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RESEARCH ARTICLE

The cholesterol depleting agent, (2-Hydroxypropyl)-ß-cyclodextrin, does not affect disease progression in SOD1^{G93A} mice

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Abstract

Objective: Previously, we demonstrated that Amyloid Precursor Protein (APP) contributes to pathology in the SOD1^{G93A} mouse model of ALS and that genetic ablation of APP in SOD1^{G93A} mice significantly improved multiple disease parameters, including muscle innervation and motor neuron survival. We also observed elevated levels of potentially neurotoxic Aß peptides that have been implicated in Alzheimer's Disease (AD) pathogenesis, within motor neurons and astrocytes in SOD1^{G93A} mice. More recently, it has been shown that blocking Aß production improves outcome measures in SOD1^{G93A} mice. The cyclodextrin, (2-Hydroxypropyl)-ß-cyclodextrin (HP-β-CD), has previously been shown to deplete intraneuronal unesterified cholesterol, resulting in effective reduction of Aß production and amelioration of disease progression in mouse models of AD and Niemann Pick Type C (NPC) disease. Here, we tested whether HP-β-CD could also improve phenotypic progression in SOD1^{G93A} mice. *Methods:* Pre-symptomatic male SOD1^{G93A} mice were randomly assigned to the following treatment groups: HP-β-CD (4000mg/kg, $n = 9$) or vehicle (saline; $n = 10$), delivered by weekly subcutaneous injection, commencing at 67 days of age. Longitudinal grip-strength and body mass analysis was performed until late-stage disease (120 days of age), followed by *in vivo* bilateral isometric muscle tension analysis of *tibialis anterior* (TA) and *extensor digitorum longus* (EDL) muscles. *Results:* HP-β-CD administration had no effect on body mass or grip-strength compared to vehicle treated SOD1^{G93A} mice. Similarly, HP-β-CD treatment had no effect on muscle force, contractile properties or motor unit number estimates (MUNE) at late-stage disease in SOD1^{G93A} mice. *Conclusion:* This study shows that HP-β-CD does not confer any therapeutic benefit in SOD1^{G93A} mice. However, the absence of detrimental effects is informative, given the common use of cyclodextrins as complexing agents for other pharmaceutical products, their standalone therapeutic potential and the emerging association between dyslipidaemia and ALS progression.

Keywords: *Motor neuron disease (MND), amyotrophic lateral sclerosis (ALS), amyloid precursor protein (APP), dyslipidaemia, cholesterol sequestration, cyclodextrin*

Introduction

The progressive degeneration of upper and lower motor neurons that occurs in amyotrophic lateral sclerosis (ALS), the most common adult-onset motor neuron disease (MND), leads to inexorable blockade of motor signal transmission. In turn, this leads to rapid decline of motor function and, ultimately, complete muscle paralysis that has invariably fatal consequences. The average life expectancy of ALS patients from symptom onset to death, usually as a result of respiratory muscle weakness and associated complications (1), is only 20-48 months (2).

The pathomechanisms involved in motor neuron loss remain are highly complex and include excitotoxicity, RNA transport and splicing defects, neuroinflammation, mitochondrial dysfunction, autophagy dysregulation (3), metabolic disturbances and dyslipidaemia (4). Moreover, the pathomechanisms leading to motor neuron degeneration are known to be non-cell autonomous, with evidence demonstrating a role for astrocytes, microglia and other CNS-resident macrophages, as well as other neuronal subtypes (5) and, potentially, muscle fibers (6).

We previously demonstrated that the Amyloid Precursor Protein (APP) contributes to motor

neuron degeneration in the SOD1^{G93A} mouse model of ALS and that Aß$_{42}$ peptide levels are significantly elevated in the spinal cord and accumulate within motor neurons in SOD1^{G93A} mice (7). Expression of the amyloid precursor-like protein 2 (APLP2) protein has also been shown to contribute to disease progression in female, but not male, SOD1^{G37R} mice (8). Moreover, Aß$_{42}$ peptides have been reported to accumulate within spinal motor neurons in ALS patients (9) and a more recent study demonstrated that a β-secretase targeting monoclonal antibody, which prevents amyloidogenic processing of APP, ameliorates aspects of disease progression in SOD1^{G93A} mice (10). Given the accepted role of Aß$_{42}$ peptides in the neurodegeneration that occurs in Alzheimer's disease (AD), these findings raise an interesting link between AD and ALS pathology and could enable repurposing of therapeutics.

There is a well-established link between lipid processing and AD pathology, which could also have implications for ALS pathology. Amyloidogenic processing of APP is known to be increased as a result of elevated cholesterol content in the cell membrane (11). Importantly, the Apolipoprotein E variant, APOEε4, which has lower binding affinity for unesterified cholesterol, is associated with hypercholesterolemia and reduced cholesterol clearance from the brain; the APOE4 isoform is also the most common risk factor for developing late-onset AD (12). Moreover, knockdown of the Cyp46a1 gene, which encodes cholesterol 24-hydroxylase that is responsible for conversion of cholesterol to 24S-hydroxcholesterol (24S-HC), in the hippocampus of wild-type mice has also been shown, experimentally, to cause intracellular cholesterol accumulation that triggers hallmarks of AD pathology, including elevated Aß$_{42}$ peptide production and neuronal apoptosis (13). Furthermore, a related neurological disorder, Niemann Pick Type-C disease (NPC), is known to be caused by null mutations in the NPC-1 gene, which encodes a glycoprotein that is involved in intracellular trafficking and clearance of cholesterol (14). NPC is characterized by excessive lysosomal accumulation of low-density lipoprotein (LDL) cholesterol and also results in AD-like pathology, including neurofibrillary tangle formation and elevated Aß$_{42}$ peptide levels (15). Moreover, overexpression of human APP containing an Alzheimer's disease (AD) associated mutation, dramatically accelerates neurodegeneration and decreases survival in Npc1-/- mice (16).

Importantly, sequestration of unesterified cholesterol using (2-Hydroxypropyl)-ß-cyclodextrin (hereafter, HP-β-CD) has been shown to dramatically reduce cellular Aß$_{42}$ peptide production and ameliorates disease progression in both the Tg19959 mouse model of AD (17) and the Npc1$^{-/-}$ mouse model of NPC (16,18,19).

Strikingly, hypercholesterolemia, including elevated total, high-density lipoprotein (HDL) and LDL cholesterol levels and lower intermediate density lipoprotein-B (IDL-B) levels, has been observed at diagnosis in 76% of ALS patients (20). Moreover, evidence of nonalcoholic fatty liver disease (NAFLD), associated with chronic hyperlipidemia, has been reported in up to 77% of a small cohort of 30 patients with disorders affecting upper and lower motor neurons, including hereditary spastic paraplegia (HSP), as well as sporadic and familial cases of ALS (21). Moreover, dyslipidaemia and accompanying liver steatosis was also observed in 77% of ALS patients in a larger study (4); this study by Dupuis and colleagues, also reported an association between low LDL:HDL ratio as being negatively correlated with survival in ALS. Furthermore, total levels (*i.e.* relatively inert esterified form and the more biologically active unesterified form) of two cholesterol metabolites, 24S-HC and 25-hydroxycholesterol (25-HC), have been shown to be elevated in plasma from AD patients (22) and in CSF from ALS patients (23), respectively, and are associated with disease severity. However, Abdel-Khalik and colleagues showed that although total levels of unesterified cholesterol are significantly elevated in CSF from ALS patients, there is actually a decrease in 24S-HC, once levels are normalized to total unesterified cholesterol (24); this is significant because 24S-HC is not only the major metabolite responsible for clearance of excess cholesterol from the CNS, but it also acts as a ligand for the Liver-X Receptor-β (LXR-β), knockout of which leads to adult onset motor neuron degeneration in mice (25), as well as inhibiting sterol regulatory element binding proteins, which are master regulators of cholesterol biosynthesis (26). Altered cholesterol homeostasis has also been implicated in Huntington's disease (HD) progression (27), whilst knockdown of the Cyp46a1 gene also induces striatal neuron loss, motor deficits and HD-like pathology (28).

Given our previous findings of a contribution of APP and elevated levels of Aß$_{42}$ peptides in SOD1^{G93A} mice and evidence that dysregulation of cholesterol metabolism may contribute to a broad range of neurodegenerative diseases, we hypothesized that cholesterol depletion, using HP-β-CD, may exert a therapeutic effect in this mouse model of ALS.

Methods

Animal breeding and maintenance

All procedures and experiments involving animals were carried out under License from the UK Home Office in accordance with the Animals (Scientific Procedures) Act 1986 (Amended Regulations 2012), following ethical approval from

the UCL Queen Square Institute of Neurology Animal Welfare Ethical Review Body (AWERB). A total of 19 male BL6SJL.SOD1^{G93A} mice were purpose bred for this study by crossing SOD1^{G93A} transgenic males with BL6xSJL F1female mice (produced by crossing C57BL6/J females with SJL males). SOD1^{G93A} transgenic males were maintained under identical conditions, with 12hr light/-dark cycle, ad libitum access to standard rodent chow and water, with environmental enrichment. Only male mice were included in this study, in order to reduce the total number of mice required, in accordance with NC3R guidelines.

Cyclodextrin treatment

At 60 days of age, SOD1^{G93A} mice were randomly assigned to either vehicle ($n = 10$) or HP-β-CD ($n = 9$) treatment groups. Vehicle (saline) or HP-β-CD (Sigma H5784), 4000mg/kg, (diluted to appropriate dose in saline) was administered in a blinded manner by subcutaneous injection at the scruff of the neck as previously described (16,29), once every 7 days, commencing at 67 days of age.

Behavioral analysis

Longitudinal grip-strength testing was performed from 60 days of age, to establish a baseline prior to initiation of HP-β-CD or vehicle treatment, as previously described (7). Briefly, mice were placed on a horizontal grid attached to a strain gauge (BioSeb) and once all 4 paws were engaged, gentle but firm pressure was applied to the base of the tail to provide resistance against the escape response. A total of 4 recordings were obtained for each timepoint and average values were used for data analysis. Longitudinal body mass data was collected at the same time as grip-strength analysis.

Physiological analysis of muscle force and motor unit survival

Isometric muscle tension physiology data was acquired from the tibialis anterior (TA) and extensor digitorum longus (EDL) muscles at 120d of age and was performed as previously described (7). Data was acquired bilaterally from individual hindlimb muscles from each animal in parallel, using independent stimulators and force-transducers, hence, each muscle was considered an independent data point. Motor unit number estimates (MUNE) were acquired from the EDL muscle. Vehicle treated group, $n = 16$ muscles versus HP-β-CD treated group, $n = 12$ muscles.

Statistical analysis

All data are presented as mean ± SEM unless otherwise indicated. GraphPad Prism 9 (Prism) was used for statistical analyses. No out-liners or data points were eliminated. Differences between two groups were assessed using multiple two-tailed unpaired t tests, with Bonferroni post hoc correction, as stated in the figure legends. Significance was defined as $^*P < 0.05$, $^{**}P < 0.01$.

Results

HP-β-CD does not affect phenotypic changes in grip-strength and body mass decline in SOD1^{G93A} mice

Weekly subcutaneous administration of HP-β-CD between 67-120 days of age did not significantly affect the characteristic progressive decline of grip-strength, in vehicle treated versus HP-β-CD treated SOD1^{G93A} mice (Figure 1(a)). Consistent with our previous study in SOD1^{G93A} mice on the same genetic background (7), within-group analysis of grip-strength decline from peak values reached significance at 95 days ($p = 0.0045$) and 102 days ($p = 0.0055$) in HP-β-CD and vehicle treated groups, respectively. Although this suggests that HP-β-CD may modestly accelerate grip-strength decline, no statistically significant difference was observed between groups at any timepoint.

Similarly, analysis of longitudinal body mass data indicated that HP-β-CD treatment did not significantly modify the progressive loss of body mass that occurs in SOD1^{G93A} mice, compared to vehicle treated controls (Figure 1(b)). Both HP-β-CD and vehicle treated groups exhibited a significant loss of body mass, from peak values, at 120d ($p = 0.025$ and 0.018, respectively). However, HP-β-CD reached peak body mass slightly later, at 102 days, compared to 95 days for vehicle treated SOD1^{G93A} mice, indicating a subtle delay in this disease parameter, although once again, no statistically significant difference was observed between groups at any timepoint.

Previously, in our APP knockout study in SOD1^{G93A} mice (7), we showed that normalization of grip-strength (GS) values as a ratio of body mass (BM) provided a clearer readout of disease modifying effects of intervention. However, in the present study, we did not observe any significant improvement in GS:BM ratio between HP-β-CD treated and vehicle treated SOD1^{G93A} mice at any timepoint examined (Figure 1(c)). Indeed, within-group analysis indicated that the GS:BM values for HP-β-CD treated mice declined slightly faster from peak values, occurring at 95 days ($p = 0.0041$), compared to 102 days ($p = 0.0053$) for vehicle treated SOD1^{G93A} mice.

HP-β-CD treatment does not affect muscle strength in late-stage SOD1^{G93A} mice

Next, we assessed whether weekly HP-β-CD treatment exerts any therapeutic effect on muscle strength and contractile characteristics, using *in vivo* physiological analysis of isometric muscle
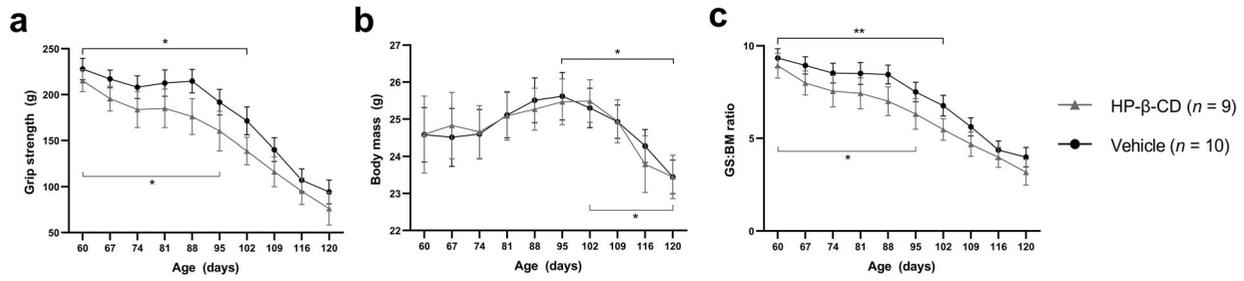
Figure 1. Analysis of the effect of HP-β-CD on disease progression in SOD1^G93A mice. Longitudinal analysis of (a) grip-strength, (b) body mass and (c) grip-strength:body mass ratio (GS:BM) in SOD1^G93A mice treated weekly with either HP-β-CD or vehicle, commencing at 67 days of age. Data is shown as mean ± SEM; comparisons between groups (no significance difference was detected) and within groups, to determine significant decline from peak values for each parameter, was assessed by multiple t-tests with Bonferroni *post hoc* correction; * denotes p = <0.05, ** denotes p = <0.005.

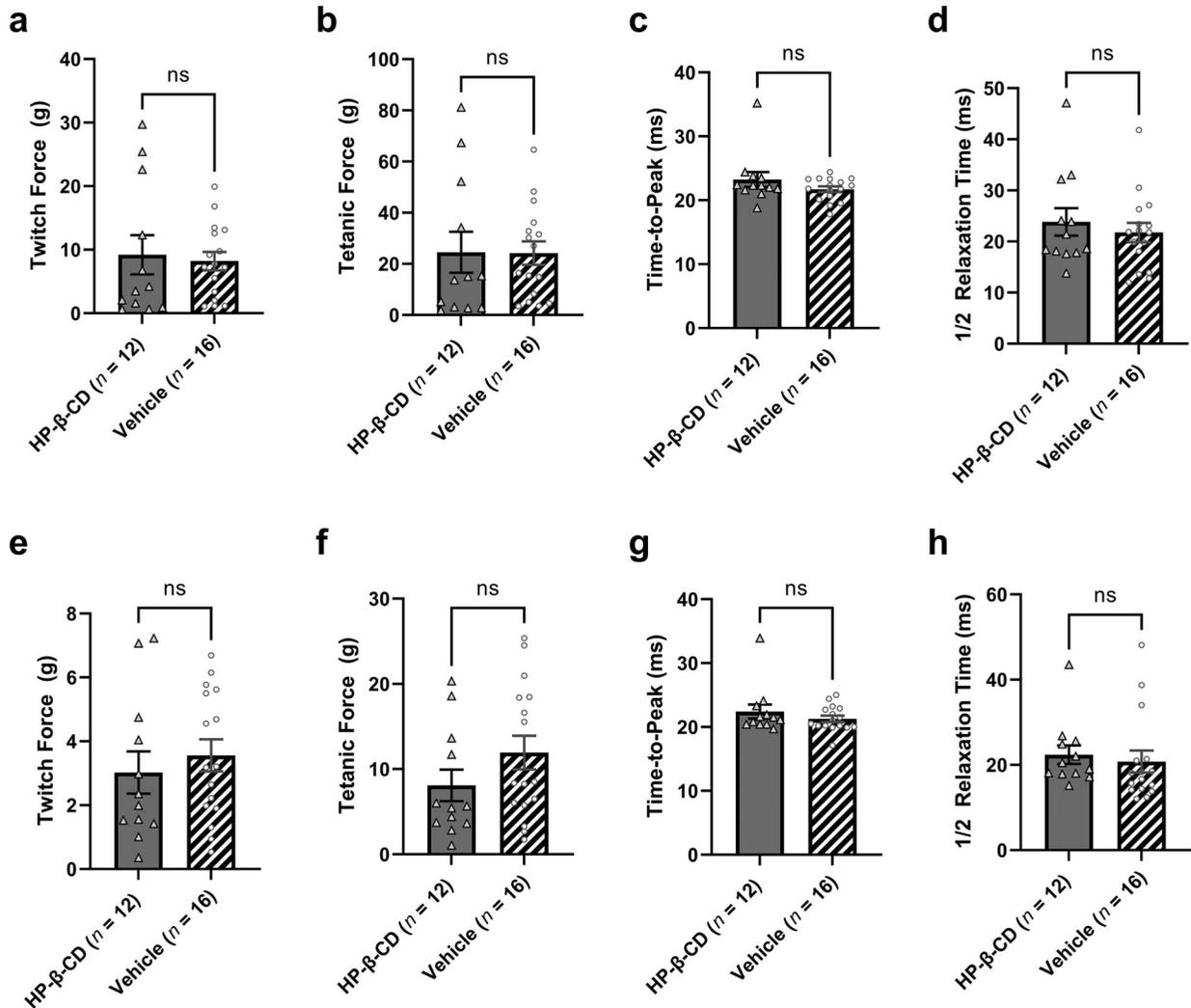

Figure 2. *In vivo* isometric muscle tension analysis of the effect of HP-β-CD on muscle properties in late-stage SOD1^G93A mice. Bilateral physiological recordings were obtained from the *tibialis anterior* (TA) muscles (a-d) and *extensor digitorum longus* (EDL) muscles from SOD1^G93A mice, following weekly treatment with either HP-β-CD or vehicle, commencing at 67 days of age, at the experimental endpoint (120 days). Parameters include maximal contractile force in response to twitch (a and e) and tetanic stimuli (b and f), as well as rate characteristics for muscles to achieve peak force (Time-to-Peak; c and g) and to relax to 50% of maximum values (1/2 Relaxation Time; d and h), following a single twitch stimuli. Data is shown as mean ± SEM; comparisons between groups were assessed by multiple t-tests with Bonferroni *post hoc* correction; "*ns*" denotes not significant.

tension recordings of the *tibialis anterior* (TA; Figure 2(a-d)) and *extensor digitorum longus* (EDL; Figure 2(e-h)) muscles, at 120 days of age. No significant difference was observed between maximal contractile force in response to either supramaximal twitch (Figure 2(a,e)) or tetanic (Figure 2(b,f)) stimuli, in either the TA or EDL muscle, as a result of HP-β-CD treatment, compared to
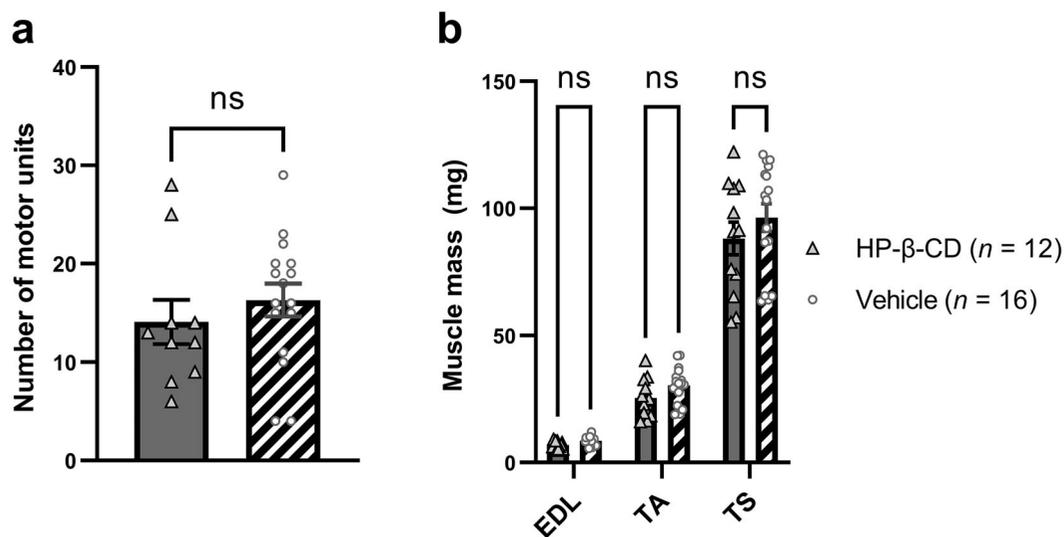
Figure 3. Motor unit number estimates and muscle mass data from late-stage SOD1^{G93A} mice following HP-β-CD or vehicle treatment. Following weekly treatment with either HP-β-CD or vehicle, (a) motor unit number estimates were obtained from the *extensor digitorum longus* (EDL) muscle using isometric muscle tension analysis and (b) total wet muscle mass was recorded from the EDL, *tibialis anterior* (TA) and triceps surae (TS) muscles from 120 day SOD1^{G93A} mice. Data is shown as mean ± SEM; comparisons between groups were assessed by multiple *t*-tests with Bonferroni *post hoc* correction; "*ns*" denotes not significant.

vehicle treated SOD1^{G93A} mice. Similarly, the time taken for the TA and EDL muscles to reach peak force (Time-to-Peak; Figure 2(c,g)) and to relax to half of peak values (1/2 Relaxation Time; Figure 2(d,h)), following a single twitch stimuli, were not significantly altered as a result of weekly HP-β-CD treatment. The contractile rate data indicates that there was no therapeutic effect on phenotypic conversion of these normally fast-twitch muscles to a slower-twitch phenotype that occurs in late-stage SOD1^{G93A} mice, as the preferentially vulnerable fast-firing motor neurons are lost early in the course of disease progression and the muscles become progressively denervated.

HP-β-CD treatment does not affect muscle innervation or prevent muscle atrophy in late-stage SOD1^{G93A} mice

In order to specifically assess the effect of weekly HP-β-CD treatment on muscle innervation, we investigated the number of functional motor units within the EDL muscle of 120d SOD1^{G39A} mice, using isometric muscle tension physiology. Motor unit number estimates (MUNE) were not significantly different between HP-β-CD treated and vehicle treated SOD1^{G93A} mice (14.1 ± 2.24, versus 16.3 ± 1.66; *p* = 0.43), demonstrating that HP-β-CD treatment had no beneficial effect on innervation of the EDL muscle (Figure 3(a)). Moreover, analysis of the EDL, TA and triceps surae (TS) muscle mass at 120 days also indicated that HP-β-CD treatment had no therapeutic effect on prevention of muscle atrophy (Figure 3(b)).

Discussion

The primary objective of this study was to assess the disease modifying effects of weekly HP-β-CD treatment, based on its established ability to ameliorate neuropathology due to APP and Aβ_{42} in other neurological disease models. Indeed, elevated cellular cholesterol has been widely shown to enhance Aβ_{42} production and induce AD-like pathology in mice. Additionally, given the extensive evidence of a contributory role of altered neuronal clearance of cholesterol in a wide range of neurodegenerative diseases, including AD, HD and ALS, we hypothesized that the ability of HP-β-CD to sequester cellular cholesterol could potentially exert additional therapeutic effects in the SOD1^{G93A} mouse model of ALS, independent of APP processing. The ability of HP-β-CD to sequester cholesterol is well established (16–18,30). The findings of this study clearly demonstrate that HP-β-CD treatment does not positively modify disease progression in SOD1^{G93A} mice, at least at the dose (4000mg/kg) and subcutaneous administration route, a treatment regime that has previously been shown to be effective at preventing intraneuronal accumulation of unesterified cholesterol in the CNS of NPC mouse models. Indeed, the dosing regimen employed in this study, commencing around the time of pathological onset until late-stage disease, mirrored the highly-effective therapeutic dosing regimen used by Davidson and colleagues (19). This suggests that intraneuronal accumulation of unesterified cholesterol, *per se*, may not contribute to disease progression in SOD1^{G93A} mice. Importantly, however, HP-β-CD treatment did not appear to detrimentally affect disease progression, a finding which has

implications for other therapeutic strategies for ALS.

In contrast to the hypothesis explored in this study, that reducing intracellular cholesterol may be beneficial in SOD1^{G93A} mice, it has previously been shown that a high fat diet can exert beneficial effects in SOD1^{G93A} mice (31) and that hyperlipidemia has been shown to be associated with longer survival in ALS patients (4). Moreover, Simvastatin, which inhibits the key enzyme involved in cellular cholesterol production, has been shown to accelerate motor neuron degeneration in SOD1^{G93A} mice (32). Simvastatin increases arachidonic acid production and prostaglandin I2 production in rat liver cells (33), both of which have been associated with disease progression in SOD1^{G93A} mice and restoration of a normal lipid profile, using caffeic acid treatment, improves multiple disease parameters in ALS models (34). Although our results indicate that intracellular cholesterol depletion, using HP-β-CD, does not alter disease progression in SOD1^{G93A} mice, this finding may still be of clinical importance as cyclodextrins are commonly used as an adjuvant or excipient for other therapeutic agents, to aid solubility. Therefore, the results of this study indicate that the use of cyclodextrins is likely to be safe for use in other combination therapies for ALS, although interaction between cyclodextrins and other therapeutic agents would obviously have to undergo rigorous safety testing before clinical implementation.

Limitations of study

Given that there is a strong body of evidence that HP-β-CD is capable of effectively reducing accumulation of free cholesterol in neurons from the NPC field, in particular the study by Davidson *et al.*, (19) which employed a similar dosing regimen to this study, we did not undertake a dose-response investigation. Similarly, we did not assess changes in APP expression or levels of Aβ_{42} peptides as a result of HP-β-CD treatment, as the therapeutic dose of HP-β-CD that results in decreased expression of mutant APP had already been established by a previous study (17).

HP-β-CD has been shown to effectively prevent intraneuronal accumulation of unesterified cholesterol in male and female $Npc1^{-/-}$ and $Npc2^{-/-}$ mice, resulting in significant therapeutic amelioration of disease phenotype (19), it is possible that sex-specific differences, downstream of intracellular cholesterol depletion, may be differentially involved in motor neuron degeneration in this mouse model of ALS. Indeed, a recent study (currently available as a non-peer reviewed preprint) demonstrated that increased activity levels of Cyp46a1, the enzyme responsible for 24S-HC production, has beneficial effects in a female mouse model of AD but not in male mice (35). Nonetheless, given the complete absence of positive effect on disease progression in the cohort of male included in this study, we did not feel it was justified to repeat the study with an additional cohort of female mice.

Author contributions

All experimental procedures, data collection, analysis and manuscript preparation were performed by JBB. JBB and LG were responsible for conception and design of the study and finalized the manuscript.

Declaration of interests

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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