Apo-Ferritin as a Therapeutic Treatment for Amyotrophic Lateral Sclerosis

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Apo-Ferritin as a Therapeutic Treatment for Amyotrophic Lateral Sclerosis

Iron accumulation and deposition have been reported in patients with amyotrophic lateral sclerosis (ALS). Previous work in mutant SOD1 mice mouse models of ALS have indicated that iron chelation with a chemical agent extends lifespan. Therefore, we propose the use of apo-ferritin, the iron-storage protein ferritin that is iron-poor, as a natural ionophore to sequester excess iron and redistribute it. The overall hypothesis is that infusion of apo-ferritin protein into the brain will provide neuroprotection by limiting the availability of excess iron to catalyze free-radical production.

The most significant findings from this project are: 1.) Infusion of artificial cerebrospinal fluid (aCSF) containing nutrients, including H-ferritin, increases lifespan and delays onset of disease in SOD1<sup>G93A</sup> mice; 2.) This effect is not achieved by infusion of saline, suggesting that there is more than just a mechanical benefit to increasing flow of CSF 3;) In an accelerated disease model of ALS where mice carry both the HFE<sup>H67D</sup> allelic variant, present in 30% of ALS patients, and the SOD1<sup>G93A</sup> mutation, infusion of aCSF with or without H-ferritin is not beneficial. The high rate of oxidative stress in these animals may be too great for an infusion strategy; 4.) Infusion of H-ferritin protein encapsulated in liposomes is even more effective at delaying onset and extending lifespan than H-ferritin protein directly infused. The potential clinical significance of this work is that increasing turnover of cerebrospinal fluid, and providing H-ferritin in a manner that may be more likely to be taken up by cells, appear to be viable therapeutic options for ALS patients.
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INTRODUCTION

Iron misregulation and oxidative stress have been implicated in the pathogenesis of amyotrophic lateral sclerosis (ALS) (1) and are consistent features of the disease in humans (2, 3) and in animal models (4). Therefore, strategies to limit oxidative stress by iron sequestration are potentially effective methods to delay or attenuate ALS symptomology. In this project, our conceptual framework is that infusion of the iron storage protein, ferritin, into the murine brain is a means to provide neuroprotection by limiting the availability of excess iron to catalyze free-radical production. Overexpression of ferritin has been reported to be effective in \textit{in vivo} (5) and \textit{in vitro} (6) systems. The purpose of this work is to address the hypothesis that apo-ferritin (ferritin that is iron poor) is a viable therapy to attenuate symptom progression and prolong survival. The scope of the research is to investigate the effect of intracranial infusion of apo-ferritin in ALS mouse models to study the effects of removal and redistribution of iron from the site of tissue damage. Our work encompassed the use of two animal models and two methods of delivery of apo-ferritin. The first animal model is the superoxide dismutase 1 (SOD1) transgenic mouse (7), a model that is widely utilized in ALS research. The other animal model examines the contribution of SOD1 and an allelic variant of the \textit{HFE} gene; this gene variant causes intracellular iron accumulation, and the variant is present in at least one-third of ALS patients. In addition to direct infusion of apo-ferritin protein (Aims 1 and 2), we examined the effect of directed delivery of apo-ferritin via liposome-encapsulation (Aim 3); these liposomes contained lipopolysaccharide on the surface and allowed for targeted delivery of apo-ferritin to microglia. Our primary outcomes are examination of symptom onset and survival in the animals; secondary outcomes are biochemical and histological assessment of our intervention.

BODY

Project progress on each of the tasks is outlined per the approved Statement of Work.

Task 1. Intracerebroventricular infusion of apo-ferritin into SOD1\textsuperscript{G93A} mice.

1a. Purchase mice and supplies. Perform surgery at 70 days of age to implant cannula and osmotic pump into mice

\textbf{Progress}: We were able to successfully express, purify, and concentrate apo-H-ferritin in the quantities required to perform the work. This was a major challenge during the first 6 months of funding and required that we utilize a new plasmid for H-ferritin lacking a poly-histidine tag and use of gel filtration to purify the protein. We did not anticipate the technical hurdle in concentrating ferritin that would require an entirely different method of purification, and this caused a slight delay in our timeline.

In January of 2012, the first group of SOD1\textsuperscript{G93A} mice was purchased from Jackson Laboratories. Animals were obtained in small batches rather than the entire approved amount so that we could generate proof-of-concept data. To our knowledge, this is the first time that H-ferritin has been infused into the brain of the SOD1\textsuperscript{G93A} mice and there are no pre-existing toxicity data.

Upon arrival, mice underwent training sessions on the rotarod apparatus prior to cannula and Alzet mini-osmotic pump surgeries at 70 days, which is historically a minimum of 30 days prior to disease onset. Pumps were replaced in a second surgery at 98 days of age to ensure continuous infusion, as the duration of the pump is 28 days. Initially, we experienced an unusually high mortality rate from the surgical procedure itself due to the ketamine and xylazine anesthesia. After consultation with the veterinary staff and approval by the Institutional Animal Care and Use Committee, we utilized a
ketamine, xylazine, and acepromazine mixture and had very high survival rates. There was no decrease in locomotor ability due to the presence of the osmotic pump, and the infusion of H-ferritin at a concentration of 2.0 mg/ml or artificial cerebrospinal fluid (CSF) did not have any adverse effect on the animals. Subsequent groups of mice were purchased and the experiments were expanded after it had been established that our intervention was not detrimental.

1b. Perform behavioral analysis of mice during disease phase. Mice that reach the predefined endpoint will be sacrificed during this time.

**Progress:** The behavioral measure of motor coordination was assessed via use of the rotarod apparatus. Latency to first fall is assessed using rotarod data collected from each individual mouse. When a mouse is not able to stay on the rotating rod for greater than one Standard Error of the Mean of its ability to stay on the rod during the pre-symptomatic phase, disease onset has occurred (8).

Disease onset was not significantly different in the animals treated with 2.0 mg/ml H-ferritin as compared to the No Surgery control group (median values 114 days versus 108.5 days, respectively) (Figure 1). It was noted that infusion with artificial CSF (aCSF), which was the control group for the H-ferritin infusion, significantly delayed disease onset as compared to the No Surgery group (median value 118 days vs. 108.5 days). The combined data suggest that our infusion paradigm is not toxic to the animals and the concept of infusing a trophic or “cleansing” solution may provide a meaningful delay in disease onset.

In terms of survival, endpoint is defined be the inability of the mouse to right itself within 30 seconds of being placed on its side or the inability to maintain basic grooming and feeding behavior (9, 10). The data in Figure 2 demonstrate that infusion with 2.0 mg/ml H-ferritin modestly extends lifespan as compared to the No Surgery group, but there appears to be a greater benefit due to infusion of aCSF.

The result of infusion with 2.0 mg/ml H-ferritin was not sufficiently robust; therefore, we doubled the concentration of H-ferritin in another group of mice, as proposed as an alternative strategy in our grant application. The initial concentration of H-ferritin that was used for infusion was selected based on in vitro work (11), and 2.0 mg/ml of ferritin was the lowest dose reported to be effective. The data in Figure 3 indicate that treatment with CSF or 4.0 mg/ml H-ferritin did not significantly delay median disease onset as compared to the No Surgery control group; the data suggest that infusion with a higher concentration of H-ferritin was detrimental to the mice. Similarly, there was no extension of lifespan due to infusion of H-ferritin 4.0 mg/ml (Figure 4). One of the challenges in interpreting our results is the low number of animals in our groups; therefore, we have also evaluated the data by combining what was obtained from infusion of H-ferritin of both 2.0 mg/ml and 4.0 mg/ml concentrations. The compilation of the data resulted in larger groups of eight or nine animals per condition and onset data are shown in Figure 5. In this figure, median survival for the No Surgery Group is 108.5 days, 118 days for the CSF group, and 112.5 days for the H-ferritin group; there is a significant delay in disease onset of 9.5 days due to aCSF infusion. As before, lifespan was increased by infusion with H-ferritin or CSF as compared to the No Surgery control group by a maximum of 4 or 23 days, respectively (Figure 6), although this was not statistically significant.
An interesting finding throughout our studies was that infusion of aCSF was consistently beneficial to the animals over no intervention. Because H-ferritin was diluted to the appropriate concentration using aCSF, we combined all data from animals that received infusion of aCSF and those infused with H-ferritin. As shown in Figure 7, infusion with an aCSF-based solution increased caused an average of a 9.2 day delay in disease onset, which was significantly significant, $p < 0.05$. Similarly, comparison of animals that received no surgery to animals that received aCSF-based infusion showed a significant extension of lifespan (Figure 8). The data suggest that aCSF is a trophic solution that delays disease onset and prolongs life in the SOD1$^{G93A}$ mice.

Although our results from aCSF infusion were quite encouraging, enthusiasm was tempered by the possibility that the simple act of diluting toxic factors in the CSF with a non-specific solution could also be beneficial. The concept that a general addition of fluid to the ventricles as a potential therapy to treat neurodegenerative disease has previously been purported (12), but not for ALS. There have been studies demonstrating that CSF from ALS patients causes motor neuron loss in vitro (13, 14) and that CSF obtained from ALS patients contains factors that promote cell death. A recent article notes that CSF flow dynamics is altered in ALS (15). These data suggest a buildup of toxic factors in the CSF in ALS. Moreover, protein content increases in CSF with age (16), most likely due to a decrease in CSF production (17).

Therefore, to demonstrate the specificity of our infusate solution over a simple pH balanced salt solution, we infused cell culture-grade phosphate buffered saline (PBS) in the same manner in which we infused aCSF via an osmotic pump. An additional surgical control was added in which an osmotic pump was implanted but the contents (PBS) exited to the body cavity; this group allowed for verification that the act of implanting a pump did not alter locomotor behavior. As shown in Figure 9, the data indicate that PBS infusion into the brain was of no different from the No Surgery group and was clearly of no benefit.

Given that the only approved drug for ALS increases life-span by just 10%, our aCSF infusion studies may have clinical relevance. Median disease onset in the No surgery group was 108.5 days and the fifty percent survival rate was 125.75 days, with a calculated disease duration of 17.25 days. Given that infusion therapy extended lifespan to 129 days (fifty percent survival), our therapy delayed endpoint by 3.25 days, which correlates to a 19% increase in lifespan after disease onset (3.25 divided by 17.25). In the clinic, average survival post-diagnosis is 4 years. A 19% extension of life due to our infusion therapy would correlate to 277 days – slightly over 9 months.

A provocative outcome of our findings is to suggest that increasing CSF flow in the central nervous system could be a therapy for ALS. This could be accomplished by increasing CSF turnover clinically by infusing trophic solutions, such as our aCSF, through intrathecal pumps into the lumbar region similar to the current use of morphine pumps used clinically for pain. There are data indicating that an increase of CSF production via caffeine, which is a known stimulant of CSF production, shortens survival in the SOD1$^{G93A}$ mouse (18). These data suggest that pharmacological stimulation of CSF production is not likely to be a clinical option and may point to an underlying defect in the choroid plexus in ALS, further strengthening the case for infusion therapy.

The turnover rate of CSF has been one of the challenges associated with using murine models of the disease, as the rates differ drastically between humans and mice. In
humans, total CSF volume is 125-150 ml and turns over 3-4 times a day. In mice, the
total CSF volume is approximately 35 µl and the turnover rate is 18 µl per hour. In two
hours, CSF is completely overturned in the mouse, which is approximately three times
faster than in humans. Although our paradigm utilizes a constant infusion at a rate of 0.25
µl per hour, it is possible that this rate is insufficient to maintain an appropriate level of
ferritin in the CSF given the high turnover rate.

In order to understand why our H-ferritin infusion data were not as effective as
treatment with a chemical iron chelator (4), a number of factors need to be taken into
account, such as mouse strain, method of treatment, and gene copy number. Jeong et al.
(2009) treated SOD1<sup>G37R</sup> mice with a chemical chelator through intraperitoneal injection;
our experiments utilize SOD1<sup>G93A</sup> mice that receive direct infusion of H-ferritin into the
lateral ventricle. The course of disease progression varies greatly among mutant SOD1
mouse strains, with onset in SOD1<sup>G37R</sup> mice occurring at 4-6 months of age with slow
progression over the course of 4-6 weeks (19); Jeong et al. reported survival in vehicle-
treated SOD1<sup>G37R</sup> mice ranging from 45-55 weeks – approximately one year – which is
greatly beyond the life expectancy for a control group that received intraperitoneal
injections of saline. Based on our experience and elsewhere, onset in SOD1<sup>G93A</sup> mice
occurs at 3-4 months of age and progression lasts approximately 3 weeks (7). Gene copy
number can vary from animal to animal within mutant SOD1 mouse strains, particularly
within the SOD1<sup>G93A</sup> mouse line (20). Consequently, gene copy number will need to be
analyzed in our mice and, when paired with treatment and behavioral data, is expected
give insight to evaluate the efficacy of our infusion paradigm.

1c. Biochemical analysis of mouse tissue.
Progress: Immunoblotting was performed on lumbar spinal cord homogenates to determine
if there are changes in proteins of iron management: H-ferritin, L-ferritin, mitochondrial
ferritin and transferrin receptor. Protein markers of apoptosis and stress, such as Caspase-
3 and heme oxygenase-1, were also examined. In terms of groups of animals that were
analyzed, we had tissue from non-symptomatic 70-day-old SOD1<sup>G93A</sup> mice to serve as a
slightly younger control; these tissues were obtained from the animals that died due to
complications from anesthesia. We examined tissue from animals at endpoint that did not
receive surgery, animals that received infusion of CSF, and animals infused with H-
ferritin. As shown by our behavioral data, the concentration of H-ferritin that was infused
did not alter outcome and the two concentrations were collapsed into one group for
biochemical analyses.

We were not able to detect differences in L-ferritin levels among the three
endpoint groups, although there was a significant decrease in L-ferritin levels in the 70-
day-old mice when compared to the CSF-treated group (Figure 10). The relative increase
in L-ferritin levels in mice at endpoint as compared to asymptomatic 70-day-old mice
may be indicative of microgliosis and an inflammatory response due to the disease
progression. Survival was the longest in the aCSF group, which may indicate prolonged
microglial activation as compared to the other groups.

We failed to detect significant changes in H-ferritin, mitochondrial ferritin, or
transferrin receptor (Figures 11-13). The lack of change in these proteins that manage
iron, which are direct indicators of iron status, suggests that infusion therapy with H-
ferritin did not create an iron-deficient profile in the central nervous system, nor was the rate in infusion therapy so great that normal cellular processes were interrupted.

As with the proteins of iron management, we did not detect significant changes in uncleaved caspase-3, nor were we able to detect cleaved caspase-3 in our immunoblots (Figure 14). The lack of change in caspase-3 levels could potentially be an indirect indicator of the apoptotic marker cleaved caspase-3: unchanged amounts of the parent molecule would denote unchanged amounts of the cleaved form. Likewise, we were unable to detect significant changes in the oxidative stress-induced protein heme oxygenase-1 (Figure 15), although there was a strong trend for a decreased level of this protein in the young mice (p = 0.058), but there was no difference between the No Surgery, aCSF, or H-ferritin groups. The lack of protein changes in the lumbar spinal cord among the treated groups is not surprising, given that all animals in the treatment groups had reached end-stage and had undergone significant motor decline, decreased ability to ambulate, and inability to maintain regular feeding behavior.

**Id. Histological analysis of mouse tissue.**

**Progress:** We have performed immunostaining using SMI-32 (Neurofilament H Non-Phosphorylated) antibody, which detects motor neurons in the spinal cord and have counted the number of neurons that survive at endpoint. Counting criteria included a minimum size requirement (an area of 100 µm²) and a clearly identifiable nucleus. The data presented in Figure 16 demonstrate that the number of surviving neurons at endpoint do not differ due to infusion with aCSF or H-ferritin. As mentioned above, animals are sacrificed at behaviorally-defined endpoint. In this way, the consistency in the number of motor neurons at endpoint serves as a validation of our assessment of endpoint in support of the behavioral data.

In order to examine changes in inflammation due to the disease and the possible effect of our infusion intervention on this process, we performed immunohistochemistry to co-localize the microglial marker, IBA-1, with SMI-32. The data presented in Figure 17 demonstrate the thickened processes and enhanced IBA-1 staining, which is consistent with an inflammatory response and phagocytic action, in all groups of mice. Although the number of motor neurons in each of the treatment groups is relatively consistent, the neurons appear to be in various stages of survival, with some neurons large and healthy with cytoplasm that strongly stains for SMI-32, while other neurons are nearly vacuolated, with vestiges of SMI-32-positive cytoplasm still evident (Figure 17, Panel D).

**Task 2. Intracerebroventricular infusion apo-ferritin into HFE\(^{\text{wt/H67D}}\) x SOD1\(^{G93A}\) mice.**

**2a. Purchase SOD1\(^{G93A}\) mice and crossbreed with existing homozygous HFE\(^{H67D/H67D}\) mice to generate experimental animals.**

**Progress** The mice that were purchased in April 2012 for Task 1 were used as breeders to the homozygous HFE\(^{H67D/H67D}\) mice that exist in our laboratory. We elected to use male and female offspring as experimental animals to determine if one gender responds better to H-ferritin infusion than the other. The first group of HFE\(^{H67D}\) x SOD1\(^{G93A}\) double-transgenic mice was born in May 2012.
2b. Allow animals to age to 70 days, at which time surgery to implant cannula and osmotic pump will be performed.

**Progress** We performed surgeries on animals using H-ferritin at a concentration of 2.0 mg/ml. As in Aim 1, surgeries occurred at 70 and 98 days of age.

2c. Perform behavioral analysis of mice during disease phase. Mice that reach the pre-defined endpoint will be sacrificed during this time.

**Progress** Behavioral performance on the rotarod apparatus was assessed in these mice to determine disease onset using the same parameters described in Aim 1. As shown in Figure 18, neither infusion of H-ferritin at 2.0 mg/ml nor infusion with aCSF was effective in delaying disease onset in these double transgenic mice, with median values of 111, 112.5, and 106 days for the No Surgery, aCSF, and H-ferritin groups, respectively. These data were obtained from mice of both sexes, although the data obtained in Aim 1 was from male SOD1\(^{G93A}\) mice only.

To determine if infusion therapy had different effects on disease onset based on the sex of the animal, we performed additional analyses. In female HFE\(^{H67D}\) x SOD1\(^{G93A}\) mice, infusion with aCSF or H-ferritin at 2.0 mg/ml does not delay disease onset, with median onset values of 137, 124, and 120 days for the No Surgery, aCSF, and H-ferritin groups, respectively (Figure 19). These non-significant data would suggest that H-ferritin infusion hastens disease onset in female double transgenic mice.

In Figure 20, disease onset data in male HFE\(^{H67D}\) x SOD1\(^{G93A}\) indicate that infusion with aCSF accelerates onset of the disease (median value of 117.5 days) and that infusion with H-ferritin causes no change in onset as compared to No Surgery (133 day median onset for both groups). Although there is no significant difference between the groups, there is clearly no benefit from infusion therapy to delay disease onset.

The disease onset data presented in Figures 18-20 argue the point that infusion therapy with aCSF or H-ferritin is not likely to be effective in delaying disease onset in HFE\(^{H67D}\) x SOD1\(^{G93A}\) mice (regardless of gender), which is quite different from the data presented in SOD1\(^{G93A}\) mice in Aim 1. It should be noted that the mice in Aim 1 were all male and aCSF infusion was significantly effective in delaying disease onset by 9.5 days (Figure 6). In Figure 20, infusion with aCSF caused a more rapid disease onset by 15.5 days in the male double transgenic mice, although this acceleration in disease onset is not statistically significant.

Infusion with aCSF or H-ferritin 2.0 mg/ml shortened lifespan in HFE\(^{H67D}\) x SOD1\(^{G93A}\) mice (males and females combined), as shown in Figure 21 and in female mice alone (Figure 22). In Figure 23, the data indicate that infusion extends lifespan in male HFE\(^{H67D}\) x SOD1\(^{G93A}\) mice, which holds for maximum survival points and fifty-percent survival rates; this result is reminiscent of the survival data from Aim 1, although the infusion of H-ferritin in the double transgenic mice is more effective than aCSF, which is a difference as compared to the data from Aim 1.

Admittedly, these data are ambiguous as to the possible benefit of infusion therapy in HFE\(^{H67D}\) x SOD1\(^{G93A}\) double transgenic mice. However, the data have demonstrated two key concepts that may be of clinical relevance: differential responses to therapy due to HFE genotype and differential responses to therapy due to gender.

Genetic variations in the \textit{HFE} gene have been associated with the iron-overload disorder hereditary hemochromatosis. In the human population, genetic variations in the
HFE gene, particularly H63D, increase the incidence of ALS (21-24); indeed, the reported prevalence of variations in the HFE gene in ALS is higher than that of SOD1 (25) and to date is the second most abundant gene variation reported in ALS. The stratification of treatment strategies due the presence of one copy of an allelic variant of HFE would be of great importance if infusion therapy were to transition to the clinic; patients with a variant of the HFE gene would not respond well to infusion therapy based on the animal data.

In terms of gender differences, infusion therapy does not delay onset nor does it extend lifespan in females; we would not expect this therapy to be of benefit to women with ALS. In male double transgenic mice, the data are less clear, given that aCSF infusion may accelerate disease onset but extend lifespan as compared to No Surgery mice of the same sex. H-ferritin infusion in these male double transgenic mice does not delay disease onset but appears to extend lifespan as compared to No Surgery animals, which may suggest that the timing of intervention is another facet to treating this disease.

**2d. Biochemical analysis of mouse tissue.**

**Progress** Immunoblot analyses were performed to examine changes in proteins that manage iron as well as proteins related to cellular distress. We detected increases in H-ferritin protein and mitochondrial ferritin protein in the aCSF-infused group as compared to the H-ferritin 2.0 mg/ml-infused group but did not identify changes in L-ferritin, transferrin receptor, caspase-3, or heme oxygenase-1 (Figures 24-29). Infusion of H-ferritin did not cause any significant changes as compared to the No Surgery group for the six proteins examined.

It is known that the HFE protein interacts with the transferrin receptor on the plasma membrane to control intracellular iron uptake (26). The H63D genetic polymorphism of the HFE gene mutation alters the HFE repressor function for transferrin uptake and results in increased cellular uptake of iron (27), so it is possible that the increased cellular uptake in iron present in our HFE\textit{H67D} x SOD1\textit{G93A} mice stimulates ferritin expression as a compensatory response, albeit ineffective. It is uncertain why L-ferritin expression is not significantly increased to match the increase in the other ferritins, although it may be possible that L-ferritin is already expressed at the maximum level in the microglia. Furthermore, increases in cytosolic H-ferritin or mitochondrial ferritin would indicate an increased buffering capacity of neurons to handle iron. Only aCSF infusion results in changes in expression of ferritins with ferroxidase activity (H- and mitochondrial ferritin) while infusion of H-ferritin itself does not change these levels. Perhaps the extracellular ferritin decreases the need for intracellular iron buffering capacity of these cells.

**2e. Histological analysis of mouse tissue.**

**Progress** Motor neuron count was assessed by counting SMI-32-positive neurons in the lumbar spinal cord with the size and clearly identifiable nucleus requirements used previously (Figure 30). There is no difference in the number of motor neurons at endpoint, regardless of infusion. However, as compared to age-matched HFE\textit{H67D} mice (normal SOD1), there is a significant decrease in motor neuron number in HFE\textit{H67D} x SOD1\textit{G93A} mice. HFE\textit{H67D} mice have a normal lifespan and no behavioral abnormalities,
and we would expect that they have a normal number of healthy motor neurons, particularly at ~140 days of age (28).

Co-localization of microglia with motor neurons illustrates the robust microglial response to the disease state (Figure 31, Panels A-C) while HFE$^{H67D}$ mice have much fewer microglia in general and they are in the resting state (Panel D). It is also evident that the motor neurons in the mice with the non-mutated SOD1 are abundant and have numerous neurites, which are elongated and extend throughout the lumbar region; motor neurons that fulfill the counting requirement are fewer in the diseased animals, regardless of infusion, and have fewer neuronal processes.

**Task 3. Intracerebroventricular infusion liposome-encapsulated apo-ferritin into SOD1$^{G93A}$ mice.**

**3a. Generate and characterize liposomes encapsulating apo-ferritin.**

**Progress**

The data to this point suggest two things that need to be taken into consideration regarding the use of H-ferritin infusion as a potential therapy for ALS: the concentration of H-ferritin that is infused and the timing of intervention. The data in Aim 1 demonstrate that doubling the amount of H-ferritin from 2.0 mg/ml to 4.0 mg/ml had no effect, which would suggest that the protein was infused in excess and that a “less is more” approach may be more beneficial. Therefore, we decreased the amount of H-ferritin by ten-fold to 0.2 mg/ml in this aim for liposome encapsulation.

The data from Aim 2 suggest differential benefits of H-ferritin infusion, and may speak to a greater issue regarding the timing of the intervention. To address this issue and to increase the clinical relevance of the timing of our infusion paradigm, we began infusion at 90 days of age in the mice in Aim 3 instead of the 70 day start-time for infusion that was used in Aims 1 and 2. This 90 day timepoint was chosen because is slightly prior to rotarod-defined onset. The use of the rotarod apparatus is the gold-standard in the field and allows for quantitative assessment of behavioral performance (8). There is a shortcoming of this method, however, as it does not evaluate the ease in which the animal can maintain locomotion; we have observed animals that can complete the defined rotarod task successfully, but this occurs with increasing difficulty as animals approach onset (as defined by the conventional falling from the rotarod apparatus). The increased effort by the animal required to successfully complete the rotarod task is perhaps the true onset of disease; this occurs near 90 days of age, which is the approximate midpoint between our neuroprotective 70 day infusion (Aims 1 and 2) and conventionally-defined disease onset.

**3b. Generate and characterize liposomes encapsulating apo-ferritin that are conjugated to lipopolysaccharide.**

**Progress**

Liposomes that encapsulate H-ferritin at a concentration of 0.2 mg/ml were generated. Approximately half of the liposomes were conjugated with lipopolysaccharide (LPS). This created liposomes that can target delivery of H-ferritin to the microglia through interaction with the Toll-Like Receptor 4 (TLR4) on the cell surface that exists on that cell type. The remaining unconjugated liposomes were used as an additional experimental group in which non-targeted liposomes containing ferritin were infused.
3c. Purchase mice and perform surgery to implant cannula and osmotic pump into mice.

**Progress** Mice were purchased in the Spring of 2013 and underwent surgery at 90 days of age. In addition to the two liposome groups, we also added a non-liposome H-ferritin 2.0 mg/ml group and an aCSF group to examine the effects of intervention at 90 days with these same treatments that were used in Aim 1; the difference is the shift in timeline of intervention.

3d. Perform behavioral analysis of mice during disease phase. Mice that reach the pre-defined endpoint will be sacrificed during this time.

**Progress** Behavioral data were collected prior to surgery and mice (with the exception of a few) had not reached conventional rotarod-defined endpoint at the time of surgery. Rotarod assessment continued with the same onset criteria used in Aims 1 and 2, and endpoint determination followed the same criteria.

Infusion beginning at 90 days of age had significantly different effects as compared to 70-day infusion (Figure 32). Infusion with aCSF at 90 days caused a significant acceleration of disease onset as compared to the No Surgery group, and infusion using same the H-ferritin 2.0 mg/ml that was used in Aims 1 and 2, had no effect on onset as compared to the No Surgery group. There was no clear benefit to infusion of 0.2 mg/ml H-ferritin encapsulated by LPS-targeted liposomes; however, infusion of 0.2 mg/ml H-ferritin encapsulated by liposomes that were not directed to a specific cell-type was associated with a 17.3 day delay in disease onset as compared to the aCSF group, which was statistically significant, \( p < 0.01 \). Median onsets for all groups are shown in the table in Figure 32.

The survival data follow a similar pattern as the onset data (Figure 33). Based on fifty-percent survival values, infusion with non-targeted liposomes containing H-ferritin caused a 9.5 day extension of lifespan as compared to No Surgery; this effect is trending strongly towards significance, \( p = 0.06 \). LPS-targeted liposomes also caused an extension in lifespan as compared to the No Surgery groups (fifty-percent survival), but this effect was less robust than untargeted liposomes.

Collectively, these data suggest that timing of infusion and concentration of H-ferritin are critical factors in determining the best strategy to treat the disease. Liposomal delivery of H-ferritin may enhance cellular uptake, as evidenced by delayed disease onset and extended lifespan in the non-targeted liposomes containing H-ferritin group. Specific targeting to microglia through interaction of the LPS-moiety with the Toll-Like Receptor-4 (TLR4) is of no benefit to delay disease onset and is of modest benefit in extending lifespan. It is possible that the use of TLR4 as the method for targeted uptake is ineffective. The data may also suggest that directed delivery of H-ferritin encapsulated by liposomes to other cell-types, such as neurons or astrocytes, should be examined; if neuronal uptake of iron were shown to be effective, it would indicate that this is a neuronally-focused disease, and the glial response is merely in reaction to neuronal distress.

The data obtained from intervention closer to disease onset have demonstrated that our infusion with non-targeted liposomes has a positive effect on the disease process at a time that is most clinically relevant. Obviously, patients would not seek treatment if they are not experiencing loss of movement. That our data demonstrate that we are
successfully attenuating disease progression at the earliest signs of symptoms in the murine model is exciting.

3e. Biochemical analysis of mouse tissue.

**Progress** Immunoblot analyses were performed on lumbar spinal cord homogenates and there were no differences in levels of L-ferritin, mitochondrial ferritin, transferrin receptor, caspase-3, or heme oxygenase-1, although there was a significant change in H-ferritin protein levels in this group of animals that received infusion at 90 days (Figures 34-39). H-ferritin protein levels are increased in the animals infused with “free” H-ferritin protein diluted in aCSF as opposed to either of the liposomal H-ferritin groups. Given that the concentration of the non-liposomal H-ferritin is ten-fold higher than liposomal H-ferritin, the increased H-ferritin levels in the non-liposomal group is not surprising. It may also be important to note that levels of H-ferritin in the lumbar spinal cord in the liposomally-delivered H-ferritin groups are not different than the No Surgery group, which may suggest increased efficiency of H-ferritin delivery and enhanced sequestration of iron.

3f. Histological analysis of mouse tissue.

**Progress** Due to our initial problems with expressing H-ferritin at the beginning of our project, our timeline was delayed. Liposome generation is costly and the best use of animals and funds required that we have data from the first two aims in-hand to better direct our efforts in the third aim. As a result, histological analysis is ongoing.

Task 4. Communication of scientific findings via presentation and/or manuscript. We anticipate 2-3 presentations and/or manuscripts as a result of this research.

**Progress** Data have been presented as a poster at the Pennsylvania Neurosurgical Society Scientific Meeting. Abstracts have been submitted and approved for presentation at two meetings (the Society for Neuroscience Meeting and the International Symposium on ALS/MND) scheduled later this calendar year. At least one manuscript is in preparation, with plans to submit by the end of the calendar year.

**KEY RESEARCH ACCOMPLISHMENTS**

- Infusion of artificial cerebrospinal fluid (aCSF) containing nutrients, including H-ferritin, increases lifespan and delays onset of disease in SOD1<sup>G93A</sup> mice.

- The behavioral improvements due to infusion with aCSF are not achieved by infusion of saline, suggesting that there is more than just a mechanical benefit to increasing flow of CSF.

- In an accelerated disease model of ALS where mice carry both the HFE<sup>H67D</sup> allelic variant and the SOD1<sup>G93A</sup> mutation, infusion of aCSF with or without H-ferritin is not beneficial and may be detrimental in females. The high rate of oxidative stress in these animals may be too great for an infusion strategy.

- Infusion of H-ferritin protein encapsulated in liposomes is even more effective in delaying onset and extending lifespan than H-ferritin protein that is directly infused.
REPORTABLE OUTCOMES

Poster Presentations:

Scheduled Poster Presentations (abstracts submitted and approved, conference upcoming):

Funding applied for based on work supported by award:
The Amyotrophic Lateral Sclerosis Association (ALSA), Multi-Year Award
Letter of Intent was submitted (on 1/11/13) and the ALSA extended an invitation to submit a full proposal (submitted 3/14/13); project was not funded

Department of Defense, Therapeutic Development Award
Letter of Intent submitted (on 6/5/13) but we were not invited to submit a full Proposal
CONCLUSIONS

Our results suggest that intracerebroventricular infusion of artificial CSF with or without H-ferritin into SOD1<sup>G93A</sup> mice may be of therapeutic potential in ALS. Infusion of artificial CSF delays onset of symptomology and increases lifespan in the murine model. A parsimonious explanation for our results is that increased flow of CSF (and subsequent dilution/removal of cytotoxic factors) out of the central nervous system is a component of neuroprotection afforded by infusion in our model. However, the absence of a positive effect on the disease when animals were infused only with saline makes such an interpretation unlikely and suggests the composition of the infusate solution is of critical importance.

Stratification of patients based on genotype and gender are critical in interpreting the results of infusion therapy. The presence of the H67D allelic variant of the <i>HFE</i> gene in the mouse, analogous to H63D in human patients, resulted in an altered response to infusion. Survival was extended in males but was shortened in females. These data underscore the importance of tailoring therapy to fit the unique needs of the patient, giving strong support for the notion of “personalized medicine”.

Timing of infusion and method of H-ferritin delivery at the nanoparticle level are of key significance in our infusion therapy paradigm. Infusion with H-ferritin encapsulated by liposomes at the time of disease onset is associated with an extension of lifespan.

In terms of impact to humans struggling with ALS, surgical implantation of a pump that would deliver a trophic solution into the lumbar spinal space would be similar in approach to that currently used with morphine pumps to treat chronic pain or baclofen for treatment of spastic muscular disease. Although still in its infancy, our study has presented an exciting, unexplored—but easily clinically implemented—method to improve outcome in patients with ALS.
REFERENCES
APPENDICES

List of Personnel Receiving Pay from the Research Effort:

James R. Connor, Ph.D.
Amanda M. Snyder, Ph.D.
A. B. Madhankumar, Ph.D.
Elizabeth B. Neely
Figure 1. Disease onset as assessed by rotarod performance in SOD1<sup>G93A</sup> mice infused with H-ferritin at a concentration of 2.0 mg/ml. Onset was earliest in the No Surgery group as well as first individual animal to fail the rotarod test (data not shown). Infusion with H-ferritin is not toxic to the mice and modestly increases latency to disease onset. Infusion with artificial CSF (aCSF) significantly delays disease onset as compared to the No Surgery group ($p < 0.05$, Kruskal-Wallis test). Median values are displayed by horizontal lines.
**Figure 2**

![The Effect of Infusion with H-ferritin 2.0 mg/ml on Lifespan](image)

Survival is extended in SOD1<sup>G93A</sup> mice that received infusion of 2.0 mg/ml H-ferritin. Fifty percent survival rates are 125.75 days, 131 days, and 129 days in the No Surgery, aCSF, and H-Ferritin 2.0 mg/ml groups, respectively. There is a trend towards significance in overall survival, \( p = 0.09 \).
Figure 3. Disease onset as assessed by rotarod performance in SOD1\textsuperscript{G93A} mice infused with H-ferritin at a concentration of 4.0 mg/ml. Onset was earliest in the No Surgery group as well as first individual animal to fail the rotarod test (data not shown). Infusion with H-ferritin is not toxic to the mice. Infusion with artificial CSF (aCSF) significantly delays disease onset as compared to the No Surgery group (\( p < 0.05 \), Kruskal-Wallis test). Median values are displayed by horizontal lines.
Figure 4. Survival is shortened in SOD1<sup>G93A</sup> mice that received infusion of 4.0 mg/ml H-ferritin. Fifty percent survival rates are 125.75 days, 131 days, and 126 days in the No Surgery, aCSF, and H-Ferritin 4.0 mg/ml groups, respectively. There is a trend towards a significant extension of lifespan with aCSF, $p = 0.09$. 
**Figure 5.** Disease onset as assessed by rotarod performance in SOD1<sup>G93A</sup> mice infused with H-ferritin. Data for H-ferritin infusion contain animals infused with 2.0 mg/ml and those infused with 4.0 mg/ml. Infusion with H-ferritin is not toxic to the mice but appears to do little to delay disease onset. Infusion with artificial aCSF significantly delays disease onset as compared to the No Surgery group (\( p < 0.05 \), Kruskal-Wallis test). Median values are displayed by horizontal lines.
Figure 6. Survival in SOD1<sup>G93A</sup> mice that received infusion of H-ferritin or aCSF. The data shown here include survival data from mice infused with 2.0 and 4.0 mg/ml H-ferritin combined into one group. Fifty percent survival rates are 125.75 days, 131 days, and 128 days in the No Surgery, aCSF, and H-Ferritin groups, respectively. There is a trend towards significance, \( p = 0.11 \).
Figure 7. Infusion of an aCSF-based solution significantly increases survival rate in SOD\textsuperscript{G93A} mice. Data collected from animals infused with CSF, H-ferritin 2.0 mg/ml, and H-ferritin 4.0 mg/ml were combined to generate an overall aCSF Infusion group. Average lifespan in the control (No Surgery) group was 108.8 days while the Infusion group’s average lifespan was 118 days. This was a significant difference, as assessed by t-test ($p < 0.05$).
Figure 8. Survival is extended in SOD1\textsuperscript{G93A} mice that received infusion an artificial CSF-based solution (aCSF). The data shown here include survival data from mice infused with 2.0 and 4.0 mg/ml H-ferritin because aCSF was used to dilute the protein to the appropriate concentration. This result is significant, $p < 0.05$. 
Figure 9

Infusion with Artificial Cerebrospinal Fluid Delays Disease Onset in the SOD1\textsuperscript{G93A} Mouse Model

<table>
<thead>
<tr>
<th></th>
<th>No Surgery</th>
<th>PBS pump to body</th>
<th>PBS Infusion</th>
<th>aCSF Infusion</th>
<th>H-ferritin 2.0 mg/ml</th>
<th>H-ferritin 4.0 mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median</td>
<td>108.5</td>
<td>111</td>
<td>107</td>
<td>118</td>
<td>114</td>
<td>111</td>
</tr>
<tr>
<td>N</td>
<td>14</td>
<td>5</td>
<td>5</td>
<td>9</td>
<td>4</td>
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</table>

Figure 9. Infusion with aCSF delays onset in SOD1\textsuperscript{G93A} mice but infusion with PBS has no effect. Median values are displayed by horizontal lines and shown in the table below the graph. There is a very strong trend towards significance, $p = 0.059$. 
Figure 10. L-ferritin levels in the lumbar spinal cord in SOD1$^{G93A}$ mice. The amount of L-ferritin in the lumbar spinal cord is significantly lower in 70-day-old mice than the CSF-treated mice at endpoint, as assessed by One-way ANOVA with Tukey post-test, $p < 0.01$. Protein values were normalized to β-actin for each animal before each group mean was calculated. Data are represented as mean ± S.E.M. $N = 3-4$ in each group.
Figure 11. H-ferritin levels in the lumbar spinal cord in SOD1\textsuperscript{G93A} mice. There is no significant difference in H-ferritin protein in the lumbar spinal cord in animals that received infusion as compared to age-matched No Surgery mice or younger asymptomatic SOD1\textsuperscript{G93A} 70-day old mice. Protein values were normalized to β-actin for each animal before each group mean was calculated. Data are represented as mean ± S.E.M. \(N = 3-4\) in each group. One-way ANOVA with Tukey post-test, differences not significant.
Figure 12. Mitochondrial ferritin protein levels in the lumbar spinal cord in SOD1\textsuperscript{G93A} mice. There is no significant difference in mitochondrial ferritin protein in the lumbar spinal cord in animals that received infusion as compared to age-matched No Surgery mice or younger asymptomatic SOD1\textsuperscript{G93A} 70-day old mice. Protein values were normalized to β-actin for each animal before each group mean was calculated. Data are represented as mean ± S.E.M. \(N = 3-4\) in each group. One-way ANOVA with Tukey post-test, differences not significant.
Figure 13. Transferrin receptor protein levels in the lumbar spinal cord in SOD1<sup>G93A</sup> mice. There is no significant difference in transferrin receptor protein in the lumbar spinal cord in animals that received infusion as compared to age-matched No Surgery mice or younger asymptomatic SOD1<sup>G93A</sup> 70-day old mice. Protein values were normalized to β-actin for each animal before each group mean was calculated. Data are represented as mean ± S.E.M. N = 3-4 in each group. One-way ANOVA with Tukey post-test, differences not significant.
Figure 14. Caspase-3 protein levels in the lumbar spinal cord in SOD1<sup>G93A</sup> mice. There is no significant difference in caspase-3 protein in the lumbar spinal cord in animals that received infusion as compared to age-matched No Surgery mice or younger asymptomatic SOD1<sup>G93A</sup> 70-day old mice. Protein values were normalized to β-actin for each animal before each group mean was calculated. Data are represented as mean ± S.E.M. N = 3-4 in each group. One-way ANOVA with Tukey post-test, differences not significant.
Figure 15. Heme oxygenase-1 protein levels in the lumbar spinal cord in SOD1$^{G93A}$ mice. There is no significant difference in heme oxygenase-1 protein in the lumbar spinal cord in animals that received infusion as compared to age-matched No Surgery mice or younger asymptomatic SOD1$^{G93A}$ 70-day old mice. However, there is a strong trend ($p = 0.058$) that the level of this stress protein is lower in the 70-day old mice than the mice sacrificed at endpoint who had developed the motor symptoms of ALS. Protein values were normalized to β-actin for each animal before each group mean was calculated. Data are represented as mean ± S.E.M. $N = 3-4$ in each group. One-way ANOVA with Tukey post-test, differences not significant ($p = 0.058$).
Figure 16. SMI-32 cell count in lumbar spinal cord sections in SOD1<sup>G93A</sup> mice infused with H-ferritin. SMI-32-positive motor neurons on the left side of the ventral lumbar spinal cord with an area greater than 100 um<sup>2</sup> were counted. Infusion failed to cause a difference in motor neuron count at endpoint in mice with SOD1<sup>G93A</sup>. Data are represented as means ± S.E.M., analyzed by One-way ANOVA with Tukey post-test.
Figure 17

Figure 17. Colocalized immunostaining of SMI-32 and IBA-1 in the lumbar spinal cord in SOD1<sup>G93A</sup> mice. Immunostaining for SMI-32, a marker of motor neurons, is shown in red, IBA-1, a microglial marker, is shown in green, and the nuclear marker, DAPI, is in blue. Panel A: No Surgery, Panel B: aCSF infusion, Panel C: H-ferritin infusion. The scale bar in Panel B is 50 µm and applies for Panels A-C. The images in panels A-C show enhanced the morphology of activated microglia, which is consistent with an inflammatory status due to the effects of the disease. Motor neurons have extensive staining throughout the cell body (Panels A-C), although they are smaller in the aCSF-infused section (Panel B).

In Panel D, the image shows an increased magnification of a motor neuron that is dying, as demonstrated by decreased SMI-32 positive cytoplasm, a loss of DAPI staining in the nucleus, and infiltrating microglia to the site of damage. The scale bar in Panel D is 25 µm in length.
Figure 18. Disease onset as assessed by rotarod performance in HFE$^{H67D}$ x SOD1$^{G93A}$ mice infused with H-ferritin at a concentration of 2.0 mg/ml. Infusion with either aCSF or H-ferritin was of no benefit to these double transgenic mice. Median values for the No Surgery, aCSF, and H-ferritin groups are 111, 112.3, and 106 days, respectively, and are displayed by horizontal lines. Data from both male and female mice are shown in this figure.
Figure 19. Disease onset as assessed by rotarod performance in female HFE$^{H67D} \times SOD1^{G93A}$ mice infused with H-ferritin at a concentration of 2.0 mg/ml. Infusion with either aCSF or H-ferritin was of no benefit to these double transgenic mice, with the data indicating that H-ferritin infusion causes an acceleration of disease onset, although this was not a statistically significant finding. Median values for the No Surgery, aCSF, and H-ferritin groups are 137, 134, and 120 days, respectively, and are displayed by horizontal lines.
Figure 20. Disease onset as assessed by rotarod performance in male HFE$^{H67D}$ x SOD1$^{G93A}$ mice infused with H-ferritin at a concentration of 2.0 mg/ml. Infusion with either aCSF or H-ferritin was of no benefit to these double transgenic mice; aCSF infusion appeared to accelerate disease onset, although this was not a statistically significant finding. Median values for the No Surgery, aCSF, and H-ferritin groups are 133, 117.5, and 133 days, respectively, and are displayed by horizontal lines.
Figure 21. Survival is shortened in HFE$^{H67D} \times$ SOD1$^{G93A}$ mice that received infusion of 2.0 mg/ml H-ferritin or aCSF. Fifty percent survival rates are 135.5 days, 135.5 days, and 134 days in the No Surgery, aCSF, and H-Ferritin groups, respectively. This effect is not statistically significant, but the data suggest that infusion is limited benefit given that the No Surgery mice had a greater maximum lifespan of over 10 days as compared to infusion.
Figure 22. Survival is shortened in female HFE$^{H67D}$ x SOD1$^{G93A}$ mice that received infusion. Fifty percent survival rates are 139 days, 136 days, and 125 days in the No Surgery, aCSF, and H-Ferritin groups, respectively. The decreased survival in the infused groups is not significant, $p = 0.17$. 
Figure 23. Survival is extended in male $HFE^{H67D} \times SOD1^{G93A}$ mice that received infusion of 2.0 mg/ml H-ferritin or aCSF. Fifty percent survival rates are 133.5 days, 135 days, and 136 days in the No Surgery, aCSF, and H-Ferritin groups, respectively. This effect is not statistically significant.
Figure 24. L-ferritin levels in the lumbar spinal cord in HFE$^{H67D}$ x SOD1$^{G93A}$ mice. There are no significant differences in L-ferritin protein in the lumbar spinal cord due to infusion with aCSF or with H-ferritin 2.0 mg/ml. Protein values were normalized to β-actin for each animal before each group mean was calculated. Data are represented as mean ± S.E.M. $N = 3-4$ in each group. One-way ANOVA with Tukey post-test, differences not significant.
Figure 25. H-ferritin levels in the lumbar spinal cord in HFE$^{H67D} \times SOD1^{G93A}$ mice. There is a significant increase in H-ferritin protein in the lumbar spinal cord in animals that received aCSF infusion as compared to animals infused with H-ferritin. Protein values were normalized to β-actin for each animal before each group mean was calculated. Data are represented as mean ± S.E.M. $N = 3-4$ in each group. One-way ANOVA with Tukey post-test, $p < 0.05$. 
Figure 26. Mitochondrial ferritin protein levels in the lumbar spinal cord in HFE$^{H67D}$ x SOD1$^{G93A}$ mice. There is a significant increase in mitochondrial ferritin protein in the lumbar spinal cord in animals that received aCSF infusion as compared to animals infused with H-ferritin. Protein values were normalized to β-actin for each animal before each group mean was calculated. Data are represented as mean ± S.E.M. $N = 3-4$ in each group. One-way ANOVA with Tukey post-test, $p < 0.05$. 
Figure 27. Transferrin receptor protein levels in lumbar spinal cord in HFE$^{H67D} \times \text{SOD1}^{G93A}$ mice. Levels of transferrin receptor protein in the lumbar spinal cord are unchanged due to infusion. Protein values were normalized to β-actin for each animal before each group mean was calculated. Data are represented as mean ± S.E.M. $N = 3-4$ in each group. One-way ANOVA with Tukey post-test, differences not significant.
Figure 28. Caspase-3 protein levels in the lumbar spinal cord in HFE$^{H67D}$ x SOD1$^{G93A}$ mice. There are no significant differences in uncleaved caspase-3 protein in the lumbar spinal cord. Protein values were normalized to β-actin for each animal before each group mean was calculated. Data are represented as mean ± S.E.M. N = 3-4 in each group. One-way ANOVA with Tukey post-test, differences not significant.
Figure 29. Heme oxygenase-1 protein levels in the lumbar spinal cord in HFE$^{H67D} \times$ SOD1$^{G93A}$ mice are unchanged due to infusion with aCSF or H-ferritin. Protein values were normalized to β-actin for each animal before each group mean was calculated. Data are represented as mean ± S.E.M. $N = 3-4$ in each group. One-way ANOVA with Tukey post-test, differences not significant.
Figure 30. SMI-32 cell count in lumbar spinal cord sections in HFE$^{H67D}$ x SOD1$^{G93A}$ mice infused with 2.0 mg/ml H-ferritin. SMI-32-positive motor neurons on the left side of the ventral lumbar spinal cord with an area greater than 100 $\mu$m$^2$ were counted. Infusion failed to show a difference in motor neuron count at endpoint in mice with SOD1$^{G93A}$ (No surgery, aCSF, and H-ferritin 2.0 mg/ml groups); however, the number of motor neurons in mice that do not carry the SOD1$^{G93A}$ gene but do carry the HFE$^{H67D}$ variant was significantly higher than mice that do have SOD1$^{G93A}$. Data are represented as means ± S.E.M., analyzed by One-way ANOVA with Tukey post-test. * represents $p < 0.05$. 
Figure 31

Figure 31. Colocalized immunostaining of SMI-32 and IBA-1 in the lumbar spinal cord in HFE<sup>H67D</sup> x SOD1<sup>G93A</sup> mice. Immunostaining for SMI-32, a marker of motor neurons, is shown in red, IBA-1, a microglial marker, is shown in green, and the nuclear marker, DAPI, is in blue. Panel A: No Surgery, Panel B: aCSF infusion, Panel C: H-ferritin infusion, Panel D, Healthy control mice of the same age.

The images in panels A-C show enhanced microglial number and the morphology of activation, consistent with an inflammatory status due to the effects of the disease. In contrast, panel D has much less microglial immunostaining, and the morphology is resting, as evidenced by thin projections emanating from the cell body rather than the thickened appearance in diseased animals (A-C).

Motor neurons are large and healthy with extensive staining throughout the cell body and a clearly defined nucleus in the non-diseased section (Panel D). Many of the remaining motor neurons in the diseased animals have small cell bodies (Panels A and B), fragmented neurites (Panel B), or no identifiable neurites (Panel C).
Figure 32. Disease onset as assessed by rotarod performance in SOD1<sup>G93A</sup> mice infused at 90 days with H-ferritin contained in liposomes. Infusion with aCSF at 90 days of age caused a significantly earlier onset of disease as compared to the No Surgery group. Infusion with non-targeted liposomes that contain H-ferritin caused a significant delay in disease onset as compared to the aCSF group. Median values are displayed by horizontal lines on the graph and are represented as days in the table. * denotes $p < 0.05$, ** denotes $p < 0.01$, Kruskal-Wallis test.
Figure 33. Survival is extended in SOD1<sup>G93A</sup> mice that received infusion of liposomes containing H-ferritin at 90 days of age. Fifty percent survival rates are shown in the table below the graph. There is a very strong trend towards a significant benefit of infusion with non-targeted liposomes containing H-ferritin, \( p = 0.06 \).
Figure 34. L-ferritin protein levels in the lumbar spinal cord in SOD1$^{G93A}$ mice that received infusion therapy at 90 days. There are no significant differences in L-ferritin protein in the lumbar spinal cord due to infusion with H-ferritin protein diluted in aCSF, H-ferritin encapsulated by non-targeted liposomes, or by H-ferritin encapsulated by lipopolysaccharide (LPS) for targeted delivery to microglia. Protein values were normalized to β-actin for each animal before each group mean was calculated. Data are represented as mean ± S.E.M. $N = 3-4$ in each group. One-way ANOVA with Tukey post-test, differences not significant.
Figure 35. H-ferritin protein levels in the lumbar spinal cord in SOD1\textsuperscript{G93A} mice that received infusion therapy at 90 days. There is a significant increase in H-ferritin protein in the lumbar spinal cord in animals that received H-ferritin infusion as compared to animals infused liposomes containing H-ferritin, regardless of targeting to microglia with lipopolysaccharide (LPS). Protein values were normalized to β-actin for each animal before each group mean was calculated. Data are represented as mean ± S.E.M. \( N = 3-4 \) in each group. One-way ANOVA with Tukey post-test, \( p < 0.05 \).
Figure 36. Mitochondrial ferritin protein levels in the lumbar spinal cord in SOD1\textsuperscript{G93A} mice were unchanged due to infusion at 90 days. Protein values were normalized to β-actin for each animal before each group mean was calculated. Data are represented as mean ± S.E.M. \textit{N} = 3-4 in each group. One-way ANOVA with Tukey post-test, not significant.
Figure 37. Transferrin receptor protein levels in the lumbar spinal cord in SOD1^{G93A} mice that received infusion therapy at 90 days were not significantly changed. Protein values were normalized to β-actin for each animal before each group mean was calculated. Data are represented as mean ± S.E.M. N = 3-4 in each group. One-way ANOVA with Tukey post-test, differences not significant.
Figure 38. Caspase-3 protein levels in the lumbar spinal cord in SOD1<sup>G93A</sup> mice that received infusion therapy at 90 days. There are no significant differences in uncleaved caspase-3 protein in the lumbar spinal cord. Protein values were normalized to β-actin for each animal before each group mean was calculated. Data are represented as mean ± S.E.M. <i>N</i> = 3-4 in each group. One-way ANOVA with Tukey post-test, differences not significant.
Figure 39. Heme oxygenase-1 protein levels in the lumbar spinal cord in SOD1\textsuperscript{G93A} mice that received infusion therapy at 90 days are unchanged. Protein values were normalized to β-actin for each animal before each group mean was calculated. Data are represented as mean ± S.E.M. \( N = 3-4 \) in each group. One-way ANOVA with Tukey post-test, differences not significant.