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TITLE: The Development of an Astrocyte Hemichannel Blocker to Delay Spatial and Temporal Progression in ALS

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14. ABSTRACT
The mechanisms by which disease progresses in ALS, both anatomically and temporally, is unknown. Halting or slowing disease progression after onset, when patients are typically diagnosed, would offer enormous practical therapeutic potential. A consistent theme in our understanding of disease progression, after onset, in ALS suggests that astrocytes play a role in this progression. Our most recent work, implicates the astrocyte connexin, Cx43, as a mediator of MN toxicity using in vitro mouse ALS modeling. We hypothesize that the network of astrocytic hemichannels (HC), composed of connexin 43, may modulate the spatial and temporal progression of disease which ultimately results in MN death or dysfunction. Therefore, modulating Cx43 HC may be an excellent target for an ALS therapeutic. Tonabersat is a drug that has been shown to block Cx43 hemichannels in several studies and this hypothesis provides the foundation for this program. We will use human induced pluripotent stem cells from ALS patients along with animal models of ALS to study Cx43 HC mediated toxicity in ALS and to examine the ability of Cx43 HC blockers to block neurotoxicity in these models.

15. SUBJECT TERMS
Amyotrophic lateral sclerosis, induced pluripotent stem cells, astrocytes, motor neurons, human, connexin, hemichannels, gap junction, biomarker, therapeutics

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1. INTRODUCTION

The mechanisms by which disease progresses in ALS, both anatomically and temporally, is unknown. Halting or slowing disease progression after onset, when patients are typically diagnosed, would offer enormous practical therapeutic potential. A consistent theme in our understanding of disease progression, after onset, in ALS suggests that astrocytes play a role in this progression. Our most recent work, implicates the astrocyte connexin, Cx43, as a mediator of MN toxicity using in vitro mouse ALS modeling. We hypothesize that the network of astrocytic hemichannels (HC), composed of connexin 43, may modulate the spatial and temporal progression of disease which ultimately results in MN death or dysfunction. Therefore, modulating Cx43 HC may be an excellent target for an ALS therapeutic. Tonabersat is a drug that has been shown to block Cx43 hemichannels in several studies and this hypothesis provides the foundation for this program. Because tonabersat can be taken by mouth and is well-tolerated by patients with few side effects, it is a drug that could be used in diseases like ALS. First, this proposal will utilize several strategies to help us understand the importance of Cx43 HC in ALS disease progression. We will accomplish this by using human induced pluripotent stem cells (hiPSC) from ALS patients. By making these hiPSC into astrocytes (hiPSC-A) or motor neurons (hiPSC-MN) we can begin to understand how Cx43 HC from ALS patient astrocytes influence motor neuron death. In order to begin treating ALS patients with tonabersat, we also need to understand its effect in an ALS mouse model. Therefore, we will dose ALS mice with several different concentrations of tonabersat to see how it is distributed in the brain, the level that produces the fewest side effects, and also measure whether tonabersat can help slow or stop the progressive weakness that these ALS mice develop. Finally, this proposal will investigate whether the Cx43 protein can be a biomarker of disease. Because we know that we can measure this protein in brain tissues as well as cerebrospinal fluid (CSF) from ALS patients, we will investigate the relationship of Cx43 with the temporal progression of ALS to help us understand which patients might be most responsive to our drug. Based upon the known data using tonabersat in patients with migraine, we believe that if the proposal demonstrates that tonabersat works in our ALS animal models and human astrocyte/motor neuron cultures, a rapid filing of a FDA IND could be achieved within a two-year time frame.

2. KEYWORDS

Amyotrophic lateral sclerosis, induced pluripotent stem cells, astrocytes, motor neurons, human, connexin, hemichannels, gap junction, biomarker, therapeutics

3. ACCOMPLISHMENTS

Major Goals--SPECIFIC AIMS

Aim #1: Establish the correlation between the degree of Cx43 HC expression in ALS hiPSC-Astrocyte and hiPSC-MN toxicity.

Accomplishments

1. We have already cultured several ALS human iPSC-Astrocyte (hiPSC-A) lines and plan to add to our data suggesting that Cx43 HC expression is enriched at the membrane of both SOD1G93A mouse astrocytes as well as both familial ALS (FALS) and sporadic ALS (SALS) hiPSC-A (**Figure 1**). **2.** We used a gene corrected $SOD1^{A4V}$ line (lines 39B and 39B2.5, provided by Kevin Eggan) to establish whether Cx43 is reduced after gene correction (as demonstrated in preliminary data) and whether hiPSC-A mediated MN toxicity is lost after gene correction and following additional blockade of Cx43 HC. As hypothesized, our initial results suggest that Cx43 expression is partially normalized in the hiPSC-A from isogenic $SOD1^{A4V}$ line (**Figure 2**).

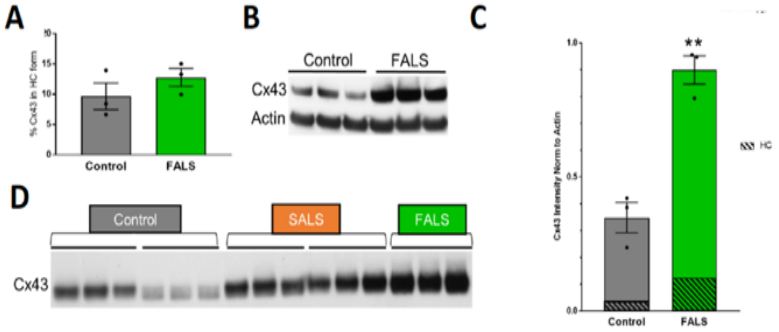
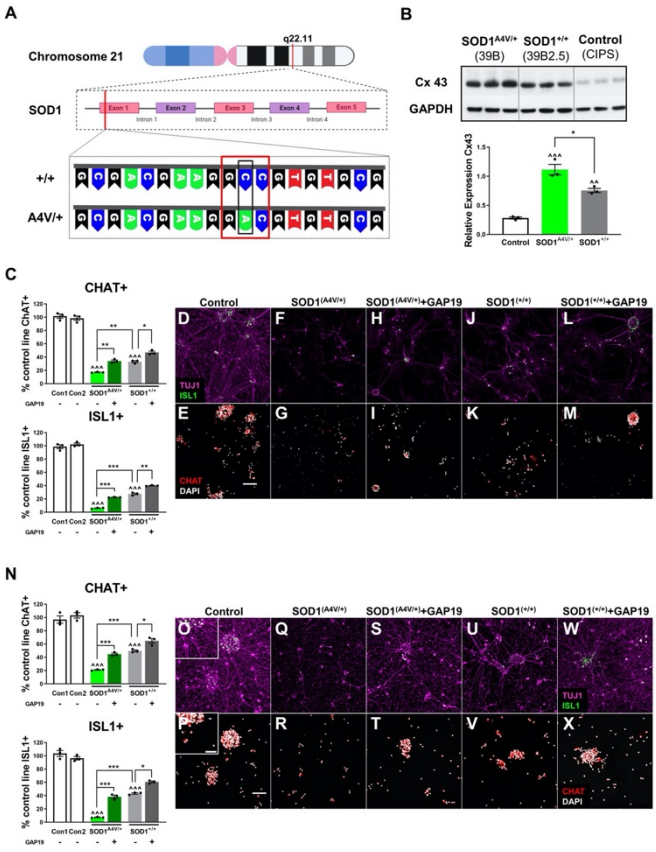


Figure 1. A. There is not a statistically significant shift in the percent of Cx43 as HC between ALS and control hiPSC-A. **B.** Western blot of total protein for hiPSC-A control and FALS lines. **C.** FALS has significantly more Cx43 than control (** $p < 0.01$, n=3). When percentages from 4e are mapped against total protein, FALS has more Cx43 in HC format by absolute calculation than control. **D.** Western blot of two lines from control and SALS, and one line of FALS patients stained for Cx43 from the biotin fraction in vitro.

Figure 2. Gene correction of $SOD1^{A4V}$ in hiPSC-A results in reduced Cx43 expression, and residual non-SOD1-mediated MN toxicity which is partially rescued by Gap19. (A) Schematic representation of SOD1 gene A4V correction. **(B)** The $SOD1^{A4V}$ hiPSC-A line (39B $SOD1^{A4V/+}$) shows an increase in Cx43 expression that is partially normalized by gene correction seen in line 39B2.5($SOD1^{+/+}$). The residual Cx43 expression in the isogenic ($SOD1^{+/+}$) line is significantly higher than control line. One-way ANOVA, * $p<0.05$; ^^^ $p<0.001$, n=3 **(C-M)** The isogenic gene-corrected line 392.5B ($SOD1^{+/+}$) shows less toxicity to $ChAT^{+}$ and $Isl1^{+}$ hiPSC-MN when compared to uncorrected line $SOD1^{A4V/+}$ in a co-culture paradigm. In both lines, however, additional neuroprotection was obtained following addition of Gap19. **(N-X)** Transwell co-culture of $SOD1^{A4V/+}$ astrocytes with hiPSC-MN shows expected neurotoxicity which is less evident in the $SOD1^{+/+}$ hiPSC-A/control MN co-culture. Additional neuroprotection was also evident following Cx43 HC specific blockade with Gap19. Con1 = CIPS, Con2 = GM01582. One-way ANOVA, * or ^ $p<0.05$; ** or ^^ $p<0.01$; *** or ^^^ $p<0.001$, n=3. Data are represented as mean ± SEM.



Aim #2. Correlate Cx43 expression in ALS brain/spinal cord tissue, CSF, and hiPSC-A with clinical disease progression and MN toxicity—Investigating a therapeutically relevant biomarker.

Accomplishments

We have been able, in some brain and spinal cord tissues, to demonstrate that differences in Cx43 expression may correlate with the rapidity of disease progression. While we did not find that we could differentiate the rapidity of disease in human ALS CSF, we did note that Cx43 is elevated in CSF from ALS patients, making it a potential biomarker of disease.

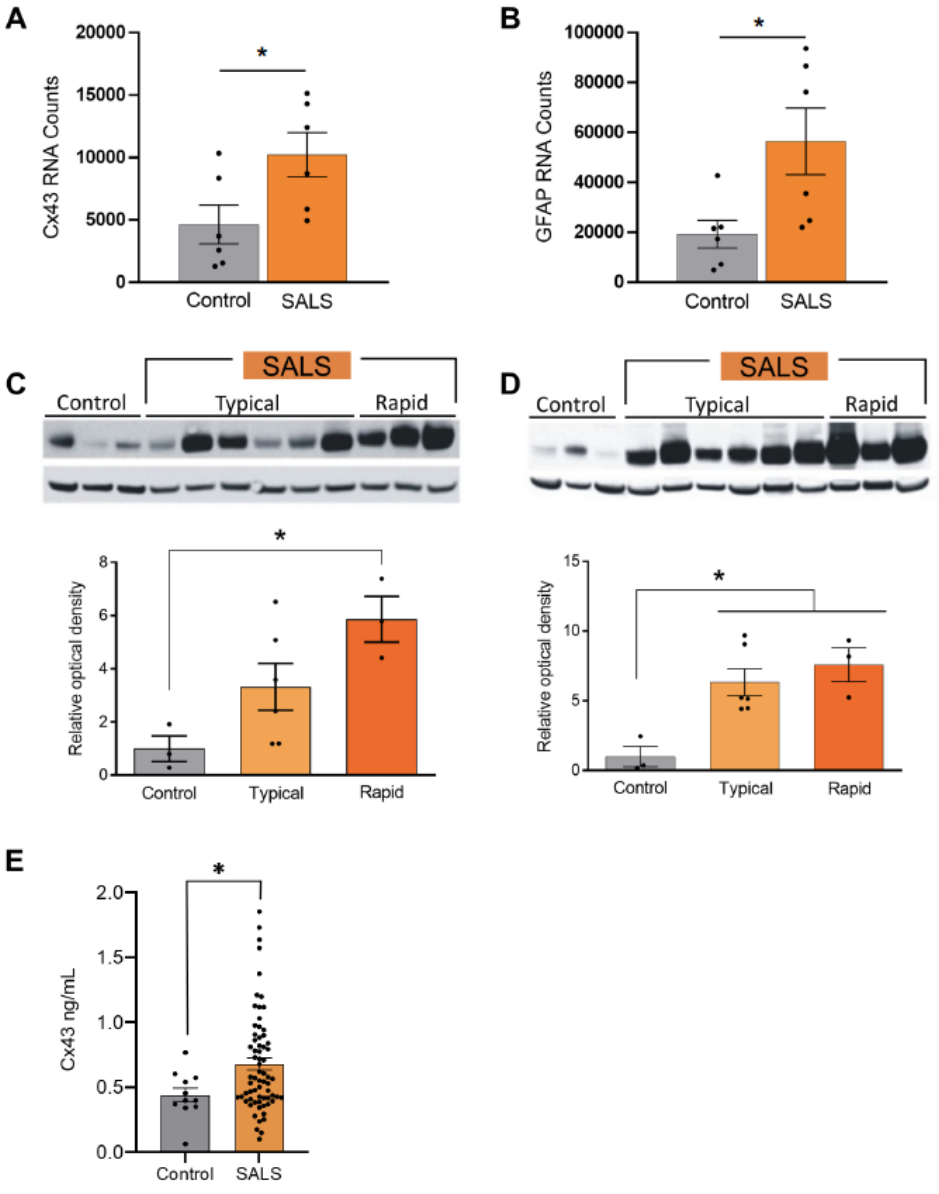


Figure. 3: Cx43 RNA and protein expression is increased in human ALS post-mortem tissue and CSF. **(A)** Gene expression using NanoString analysis of Cx43 motor cortex from SALS patients and controls shows significant increase (* $p < 0.05$, t-test, n=6/condition). **(B)** Similarly, NanoString analysis of GFAP in motor cortex shows significant changes (* $p < 0.05$, t-test, n=6/condition). **(C)** Post-mortem motor cortex tissue shows significant increase of Cx43 by Western blot of fast sporadic patients compared to healthy controls. Quantification pictured below (* $p < 0.05$, one-way ANOVA, n=3-6/condition). **(D)** In both typical and fast sporadic patient cervical cords Cx43 is significantly higher. Quantification pictured below (* $p < 0.05$, one-way ANOVA, n=3-6/condition). **(E)** CSF from sporadic ALS patients and healthy volunteers shows significantly increased Cx43 levels by ELISA in SALS samples (* $p<0.05$, ** $p<0.01$, t-test, n= 11 controls and 68 ALS). Data are represented as mean ± SEM.

Aim #3. Determine whether the Cx43- hemichannel blocker Tonabersat can reduce ALS human iPSC-derived astrocyte toxicity to motor neurons (MN)--An eye towards translation.

Accomplishments

We have now demonstrated in both SALS and FALS hiPSC-derived astrocyte/MN co-cultures, that tonabersat provides dose-dependent neuroprotection.

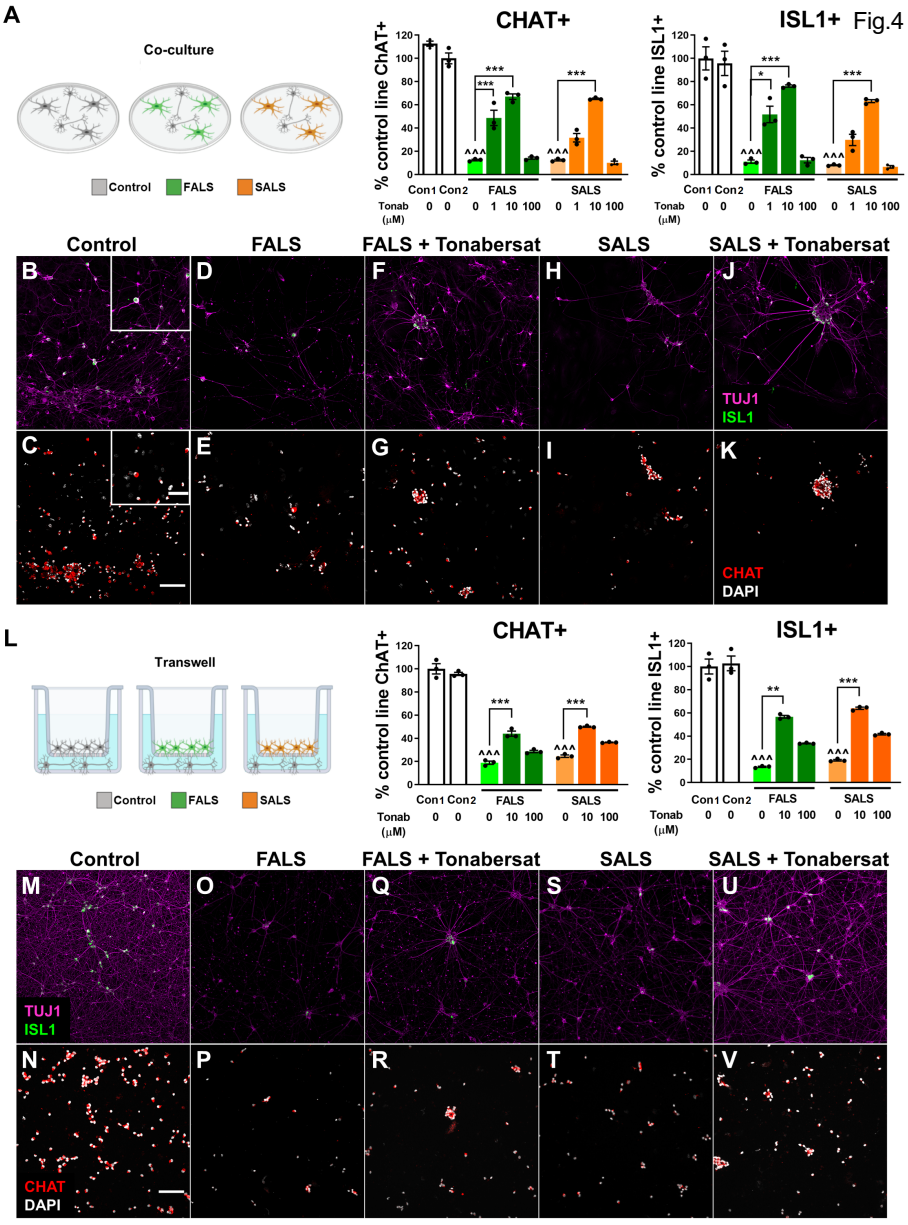


Figure. 4: The Cx43 HC blocker tonabersat provides dose-dependent neuroprotection to hiPSC-MN. (A-K) FALS and SALS hiPSC-A/MN co-culture immunostained for ChAT and Isl-1 shows dose-dependent neuroprotection from tonabersat (1μM, 10μM and 100μM), a small molecule that acts as Cx43 blocker, after a 14 day incubation period. **(L-V)** Following transwell co-culture of FALS and SALS hiPSC-A with hiPSC-MN, immunostainining for ChAT$^+$ MN and Isl1$^+$ MN confirms dose-dependent neuroprotection with tonabersat. Con1 = CIPS, Con2 = GM01582, FALS = GO013, SALS = JH058. Significant comparisons (one-way ANOVA) between untreated control and ALS co-cultures are marked with (^), while significant effects of tonabersat on co-cultures containing ALS astrocytes are marked with (*). * or ^ $p<0.05$; ** or ^^ $p<0.01$; *** or ^^^ $p<0.001$, n=3/condition Scale bar=50μm, inset scale bar 20μm. Data are represented as mean ± SEM.

Aim #4. Perform a dose ranging study of safety, exploratory biomarkers, and therapeutic efficacy of tonabersat in SOD1^{G93A} and SOD1^{G37R} mice.

Accomplishments

We have demonstrated that treatment of SOD1G93A mice at the presymptomatic stage protects motor neurons and has a trend towards improvements in forelimb strength.

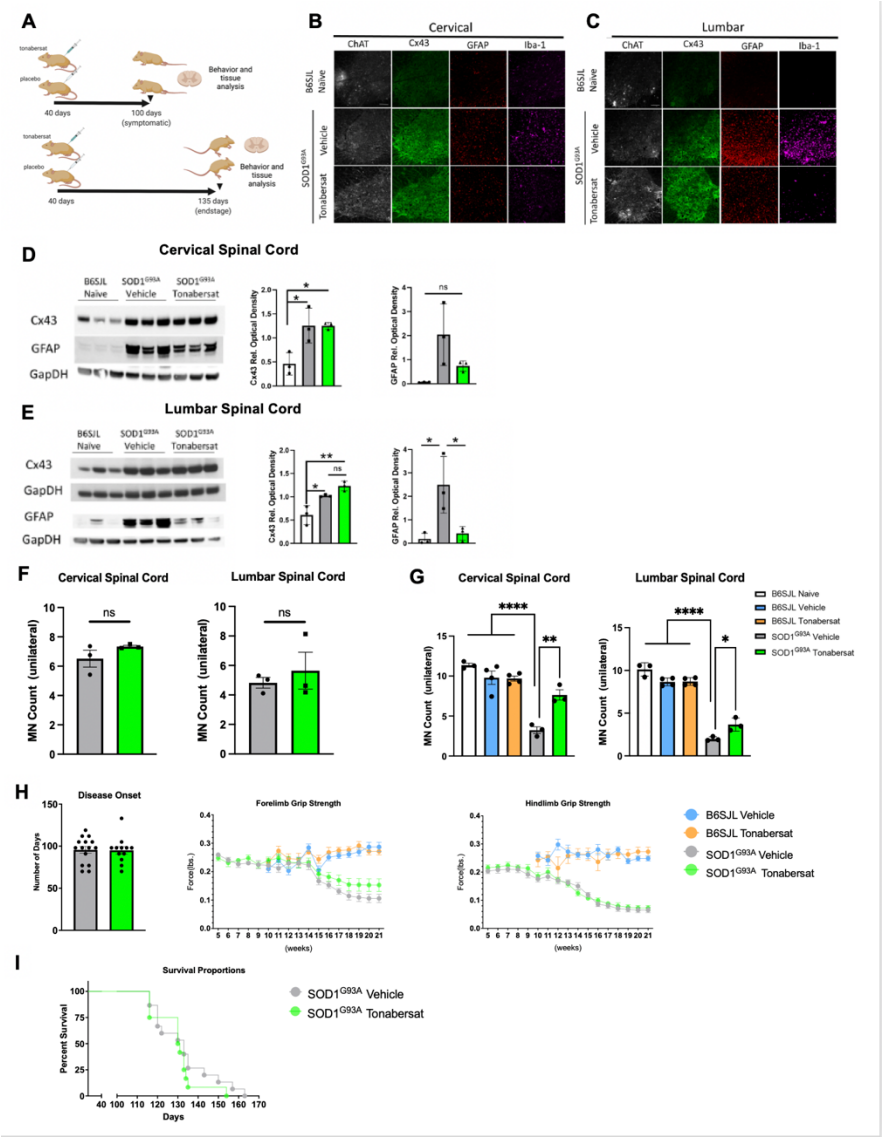


Figure 5. Tonabersat provides in vivo neuroprotection in SOD1^{G93A} mice with presymptomatic treatment. (A) Schematic of tonabersat dosing regimen starting at 40 days of age **(B, C)** Immunohistochemistry using ChAT antibody to identify motor neurons, Cx43 (astrocytes), GFAP (astrocytes), and Iba-1 (microglia) in ventral horn of B6SJL WT mice, vehicle treated SOD1^{G93A} mice, and tonabersat treated SOD1^{G93A} mice in the cervical and lumbar spinal cord at endstage. **(D)** Motor neuron survival is significantly improved following chronic tonabersat treatment when animals are treated from 40 days to endstage. **(E)** Ventral horn motor neurons for SOD1G93A mice treated at 40 days and analyzed at early symptomatic stage (100days) show no benefits from tonabersat. Notably, GFAP and Iba-1 immunoreactivity are reduced following tonabsersat treatment. **(F, G)**. Cervical spinal cord **(F)** and lumbar spinal cord **(G)** immunoblot quantification of Cx43 shows an increase in Cx43 expression in SOD1^{G93A} mice which is not affected by treatment with tonabersat. GFAP shows a reduction in protein expression following chronic tonabersat treatment. **(H)**. Disease onset is unaffected by tonabersat treatment but trends in maintenance of forelimb function in SOD1G93A mice treated with tonabersat is observed. Chronic treatment with tonabersat does not induce toxicity to B6SJL WT mice. **(I)** Survival in this small cohort is not significantly changed with tonabersat treatment (n=12 tonabersat treated, n=14 vehicle). One-way ANOVA, * $p<0.05$; ** $p<0.01$; *** $p<0.001$, n=3. Data are represented as mean ± SEM.

Opportunities for professional development

Nothing to report

Dissemination

Speaker: ALS One Research Symposium: "Astrocyte Hemichannels as Targets for ALS Therapeutics". Virtual Meeting

Plans

1. Examine Cx43 in serum of ALS patients by new ELISA method
2. Examine the electrophysiological properties of ALS hiPSC-A induced hyperexcitability of hiPSC/MN using multielectrode array (MEA).
3. Establish whether tonabersat influences electrophysiological activity of hiPSC-A induced hyperexcitability of hiPSC/MN using MEA.
4. Examine Cx43 from serum of SOD1G93A mice treated with vehicle and tonabersat
5. Treat SOD1G93A mice after symptom onset at 100 days of age to establish whether neuroprotection is observed.
6. Perform dosing of tonabersat in G37R mice

4. IMPACT

Impact on development of principle discipline of the project: Our identification of Cx43 HC-mediated toxicity to motor neurons represents a novel mechanism by which astrocytes influence cell death and has already been identified as a drug target. Our proposal utilizes several novel strategies which may afford greater confidence in the importance of this HC protein in ALS: 1. We employ a fully humanized, spinal cord specific hiPSC model which is relevant to ALS. 2. We have a precision medicine approach using the Answer ALS database to access iPSC-A (human induced pluripotent stem cell-derived astrocytes) derived from both familial ALS (FALS) and sporadic ALS (SALS) patients allowing us to address the importance of this mechanism in ALS patient subgroups. 3. We will investigate Cx43 as a therapeutically-relevant biomarker of astrocyte pathobiology in ALS by leveraging the Answer ALS, Northeast ALS Consortium, and Target ALS databases. 4. Given the abundant evidence that astrocytes participate in disease progression, targeting this Cx43 HC may be of particular relevance in treating ALS after disease onset. 5. Understanding the biological relevance of these HC is particularly timely as we are working with compounds, like tonabersat, which have activity at the Cx43 HC. Given that tonabersat has been used in humans for another indication, there are abundant PK and safety data on this compound—suggesting that it could be translated to ALS patients in a timely fashion. The success of tonabersat in ALS patients may offer new directions into developing molecularly-targeted disease-modifying agents for ALS. Coupled with molecular mechanism insights and the completion of our proposed studies, we will file an FDA IND application for first-in-human studies of tonabersat in ALS patients.

Impact on other disciplines: Nothing to report

Impact on technology transfer: Nothing to report

Impact on society beyond science and technology: Nothing to report

5. CHANGES/PROBLEMS

The major obstacle has been the availability of many products for human iPSC culture including severe shortages of pipets, plastics, and Matrigel. Many items are on backorder which has slowed in vitro work using these cells. However, we are continuing to work on other aspects of the project including biomarker development, in vivo mouse studies while these delays are resolved.

Scientifically, we have not encountered any issues. The hypotheses remain strong and the specific aims on track.

No significant changes in use or care of human subjects or vertebrate animals. No changes in use of select agents.

6. PRODUCTS

Nothing to report

7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

Name	**Nicholas J. Maragakis**
Project Role:	**PI**
Researcher ID	0000-0002-7311-9614
Nearest person month worked	**3.6**
Contribution to project	Correlate total Cx43 expression with Cx43 HC expression and function at the membrane—validating a drug-relevant target.
Funding support	**ALSRP, NIH**

Name	**Sarah Gross**
Project Role:	**Lab Manager**
Researcher ID	
Nearest person month worked	**12**
Contribution to project	**In vitro human iPSC** Establishing a correlation between Cx43 HC expression and function using ALS hiPSC-A/MN co-culture
Funding support	**ALSRP**

Name:	**Norman Haughey**
Project Role:	**Co-I**
Researcher ID:	0000-0001-5194-4122
Nearest Person Month Worked:	**0.60**
Contribution to project:	Examine whether there is a dose-dependent electrophysiological response to Cx43 HC blockade using multi-electrode array analyses—providing a mechanistic link to neuroprotection
Funding Support:	**ALSRP, NIH, TEDCO**

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report

9. APPENDICES

N/A