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

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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					
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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

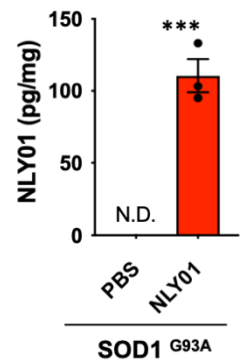
3. OVERALL PROJECT SUMMARY

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 SOD1^{G93A} 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 \pm 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).

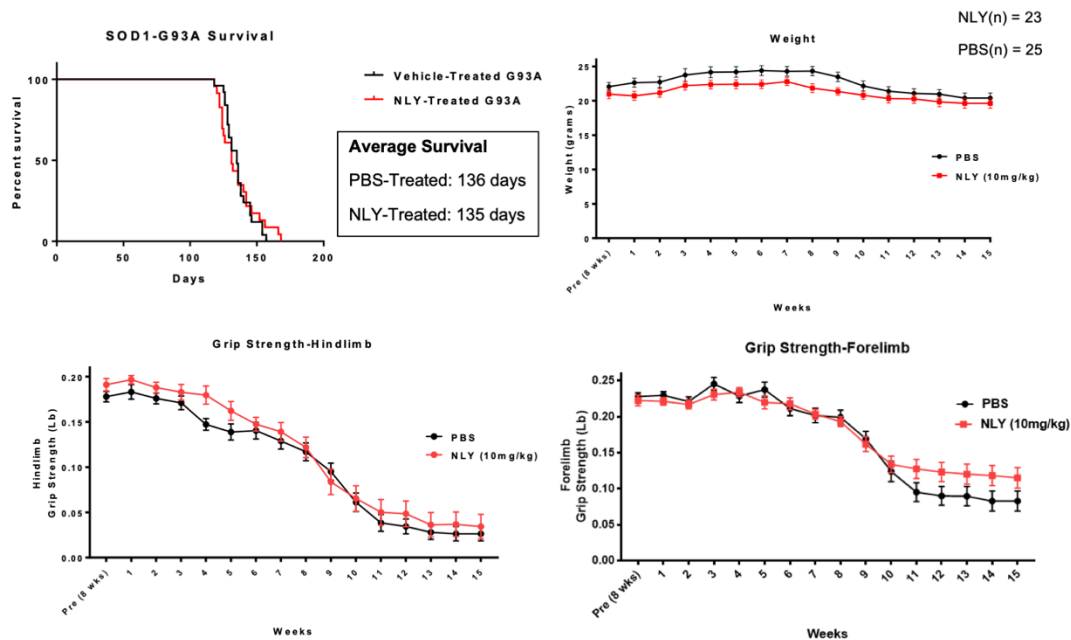


Figure 2. Behavioral analysis of NLY01 treated SOD1G93A mice

- 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-1 α , IL-1 β and IL-6 were significantly increased in the cervical region of presymptomatic stage by qRT-PCR. In the symptomatic stage, most of neurotoxic

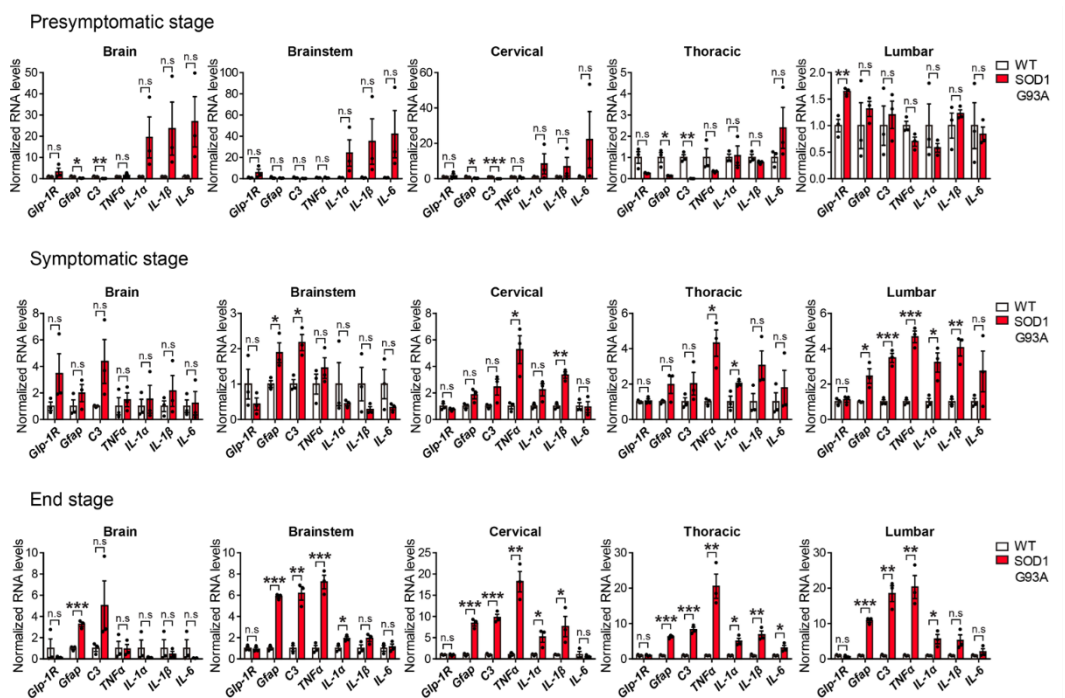


Figure 3. Analysis of spinal cord from SOD1 G93A mice for microglia activation

cytokines including C3, TNF- α , IL-1 α , IL-1 β 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 α 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).

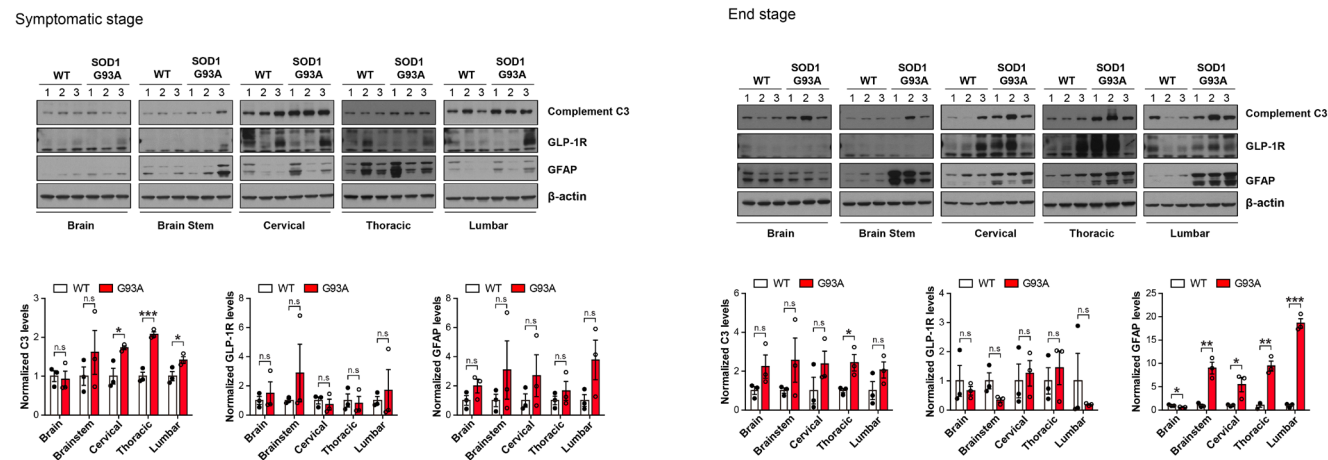


Figure 4. Analysis of spinal cord from symptomatic SOD1 G93A mice for C3 expression by WB.

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).

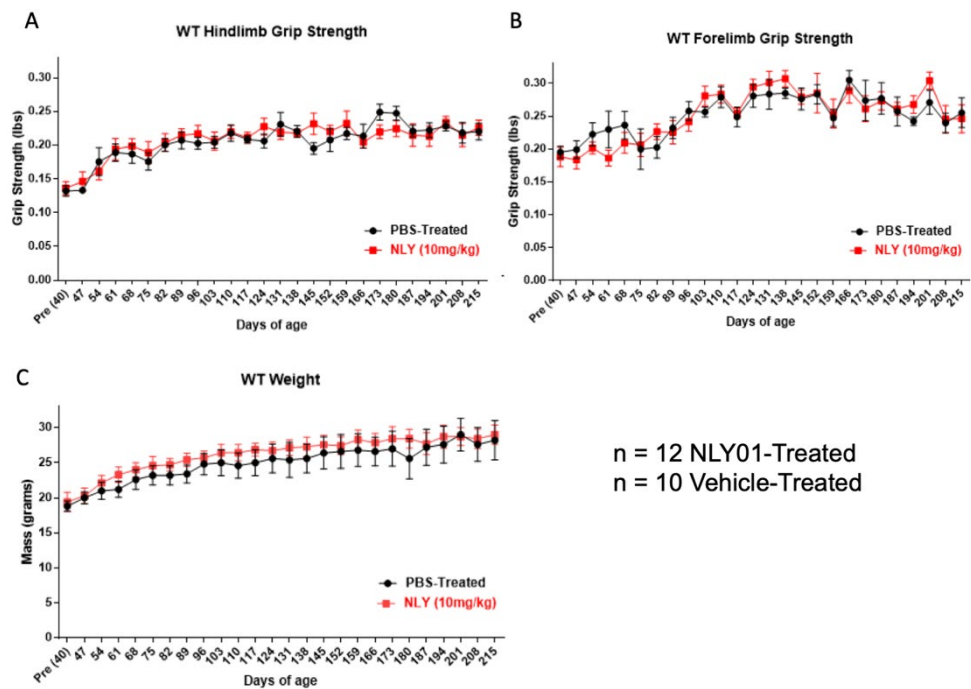
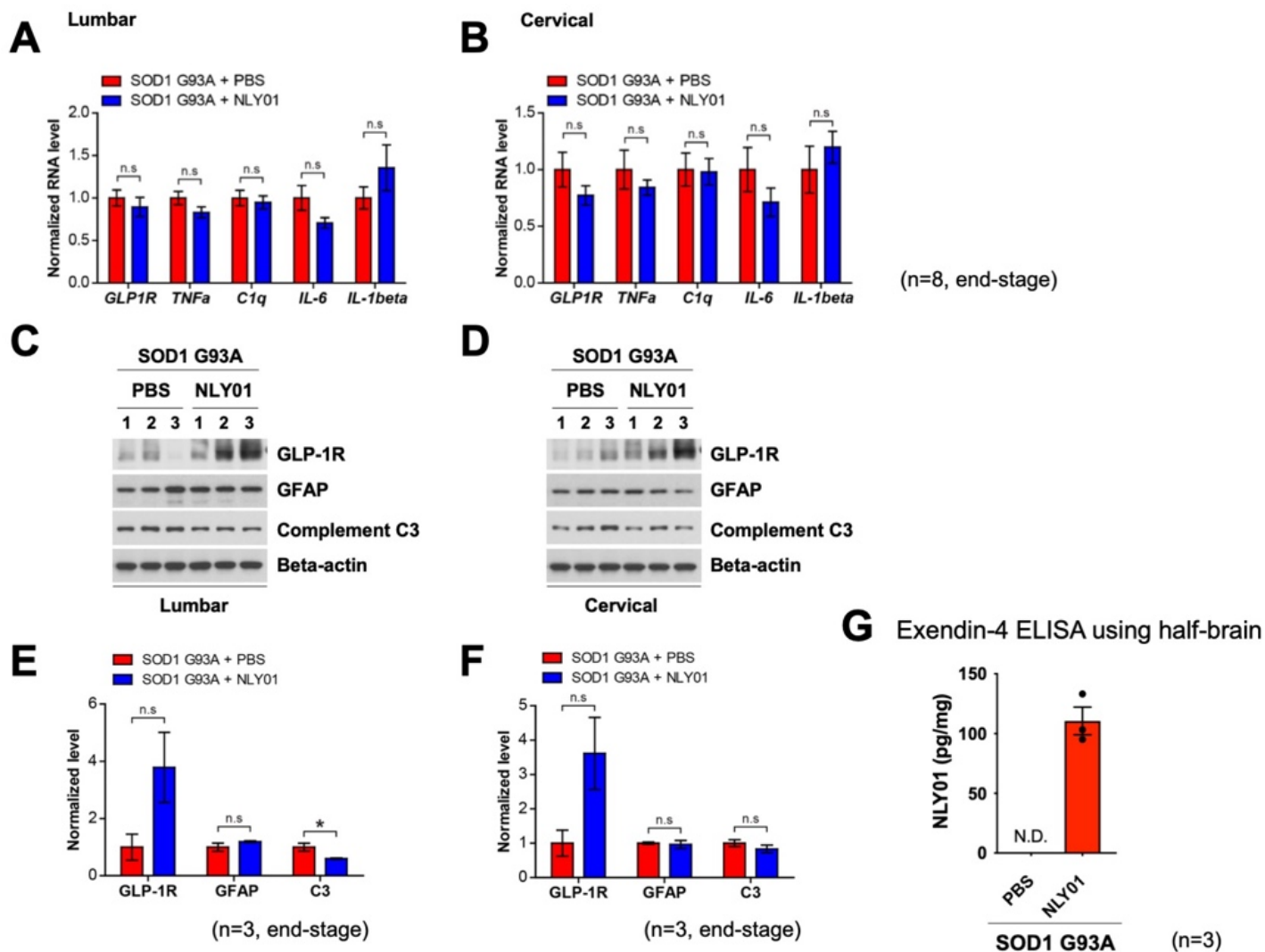
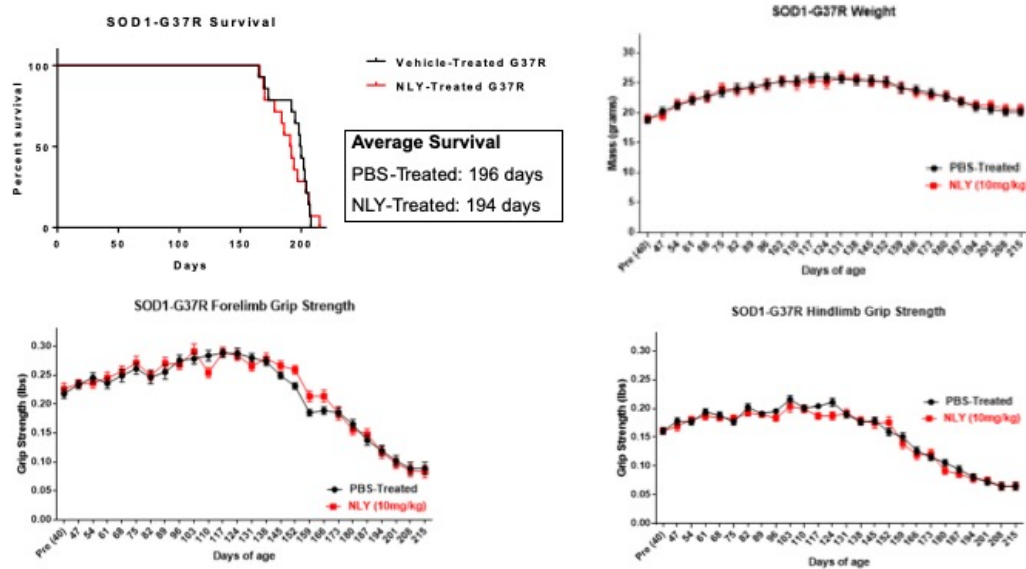


Figure 5. NLY01 has no adverse behavioral effects on WT mice

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 SOD1^{G93A} 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).

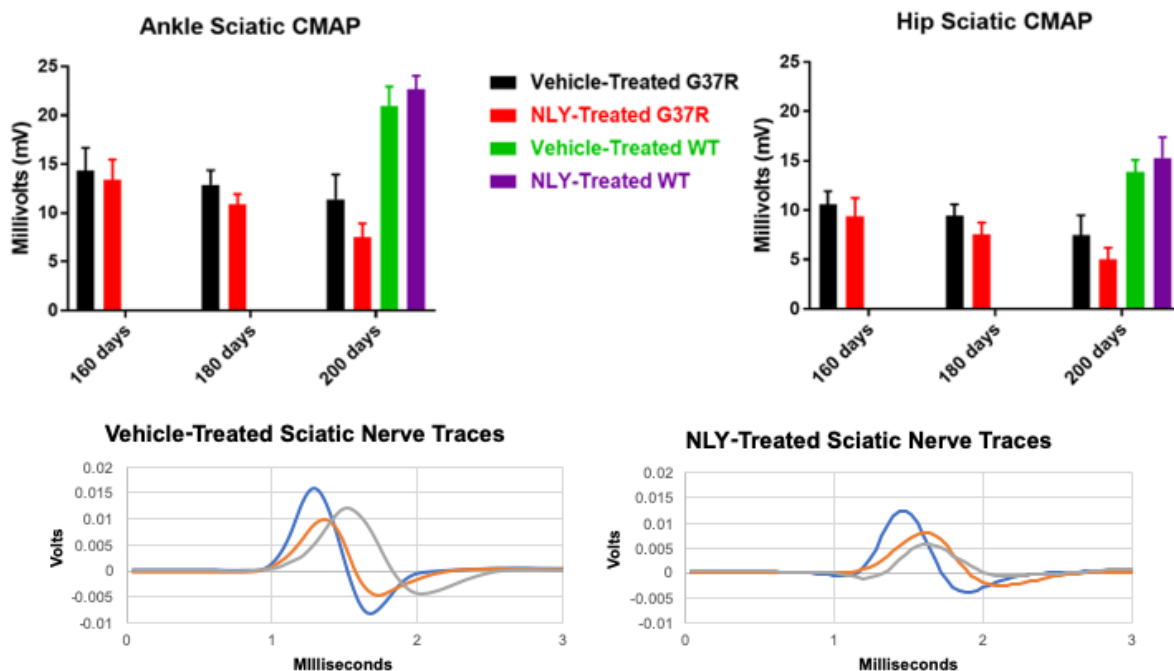


Figure 8. Electrophysiological measures of motor activity in SOD1^{G37R} mice following NLY01 treatment.

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.

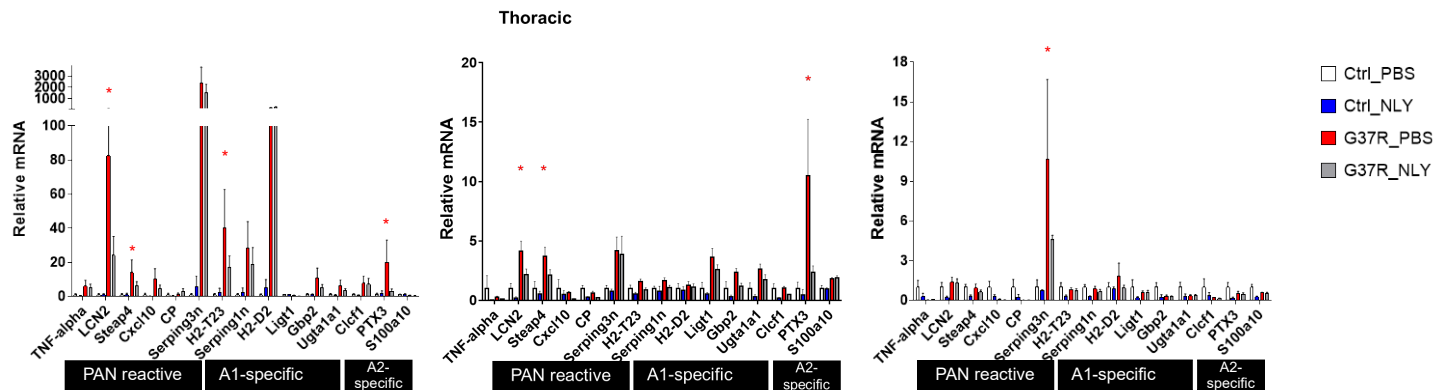


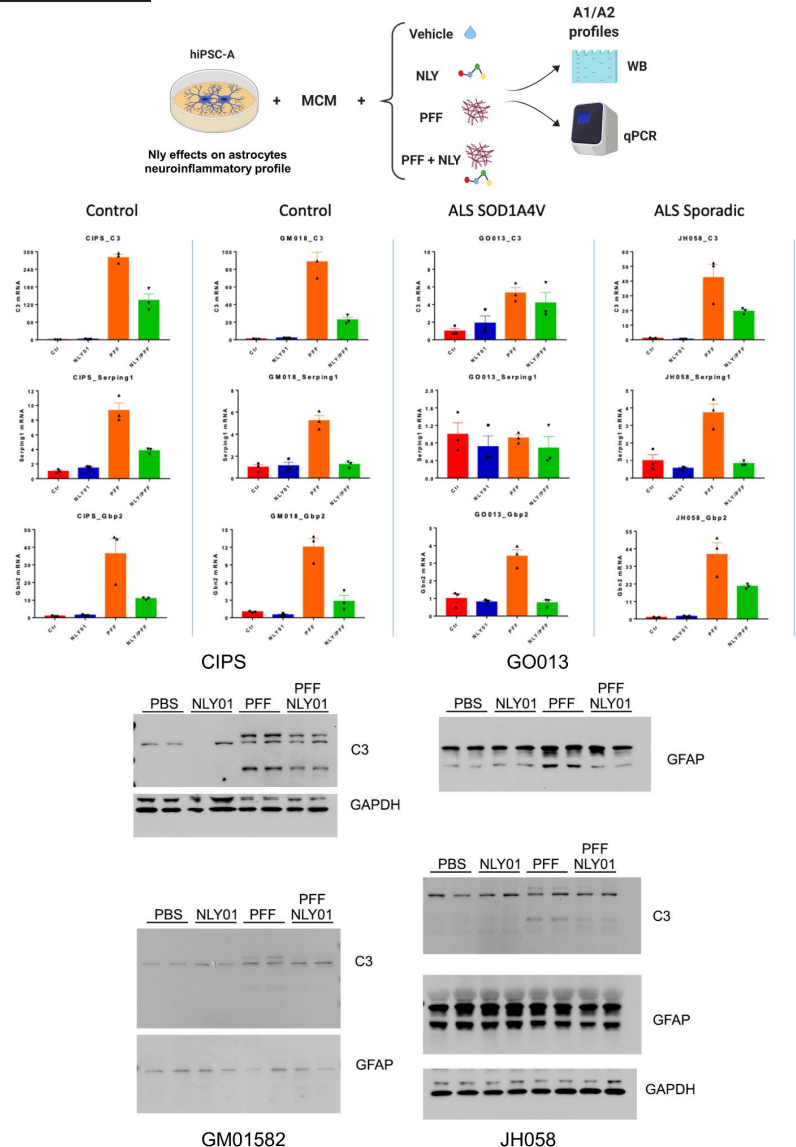
Figure 9. Analysis of isolated astrocyte in SOD1^{G37R} mice following NLY01 treatment.

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

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

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-1 α , tumor necrosis factor alpha (TNF α) 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



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 have designed and are now recruiting for the trial:

TITLE: 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

ClinicalTrials.gov Identifier: NCT04749433

Phase of Development: Phase 1

Number of Study Centers: 1 in the US

Johns Hopkins University

IRB #: IRB00263068

[¹¹C]CPPC FDA IND #: IND 143883

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, such as Alzheimer's disease (AD). [¹¹C]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 [¹¹C]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 [¹¹C]CPPC in patients with ALS, and, ultimately, to determine if use of [¹¹C]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: Once the safety and tolerability of the PET imaging biomarker has been established, we will use this as a biomarker of neuroprotection for the further development of NLY01. Using this biomarker protocol, we anticipate that we will be able to stratify patients for a therapeutic trial of NLY01 based upon their level of microglial activity as measured by [¹¹C]CPPC. This will allow us to see potential pharmacodynamic effects of NLY01 in a smaller subset of patients prior to the development of a broader Phase 2 or Phase 3 study of the drug.

***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 now begun to enroll patients into a Phase 1 PET imaging study (see Aim #3) to examine microglial activation as a biomarker that will eventually be able to measure NLY01 pharmacodynamic activity. This is an important step in targeted therapeutics for the development of NLY01.

5. CHANGES/PROBLEMS

Overall, COVID-19 slowed down our progress with all our in vivo animal studies and in vitro iPSC had to be shut down in the first approximately 6 months of 2020 and a reduction to approximately 50% of capacity into the latter half of 2021 so this has delayed our progress towards completing the aims detailed above. Because of COVID-19, all clinical trials were also halted and therefore, this has resulted in a delay of the PET imaging study that will help in our designing an outcome measure (microglial activity) that can be mediated by NLY01. However, the clinical trial is now open for enrollment

6. PRODUCTS

No products to be reported related to the current award.

7. Participants & Other Collaborating Organizations

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<u>Contribution to project</u>	<u>Oversees project</u>
<u>Funding support</u>	<u>ALSRP, ALS Association</u>

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<u>Contribution to project</u>	<u>Oversees project related to NLY01</u>
<u>Funding support</u>	<u>ALSRP, PRMP</u>

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<u>Nearest person month worked</u>	<u>3.6</u>
<u>Contribution to project</u>	<u>In vitro human iPSC</u>
<u>Funding support</u>	<u>ALSRP</u>

<u>Name</u>	<u>Katherine Marshall</u>
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<u>Nearest person month worked</u>	<u>6</u>
<u>Contribution to project</u>	<u>In vitro human iPSC</u>
<u>Funding support</u>	<u>ALSRP</u>

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<u>Project Role:</u>	<u>Instructor</u>
<u>Nearest person month worked</u>	<u>12</u>

<u>Contribution to project</u>	<u>Analysis of cytokines/A1 phenotypes in vitro/vivo</u>
<u>Funding support</u>	<u>Thome Memorial Foundation</u>

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<u>Nearest person month worked</u>	<u>12</u>
<u>Contribution to project</u>	<u>Protein and molecular assays</u>
<u>Funding support</u>	<u>Neuraly</u>

8. Special Reporting Requirements

N/A

9. Appendices

None