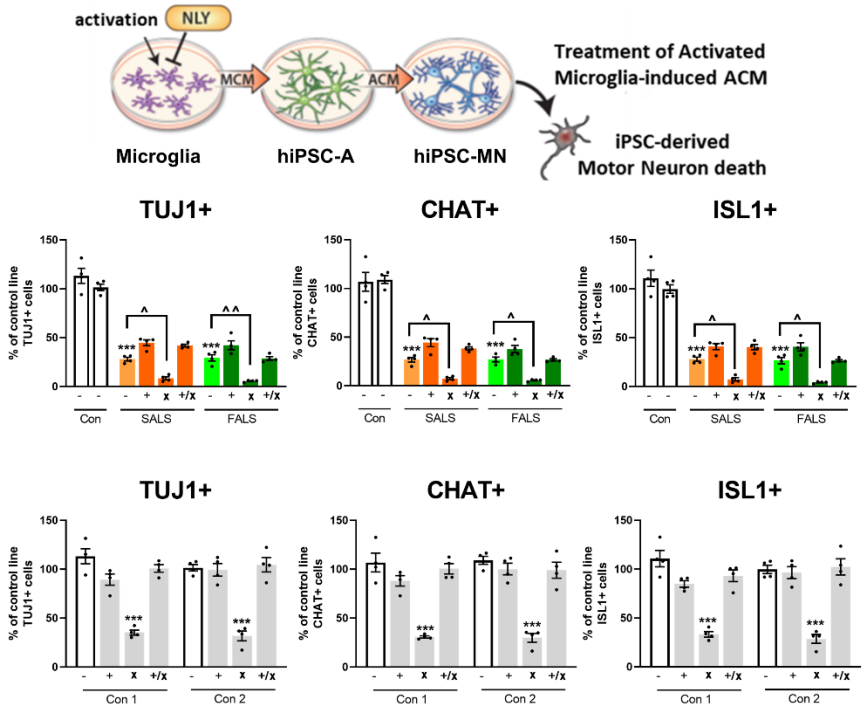
Interestingly, similar changes in transcriptional profiles and corresponding protein levels were seen when control instead of ALS hiPSC-A were treated with MCM suggesting that PFF- and microglia-mediated activation of astrocytes may not be unique to ALS, at least with regard to A1/A2 markers, but may be a broader mechanism of microglia-astrocyte crosstalk. As for ALS samples, NLY01 prevented control hiPSC-A conversion to a neuroinflammatory phenotype.

b. <u>NLY01 prevents microglia-mediated neurotoxicity of human astrocytes from control and ALS patients (Figure 11)</u>

Astrocyte-conditioned media (MCM) was collected from control, SALS and FALS hiPSC-A cultures that have been treated with vehicle, NLY01, PFF or PFF+NLY01 MCM and was then applied to cultures of a control hIPSC-derived motor neurons (hiPSC-MN).

ACM from both sporadic and familial ALS astrocytes was neurotoxic toward ChAT+ MN, including a subgroup of ISL1+ MN, suggesting that astrocyte neurotoxicity is at least partially mediated by the release of soluble molecules. The treatment of hiPSC-A with MCM from PFF activated microglia resulted in even higher degrees of MN toxicity. These effects microglia were completely rescued by treatment with NLY01, which, however, did not have any significant effect on non-activated microglia (NLY01 alone).

Control astrocytes did not display cell-autonomous toxicity toward hiPSC-MN, but when exposed to PFF MCM from activated microglia, these cells were converted to a neurotoxic phenotype, as suggested by significant decrease of ChAT+ and ISL1+ MN survival. This phenomenon was completely rescued when PFF-activated microglia but not inactive microglia were treated with NLY01.



Aim #3: Develop clinical trial protocol and compose an IND application for NLY01 in ALS patients (Neuraly).

We have made significant progress since our last report. We have now developed a research trial design with our initial biomarker study for further development to eventually study the capacity of NLY01 to reduce microglial activation in ALS. We began with a A Phase 1 Study to Assess the Safety and Tolerability of PET Imaging with [11C]CPPC [5-cyano-N-(4-(4-[11C]methylpiperazin-1-yl)-2-(piperidin-1-yl)phenyl)furan-2-carboxamide] Radioligand in Patients with Amyotrophic Lateral Sclerosis and are analyzing the data from control patient enrolled in the study. Based on our experience with this study and safety of the [^{11}C]CPPC PET ligand, we recently were recently able to expand the effort to include a larger Phase 1-2 study that would examine the CSF1 receptor PET ligand in a longitudinal fashion in a larger cohort of patients. This protocol received DOD ALSRP funding AL210044 Development of [^{11}C]CPPC as a Clinical PET Radioligand Biomarker of Microglial Activation in ALS.

This newly funded Phase 2 trial, based upon our experience from the current proposal, is awaiting final IRB approval.

Phase of Development: Phase 1-2
Number of Study Centers: 1 in the US
Johns Hopkins University
IRB #: IRB00343494

IND Sponsor-Investigator: Martin Pomper, M.D., Ph.D.

Principal Investigator: Nicholas Maragakis, MD.

Abstract for Research Study:
Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease of motor neurons, leading to progressive disability and ultimately death as there is no definitive treatment. One of the biological processes that is involved in the pathophysiology of ALS is microglial dysfunction. Microglia express the colony stimulating factor 1 receptor (CSF1R), which has been shown to be elevated in patients with ALS. Other studies looking at CSF1R have shown it to be possibly involved in other neuroinflammatory diseases, including Alzheimer's disease (AD). 5-cyano-N-(4-(4-[11C]methylpiperazin-1-yl)-2-(piperidin-1-yl)phenyl)furan-2-carboxamide **(**[^{11}C]CPPC) is a radiotracer ligand that specifically binds to CSF1R. Recent work by Dr. Pomper and colleagues in animal models of AD have shown that when the radioligand tracer [^{11}C]CPPC, is used in PET neuroimaging, there are differences in the images between animals affected by neuroinflammatory disease and controls. We would like to determine the safety of use of [^{11}C]CPPC in patients with ALS, and, ultimately, to determine if use of [^{11}C]CPPC in PET imaging can be used to differentiate between patients with and without ALS, to improve patient care, and to better understand the disease process.

Future Plans: What we learn from this new effort in a longitudinal fashion that correlates neuroinflammation with ALS progression will help with our better understanding of potential timing for the future administration of NLY01.

***Note:** DOD funds are not being utilized for the clinical trial but rather what was learned from this proposal (Aims #1 and #2) was incorporated into the development and design of the trial (Aim #3).

4. IMPACT

We have established that NLY01 has a biodistribution that can allow it to exert an effect on the brain and spinal cord in ALS models. We have also demonstrated that NLY01 can effectively reduce astrocyte A1 toxic phenotypes. This is important because there are parallels to the observed effects of NLY01 in Parkinson's and Alzheimer's disease models. While NLY01 did not show improvement in survival or behavioral phenotypes in

our 2 SOD1 mouse models of ALS we have now also demonstrated that NLY01 prevents microglia-mediated neurotoxicity of human astrocytes from control and ALS patients in our in vitro iPS cell platform. We have sought to have a better understanding of the degree and timing of activation of the CSF1R during the course of ALS in order to understand if there is a particular window in which NLY01 might be most effective. This is an important step in targeted therapeutics for the development of NLY01.

5. CHANGES/PROBLEMS

N/A

6. PRODUCTS

No products to be reported related to the current award.

7. Participants & Other Collaborating Organizations

Name	**Nicholas J. Maragakis**
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Researcher ID	0000-0002-7311-9614
Nearest person month worked	**12**
Contribution to project	**Oversees project**
Funding support	**ALSRP, ALS Association**

Name	**Seulki lee**
Project Role:	**Co-PI**
Researcher ID	
Nearest person month worked	**0**
Contribution to project	**Oversees project related to NLY01**
Funding support	**ALSRP, PRMP**

Name	**Khalil Rust**
Project Role:	**Research Technician**
Researcher ID	
Nearest person month worked	**11**
Contribution to project	**Performs SOD1 mouse study**
Funding support	**ALSRP**

8. Special Reporting Requirements

N/A

9. Appendices

None