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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 by validating its potential as a target using both ALS animal and human ALS iPSC-based models.						
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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

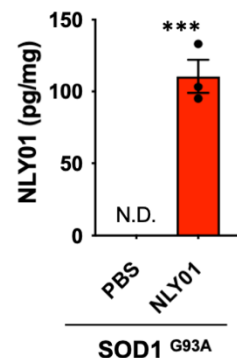
3. OVERALL PROJECT SUMMARY

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).

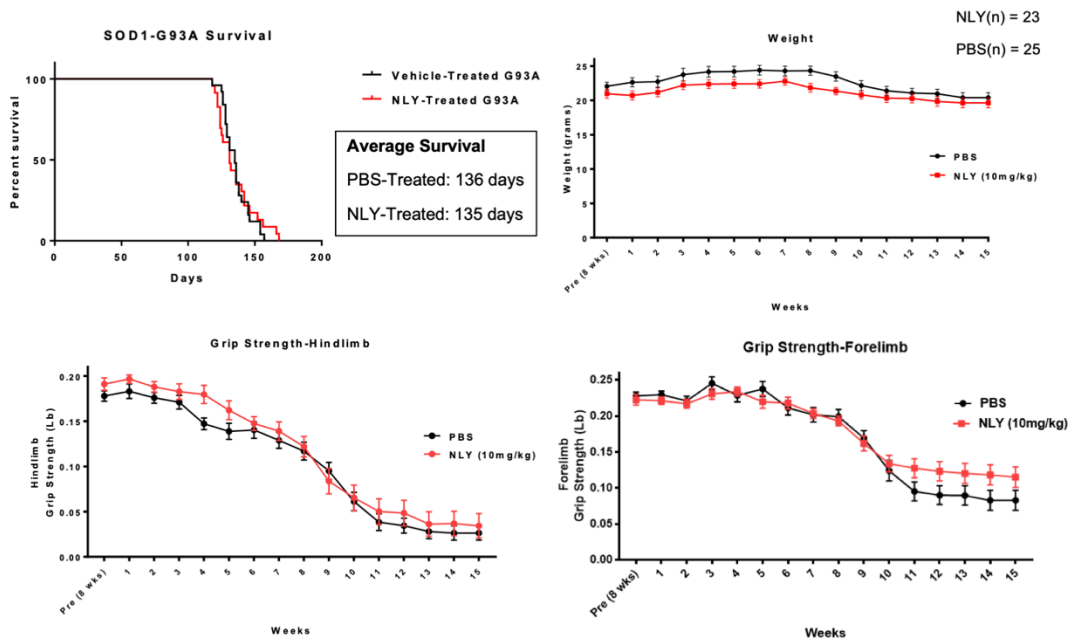


Figure 2. Behavioral analysis of NLY01 treated SOD1G93A mice

- 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 cytokines including C3, TNF-A, IL-1A, IL-

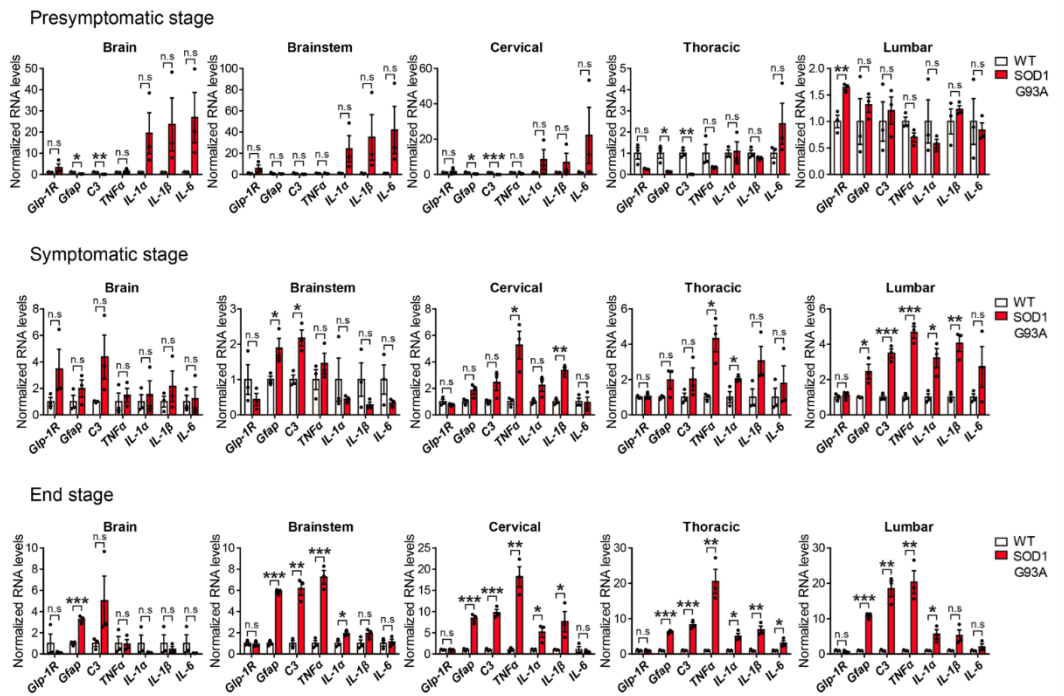


Figure 3. Analysis of spinal cord from SOD1 G93A mice for microglia activation

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 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).

Symptomatic stage

End stage

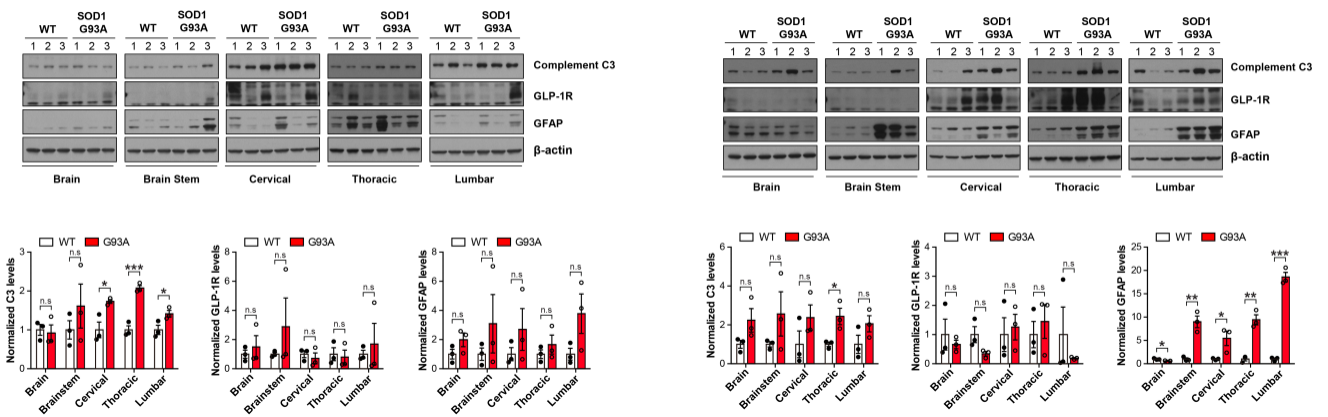


Figure 4. Analysis of spinal cord from symptomatic SOD1 G93A mice for C3 expression by WB.

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).

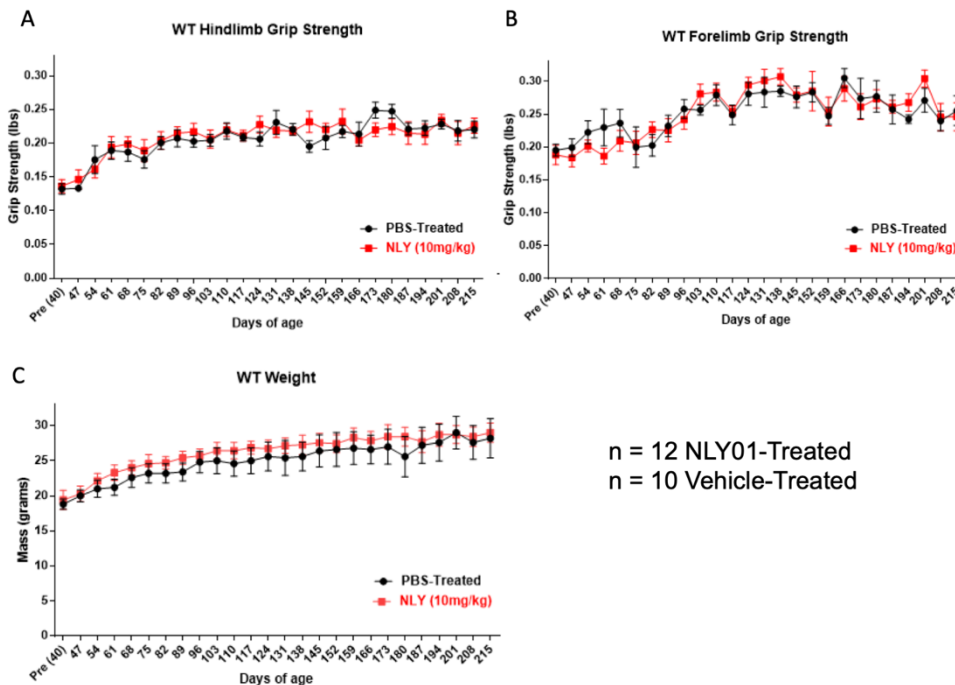
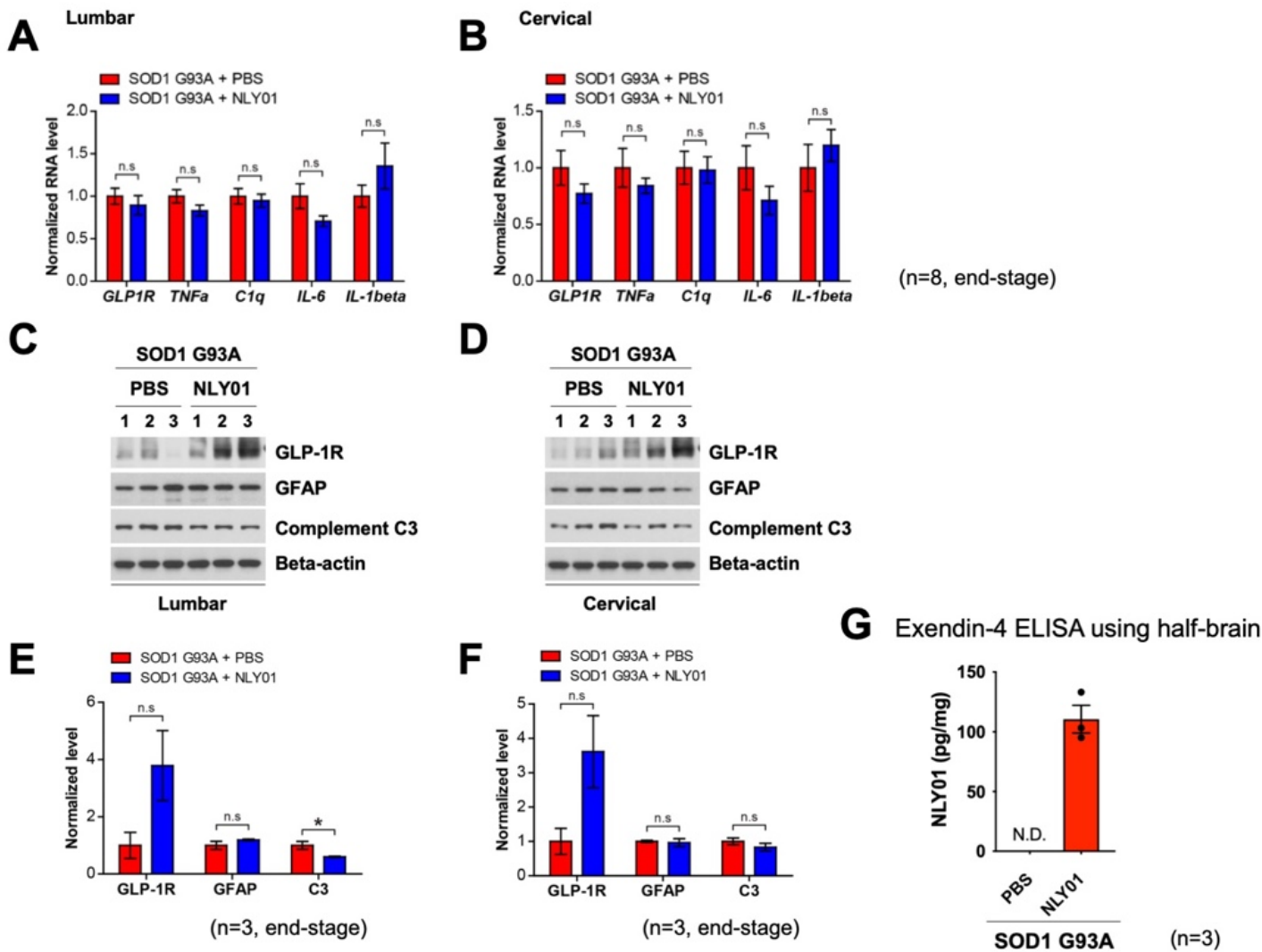
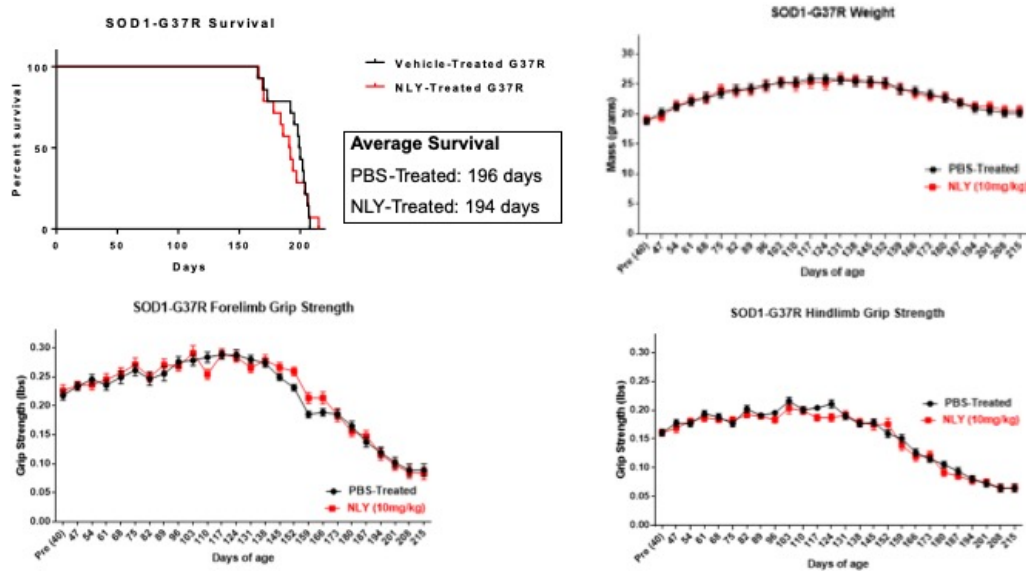


Figure 5. NLY01 has no adverse behavioral effects on WT mice

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).

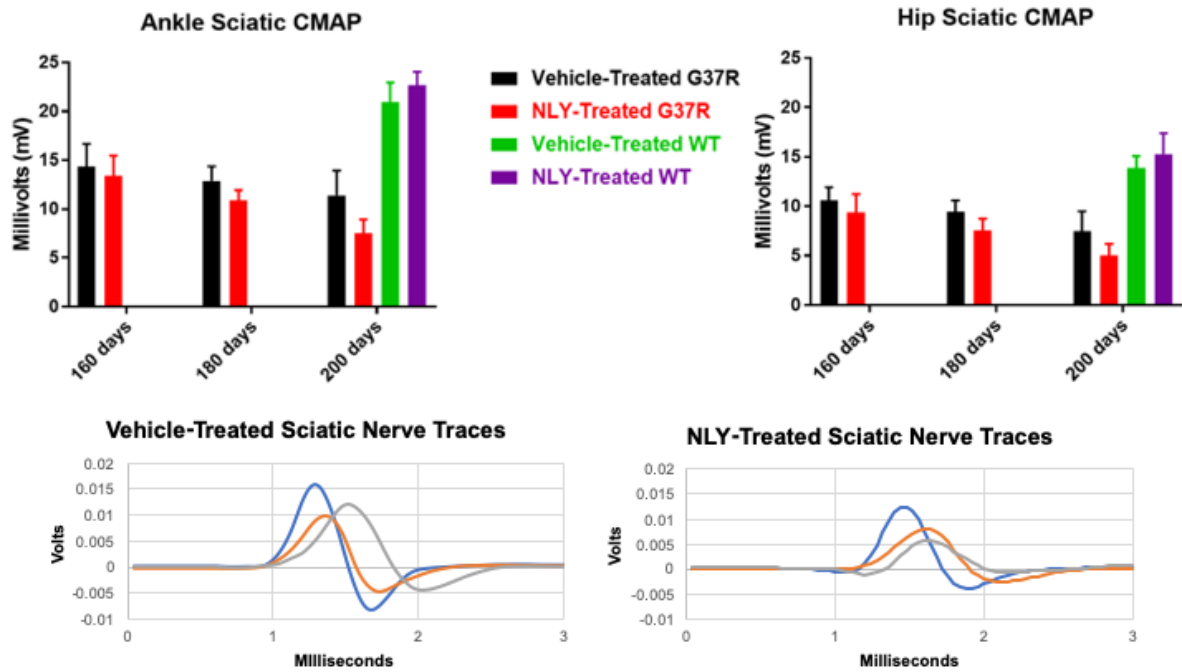


Figure 8. Electrophysiological measures of motor activity in $SOD1^{G37R}$ mice following NLY01 treatment.

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.

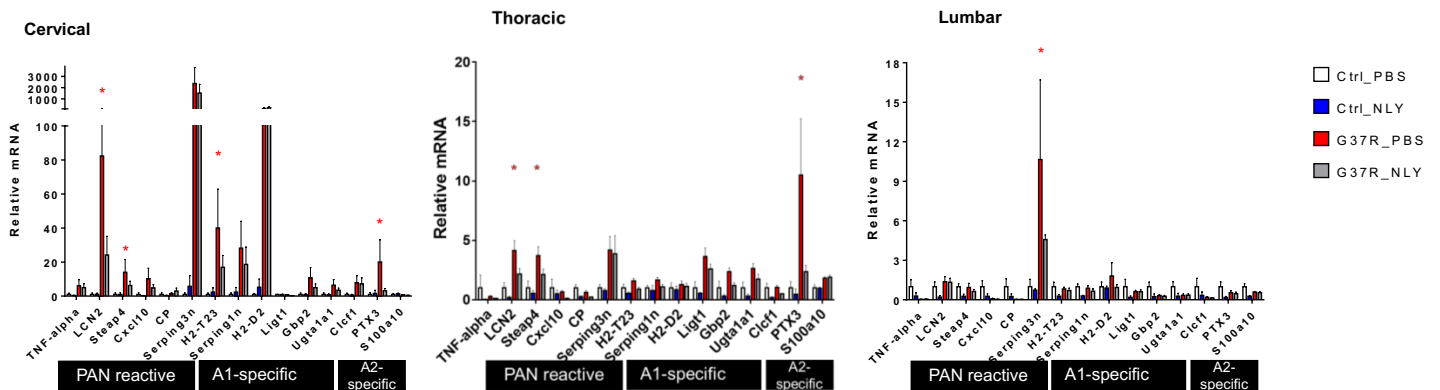


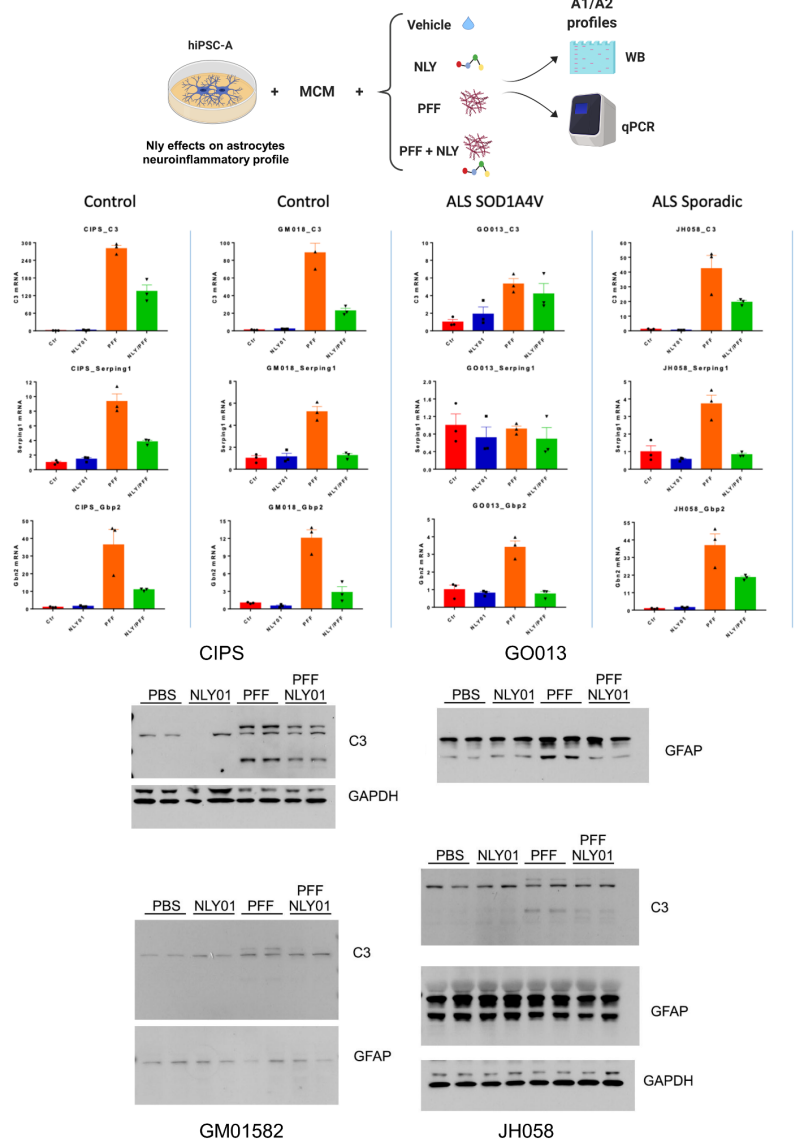
Figure 9. Analysis of isolated astrocyte in $SOD1^{G37R}$ mice following NLY01 treatment.

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 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. NLY01 treatment 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}).



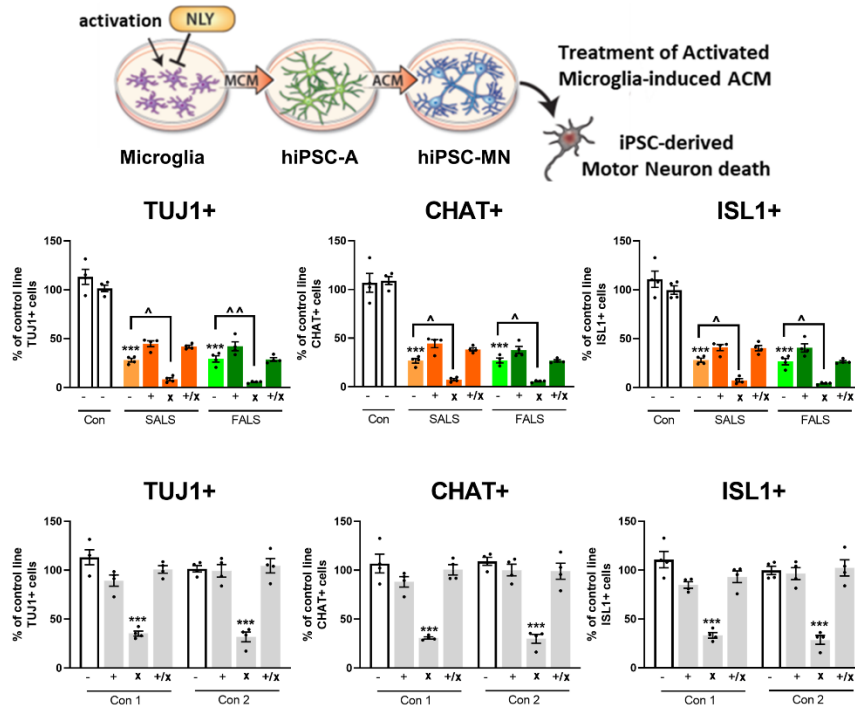
Interestingly, similar changes in transcriptional profiles and corresponding protein levels were seen when control instead of ALS hiPSC-A were treated with MCM suggesting that PFF- and microglia-mediated activation of astrocytes may not be unique to ALS, at least with regard to A1/A2 markers, but may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

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

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.



The neurotoxic effect of PFF ACM as well as the neuroprotective effects of PFF+NLY01 ACM on ChAT+ and ISL1+ MN survival were comparable between control and ALS, suggesting that PFF-activated microglia conversion of astrocytes may not be an ALS-specific phenomenon. These observations paralleled the effects of MCM on hiPSC-A A1/A2 profiles. (- No NLY01, + NLY01 treated, X=MCM treated, +/X NLY01 treated MCM)

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

a. PET imaging of Microglial Activity as an Imaging Biomarker for NLY01 Activity. In collaboration with Dr. Marty Pomper, M.D., Ph.D. we are designing a study of NLY01 using PET imaging. We are planning a Phase 1 clinical trial to examine microglial activity in the brains of ALS patients titled: A Phase 1, Open-label Study of [11C]CPPC [5-cyano-N-(4-(4-[11C]methylpiperazin-1-yl)-2-(piperidin-1-yl)phenyl)furan-2-carboxamide] to Assess the Safety and Tolerability of microglial activation in the brains of patients with ALS. This will allow us to measure target engagement of NLY01 longitudinally in ALS patients as their disease progresses.

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 iPSC cell platform.

CHANGES/PROBLEMS: Dr. Arens Taga, a postdoctoral fellow in our laboratory, took over the project from Dr. Richard who had left the lab to take a position with a company focused on stem cell research. However, due to COVID-19 all our in vivo animal studies and in vitro iPSC had to be shut down. All iPSC lines were cryopreserved. This resulted in a 4 month period of inactivity March-June 2020. The laboratory was recently allowed to resume research activities at approximately 50% of capacity so this has significantly 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.

PRODUCTS: No products to be reported related to the current award.

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<u>Contribution to project</u>	<u>In vitro human iPSC</u>
<u>Funding support</u>	<u>ALSRP</u>

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<u>Nearest person month worked</u>	<u>11</u>
<u>Contribution to project</u>	<u>Performs SOD1 mouse study</u>
<u>Funding support</u>	<u>ALSRP</u>

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<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>

4. KEY RESEARCH ACCOMPLISHMENTS

- We have verified that NLY01 crosses the blood brain barrier.
- The SOD^{G93A} and SOD1^{G37R} mouse studies (twice weekly injections with 10mg/kg NLY01) is completed.
- Initial analyses have shown the upregulation of some A1 markers in human iPSC-astrocytes following incubation with microglia conditioned media (MCM). These elevations can be reduced following the incubation with NLY01.
- We have now been able to demonstrate that NLY01 can reduce microglially-mediated human astrocyte toxicity to motor neurons using our human ALS iPSC-based platform. This is a significant step forward in being able to demonstrate the potential of NLY01 in the context of human ALS.

5. CONCLUSION

We have made significant advances and have largely completed the in vivo studies of NLY01 in 2 models of SOD1 mice but were not able to demonstrate efficacy with regard to survival and behavioral outcomes. However, as is relevant to future studies, we were able to demonstrate that NLY01 was able to cross the BBB and reduce levels of C3 (important in demonstrating target engagement). Most importantly, we have been able to utilize our human iPSC platform to demonstrate that NLY01 can afford protection from microglially-mediated astrocyte toxicity to motor neurons.

6. PUBLICATIONS (Peer Reviewed)

The following manuscript was not funded by the CDMRP but is relevant to the current work that is proposed in this grant. Drs. Lee and Oh are authors on this manuscript and describes the use of NLY01 in a model of Parkinson's disease.

[Nat Med.](#) 2018 Jun 11. doi: 10.1038/s41591-018-0051-5. [Epub ahead of print]

Block of A1 astrocyte conversion by microglia is neuroprotective in models of Parkinson's disease.

7. INVENTIONS, PATENTS

None related to the current award.

8. REPORTABLE OUTCOMES

Nothing to report

9. OTHER ACHIEVEMENTS

Neuraly Inc, Johns Hopkins spinoff biotech, currently developing NLY01 for both Parkinson's and Alzheimer's diseases. The nonclinical chronic toxicity studies in rats and cynomolgus monkeys were completed and no adverse clinical, laboratory or histopathologic signs at any dose were observed. In a first-in-man, Phase 1 safety study 96 healthy subjects have been sequentially enrolled in dose-escalating single and repeat-dose cohorts (<https://clinicaltrials.gov/ct2/show/NCT03672604>). In the Phase 1 study, NLY01 was well-tolerated, providing near continuous exposure to therapeutic doses with once-weekly dosing. The half-life of NLY01 was approximately 12.5 days and when administered weekly, achieved levels three times higher than shorter-acting GLP-1R agonists which are often limited by side effects. In 2020, Neuraly has initiated Phase 2 study in patient with Parkinson's disease (<https://clinicaltrials.gov/ct2/show/NCT04154072>). The Phase 2 study is a multicenter, randomized, double-blind, placebo-controlled trial that is expected to enroll approximately 240 patients in the US and Canada. The study is designed to assess the safety, tolerability and efficacy of NLY01 in subjects with early Parkinson's disease. In addition, Neuraly plans to initiate Phase 2b study in patients with mild cognitive impairment and Alzheimer's disease in the fourth quarter 2020.

10. REFERENCES

None

11. APPENDICES

None