

AWARD NUMBER: W81XWH-20-1-0161

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

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REPORT DATE: October 2022

TYPE OF REPORT: Annual

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

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REPORT DOCUMENTATION PAGE

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1. REPORT DATE October 2022		2. REPORT TYPE Annual		3. DATES COVERED 15Sep2021-14Sep2022	
4. TITLE AND SUBTITLE The Development of an Astrocyte Hemichannel Blocker to Delay Spatial and Temporal Progression in ALS				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-20-1-0161	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Nicholas J. Maragakis, M.D. E-Mail: nmaragak@jhmi.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Johns Hopkins University, School of Medicine Baltimore, MD, 21205				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 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					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Unclassified	18. NUMBER OF PAGES 23	19a. NAME OF RESPONSIBLE PERSON USAMRDC
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified			19b. TELEPHONE NUMBER (include area code)

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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 Eggen) 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).

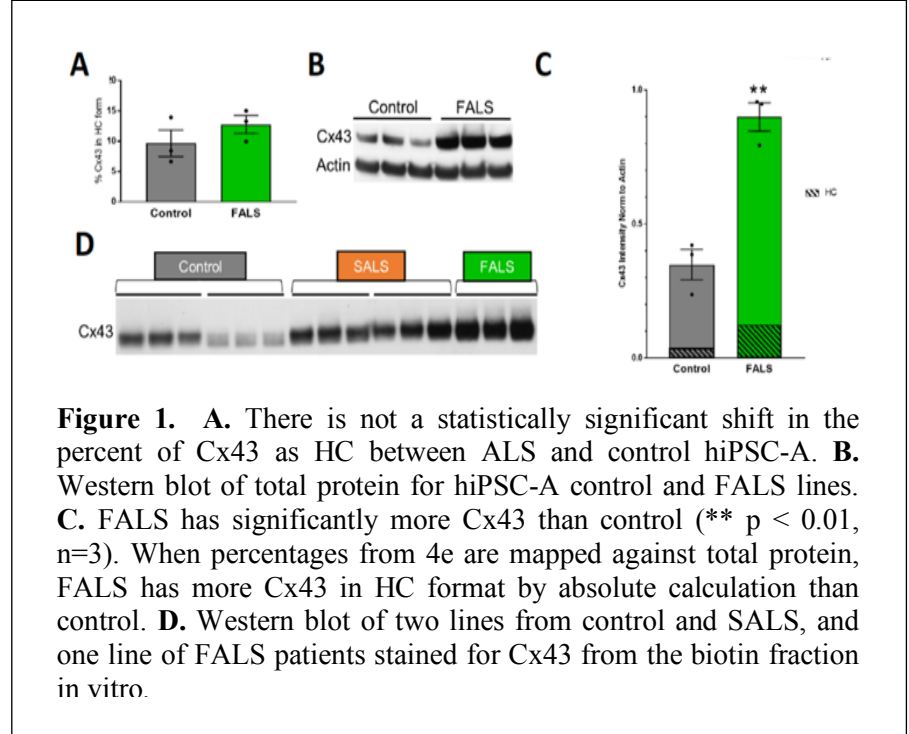
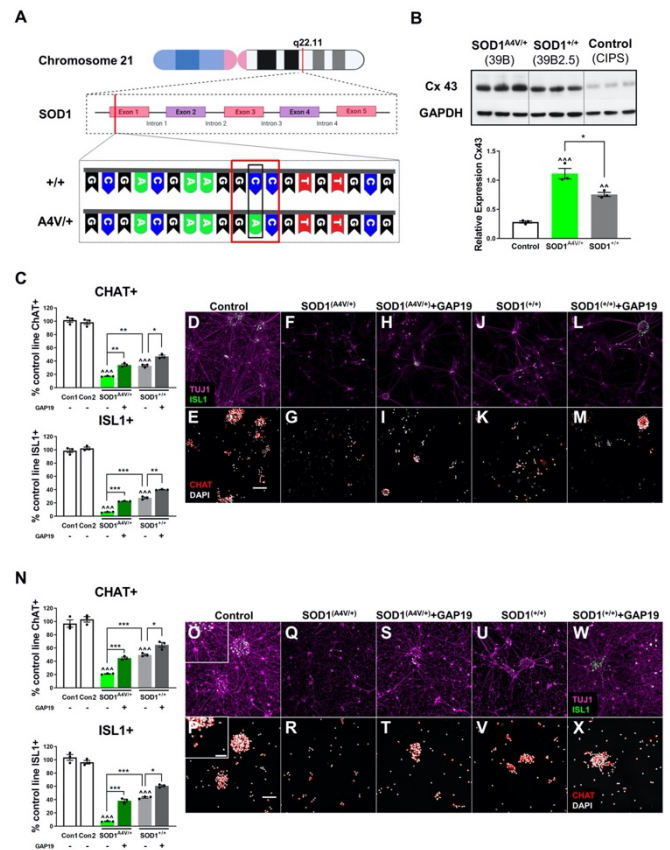


Figure 2. Gene correction of SOD1^{A4V} in hiPSC-A results in reduced Cx43 expression, and residual non-SOD1-mediated MN toxicity which is partially rescued by Gap19. (A) Schematic representation of SOD1 gene A4V correction. (B) The SOD1^{A4V} hiPSC-A line (39B SOD1^{A4V/+}) shows an increase in Cx43 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^{+/+} 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.



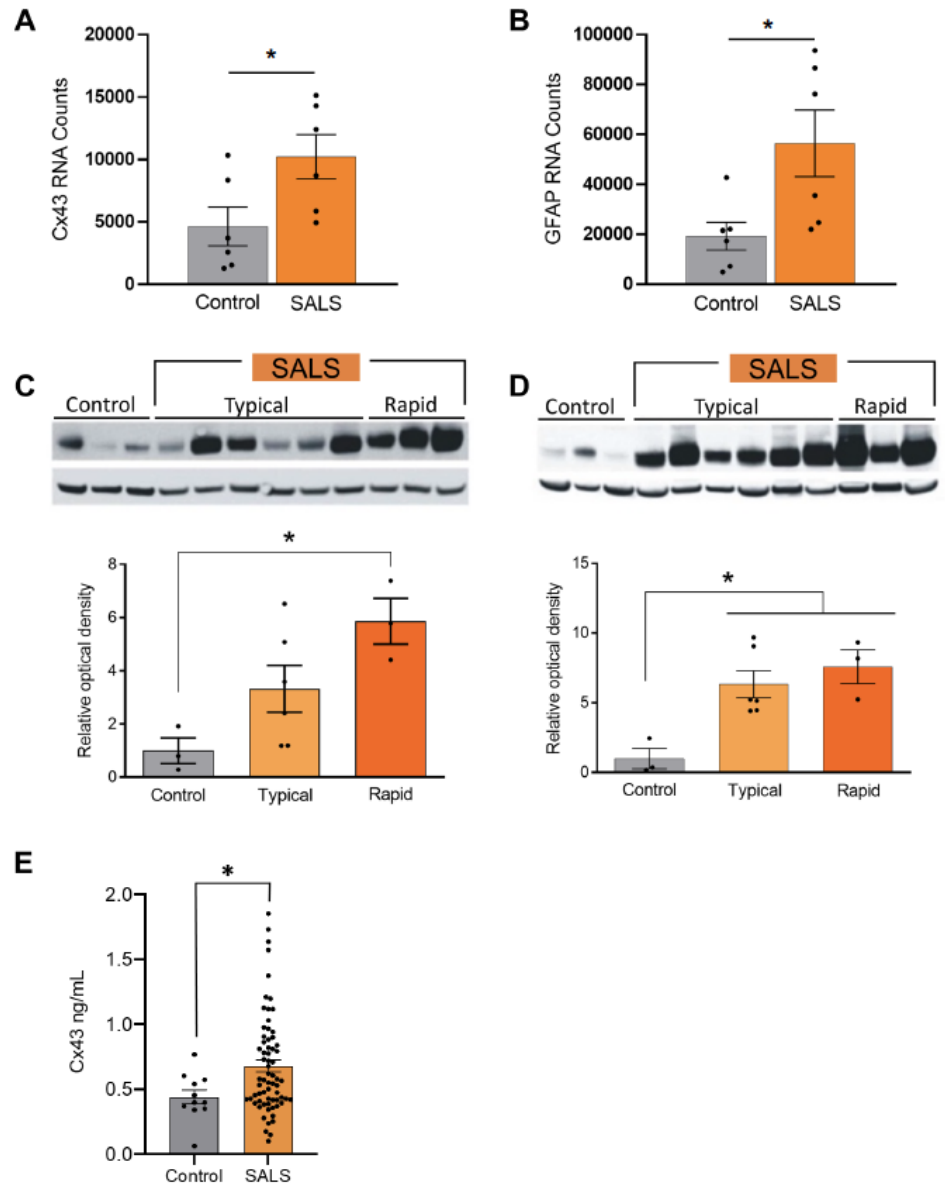
Aim #2. Correlate Cx43 expression in ALS brain/spinal cord tissue, CSF, and hiPSC-A with clinical disease progression and MN toxicity—Investigating a therapeutically relevant biomarker.

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 \pm SEM.

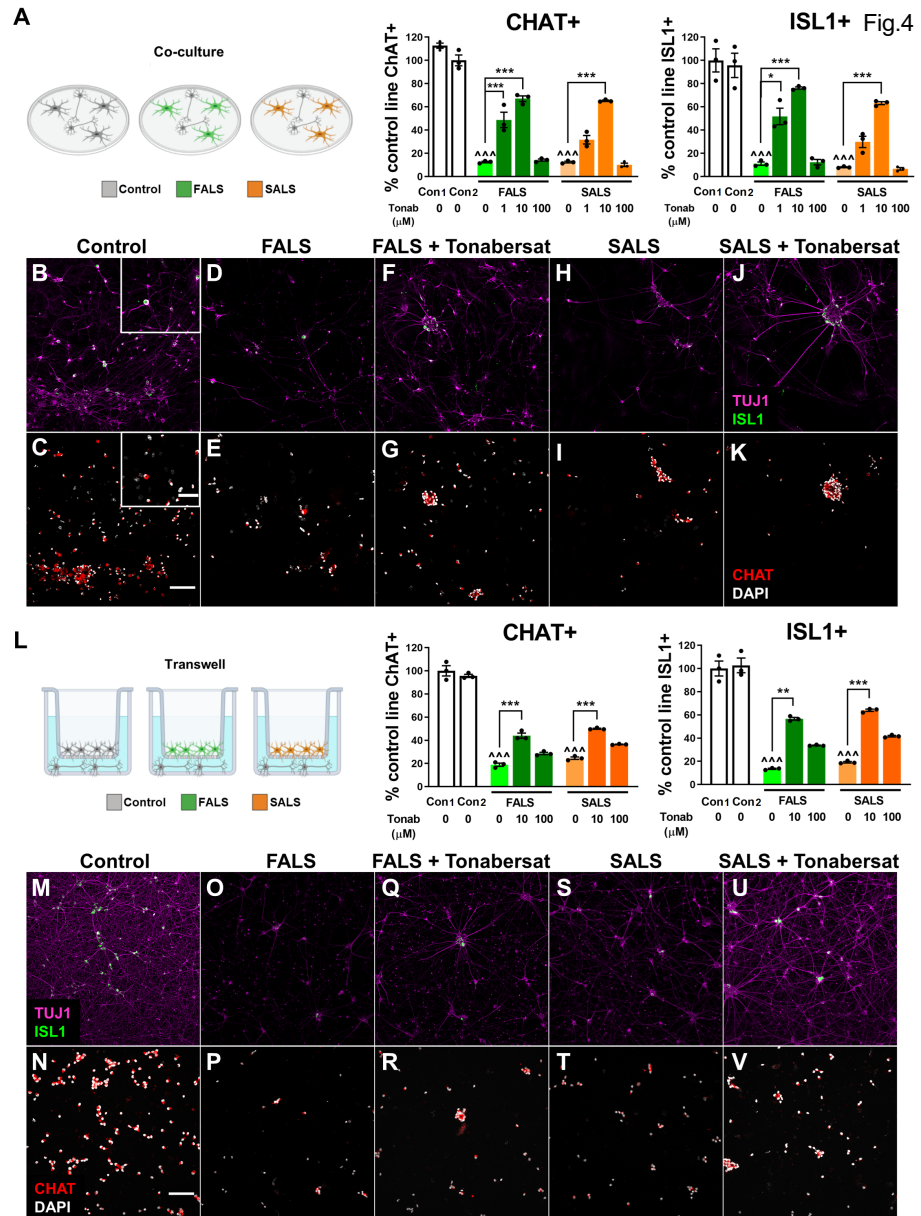


Aim #3. Determine whether the Cx43- hemichannel blocker Tonabersat can reduce ALS human iPSC-derived astrocyte toxicity to motor neurons (MN)--An eye towards translation.

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 (1 μ M, 10 μ M and 100 μ M), 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, immunostaining 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=50 μ m, inset scale bar 20 μ m. Data are represented as mean \pm SEM.



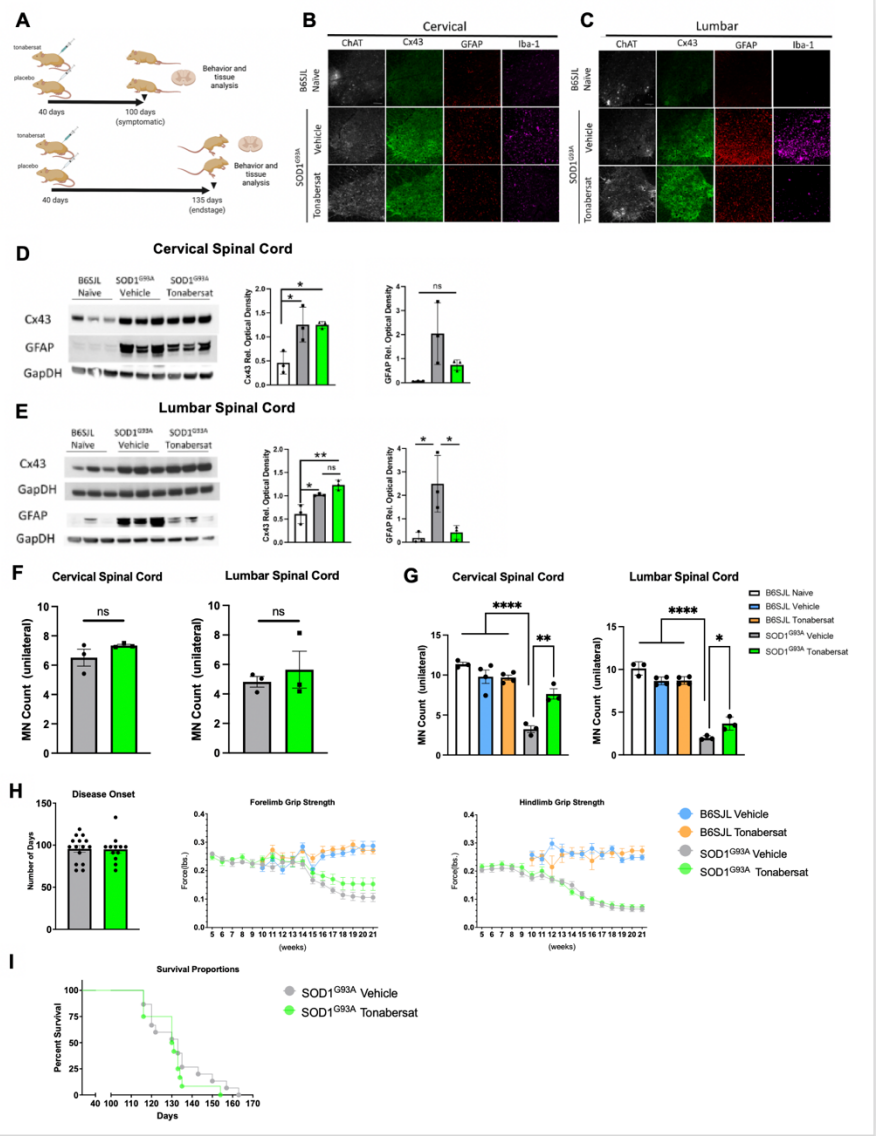
Aim #4. Perform a dose ranging study of safety, exploratory biomarkers, and therapeutic efficacy of tonabersat in SOD1^{G93A} and SOD1^{G37R} mice.

Accomplishments

We have demonstrated that treatment of SOD1^{G93A} 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 SOD1^{G93A} 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 tonabersat 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 SOD1^{G93A} 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.



(H). Disease onset is unaffected by tonabersat treatment but trends in maintenance of forelimb function in SOD1^{G93A} 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

Packard Center for ALS Research Annual Symposium: “ Cx43 Hemichannels as Druggable Targets for Astrocyte-Mediated Toxicity in ALS”

Plans

1. Examine Cx43 in serum of ALS patients by new ELISA method

2. Examine Cx43 from serum of SOD1G93A mice treated with vehicle and tonabersat
3. Correlate Cx43 hemichannel function with speed of disease progression via measurement of ATP release from ALS hiPSC-Astrocytes
4. Mechanistically, attempt to detail the nature of tonabersat's mechanism of action at the Cx43 hemichannel
5. 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

We continue to have intermittent problems with regard to 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. We have also been delayed in being able to hire a new, and qualified, research specialist for human iPSC culture.

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	Khalil Rust
Project Role:	Research Specialist
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	Raha Dastgheyb
Project Role:	Research Associate
Researcher ID	
Nearest person month worked	0.6
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:	
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

Almad AA, Taga A, Joseph J, Gross SK, Welsh C, Patankar A, Richard JP, Rust K, Pokharel A, Plott C, Lillo M, Dastgheyb R, Eggan K, Haughey N, Contreras JE, **Maragakis NJ**. Cx43 hemichannels contribute to astrocyte-mediated toxicity in sporadic and familial ALS. *Proc Natl Acad Sci U S A*. 2022 Mar 29;119(13):e2107391119. Epub 2022 Mar 21



Cx43 hemichannels contribute to astrocyte-mediated toxicity in sporadic and familial ALS

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Edited by Anders Björklund, Lund University, Lund, Sweden; received May 4, 2021; accepted December 16, 2021

Connexin 43 (Cx43) gap junctions and hemichannels mediate astrocyte intercellular communication in the central nervous system under normal conditions and contribute to astrocyte-mediated neurotoxicity in amyotrophic lateral sclerosis (ALS). Here, we show that astrocyte-specific knockout of Cx43 in a mouse model of ALS slows disease progression both spatially and temporally, provides motor neuron (MN) protection, and improves survival. In addition, Cx43 expression is up-regulated in human postmortem tissue and cerebrospinal fluid from ALS patients. Using human induced pluripotent stem cell-derived astrocytes (hiPSC-A) from both familial and sporadic ALS, we establish that Cx43 is up-regulated and that Cx43-hemichannels are enriched at the astrocyte membrane. We also demonstrate that the pharmacological blockade of Cx43-hemichannels in ALS astrocytes using GAP 19, a mimetic peptide blocker, and tonabersat, a clinically tested small molecule, provides neuroprotection of hiPSC-MN and reduces ALS astrocyte-mediated neuronal hyperexcitability. Extending the in vitro application of tonabersat with chronic administration to SOD1^{G93A} mice results in MN protection with a reduction in reactive astrocytosis and microgliosis. Taking these data together, our studies identify Cx43 hemichannels as conduits of astrocyte-mediated disease progression and a pharmacological target for disease-modifying ALS therapies.

astrocyte | stem cells | connexin

Astrocytes form a highly coupled intercellular network in the central nervous system (CNS) through gap junctions (GJ) and hemichannels (HC) (1). GJ facilitate intercellular communication through the exchange of metabolites (2, 3), ions (4, 5), second messengers (6, 7), and microRNA (8). Each GJ is composed of two adjoining HC on the plasma membrane of adjacent cells and each HC is made of six connexin subunits arranged around a central pore (9). While connexins mostly form GJ, they can also exist as nonjunctional HC that open at the plasma membrane into the extracellular space. HC opening is dynamic and especially relevant as HC open upon cellular damage and under pathological conditions (10–12). Connexin 43 (Cx43) is the predominant connexin in astrocytes and the major contributor to astrocyte HC (11, 13–15). Some key roles of Cx43 include (16–19) homeostatic buffering, synchronization of astrocyte networks, metabolic support for neurons, and modulation of synaptic activity and plasticity.

In amyotrophic lateral sclerosis (ALS), astrocytes carrying disease-specific mutations contribute to disease progression after onset and exacerbate motor neuron (MN) toxicity (20–22). ALS astrocyte-mediated neurotoxicity has also been demonstrated in vitro and in vivo using both murine (22, 23) and human ALS astrocytes (24–27). Recent studies suggest cytokines can influence specific astrocyte profiles that render astrocytes to be proinflammatory causing toxicity across several neurodegenerative diseases (28).

Altered Cx43 expression and function, including GJ coupling and HC activity, occurs in a number of neurological diseases (10, 11). In ALS, we have previously reported Cx43 is increased in the lumbar spinal cord of the ALS mouse model expressing mutant superoxide dismutase (SOD1^{G93A}) (29), and demonstrated using an in vitro mouse model that blocking Cx43 in SOD1^{G93A} astrocytes is neuroprotective.

This work now addresses a central role for astrocytes in ALS, their involvement in disease progression, and whether Cx43 HC are a common mechanism contributing to MN death in both familial (FALS) and sporadic ALS (SALS) patients. To that end, we utilize an in vivo conditional Cx43 knockout (KO) mouse model to demonstrate that astrocyte Cx43 contributes to the temporal progression of disease. Using human brain and spinal cord tissues, as well as cerebrospinal fluid (CSF) from ALS patients, we demonstrate that Cx43 expression is enhanced in ALS and has some correlation with

Significance

Our results demonstrate that connexin 43 hemichannels are the conduits for amyotrophic lateral sclerosis (ALS) astrocyte-mediated motor neuron toxicity and disease spread, acting as a common mechanism that can target both familial ALS and sporadic ALS populations. Furthermore, our present work provides proof of principle that tonabersat, as a drug already studied in clinical trials for other indications, could serve as a potential ALS therapeutic.

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The authors declare a competing interest. K.E. is a founder of and consultant for Q-state Biosciences, Qralis, and Encler therapies. N.J.M. has filed US Patent Application #16868764 titled “Neuroprotective Compounds for ALS.”

This article is a PNAS Direct Submission.

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This article contains supporting information online at <http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2107391119/-DCSupplemental>.

Published March 21, 2022.

the rapidity of disease progression in tissues. Our fully humanized, spinal cord-specific platform of SALS and FALS human-induced pluripotent stem cell astrocyte (hiPSC-A) and MN (hiPSC-MN) cocultures enable us to investigate whether blocking Cx43 HC in hiPSC-A using a small molecule has functional relevance in providing neuroprotection to a broad ALS patient population. Finally, we translate these *in vitro* findings to *in vivo* treatment of SOD1^{G93A} mice, demonstrating the potential of Cx43 HC blockade as a strategy for neuroprotection.

Results

Astrocyte-Specific Deletion of Cx43 in SOD1^{G93A} Mice Slows Temporal and Anatomical Disease Progression. We have shown (29) that Cx43 expression increases during the symptomatic and end stages of disease in the SOD1^{G93A} mouse model of ALS and that astrocytes from these mice display functional increases in Cx43-mediated GJ and HC activity *in vitro*. To assess the role of astrocyte Cx43 *in vivo* in the context of ALS, we crossed the SOD1^{G93A} mouse with a Cx43fl/fl:GFAP-Cre mice to specifically delete Cx43 in astrocytes (Fig. 1). Examination of the ventral horn of the lumbar spinal cord, where loss of MN is particularly evident, shows an increase in Cx43 expression in SOD1^{G93A} mice, as expected (29). However, Cx43fl/fl:SOD1^{G93A}:GFAP-Cre (SOD1^{G93A}:Cx43 KO) mice have a near complete loss of Cx43 expression in the ventral horn, as demonstrated by immunofluorescence (Fig. 1 *A* and *B*). This decrease in Cx43 expression is accompanied by a reduction in GFAP expression in the SOD1^{G93A}:Cx43 KO mice (Fig. 1 *A* and *B*). In addition, we verified that while Cx43 expression is typically elevated at the symptomatic and end stages in the SOD1^{G93A} model, it remains knocked out through end stage (Fig. 1 *C*). The KO of Cx43 in astrocytes of SOD1^{G93A} mice was anatomically consistent across different segments of spinal cord (Fig. 1 *C*).

To assess whether the loss of astrocyte Cx43 affects MN survival and disease progression, we performed pathological and motor behavioral studies, specifically grip strength analysis, on SOD1^{G93A}:Cx43 KO mice during the course of disease. We did not observe any differences in the timing of hindlimb disease onset in SOD1^{G93A}:Cx43 KO mice (Fig. 1 *D*). However, the SOD1^{G93A}:Cx43 KO mice displayed prolonged survival compared to their littermate control SOD1^{G93A} mice (Fig. 1 *E*). While the disease onset started at the same time, as reflected in motor function using hindlimb grip strength (Fig. 1 *F*), the forelimb grip strength of SOD1^{G93A}:Cx43 KO was significantly maintained compared to the decline observed in the control SOD1^{G93A} mice (Fig. 1 *F*). We examined whether this sustained forelimb function, indicative of slower disease progression in the SOD1^{G93A}:Cx43 KO mice, was due to preservation of cervical spinal cord MNs. We observed a significant protection of cervical MN (but not lumbar MN) in the SOD1^{G93A}:Cx43 KO spinal cord compared to control SOD1^{G93A} (Fig. 1 *G*).

While we previously did not detect any substantial change in Cx30 expression (29), the other major astrocyte connexin in SOD1^{G93A} mice, SOD1^{G93A}:Cx43 KO mice show a modest up-regulation of Cx30 in the gray matter, potentially as a compensatory effect from loss of Cx43 (SI Appendix, Fig. S1A). No changes were observed in microglial reactivity measured by Iba-1 immunostaining, and the astrocyte-specific glutamate transporter, GLT-1, was also unchanged in the lumbar spinal cord (SI Appendix, Fig. S1A). We also validated that the transgene expression of mutant human SOD1 levels were similar in

SOD1^{G93A}:Cx43 KO mice and the control litter mate SOD1^{G93A} mice (SI Appendix, Fig. S1B). As MN protection was observed in the cervical cord, we examined both astrogliosis (GFAP) and microgliosis (Iba1) and a significant decrease was noted in the SOD1^{G93A}:Cx43 KO mice compared to their littermate control SOD1^{G93A} mice (SI Appendix, Fig. S1C).

We further confirmed that the neuroprotection seen *in vivo* was recapitulated in an *in vitro* SOD1^{G93A} astrocyte/WT MN cocultures (29). We first validated that not only do SOD1^{G93A} astrocytes exhibit an increase in Cx43 expression, but that the SOD1^{G93A}:Cx43 KO astrocytes lack Cx43 expression, as expected (SI Appendix, Fig. S1E and F). Upon coculturing these astrocytes with Hb9GFP MNs, we demonstrate that SOD1^{G93A} astrocytes induce MN toxicity, and this effect is rescued in cocultures with SOD1^{G93A}:Cx43 KO astrocytes resulting in neuroprotection (SI Appendix, Fig. S1G). These data show that deletion of Cx43 (both GJs and HCs) in general has a beneficial protective effect *in vivo* in the SOD1^{G93A} mice.

Previous work (28, 30) suggests that activated astrocytes can have either a proinflammatory or “A1” profile that is neurotoxic or an antiinflammatory “A2” profile that confers neuroprotection. However, our work suggests that the mechanism of toxicity to MNs from SOD1^{G93A} astrocytes and of neuroprotection from SOD1^{G93A}:Cx43 KO astrocytes is disparate from the shift in astrocyte “activation state” (SI Appendix, Fig. S1H) (28, 30).

Elevated Cx43 Is Disproportionately Incorporated into HCs in SOD1^{G93A} Mouse Astrocytes. Having established the role of Cx43 in astrocyte-mediated MN loss and disease progression in a murine ALS model, we wanted to determine the specific contribution of Cx43 GJ vs. HC to these phenomena. As we observed significant neuroprotection with Cx43 HC blocker *in vitro* (29), we hypothesized that Cx43 is primarily present as HC on the astrocyte membrane. We used a previously described (31) biotin pull-down assay to specifically target Cx43 HC, which have exposed lysine residues and can bind to biotin in monolayer cultures, thereby allowing us to determine what proportion of Cx43 is present as HC (SI Appendix, Fig. S2A–C). Interestingly, we observed that 9.6 ± 2.1% of Cx43 incorporate as HCs in WT astrocytes, whereas 32.8 ± 4.7% of Cx43 form HCs in SOD1^{G93A} astrocytes (SI Appendix, Fig. S2D). While there is an overall increase in Cx43 levels in SOD1^{G93A} (SI Appendix, Fig. S2E), the absolute levels of Cx43 HC were specifically elevated in diseased cells compared to WT astrocytes (SI Appendix, Fig. S2F). These data suggest that there is more total Cx43 protein present on the surface of SOD1^{G93A} astrocytes than in WT, and that this amount represents an abnormal shift in the percentage of total Cx43 expression to HC formation. As a negative control, we used the SOD1^{G93A}:Cx43 KO astrocytes and found that the Cx43 KO cells do not have a measurable quantity of either total Cx43 or Cx43 in HC form compared to WT and SOD1^{G93A} astrocytes (SI Appendix, Fig. S2G).

Cx43 Expression Is Increased in Human ALS Postmortem Tissue and CSF. Having demonstrated the distribution of Cx43 and its relevance to MN loss in murine ALS models, we then examined this biology in ALS patients. Previously, we demonstrated an increase in Cx43 expression in a cohort of ALS patient motor cortex and spinal cord (29). Here, we inquired if there is a correlation between Cx43 expression and the temporal course of disease. We examined an expanded cohort of SALS motor cortex and cervical spinal cord (SI Appendix, Table S1) and observed an increase in Cx43 and GFAP transcript levels in the motor cortex of post mortem SALS patients (SI

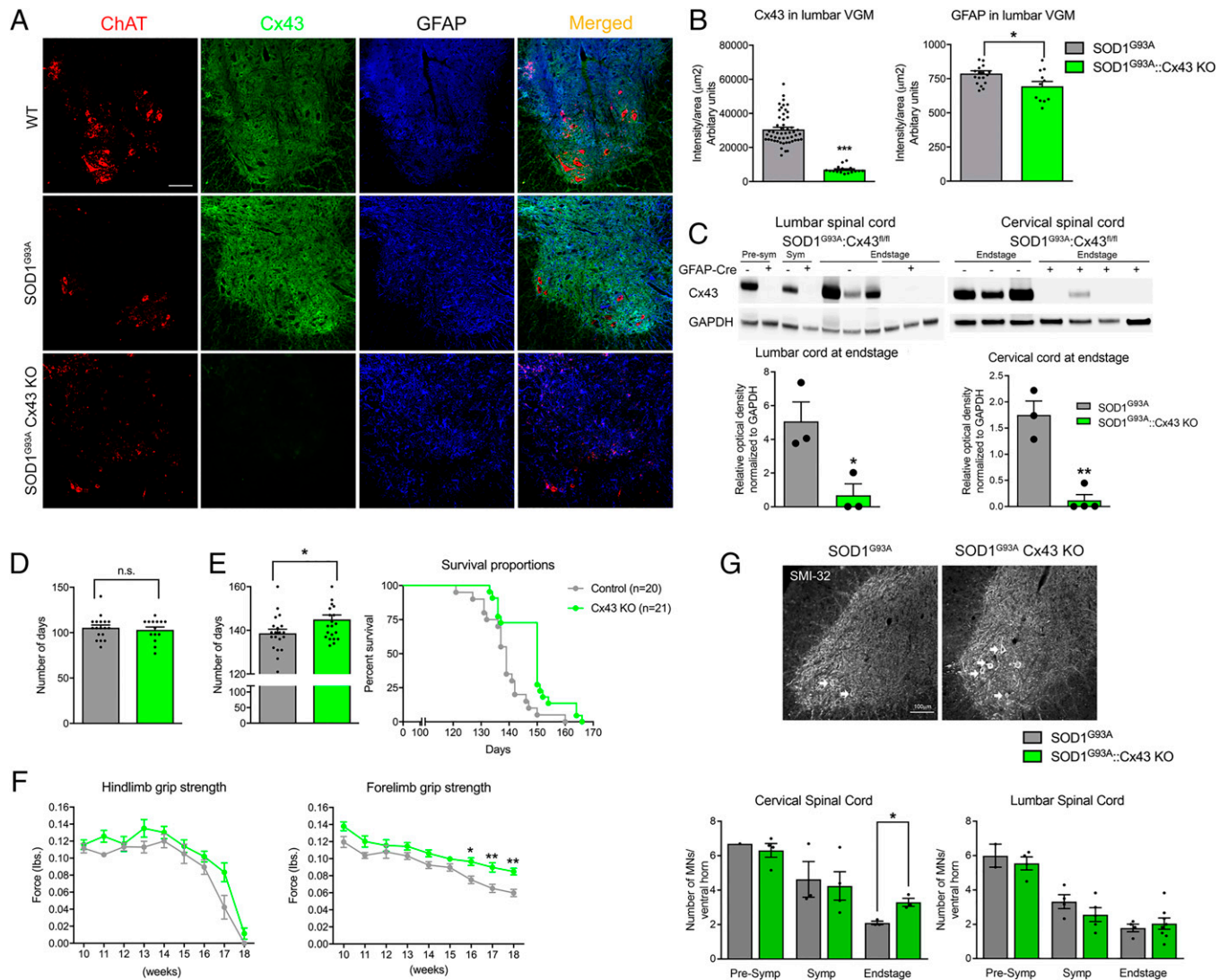


Fig. 1. Astrocyte-specific deletion of Cx43 improves survival and slows caudal rostral progression in SOD1^{G93A} mice. (A and B) Cx43^{fl/fl} mice were bred with a GFAP-Cre driver line, which were further crossed with SOD1^{G93A} mice to generate a triple transgenic mouse (SOD1^{G93A}:Cx43 KO). The MNs located in the ventral spinal cord are labeled with ChAT immunostain. The immunohistochemical staining confirms the lack of Cx43 staining in the lumbar spinal cord of the SOD1^{G93A}:Cx43 KO mice compared to WT and control SOD1^{G93A} mice. A reduction in astrogliosis was observed based on GFAP immunoreactivity in the SOD1^{G93A}:Cx43 KO mice compared to SOD1^{G93A} mice. **P* < 0.05, ****P* < 0.001, *n* = 4 animals per group, and at least three sections per animal. (Scale bar, 100 μm.) (C) Immunoblotting confirmed the deletion of Cx43 in SOD1^{G93A}:Cx43^{fl/fl}:GFAP-Cre⁺ mice in the lumbar spinal cord at presymptomatic, symptomatic, and end stage of disease. Cx43 is also absent in cervical spinal cord of the SOD1^{G93A}:Cx43^{fl/fl}:GFAP-Cre⁺ (*n* = 3 to 5 animals per group) ****P* < 0.01. (D) SOD1^{G93A}:Cx43 KO mice and littermate SOD1^{G93A} mice show similar time of disease onset. (E) SOD1^{G93A}:Cx43 KO mice survived significantly longer (145 ± 2.1, *n* = 21) compared to SOD1^{G93A} mice (138.7 ± 1.9, *n* = 20), also depicted in the Kaplan-Meier survival curve, **P* < 0.05. (F) Motor function was tested using grip-strength analysis and SOD1^{G93A}:Cx43 KO mice maintained significantly higher forelimb grip strength compared to SOD1^{G93A} mice with no overall change in hindlimb grip strength. **P* < 0.05, ****P* < 0.01. (*n* = 16 to 20). (G) No differences in MN survival were noted at presymptomatic and symptomatic stages of the disease in the cervical and lumbar spinal cord. However, a significant preservation of MNs was observed at end stage in the cervical spinal cord of SOD1^{G93A}:Cx43 KO mice (*n* = 3 per group) compared to SOD1^{G93A} mice but not in the lumbar cord. (*n* = 4 to 8 per group), **P* < 0.05. Data are represented as mean ± SEM.

Appendix, Fig. S3 A and B). This increase in Cx43 transcript parallels protein expression levels in ALS-affected brain regions, such as the motor cortex and cervical spinal cord, when compared to controls. Given the heterogeneity in disease progression, we inquired whether there is a correlation with Cx43 expression. ALS patients with rapidly progressing disease (i.e., deceased within 2 y after onset) (32, 33) display higher Cx43 protein levels in motor cortex compared to both control and patients with a more typical ALS disease progression (i.e., deceased between 2 and 5 y after onset) (32, 33) (SI Appendix, Fig. S3C). This correlation between increased Cx43 levels and ALS disease progression also holds true for cervical spinal cord samples (SI Appendix, Fig. S3D). By using ELISA, we demonstrated that Cx43 protein is detected in vivo in the CSF from control and ALS patients. As

observed in postmortem tissue, Cx43 protein level was significantly elevated in the CSF samples obtained from ALS patients (SI Appendix, Fig. S3E).

Astrocyte Cx43 HC Expression and Function Is Increased in hiPSC-A from both FALS and SALS Patients. To assess if Cx43-mediated effects in ALS extend beyond SOD1 mutations, we examined Cx43 expression in cells obtained from a cohort of patients with both SALS and FALS (SI Appendix, Table S2) and differentiated them into hiPSC-A using a spinal cord patterning protocol (34) (SI Appendix, Fig. S4 A–D). Consistent with our previous findings, similar degrees of astrocyte differentiation occur between control, FALS and SALS hiPSC-A, as indicated by astrocyte markers (SI Appendix, Fig. S4 E–H) (34).

A consistent and significant increase in Cx43 expression was observed in hiPSC-A from SALS patients (Fig. 2A). In addition, we investigated Cx43 changes in FALS hiPSC-A specifically from three different slowly progressing *SOD1*^{D90A} and from three unrelated fast-progressing *SOD1*^{A4V} patients (Fig. 2B). While *SOD1*^{D90A} samples display a trend for an increase in Cx43 expression, the fast-progressing *SOD1*^{A4V} hiPSC-A show a significant increase in Cx43 expression compared to control hiPSC-A (Fig. 2B). As hiPSC-A are differentiated in the absence of neurons (SI Appendix, Fig. S4), any change in Cx43 occurs independently of neuronal input (cell autonomous) and not as a secondary effect of neuron-dependent astrocyte “activation.” Confirming this, we observed that the expression of Cx43 in FALS and SALS hiPSC-A remains significantly elevated in hiPSC-A/MN cocultures as well (SI Appendix, Fig. S5).

Intriguingly, Cx43 levels in hiPSC-A exhibit a correlation with ALS disease progression similar to ALS postmortem samples. While Cx43 expression is increased in *SOD1*^{D90A} cases (who have a relatively slower temporal course), Cx43 levels are further significantly higher in patients with *SOD1*^{A4V} mutation (who exhibit a faster disease progression) compared to control patients (35) (Fig. 2B). Similar to the murine in vitro studies above, we determined the specific contribution of Cx43 HC to the amount of total Cx43 using ALS hiPSC-A. We used the biotinylation assay for FALS hiPSC-A (Fig. 2 C–G), as described for SI Appendix, Fig. S2, and observed that both control and the FALS hiPSC-A expressed similar proportional amounts of Cx43 as HC (Fig. 2D). Therefore, when quantified, the percentages of Cx43 present as HC were not significantly different (Fig. 2E). However, FALS astrocytes had higher absolute levels of Cx43 expression (Fig. 2 F and G). We extended our analysis to the absolute amounts of Cx43 HC between pulldowns from control and SALS patient lines, and noted that all ALS hiPSC-A lines had higher absolute amounts of Cx43 HC present on their membranes (Fig. 2H).

The release of ATP through the Cx43 HC opening has been reported in other neurodegenerative diseases (36) as a measure for Cx43 HC activity. Hence, we tested the opening of Cx43 HC by measuring ATP release via a luciferase assay in the supernatants of FALS and SALS hiPSC-A. Endogenous ATP in this instance acts as a functional marker for HC opening. At baseline, both FALS and SALS hiPSC-A had higher concentrations of ATP in their supernatant compared to hiPSC-A from controls. Upon treatment with Gap19, a Cx43 HC-specific mimetic peptide blocker (37), control ATP supernatant levels were unchanged but ATP levels in both the FALS and SALS hiPSC-A supernatants, were reduced to control ranges (Fig. 2 I and J). This suggests that, at baseline, ALS hiPSC-A have increased HC permeability to ATP that can be pharmacologically blocked.

Blocking Cx43 HC in Human ALS iPSC-A Mitigates MN Toxicity.

Given the opportunity to examine the genetic and phenotypic heterogeneity of ALS using hiPSC-A, we cocultured hiPSC-A (Fig. 3A) from control and two unrelated FALS (*SOD1*^{A4V}) patients with hiPSC-MN from controls. We found that FALS hiPSC-A induced toxicity in human iPSC-MN (ChAT⁺), including a subset of Isl1⁺ MNs, and that blocking Cx43 HC with Gap19 provided significant neuroprotection (Fig. 3 A–K). Similarly, we employed an identical coculture paradigm to examine whether hiPSC-A from SALS patients would be toxic to control hiPSC-MN (Fig. 3A). We observed a similar level of hiPSC-MN toxicity compared to the *SOD1*^{A4V} hiPSC-A and also demonstrated neuroprotection via HC blockade using Gap19 (Fig. 3 A–K).

In order to test Cx43 HC-specific neurotoxicity, we hypothesized that ALS hiPSC-A toxicity was conferred by a factor released into the medium, as might be expected for connexin HC. To this end, we performed a hiPSC-A/MN coculture study where hiPSC-A were grown on a transwell insert above and control hiPSC-MN on the base of the well, with both cell groups grown in the same media (Fig. 3L). We cocultured FALS and SALS hiPSC-A lines with hiPSC-MN derived from control patients, and observed a significant reduction in hiPSC-MN survival as defined by ChAT and Isl1 immunostaining, similar to the direct coculture paradigm (Fig. 3 L–V). We also treated the transwell cocultures with Gap19, which resulted in significant neuroprotection from both FALS hiPSC-A, as well as SALS hiPSC-A–induced toxicity (Fig. 3 L–V). These data suggest that astrocyte-mediated toxicity from these ALS lines is propagated mainly through Cx43 HC and further demonstrate the neuroprotective effect of Gap19 in FALS and SALS astrocytes. To rule out the effect of Gap19 directly on neurons, we confirmed that the addition of Gap19 to hiPSC-MN cultures alone did not affect MN survival and thus neuroprotection is specifically mediated by astrocytes (SI Appendix, Fig. S6A).

While much of ALS in vitro biology is focused on MN loss, there is in vivo evidence (38–40) that other neuronal populations are affected by neurodegeneration. We utilized our hiPSC-based platform to examine whether non-MN degeneration in ALS is astrocyte and specifically Cx43 HC-mediated. Our data utilizing human ALS hiPSC-A/MN cocultures show that ALS astrocytes from both SALS and FALS patients also induce toxicity to TUJ1⁺/ChAT[−] neurons. This effect was partially rescued by the addition of the Cx43 HC blocker Gap19 (SI Appendix, Fig. S6B). We established, by using the transwell system and Gap19, as described above, that this ALS astrocyte-mediated neurotoxicity was related to a factor released through Cx43 HC (SI Appendix, Fig. S6C). Furthermore, we have reproduced our findings on astrocyte and Cx43 HC-mediated hiPSC-A neurotoxicity toward MN and non-MN populations using a different control hiPSC-neuronal line (CS25) (SI Appendix, Fig. S7).

Gene Correction of *SOD1*^{A4V} in hiPSC-A Results in Reduced Cx43 Expression and MN Protection.

To examine if correction of the *SOD1*^{A4V} mutation attenuated Cx43 expression, we used a previously (41) characterized ALS *SOD1*^{A4V} hiPSC line and its gene-corrected isogenic control line (SI Appendix, Fig. S8A). As expected, Cx43 protein levels were higher in the ALS *SOD1*^{A4V} hiPSC-A compared to control astrocytes, while the corresponding gene-corrected *SOD1*^{A4V} line showed a partial normalization of Cx43 expression (SI Appendix, Fig. S8B). These data indicate that increases in Cx43 expression are a common feature of both SALS and FALS patients. Interestingly, when the *SOD1*^{A4V} mutation was corrected using zinc finger endonuclease, the resulting isogenic line displayed residual neurotoxicity on hiPSC-MN in a coculture paradigm (SI Appendix, Fig. S8 C–M). This neurotoxicity could be further rescued by Cx43 HC blockade with Gap19 (SI Appendix, Fig. S8C). This was confirmed in the transwell platform as well (SI Appendix, Fig. S8 N–X), where the correction of the *SOD1*^{A4V} mutation in the FALS hiPSC-A line conferred partial neuroprotection, with the residual neurotoxicity being further rescued by Cx43 HC blockade with Gap19.

The Cx43 HC Blocker Tonabersat Decreases Astrocyte-Mediated hiPSC-MN Death and Hyperexcitability in a Dose-Dependent Manner. Our findings from rodent models and human ALS samples suggest that Cx43 HC may provide an appropriate target for ALS therapeutics. Tonabersat (SB-

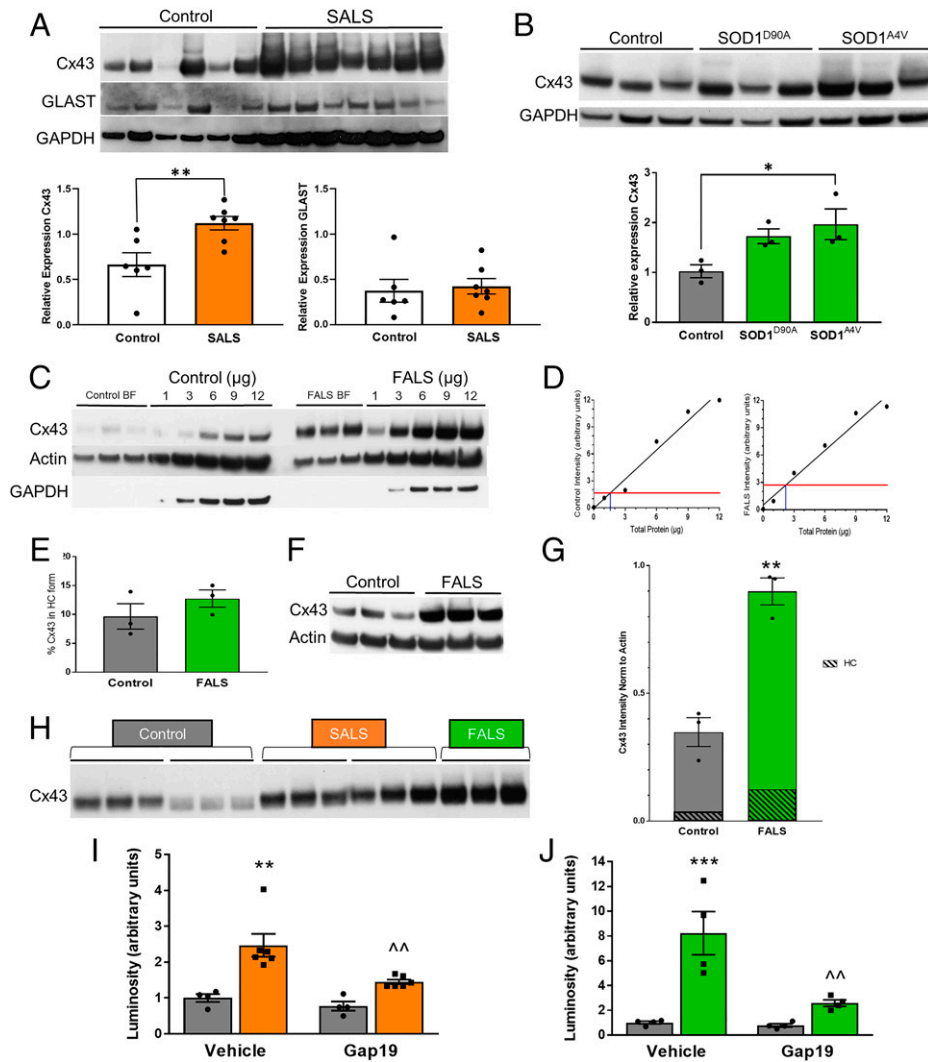


Fig. 2. Human ALS iPSC-A have increased Cx43 HC levels associated with increased cell permeability, which can be blocked by Gap19. (A) Western blots of total protein collected from various hiPSC-A control and SALS lines were compared for the expression of Cx43 and GLAST (EAAT1), a glial-specific membrane protein. Quantification shows sporadic Cx43 expression is significantly higher, while no difference was found in the expression of GLAST. Control lines from left to right: GO018, CS25, JH082, CIPS, JH003, GM01582. SALS lines from left to right: JH013, JH036, JH053, JH029, JH017, JH058, JH040 (** $P < 0.01$, t test, $n = 6$ to 7 per condition). (B) Western blots stained for Cx43 in hiPSC-A from control and familial lines shows that in SOD1^{A4V} patients, Cx43 expression is significantly higher. Control lines from left to right: CIPS, GM01582, JH082. SOD1^{D90A}-lines left to right: GO017, GO004, GO028. SOD1^{A4V} lines left to right: GO013, GO002, GO007 (* $P < 0.05$, one-way ANOVA, $n = 3$ per condition). (C) Control (CIPS) and FALS hiPSC-A (GO013) in vitro lines were subjected to biotin pulldown. Subsequent biotin fractions and total protein were immunoblotted for Cx43, with actin and GAPDH as housekeeping genes. (D) Linear quantification against the total protein standards have R^2 values of 0.97 and 0.96. (E) There is no statistically significant shift in the percent of Cx43 as HC between ALS and control hiPSC-A. (F) Western blot of total protein for hiPSC-A control and FALS lines. (G) 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 than control. (H) Western blot of two lines from control (CIPS, GM01582) and SALS (JH058, JH040), and one line of FALS (GO013) stained for Cx43 from the biotin fraction in vitro. Each bar represents one line tested in triplicate. (I) ATP levels detected in the supernatants of control and sporadic hiPSC-A show significantly higher amounts in SALS hiPSC-A compared to controls. Treatment with Gap19 significantly reduces supernatant ATP in sporadic astrocytes (two-way ANOVA, ** $P < 0.01$, $\wedge\wedge P < 0.01$, $n = 4$ to 6). (J) Supernatant ATP

of FALS hiPSC-A is significantly higher than control. Gap19 reduces supernatant ATP of FALS hiPSC-A compared to vehicle treatment (two-way ANOVA, *** $P < 0.001$, $\wedge\wedge P < 0.01$, $n = 4$). Data are represented as mean \pm SEM.

220453) was selected for its translational potential in human clinical trials (42, 43). It specifically blocked Cx43 HC at low concentrations (1 to 10 μ M), but not the astrocytic Cx30 HC, as demonstrated in a heterologous expression system (44) (*SI Appendix, Fig. S9*).

To examine the potential of tonabersat in preventing hiPSC-A and Cx43 HC-mediated neurotoxicity, we cocultured control hiPSC-MN with either control hiPSC-A, FALS hiPSC-A, or SALS hiPSC-A. We utilized direct coculture (Fig. 4 A–K) and transwell (Fig. 4 L–V) paradigms as described above, and found that tonabersat protects hiPSC-MN from ALS astrocyte-mediated death in a dose-dependent manner in both systems. At 1 and 10 μ M, tonabersat blocks Cx43 HC and rescues ChAT⁺ MNs, including a subpopulation of Isl1⁺ MN. At a higher concentration of 100 μ M, tonabersat was not neuroprotective, and likely reflects a long-term effect on reducing GJ trafficking instead of HC activity, as described previously (42) (Fig. 4 A–K). The neuroprotective effect of tonabersat was also seen in the transwell study (Fig. 4 L–V), suggesting that tonabersat's neuroprotection is HC-mediated and further confirming the importance of Cx43 HC in toxicity. We believe that the neuroprotective effects of tonabersat are specific to Cx43 HC on astrocytes, since the application of tonabersat to cultures of hiPSC-MN alone did not have any effect on hiPSC-

MN survival (*SI Appendix, Fig. S10A*). Interestingly, we also observed that tonabersat was protective to non-MN populations (ChAT[−] and Isl1[−] neurons), similar to the results seen with Gap19 treatment (*SI Appendix, Fig. S10 B and C*).

In the CNS, tonabersat's actions may translate to an astrocyte-mediated reduction of neuronal excitability (45, 46), as suggested by in vivo studies showing tonabersat's inhibitory effects on cortical spreading depression (47, 48) and seizures (49, 50). We have previously used (34) multielectrode array (MEA) in a fully human iPSC-based platform to evaluate how astrocytes influence MN electrophysiology (*SI Appendix, Fig. S11A*), and here we employ that system to evaluate the electrophysiological actions of tonabersat in vitro. We first utilized control cocultures of hiPSC-A/MN on MEA plates to study how the pharmacological blockade of hiPSC-A Cx43 HC would affect hiPSC-MN electrophysiological activity. The addition of tonabersat (*SI Appendix, Fig. S11B*) at a concentration of 10 μ M significantly reduced neuronal spiking and bursting activity within 5 min of application. The effect was dose-dependent, as a lower concentration of tonabersat (i.e., 1 μ M) was less effective (*SI Appendix, Fig. S11B*). These actions parallel those of the Cx43 HC-specific blocker Gap19 (*SI Appendix, Fig. S11C*), confirming our previous observations that Cx43 HC influences neuronal firing (34). To ensure that these effects

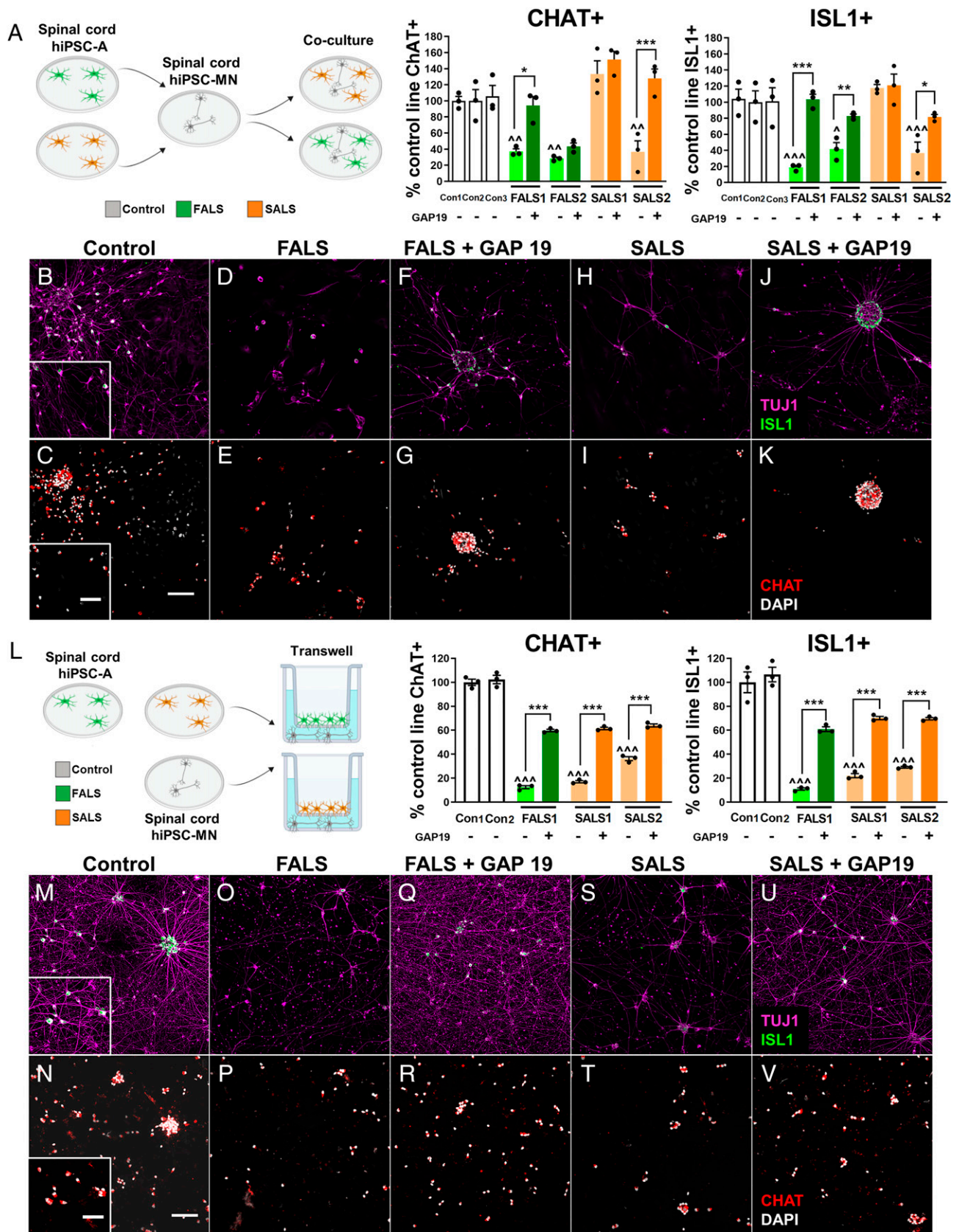


Fig. 3. Human iPSC-MN toxicity is mediated by Cx43 HC from FALS as well as SALS hiPSC-A. (A–K) MNs derived from control hiPSC were plated with hiPSC-A from either control, SALS, or FALS (SOD1^{A4V}) patients. The number of hiPSC-MN (ChAT⁺ and Isl-1⁺) plated on FALS and SALS hiPSC-A decreased over a period of 14 d compared to MN plated with control hiPSC-A. Cocultures containing ALS hiPSC-A treated with Gap19 show an increase in hiPSC-MN survival. (L–V) The coculture of hiPSC-A/MN in a transwell system allowed neurons and astrocytes to share the same medium, preventing direct cell contact. FALS (SOD1^{A4V}) and SALS hiPSC-A shows ChAT⁺ and Isl-1⁺ hiPSC-MN toxicity rescued by Gap19. Con1 = CIPS, Con2 = JH082, Con3 = GM01582, FALS1 = GO013, FALS2 = GO002, SALS1 = JH040, SALS2 = JH058 (*n* = 3 coverslips per condition). Significant comparisons (one-way ANOVA) between untreated control and ALS cocultures are marked with “^”; significant effects of Gap19 on cocultures containing ALS astrocytes are marked with “***”. **P* < 0.05 and ^*P* < 0.05; ****P* < 0.01 and ^^*P* < 0.01; ***P* < 0.001 and ^^*P* < 0.001. (Scale bars, 50 μm main panel; *Inset* is 20 μm.) Data are represented as mean ± SEM.

were astrocyte-mediated, we tested tonabersat on hiPSC-MN alone in culture and did not observe any change in neuronal electrophysiological activity (*SI Appendix, Fig. S11D*).

We then evaluated these electrophysiological findings in the context of ALS (Fig. 5) and cocultured control and ALS hiPSC-A with control hiPSC-MN and recorded them longitudinally for 2 wk. A transient increase in neuronal spiking and bursting activity was observed in the cocultures of SALS and FALS hiPSC-A when compared to control cocultures (Fig. 5A). This occurs early during the 2-wk recording period (significant at day in vitro [DIV] 6 of coculture), followed by a depression of electrophysiological activity (DIV 14 of coculture). Acute treatment with tonabersat significantly reduced this transient ALS astrocyte-mediated hyperexcitability (Fig. 5B), leading to spiking and bursting activity that are comparable to untreated control cocultures.

We then evaluated if there is a temporal correlation between astrocyte-mediated neuronal hyperexcitability and Cx43 HC-mediated neurotoxicity in ALS. To address this, we used the coculture paradigm with ALS and control hiPSC-A/MN cocultures with a shorter treatment course with tonabersat (Fig. 5C). We observed that early application of 10 μ M tonabersat (i.e., from day 5 to day 8 of coculture) confers neuroprotection of hiPSC-MN in coculture with ALS hiPSC-A, which corresponds to the time period of neuronal hyperexcitability based on MEA recordings. However, when the cocultures are treated with tonabersat at a later time-point (from day 11 to day 14 of coculture), when neuronal hypoactivity occurs in ALS hiPSC-A cocultures (based on MEA recordings), there is no neuroprotection. Examination of MN survival at day 11 of coculture confirmed that the early window of neuroprotection is critical as most MN death has occurred prior to that time, thus not allowing for neuroprotection from the later addition of tonabersat (*SI Appendix, Fig. S12*). Therefore, there appears to be a critical early window of time during which tonabersat provides this temporal neuroprotection, which is seen not only in MNs, but also in ChAT⁻ neurons (Fig. 5C).

Tonabersat Provides In Vivo Neuroprotection in SOD1^{G93A} Mice. To test whether the neuroprotective effects of tonabersat on ALS iPSC-MN translated in vivo, we treated a cohort of SOD1^{G93A} mice starting at a presymptomatic stage (40 d of age) with daily administration of tonabersat at 10 mg/kg (*SI Appendix, Fig. 13A*). As predicted, Cx43 expression was increased in the ventral horn of SOD1^{G93A} spinal cord in vehicle-treated mice but, interestingly, we did not see an altered expression of Cx43 in SOD1^{G93A} mice treated with tonabersat (*SI Appendix, Fig. S13 B–E*). Tonabersat treatment in SOD1^{G93A} mice significantly reduced both astrogliosis, measured with GFAP (*SI Appendix, Fig. S13 B–E*), as well as microgliosis (Iba-1) (*SI Appendix, Fig. S13 B and C*). While the neuroprotective effect of tonabersat was not yet evident at the early symptomatic stages for SOD1^{G93A} mice (*SI Appendix, Fig. S13F*), a significant preservation of MN in the cervical spinal cord (*SI Appendix, Fig. S13G*), and to a lesser degree the lumbar spinal cord, was noted in tonabersat-treated SOD1^{G93A} mice compared to vehicle-treated SOD1^{G93A} mice. A trend toward preservation of forelimb grip strength was noted without an effect on survival. To test whether tonabersat had any toxicity when delivered chronically from 40 d of age, WT mice were treated with tonabersat and vehicle. We did not appreciate any toxic effect on MN number, behavior, or survival in WT mice (*SI Appendix, Fig. S13 G–J*).

To examine whether treatment after symptom onset could provide neuroprotection, another cohort of SOD1^{G93A} mice was treated with tonabersat after disease onset beginning at 100 d of age (*SI Appendix, Fig. S14A*). As with the mice whose treatment began at 40 d of age, neuroprotection was again seen in the cervical spinal cord (*SI Appendix, Fig. S14B*). Trends toward maintenance in forelimb grip strength were again observed in both the 100 d of age-treated cohorts (*SI Appendix, Fig. S14C*). Together, these in vivo data suggest that effects of blocking Cx43 HC-mediated MN toxicity is most evident following disease onset.

Discussion

Cx43 is the predominant connexin in astrocytes (11, 13–15, 51) and is unique as it is the only astrocyte connexin that can be incorporated into HCs as well as GJs. Changes in Cx43 expression are reported in neurodegenerative disorders, primarily as an up-regulation of the protein (29, 52–55), although less is known about the degree to which these increases are incorporated specifically into the HC, GJ, or both (56).

In ALS, few studies have shown increased total Cx43 in lumbar spinal cord of SOD1^{G93A} mice, although the implications for the increases in total Cx43 protein were not examined (57–59). In our previous study (29), we observed an increase in expression of Cx43 in this ALS mouse model and in SOD1^{G93A} astrocytes occurring cell autonomously, in the absence of neurons or neuronal death (29).

Astrocytes have been implicated in ALS progression after disease onset (21). In order to investigate astrocyte Cx43-specific contributions in vivo and in disease progression, we generated a triple transgenic SOD1^{G93A} mouse with Cx43 KO in astrocytes using GFAP-Cre mice. While the disease onset was not affected by astrocyte-specific Cx43 KO, we observed sustained forelimb motor function and an overall increase in survival in this mouse model. These observations suggest that astrocyte Cx43 influences both the temporal course after onset and the anatomical progression of disease. The alteration of the temporal and anatomical progression following KO of astrocyte Cx43 and Cx30 has similarly been reported in a spinal cord injury model (60). Previous studies in Alzheimer's disease, however, have suggested that GJ coupling may be a mechanism for compensatory neuroprotection by buffering potentially toxic substances through the astrocyte network and away from at-risk neurons (61, 62). Therefore, it may be that the KO of Cx43, which affects both GJ and HC formation in our SOD1^{G93A}:Cx43 KO mice, may actually underestimate the potential neuroprotective effect of Cx43 HC loss alone. This would be consistent with our previous data in SOD1^{G93A} in vitro studies showing that pharmacologic blockade of Cx43 HC provided equivalent neuroprotection to the less specific pharmacological blockade of both Cx43 GJ and HC (29). Interestingly, we did not appreciate that SOD1^{G93A} astrocytes had a toxic "A1" profile nor did the KO of Cx43 in the SOD1^{G93A} astrocytes mirror what had previously been described as a neuroprotective "A2" profile (28). At least in this paradigm, our data would suggest that molecular phenotypes related to "activated" ALS astrocyte-mediated MN toxicity may be more complex among disease states.

Cx43 is incorporated into both GJs as well as HCs. Hence, we tested whether the increases in Cx43 observed in SOD1^{G93A} mice primarily affects Cx43 HC at the astrocyte membrane. Previous studies relied on pharmacologic blockade with peptide blockers to implicate Cx43 HC relevance in the context of disease (11, 29). By modifying a biotin pulldown method (31),

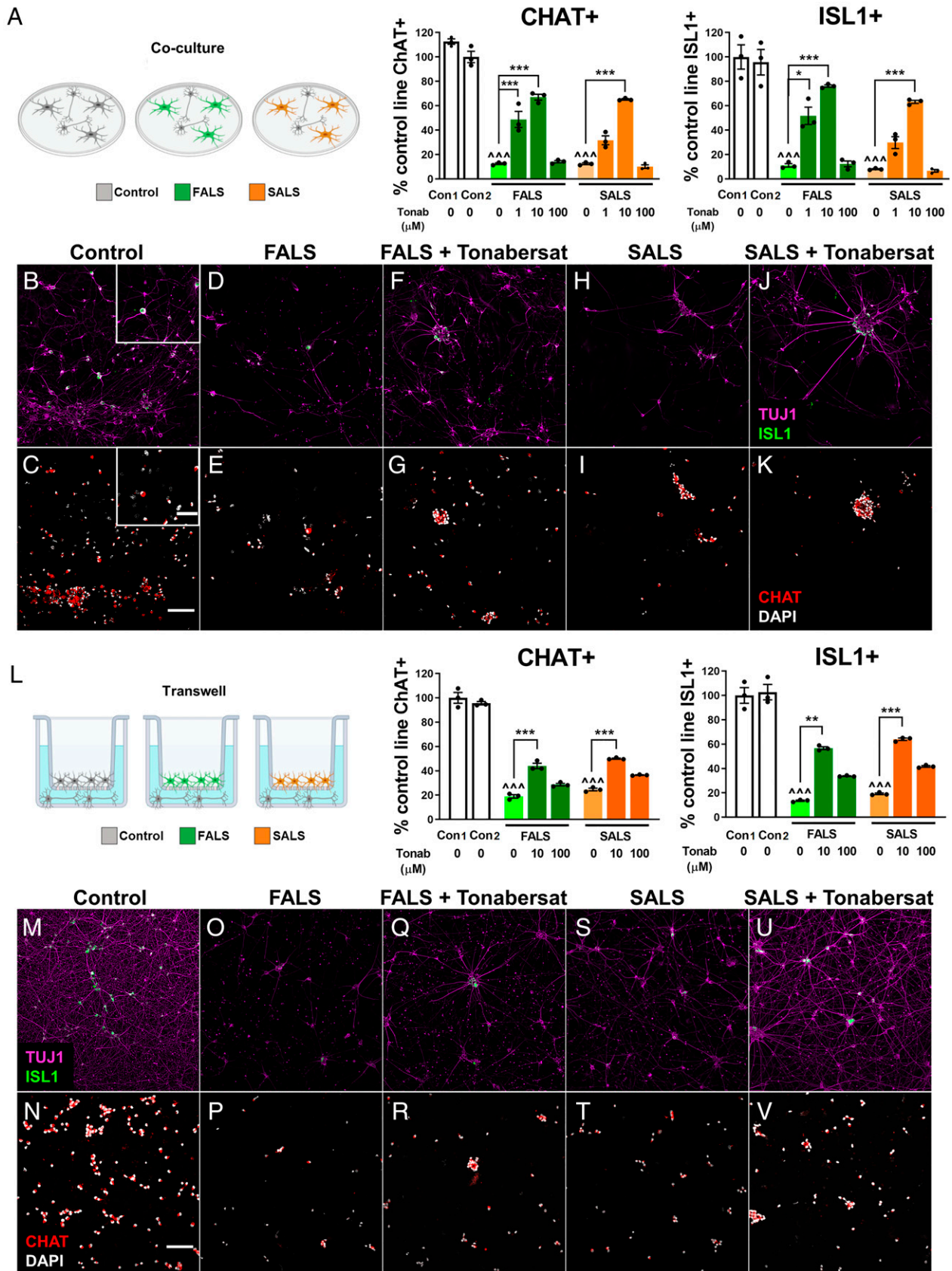


Fig. 4. The Cx43 HC blocker tonabersat provides dose-dependent neuroprotection to hiPSC-MN. (A–K) FALS and SALS hiPSC-A/MN cocultures immunostained for ChAT and Isl-1 show dose-dependent neuroprotection from tonabersat (1 μM, 10 μM, and 100 μM), after a 14-d incubation period. (L–V) Following transwell coculture of FALS and SALS hiPSC-A with hiPSC-MN, immunostaining 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 cocultures are marked with “^”; significant effects of tonabersat on cocultures containing ALS astrocytes are marked with “***”. **P* < 0.05 and ^*P* < 0.05; ****P* < 0.01 and ^^*P* < 0.01; ****P* < 0.001 or ^^*P* < 0.001, *n* = 3 per condition. (Scale bars, 50 μm main panel; *Inset* is 20 μm.) Data are represented as mean ± SEM.

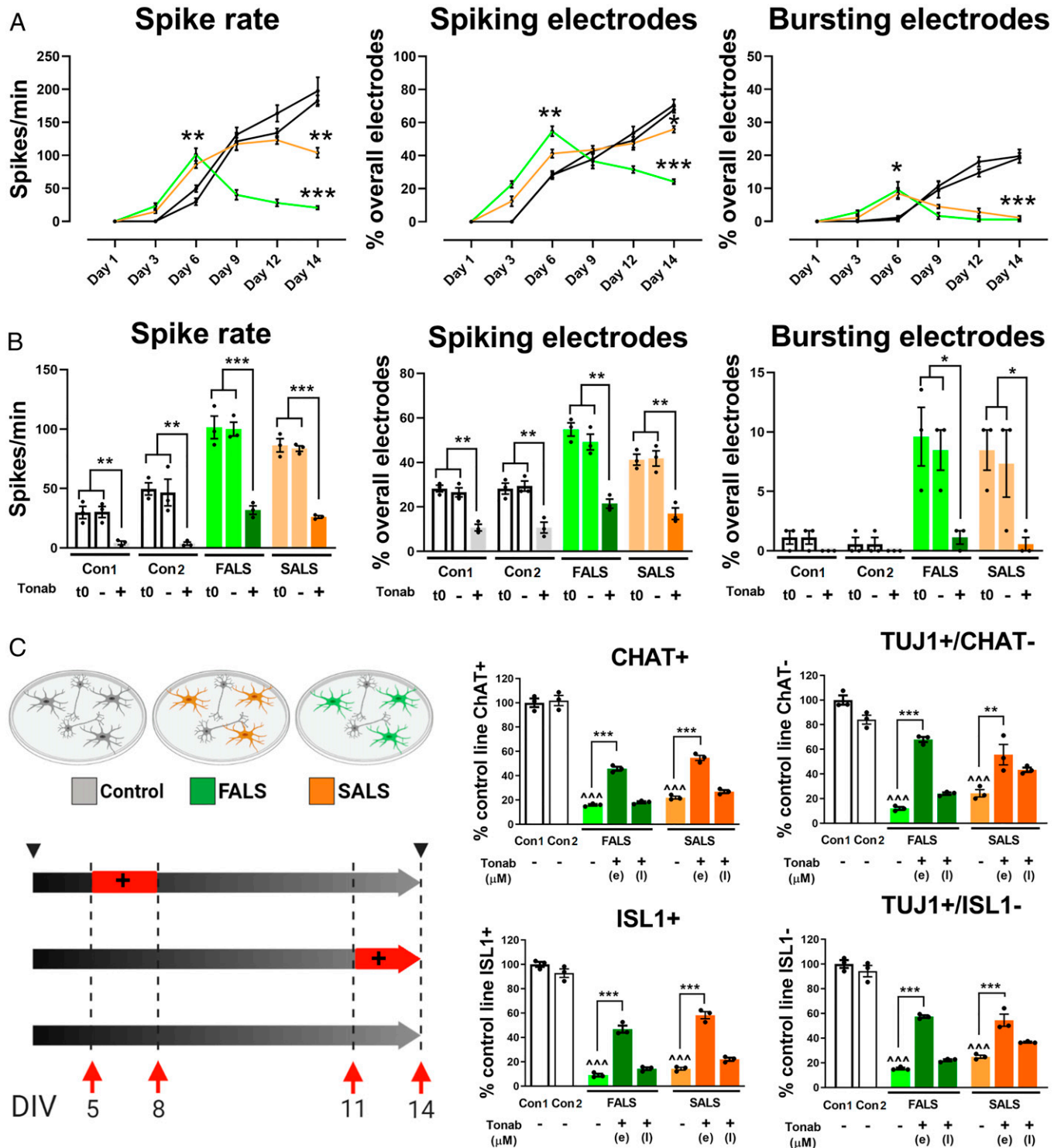


Fig. 5. MEA recordings from hiPSC-Astro/MN cultures demonstrate that tonabersat Cx43 HC-mediated effects on hiPSC-MN electrophysiology correlates with neuroprotection. (A) MEA recording of neuronal activity from cocultures between control hiPSC-MN and control or ALS hiPSC-A. The presence of SALS and FALS astrocytes when compared to control astrocytes determines early increases in spiking and bursting activity followed, at later time points, by reduced electrophysiological activity. Significant time-point comparisons (i.e., day 6 and 14 of coculture) between control and ALS conditions are marked with an asterisk (*) (black line = control, orange line = SALS, green line = FALS). (B) MEA activity within 5 min after the application of 10 μ M tonabersat (+) on cocultures with control or ALS hiPSC-A compared to baseline (t0) and vehicle (-). MEA baseline activity at day 6 shown in A. Tonabersat shows significant inhibition (*) of neuronal spiking and bursting activity. (C) The effects of 10 μ M tonabersat (+) on hiPSC-neuron survival in control and ALS cocultures was tested as outlined in the figure, but for a shorter time course of 3 d, either early (DIV 5 to 8, or "e") or late (DIV 11 to 14 or "l") during coculture. Significant neuroprotection was appreciated after early but not late exposure to tonabersat; evident for both MN (CHAT⁺ and ISL1⁺ cells) and non-MN cell types (TUJ1⁺/CHAT⁻, TUJ1⁺/ISL1⁻). Con1 = CIPS, Con2 = GM01582, FALS = GO013, SALS = JH058. Significant comparisons (one-way ANOVA) between untreated control and ALS cocultures are marked with ([^]), while significant effects of tonabersat on cocultures containing ALS astrocytes are marked with (*). **P* < 0.05 and [^]*P* < 0.05; ***P* < 0.01 and ^{^^}*P* < 0.01; ****P* < 0.001 and ^{^^^}*P* < 0.001, *n* = 3 per condition. Data are represented as mean \pm SEM.

we directly demonstrated increases in the total amount of Cx43 in its HC form. Interestingly, in the SOD1^{G93A} mice, the actual proportion of Cx43 in HC was significantly higher than the percentage seen in WT. This provides evidence that the up-regulation of Cx43 may be the result of altered HC trafficking toward or from astrocytic membranes as well.

Human iPSC-A derived from FALS patients carrying SOD1 mutations as well as from SALS patients provide a unique *in vitro* tool to investigate astrocyte contributions to neurodegeneration. Importantly, we could begin to address whether both FALS and SALS populations have Cx43 HC-mediated neurotoxicity. Similar to the SOD1^{G93A} mouse astrocytes, we noted that increases in Cx43 expression in our library of hiPSC-A lines from both FALS and SALS patients. A gene-corrected isogenic SOD1^{A4V} hiPSC-A line resulted in a reduction in Cx43 expression. Our data are particularly notable as these ALS hiPSC-A have never been in coculture with MN and, therefore, the increases in Cx43 are not determined by neuronal signals, as we have previously (34) described in hiPSC-A/-MN cocultures, nor have they been exposed to neuronal cell death as occurs *in vivo*. Previous evidence has suggested that epigenetic profiles, including age- and disease-dependent signatures, may not be affected by the reprogramming process and, therefore, may constitute a residual “epigenetic memory” (63), as demonstrated by studies utilizing hiPSC-A for modeling FALS and SALS and other neurodegenerative disorders (64). While studying the potential mechanisms regulating Cx43 expression was beyond the aims of the present study, there is evidence suggesting that intrinsic changes in Cx43 expression in ALS hiPSC-A may arise from an altered metabolic state of reprogrammed astrocytes (65), changes in transcriptional and epigenetic factors (63, 66), and differential expression of microRNAs regulating Cx43 expression (67).

Using a fully humanized, spinal cord-specific, coculture platform to study ALS astrocyte/MN interactions, we were able to recapitulate previous studies (24, 27, 68–72) showing that ALS hiPSC-A were toxic to hiPSC-MN in coculture with FALS and SALS lines. Some variability in the degrees of neurotoxicity was noted among iPSC lines, which was expected and likely reflects the *in vivo* clinical, genetic, and pathogenic heterogeneity of ALS. Importantly, we could replicate this hiPSC-A toxicity neurotoxicity in two different MN lines. To identify specifically whether toxicity was mediated by the extracellular release of relevant toxic factors, we used a transwell system separating ALS hiPSC-A from hiPSC-MN. This system proved valuable as toxicity from ALS hiPSC-A was noted among all the lines. The differences in relative hiPSC-MN toxicity from ALS hiPSC-A between the coculture and transwell experiments could suggest that there are compensatory astrocyte-mediated neuroprotective factors manifested through the direct contact of astrocytes and MN, that may be eliminated in the transwell system. This direct contact could include spatial buffering of potassium, glutamate, and other metabolites away from synapses via an astrocytic GJ network at the level of the tripartite synapse (18, 73), the transport of glutamate via astrocytic perisynaptic glutamate transporters (74), stabilization of the synapse and perineuronal net by astrocyte released chondroitin sulfate proteoglycans (75), and the phagocytosis of dead neurons following injury as a mechanism of neuroprotection (76). The transwell cultures, and pharmacological blockade by the Gap19 mimetic peptide, show significant neuroprotection and implicate the release of factors through Cx43 HC into the media as a potent mediator of toxicity. This is further supported by our observation, not only that there are more Cx43 HC on the ALS hiPSC-A

membrane, but they are functionally more active as well. We also noted, however, that blocking Cx43 HC was not sufficient to completely restore hiPSC-MN numbers to their control values. This supports previous work in the field suggesting that, in addition to Cx43 HC-mediated astrocyte toxicity, other mechanisms of ALS hiPSC-A induced MN toxicity are likely contributing as well.

To examine whether the cell-autonomous and Cx43-mediated astrocyte neurotoxicity is due to ALS mutations alone, we used a gene-correction strategy in the SOD1^{A4V} mutation carrying hiPSC-A. Gene editing did not result in a complete rescue of astrocyte-mediated neurotoxicity, which paralleled the relatively higher expression of Cx43 retained in isogenic control cells compared to control lines. This may suggest that other factors, including epigenetic changes or as of yet unidentified risk-factor genes in both SALS and FALS, may represent ALS “disease signatures” contributing to residual astrocyte-mediated neurotoxicity, which are not affected by gene correction of FALS mutations (63). It is notable that the remaining neurotoxicity induced by isogenic SOD1^{A4V} hiPSC-A was rescued by Gap19, suggesting that Cx43 HC contribute to this astrocyte-mediated phenomenon.

Several studies have suggested that astrocyte-mediated neurotoxicity in ALS appears to be specific to MN populations (23, 25, 26, 77). Our spinal cord-specific hiPSC platform of astrocyte/neuron cocultures may provide a more accurate window into spinal cord pathobiology. While the majority of our hiPSC-neurons are MN, (34), our data suggest that ALS hiPSC-A HC-mediated toxicity is not specific to MN subtypes but affects other neuronal subtypes and is consistent with ALS being increasingly recognized as a disorder not only of MN but GABAergic and glycinergic interneurons (78–80) as well.

One of the proposed strengths of using human iPSC is their potential use in translating fundamental observations to therapeutic opportunities (81). Mimetic peptides like Gap19 do not have the capacity to cross the blood brain barrier, and therefore their clinical use is limited. Tonabersat (SB-220453) (42) was selected for its specificity as a Cx43 HC blocker at low concentrations (42) as well as its ability to cross the blood brain barrier (48). Systemic delivery of tonabersat in low concentrations in a rat model of age-related macular degeneration improved functional outcomes by electroretinography and also prevented thinning of the retina through its capacity to block Cx43 HC (42). Tonabersat has been shown to reduce neurogenic inflammation, and antagonize transient neuronal hyperexcitability associated with cortical spreading depression in animal models of migraine with aura (82, 83), where a role of GJs has been proposed (84). Based upon this hypothesis and preclinical results, the oral administration of tonabersat was tested in a phase II clinical trial as a prophylaxis for migraine, where it was found to be well tolerated (85, 86). Tonabersat has also been tested on animal models of epilepsy (49, 50) and proposed as a novel antiepileptic therapy for clinical trials (87).

We therefore investigated the effect of tonabersat on MN survival and found that tonabersat displayed dose-dependent neuroprotective effects in the ALS hiPSC-A/MN coculture platform and, importantly, also in a transwell coculture model, consistent with its capacity to reduce Cx43 HC-mediated release of factors into the media. Interestingly, the neuroprotective effect of tonabersat was more robust in the direct coculture model when compared to the transwell coculture model. We hypothesize that this could reflect differences in Cx43 HC trafficking and localization to the astrocyte membrane or differences in Cx43 HC opening probability when astrocytes are separated from their MN counterparts, although these specific differences could not be definitively

identified. Since tonabersat has been shown to reduce astrocyte-mediated neuronal hyperexcitability, we used our recently established MEA recording technique (34) to test the effects of tonabersat on hiPSC-MN firing. We found that tonabersat reduces neuronal spiking and bursting activity in a dose-dependent fashion in control hiPSC-A/MN coculture. This effect was both astrocyte-specific, as the drug did not affect hiPSC-MN survival alone, and Cx43 HC-specific, as it occurred at concentrations of tonabersat known to specifically target HCs and was comparable to the electrophysiological effects of Gap19. We then translated this MEA platform for electrophysiological recording and drug testing to cultures of control and ALS hiPSC-A with control hiPSC-MN. The culture of human MN with ALS astrocytes resulted in a transient increase in neuronal firing early during the coculture period and, importantly, that this effect could be inhibited with acute addition of tonabersat. This study suggests, in an all-human iPSC model, that tonabersat can bring control MN firing rates back to normal in coculture with ALS astrocytes. Additional findings also support that treatment of ALS hiPSC-A/MN cocultures with tonabersat during a defined period of neuronal hyperexcitability was sufficient to provide long-lasting neuroprotection, suggesting that there is a temporal timeframe to the induction of astrocyte-mediated MN loss.

Having demonstrated, in our human iPSC *in vitro* platform, the efficacy of tonabersat in promoting MN neuroprotection, we examined some of its neuroprotective properties *in vivo* since this compound had not been previously administered chronically. Our data following chronic administration of tonabersat showed that Cx43 levels in SOD1^{G93A} mice were unchanged, suggesting that any neuroprotective effect would not be related to loss of Cx43 but rather blocking of Cx43 HCs. The neuroprotective effect of tonabersat, while not evident during the early symptomatic phase, was observed at end stage as MNs were preserved in both cervical and lumbar spinal cords of SOD1^{G93A} mice. This indicates that most of the neuroprotective effect of tonabersat occurred during the last 20 to 25 d leading up to end stage, and is reinforced by the observation that neuroprotection in the cervical spinal cord was also noted following tonabersat dosing beginning after the onset of disease, with treatment beginning at 100 d of age. This suggests, as seen with the SOD1^{G93A}:Cx43 KO mice, tonabersat does not alter the onset of disease but rather extends and confers preservation of MN during disease progression. Interestingly, while astrocyte Cx43 was unchanged following tonabersat treatment, GFAP expression (marker of reactive astrocytes) and Iba-1 (marker of microgliosis) were both reduced. Importantly, we did not see any detrimental effects on WT mice from tonabersat treatment beginning at 40 d of age. These observations suggest that tonabersat's *in vitro* neuroprotective effects are also translatable in the setting of chronic drug administration *in vivo*.

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Using human ALS CNS tissue and CSF and showing increased expression of Cx43, as a potential biomarker of disease combined with the use of the human iPSC *in vitro* platform demonstrates the importance of Cx43 HC as contributors to ALS astrocyte-mediated neurotoxicity. The findings of this study offer potential for using Cx43 HC blockade for mediating ALS progression.

Methods

Cx43^{fl/fl}/SOD1^{G93A}:GFAP-Cre Transgenic Mice. Transgenic SOD1^{G93A}:hGFAP-Cre: Cx43^{fl/fl} mice were generated by breeding hGFAP-Cre on FVB background (Jackson Laboratories, JAX#004600) with Cx43^{fl/fl} mice on C57BL6 background (Jackson Laboratories, JAX #008039), and those mice were further crossed with B6SJL SOD1^{G93A} Gur/J mice (Jackson Laboratories, JAX #002726). Control littermates were used for all the experimental assays. All animal procedures were performed in accordance with the NIH guidelines on the care and use of vertebrate animals and approved by the Institutional Animal Care and Use Committee of the Research Institute at The Johns Hopkins University.

Human iPSC Astrocyte and hiPSC-MN Culture. The protocols for the use of human iPSC have undergone and received Johns Hopkins University Institutional Review Board approval. The titles of the protocols are "Skin biopsies and PBMCs generate cell lines for study of ALS" (approval 10/16/2008 NA_00021979) and "Generation and characterization of cell lines for ALS" (approval 1/19/2010 NA_00033726). Informed consent was obtained from all donors. Donations of samples were voluntary.

The generation, characterization of the human iPSC lines, and their differentiation into astrocytes have been previously described by our group and the lines utilized for this study are listed in *SI Appendix, Table S2* (26, 34, 88). Human iPSC-A and hiPSC-MN coculture are described in *SI Appendix*.

MEA Culture and Recordings. MEA plates (MultiChannel Systems, 60MEA200/30iR-Ti-gr) with 60 electrodes, including 59 active and 1 reference, and an MEA2100 (MultiChannel Systems) platform were used for the recording of neuronal activity in hiPSC-A/MN cocultures, as we have previously described (34).

Data Availability. All study data are included in the main text and *SI Appendix*.

ACKNOWLEDGMENTS. This study was supported in part by ALS Association 18-DDC-436 (to N.J.M.), Maryland Stem Cell Research Fund 2019-MSCRFD-5122 (to N.J.M.), The Packard Center for ALS research at The Johns Hopkins University (N.J.M.), Department of Defense ALSRP W81XWH1810175 (to N.J.M.), 1R01NS117604-01 (to N.J.M.), and NIH R01-GM099490 (to J.E.C.). The SOD1^{ΔM^V} isogenic lines were a kind gift from K.E. Amyotrophic lateral sclerosis and control brain and spinal cord tissues were obtained from the Target ALS Human Postmortem Tissue Core (Lyle Ostrow), as well as the VA Tissue Bank. Northeast ALS Consortium Sample Repository provided cerebrospinal fluid samples (James Berry). Illustrations were created with BioRender (<https://biorender.com/>).

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