Sveriges lantbruksuniversitet
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Varför ökar inte trehalos autofagi hos musmodellen SOD1^{G93A} av familjär amyotrofisk lateralskleros?

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SUMMARY

Amyotrophic lateral sclerosis is one of the major neurodegenerative diseases, causing an ascending paralysis that usually kills the patient within a few years from disease onset. The motor neurons show aggregates of proteins which in approximately 20 % of cases of the familial form contain mutated SOD1 protein.

Trehalose is a disaccharide which has been shown to reduce protein aggregation and increase viability in cell models and alleviate symptoms in animal models of several neurodegenerative diseases associated with protein aggregation. When given orally to the $SOD1^{G93A}$ mouse model of ALS, trehalose failed to slow down the disease progression, which has led to questions about the uptake and distribution of the molecule in this mouse strain.

The aim of this study was to investigate whether significant levels of trehalose reach the central nervous system of the $SOD1^{G93A}$ mouse after oral administration. This was performed by a trehalose assay of the brain of trehalose treated animals. A glucose assay was optimised for use in small samples of brain lysate after the digestion of trehalose into glucose by trehalase, and the difference in glucose concentration before and after digestion represented the trehalose level. No significant differences were detected between digested and undigested samples in trehalose treated mice (46.58±10.39 µg/g wet tissue and 41.86±7.93 µg/g, respectively) or in control mice (60.94±11.39 µg/g wet tissue and 71.58±10.25 µg/g, respectively).

Additionally, the possible effects of trehalose were assessed by histological evaluation of spinal cord sections stained with an antibody directed towards misfolded SOD1 protein. A semi-quantitative method was used to evaluate the number of neurons in different categories of staining and significant differences were only found in the extracellular staining of female mouse spinal cord, where trehalose treated animals showed more intense extracellular staining ($p=0.031$). Since the study used small groups of animals ($n = 4$-5 mice), it is possible that the significant result is a false positive, which would support the overall conclusion that trehalose does not reach the central nervous system of the $SOD1^{G93A}$ mouse in significant amounts.

SAMMANFATTNING

Amyotrofisk lateralskleros (ALS) är en av de vanligaste neurodegenerativa sjukdomarna hos människor och påverkar övre och nedre motorneuron. Det är en sent insättande progressiv sjukom som oftast yttrar sig initialt genom asymmetrisk muskelsvaghet i ben eller armar eller sluddrande tal och utvecklas till en successivt mer utbredd förlamning. Andningsdepression är den vanligaste dödsorsaken hos ALS-patienter och sker oftast inom 2-5 år efter symptomdebut.

Fem till tio procent av ALS-patienter har en släkting med sjukdomen, varför begreppet familjär ALS (fALS) har myntats för denna grupp. Hos 20 % av fALS-patienter är SOD1-genen muterad, men funktionen av detta är oklar. Dock har man sett att modifierade SOD1-protein bildar intracellulära plack, vilka tycks påverka sjukdomsutvecklingen. Transgena musmodeller av ALS har utvecklats genom att inkorporera en muterad variant av SOD1-genen.

Syftet med den redovisade studien var att undersöka huruvida oralt intagen trehalos når centrala nervsystemet hos musmodellen $SOD1^{G93A}$. Bakgrunden till detta är att flera studier av trehalos visat lovande resultat i form av minskad proteinaggregering och ökad överlevnad hos cell- och djurmodeller av flera neurodegenerativa sjukdomar. En tidigare studie utförd på SOD1-möss visade dock inga skillnader på symptomutveckling eller överlevnad vid oralt intag av trehalos. Detta har väckt frågor angående upptag och distribution av trehalos hos dessa möss.

Vår frågeställning var huruvida trehalos tas upp från tarmen till centrala nervsystemet, vilket undersöktes genom en mätning av trehaloskoncentrationen i hjärnvävnad från oralt trehalosbehandlade möss. En kommersiell mätmetod för glukoskoncentration optimerades för analys av små volymer av hjärnlysat före och efter digerering av trehalos till glukos. Skillnaden i glukoskoncentration före och efter digerering representerade trehalos-koncentrationen. Inga signifikanta skillnader noterades mellan digererade och odigererade prover hos trehalosbehandlade möss (46,58±10,39 respektive 41,86±7,93 μg/g vävnad) eller kontrolldjur (60,94±11,39 respektive 71,58±10,25 μg/g vävnad).

Dessutom studerades eventuella effekter av trehalos genom en immunohistologisk utvärdering av ryggmärgssnitt infärgade med en antikropp riktad mot muterat SOD1-protein. En semikvantitativ metod användes för att utvärdera antalet neuron i olika kategorier av färgning. Signifikanta skillnader mellan trehalosbehandlade och kontrolldjur observerades endast i kategorin extracellulär färgning hos honor, där trehalosbehandlade honors ryggmärg färgades mer intensivt extracellulärt ($p=0,031$). Då studien var av liten storlek ($n = 4$-5 möss) är det möjligt att detta enda signifikanta resultat är falskt positivt, vilket skulle stämma överens med den övergripande slutsatsen som indikerar att trehalos vid oral administration inte når CNS hos $SOD1^{G93A}$-möss i signifikanta mängder.

CONTENT

ABBREVIATIONS

ALS = amyotrophic lateral sclerosis

CNS = central nervous system

fALS = familial amyotrophic lateral sclerosis

FUS/TLS = fused in sarcoma/translocated in liposarcoma

LC3 = microtubule-associated protein 1 light chain 3

OPMD = oculopharyngeal muscular dystrophy

PCR = polymerase chain reaction

sALS = sporadic amyotrophic lateral sclerosis

SOD1 = Cu/Zn superoxide dismutase 1

TDP-43 = TAR DNA-binding protein of 43 kDa

VAPB = vesicle-associated membrane protein B

INTRODUCTION AND LITERATURE REVIEW

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease selectively affecting upper and lower motor neurons. The degeneration of lower motor neurons seems to be ascending from the neuromuscular junctions or axons, ending with the disintegration of the entire neuron. It is a late-onset disease that most often causes asymmetric muscle weakness of the limbs (cervical or lumbar onset) or slurring of speech (bulbar onset) as a first symptom. The progression continues to paralysis and death, usually from respiratory failure, with a median age of survival of three to five years for patients with cervical or lumbar onset and two to three years after bulbar onset (Wijesekera & Leigh. 2009). ALS occurs worldwide and has a European incidence of about 2 per 100 000 person-years, which makes it one of the major neurodegenerative diseases (Logroscino *et al.*, 2010).

There have been many theories as to what causes ALS, including heavy metal exposure and viral infections, but while no external factors have been convincingly attributed to the pathogenesis, 5-10% of patients have a familial history of ALS (Rowland & Shneider, 2001). Since the majority of ALS patients do not have any obvious genetic link to another ALS patient, there is today a subdivision into familial ALS (fALS) and sporadic ALS (sALS). The familial form is predominantly inherited in an autosomal dominant fashion.

SOD1

The SOD1 gene was the first to be linked to familial ALS, and it is estimated that up to 20 % of fALS cases show mutations in this gene (Rosen *et al.*, 1993). It encodes the protein Cu/Zn superoxide dismutase 1, a 32 kDa enzyme that has a number of functions in the cell. Probably most importantly, it functions as an antioxidant by catalysing the dismutation of superoxide into hydrogen peroxide or molecular oxygen.

The SOD1 gene has been a major subject of ALS research for over 10 years, with primarily autosomal dominant inheritance and more than 150 mutations (mainly missense, but also deletions and insertions) causing ALS-like pathology (Siddique & Ajroud-Driss, 2011). Different mutations of the SOD1 gene seem to cause different disease progression and phenotypes (e.g. rapid progression caused by the A4V mutation). Since the discovery of mutated SOD1, several other genes have been implicated in familial ALS. These include VAPB (vesicle-associated membrane protein B), TDP-43 (TAR DNA-binding protein of 43 kDa), FUS/TLS (fused in sarcoma/translocated in liposarcoma) and C9orf72, the last of which carries a mutation in about one third of European familial ALS cases (Nishimura *et al.*, 2004; Sreeharan *et al.*, 2008; Vance *et al.*, 2009; Renton *et al.*, 2011; DeJesus-Hernandez *et al.*, 2011).

Despite the correlation between mutant SOD1 protein and ALS pathology, it is still unknown how the mutations cause symptoms. The leading theories involve connections between the aggregating properties of the mutated protein to its pathology, although it is still unknown how protein aggregation in itself leads to neurodegeneration. To study the effects of the

SOD1 mutations, at least 15 mouse models of ALS that express a mutated version of the human SOD1 gene have been produced (Turner & Talbot, 2008). Mouse models overexpressing wild-type human SOD1 and SOD1 knock-out mice also show neurodegenerative signs, but do not develop ALS pathology, as opposed to the SOD1 mutants (Turner & Talbot, 2008).

The SOD1^{G93A} mouse model

In ALS-related research the most commonly used model is the SOD1^{G93A} mouse strain, in which the glycine residue at position 93 of the SOD1 protein has been substituted by alanine (Gurney *et al.*, 1994). Recombination has divided the SOD1^{G93A} mouse strain into several sub-strains, with different copy numbers of the mutant transgene. The number of copies affects age of onset, disease progression and lifespan, with a more aggressive progression associated with a high copy number (Turner & Talbot, 2008). The mouse most frequently used has a high copy number (25 predicted copies) and is called B6SJL-TgN(SOD1-G93A)1Gur, but will be referred to as SOD1^{G93A} from this point. It expresses the mutant SOD1 protein in a stable and abundant manner, and the protein has dismutase activity.

The SOD1^{G93A} mouse model starts showing signs of hind limb weakness (e.g. hypoextension of the hind limbs when the mice are lifted by the base of the tail) at three to four months of age (Gurney *et al.*, 1994). After four months of age the stride length decreases and paralysis develops (Dal Canto & Gurney, 1995). A tremor of the hind limbs when suspended in the air develops, and the hind limbs and toes may lock in a hyperextended position when walking (Gurney *et al.*, 1994). The mice are generally dying or reach the commonly used endpoint, defined as not being able to right themselves within 20-30 seconds after being placed on one side, within five months of age (Dal Canto & Gurney, 1995; Kaneb, 2012).

Progressive histological changes have been observed, starting with vacuolar degeneration of the neurons in the ventral horns (from about 70 days of age to 135 days) to atrophy of the ventral horn (after 120 days), where a severe loss of motor neurons is observed, along with filamentous inclusions in the surviving neurons (Dal Canto & Gurney, 1994; Gurney *et al.*, 1994). At this stage, only a small number of the remaining neurons show signs of vacuolar degeneration.

Degeneration of neuromuscular junctions has been reported to start as early as 25 days of age (Gould *et al.*, 2006) and a decrease in the number of spinal motor neurons by 100 days of age (Fischer *et al.*, 2004).

Trehalose

Trehalose is a disaccharide which occurs in fungi, plants and invertebrates. It has stabilising properties and protects cells from heat, freezing and desiccation. Upon ingestion by mice and humans it is believed to be rapidly broken down by the enzyme trehalase which is localised to the small intestine and kidneys of most mammals (Kamiya *et al.*, 2003).

In several experiments, trehalose has been shown to have positive effects on models of prion disease, Parkinson's and Huntington's diseases, and oculopharyngeal muscular dystrophy (OPMD), on a molecular and cellular level (Béranger *et al.*, 2008; Rodríguez-Navarro *et al.*, 2010; Tanaka *et al.*, 2004; Davies *et al.*, 2006). It has been shown to reduce the aggregate formation associated with these conditions and increase cell viability (Béranger *et al.*, 2008; Tanaka *et al.*, 2004; Davies *et al.*, 2006) and it is believed to have these effects by preventing the formation of, rather than dissolving the already formed aggregates (Tanaka *et al.*, 2004; Béranger *et al.*, 2008). Since the formation of SOD1-containing aggregates has been shown in correlation with disease progression in ALS, these studies suggest that trehalose could be effective in alleviating ALS symptoms (Kaneb, 2012).

Tanaka *et al.* (2004) have studied the effects of trehalose on molecular, cellular and animal models of Huntington's disease and have published the only investigation of trehalose levels in the tissue of treated animals so far. As well as showing decreased polyglutamine aggregation and increased cell viability in culture (mouse neuroblastoma Neuro2a cells expressing a truncated N-terminal huntingtin), they treated the R6/2 mouse model of Huntington's disease with 2% of trehalose added to their drinking water. The trehalose treatment reduced weight loss, as well as the number of ubiquitin positive polyglutamine aggregates in the brains of the R6/2 mice at 8 and 12 weeks of age. Also, macroscopic neuropathology and clinical signs (motor dysfunction) were ameliorated and lifespan was extended after trehalose treatment. The concentration of trehalose in the brains of the animals was reported to be 0.11 ± 0.02 nmol/μg protein (mean ± SEM).

In a study of OPMD, trehalose ingested via the drinking water also alleviated the characteristic forelimb symptoms of the mouse model A17-1 (Davies *et al.*, 2006). Histologically, aggregate formation and DNA fragmentation (apoptosis) were decreased in the biceps of the trehalose treated animals.

A mouse model of Parkinson's disease (parkin-deleted/tau overexpressing) fed trehalose for up to 4 months exhibited alleviation of cognitive and motor signs in a number of tests, a reduction of neuritic plaques and astrogliosis (Rodríguez-Navarro *et al.*, 2010). However, weight loss was significantly exacerbated in the treated mice. Some effects were less pronounced (e.g. astrogliosis) or absent (e.g. dopaminergic effects) after 4 months of treatment, despite showing a significant effect after 2.5 months of treatment.

Since trehalose had positive effects on aggregation of misfolded proteins in other neurodegenerative diseases, and had alleviated SOD1 aggregation in a cell model (Gomes *et al.*, 2010), it was believed that it might have positive effects on the $SOD1^{G93A}$ mouse model of ALS. However, up to 5% trehalose in the drinking water from 35 days of age did not significantly affect the age of disease onset, survival, motor function or the proportion of motor neurons showing SOD1 aggregates (Kaneb, 2012). The failure to see a response to the treatment in this study has raised questions about whether the trehalose was reaching the central nervous system (CNS).

The purpose of the present study was to investigate to what extent trehalose reaches the CNS of the $SOD1^{G93A}$ mouse. This was explored by measuring the trehalose levels in the brains of trehalose treated $SOD1^{G93A}$ mice. Since different kinds of SOD1 inclusions had been noticed in the work by Kaneb (2012), a re-evaluation of existing histological material from previously trehalose treated $SOD1^{G93A}$ mice formed a second part of the study.

The hypothesis was that trehalose does not reach the CNS. That would mean that no significant differences in trehalose concentration are to be observed between treated and control $SOD1^{G93A}$ mice. Neither would any significant differences be observed between the two groups in the extent of aggregation of mutated SOD1 protein in the motor neurons of the spinal cord.

MATERIALS AND METHODS

Animals

All animals used in this study were either wildtype or hemizygous $SOD1^{G93A}$ mice. The latter were bred in a colony where male mice carrying the human mutated $SOD1^{G93A}$ gene (B6SJL-TgN SOD1G93A 1 Gur/J, Stock number 002726, purchased from the Jackson Laboratory, Bar Harbour, ME) had been bred with wild-type hybrid C57Bl/6 x CBA/Ca females. The hemizygous $SOD1^{G93A}$ offspring were maintained throughout more than 20 generations and no differences in disease progression and lifespan have been observed in the colony (Kaneb, 2012). All animals were housed according to the Home Office code of practice and treatment was conducted under a Project Licence held by Professor Dominic Wells.

Eight female $SOD1^{G93A}$ mice were used for the definitive part of this study. After genotyping by polymerase chain reaction (PCR) of ear biopsy material according to previously published protocol (Kaneb, 2012), hemizygous female $SOD1^{G93A}$ mice from the same litter were divided into a treatment group (n=4) and a control group (n=4). The mice of the treatment group were fed 5 % trehalose (MP Biomedicals, USA) in their drinking water from 35 to 90 days of age. On day 90 they were culled by spinal dislocation and their brains were collected post-mortem. The tissue was frozen in liquid nitrogen immediately after collection.

Additional $SOD1^{G93A}$ mice and wild-type mice were either not treated or treated for shorter periods and their tissues were used to optimise the assay.

Trehalose assay

Each brain was pulverised in liquid nitrogen using a pestle and mortar and suspended in deionised water (≥18 mΩ, 2.5 µl per mg of brain). The resulting homogenate was thoroughly mixed and heat treated, in aliquots of up to 500 µl, for 30 minutes in a 75°C water bath. The heat treated homogenates were then quickly frozen to -20°C. Once all the tissue had been processed, the homogenates were defrosted before being centrifuged for 20 minutes at 13,000 g, after which the supernatant was collected. The supernatant was again centrifuged for 20 minutes at 13,000 g to make a clear supernatant, which was stored in a -20°C freezer.

After defrosting, each supernatant was split into three aliquots of which one (A) was treated with trehalase (Megazyme International Ireland) at pH 7 and 40°C for 60 minutes. The two other aliquots (B and C) were diluted in the same buffer as A without trehalase and incubated at 40°C for 60 minutes. The glucose concentration was assessed in supernatants A and B using a hexokinase glucose kit (Sigma-Aldrich Co., UK) and C was used as a blank control.

The endogenous glucose concentration of each supernatant was calculated by subtracting the absorbance of C, and the absorbance of the reagent, from B and convert the difference to the concentration of glucose in the supernatant by comparison to a glucose standard curve ranging from 2.5 to 80 µg/ml (0,125 to 4 µg in a volume of 50 µl). The glucose concentration was then calculated for the entire brain. The total glucose concentration, including that added

by trehalose conversion, was calculated by subtracting the absorbance of C and the reagent from that of A and converting it into glucose concentration in the brain in the same way.

Samples were processed in duplicates with the sample identity hidden from the researcher, after which the glucose concentrations obtained were analysed using the Kruskal-Wallis one-way analysis of variance.

Histology

Spinal cord sections used in a previous study (Kaneb, 2012) were histologically analysed using a semi-quantitative method. The tissues of trehalose treated and untreated 75-day-old $SOD1^{G93A}$ mice (n=5 female and n=4 male in each group) had been sectioned and stained with the USOD antibody (Kerman *et al.*, 2010) and Nissl stain as previously reported (Kaneb, 2012). The USOD antibody (donated by Dr Avijit Chakrabartty, University of Toronto) is raised specifically to misfolded SOD1 protein where the hydrophobic core is exposed and the copper ion lost (Kerman *et al.*, 2010). The mice had been treated with 5% trehalose in the drinking water from 35 days of age until they were sacrificed at 75 days of age. A scoring system for the type and intensity of staining was set up according to the categories suggested by Kaneb (2012). All sections which could not be reliably assessed were excluded from the study. Reasons for exclusion included folding of sections on the slide (when this resulted in interference between the two levels of the sections so that it was no longer clear whether USOD staining was directed toward motor neurons) and tearing of the tissue resulting in incomplete sections. The sections had been allocated to specific levels of the spinal cord previously and this assessment was used to level the sections between the animals. The sections corresponding to levels where one or several sections could not be interpreted reliably were excluded from all the mice in order to get comparable results. This left seven sections that were present in all 18 animals.

The number of USOD positive motor neurons in each category (see below) was quantified for each section of every animal in a blinded fashion and the sum of positive neurons in the seven sections was calculated for each animal. The Kruskal-Wallis one-way analysis of variance was used to analyse the differences between the four groups (treated females, untreated females, treated males, untreated males).

The extracellular staining was categorised (see below) for every section and the result was analysed using generalised estimating equations.

Scoring criteria for intracellular staining

The number of neurons exhibiting the different degrees of intracellular staining was assessed according to the following definitions:

- Diffuse staining: all intracellular staining which lacked a particular structure. Any neuron which showed intense or focal staining would be included in the other categories.
- Faint inclusions: all motor neurons containing solid shapes and inclusions that seemed to aggregate but were less intense than "intense inclusions" below.
- Intense inclusions: all inclusions which were intensely stained at least in some area (punctae to patches, one or several) up to approximately one third of the area of the neuron cell body
- Intense staining throughout motor neuron: intense staining extending across more than one third of the area of the neuron cell body.

Scoring criteria for extracellular staining

The scoring of extracellular staining of individual sections was performed according to the following definitions:

- None: lacking staining apart from intracellular staining (in neurons, small membrane-bound compartments, erythrocytes or fungi), background staining or other obvious artefacts.
- Mild-moderate: all staining which did not appear to be intracellular or obvious artefacts, but did not meet the criteria for extensive extracellular staining (see below). Reasons for exclusion from "extensive" could be that the staining was less intense, less dense, consisted of smaller punctae or covered a smaller area.
- Extensive: staining covering at least one quarter of one ventral horn, consisting of intensely stained plaques larger than punctae or clearly stained vacuolar structures. The staining should be substantial in density throughout the assigned area.

RESULTS

Trehalose assay

Trehalase was used to convert trehalose into glucose, as the latter can be easily measured using an enzymatic assay. The difference between the glucose level of the untreated sample and that of the sample after incubation with trehalase would then be proportional to the original amount of trehalose (1.052 g of glucose per gram of trehalose).

Validation of glucose assay for use in supernatant

The glucose assay kit was validated using a 1 mg/ml glucose solution according to the technical bulletin. In short, this assay contains hexokinase and glucose-6-phosphate dehydrogenase which catalyse the conversion of glucose into 6-phosphogluconate, producing one NADH molecule per glucose molecule used. NADH absorbs light at 340 nm proportional to the original glucose concentration. Δabsorbance, defined as the absorbance of the sample with reagent minus the sum of the absorbance of the reagent blank (reagent and deionised water) and the sample blank (deionised water and sample), is used in a formula to obtain the actual concentration of glucose in the sample. The assay was first used in its recommended volumes of 1 ml reagent and 10-200 µl of sample, but when using this setup to measure the endogenous glucose content of supernatant from wild-type mouse brain it was concluded that the glucose concentration was often too low to obtain results within the recommended range. In order to assess the variability of the assay and to optimise the method for samples of low glucose concentrations, the possibility of reducing the volume was investigated.

50 µl of sample was used with 150 µl of reagent in a 96-well ELISA UV-plate. A serial dilution (1:2) of glucose in deionised water, reaching a total volume of 50 µl was used in each experiment to obtain a standard curve, usually ranging from 5 to 80 µg/ml. Also 2.5 and 160 µg/ml were proven to be in the linear range of the standard curve. All reactions were performed in duplicate.

Since the glucose levels were lower than expected, a potential breakdown of glucose in the supernatant derived from a wild-type mouse brain was investigated by adding a known amount of glucose to one half of the supernatant while leaving the other half neat. After overnight incubation at 37°C, the same amount of glucose was added to the neat supernatant right before measuring the glucose concentration of the samples. Very similar glucose concentrations were obtained (results not shown), indicating that glucose is not broken down in the supernatant.

The possible inhibition of the supernatant on the assay reagent was investigated using 40 µl of supernatant (derived from wild-type mouse brain) in different dilutions (1:1, 1:2, 1:4 and 1:8) and 10 µl of a glucose solution of known concentration. This experiment was first performed using 150 µl of reagent, which showed a small but significant decrease in glucose recovery in more concentrated supernatant. The experiment was repeated using 250 µl of reagent to explore whether the inhibitory effect was caused by too high sample:reagent ratio. This ratio of 50:250 is equivalent to the ratio when using the maximum sample volume recommended

for the assay, 200:1000. The result showed slightly higher inhibition (Figure 1), so a ratio-dependent inhibition was ruled out. However, from this point 250 µl was used in each experiment in order to get as close as possible to the recommended conditions.

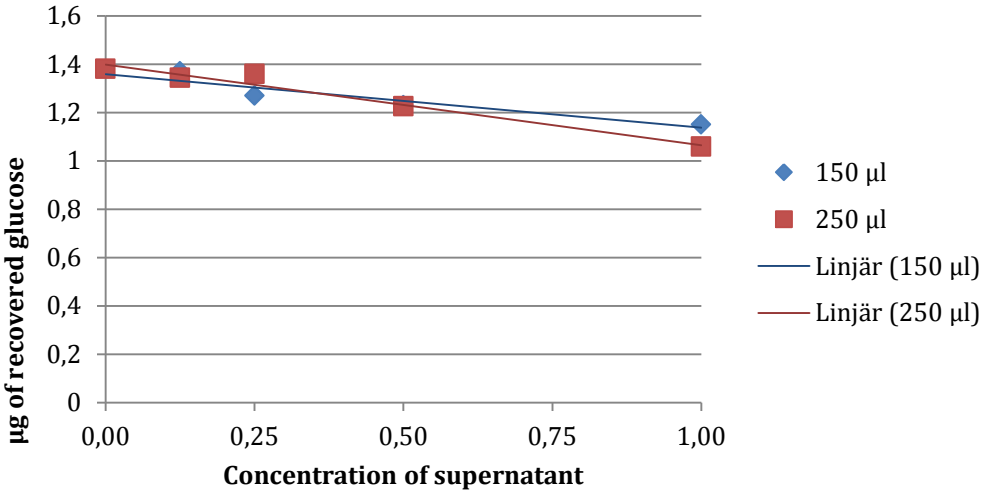


Figure 1. Inhibition of the glucose assay reagent by supernatant.

The mass of glucose recovered after adding the same amount to different concentrations of brain lysates (diluted with water). Mass of glucose recovered (m) as a function of the concentration of supernatant (c) follows the equations:

$m = -0.3341c + 1.3992$, $R^2 = 0.9646$ (250 µl)

$m = -0.2212c + 1.3587$, $R^2 = 0.8758$ (150 µl)

Different amounts of glucose, similar to the standard curve, were then added to supernatant of dilutions 1:1, 1:2 and 1:4 in order to investigate whether the increase in glucose (representing the product of trehalose after trehalase digestion) could be reliably measured. The best correlation and correspondence to the standard curve in water was obtained in the 1:2 dilution of the supernatant (Figure 2). The standard curve in the 1:4 dilution of supernatant also corresponded well to that in water, but since it was considered important to keep the sample concentration as high as possible, the 1:2 dilution was chosen for further investigations.

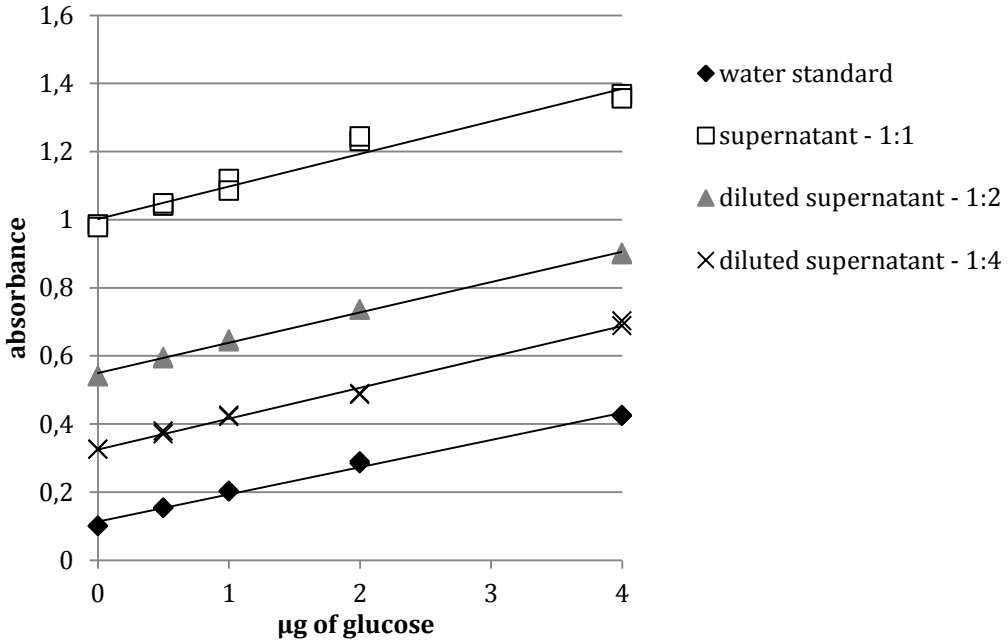


Figure 2. Glucose recovery in different dilutions of brain lysate.

The amount of detected glucose is indirectly represented by the absorbance at 340 nm. The absorbance at 0 µg of glucose represents the background absorbance, which increases with the concentration of the supernatant. The absorbance (A) as a function of the mass of added glucose in µg (m) follows the equations:

$A = 0.08m + 0.1134$, $R^2 = 0.992$ (water standard)

$A = 0.0957m + 1.0019$, $R^2 = 0.9649$ (1:1 neat supernatant)

$A = 0.0892m + 0.5493$, $R^2 = 0.9971$ (1:2 diluted supernatant)

$A = 0.0907m + 0.325$, $R^2 = 0.9933$ (1:4 diluted supernatant)

Validation of trehalase

1 µl of trehalase (corresponding to 61 U at pH 7 or 148 U at pH 5.5) was chosen for digestion of each 50 µl sample volume, as it should contain a surplus of enzyme, and any smaller volume would be hard to measure accurately.

Since the glucose standard curve worked well between 2.5 and 160 µg/ml, 126 µg/ml trehalose dihydrate (6.3 µg in a volume of 50 µl, producing 6 µg of glucose) was considered a suitable concentration for the testing of trehalase. The combination of trehalose and trehalase was assessed in 0.1 M sodium maleate buffers of pH 5.5 and pH 7 containing 0.5 mg/ml BSA, incubated in a 40°C water bath. Aliquots were collected for assessment at different time points; before incubation, after 5 minutes, 30, 60 and 120 minutes of incubation at 40°C. There was no significant difference between the different buffers or time points and no incubation in the water bath seemed to be required (Figure 3). However, an incubation time of 60 minutes was chosen since it showed the highest concentration of glucose at pH 7 (which

was chosen because it was similar to physiological pH). Controls containing all constituents except the trehalase showed no increase in glucose level, thus proving that there was no spontaneous breakdown of trehalose.

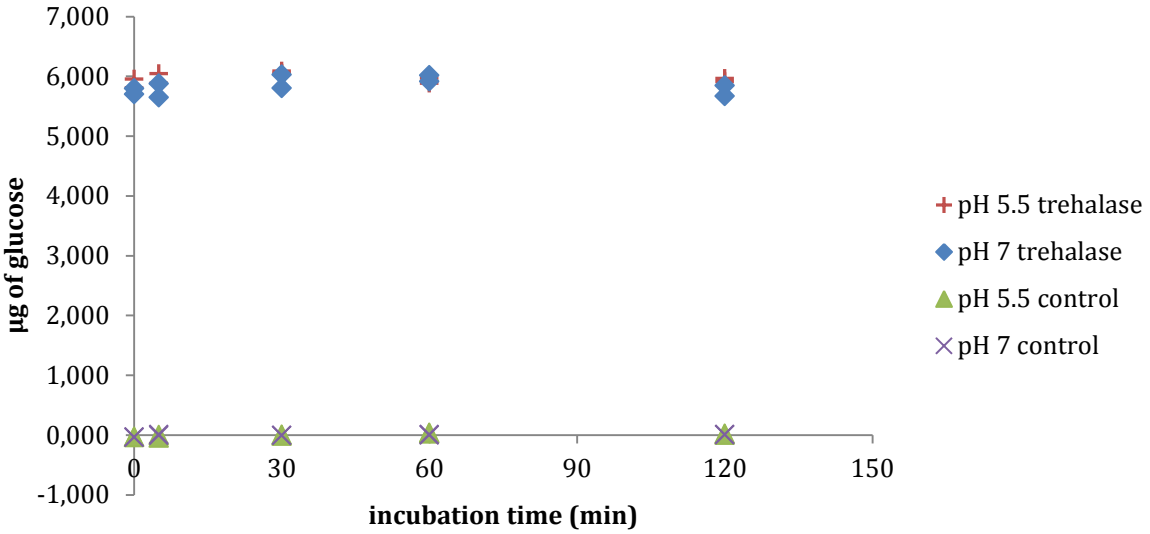


Figure 3. Reaction time of trehalase.

Amount of glucose produced from 6.3 µg of trehalose as a function of the time of incubation at 40°C. Controls were incubated without trehalase under the same conditions and showed no increase in glucose concentration.

Different amounts of trehalose were also treated with trehalase in 20 µl supernatant diluted in buffer (to correspond to 40 µl of 1:2 supernatant) or buffer only, to investigate whether the enzyme works well in this new environment, and whether the concentration can be accurately measured using the glucose assay. The results resembled the standard curve in water well (Figure 4).

Controls were set up to check that trehalose does not interfere with the glucose kit (e.g. gives false positives) and to see whether there are detectable trehalose levels in a non-treated mouse brain. These experiments showed no unexpected detection (results not shown).

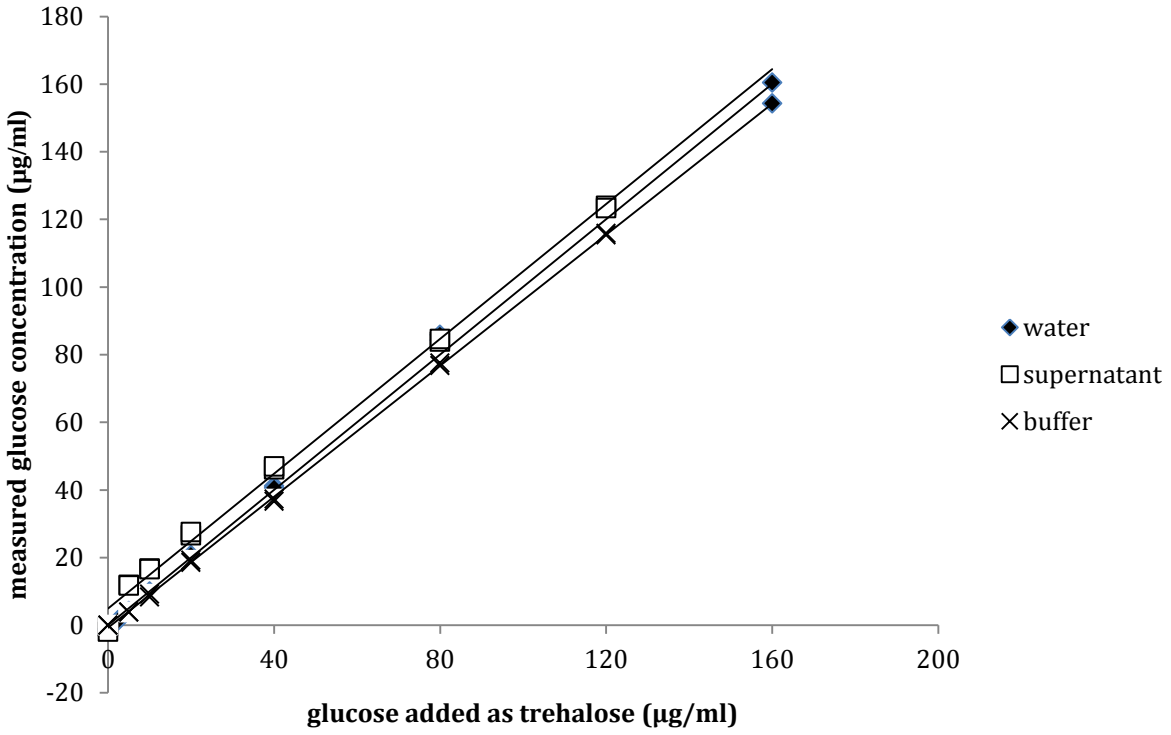


Figure 4. Trehalose digestion in water, supernatant or buffer.

The measured glucose concentration after digestion of different amounts of trehalose (calculated using the standard curve in water) as a function of the expected concentration of glucose, added in the form of trehalose.

The correlation coefficients (R^2) are 0.9978, 0.9955 and 0.9998 for water, supernatant and buffer, respectively.

Trehalose assay results

Both the endogenous glucose levels and total glucose levels (including that added through the trehalase conversion of trehalose) were lower in the trehalose treated mice than the controls (Figure 5). The mean and SEM glucose concentrations in the samples from trehalose treated mice (n=4) were 46.58±10.39 µg per g of brain tissue after trehalase digestion (representing total glucose with the addition from the digested trehalose) and 41.86±7.93 µg per g before digestion (representing endogenous glucose). The glucose concentrations (mean and SEM) in the samples from control mice (n=4) were 60.94±11.39 µg/g after trehalase digestion and 71.58±10.25 µg/g before. There was no significant difference in the glucose concentrations before and after trehalase digestion in the trehalose treated group or the control group, significance being defined as $p<0.05$ using the Kruskal-Wallis one-way analysis of variance.

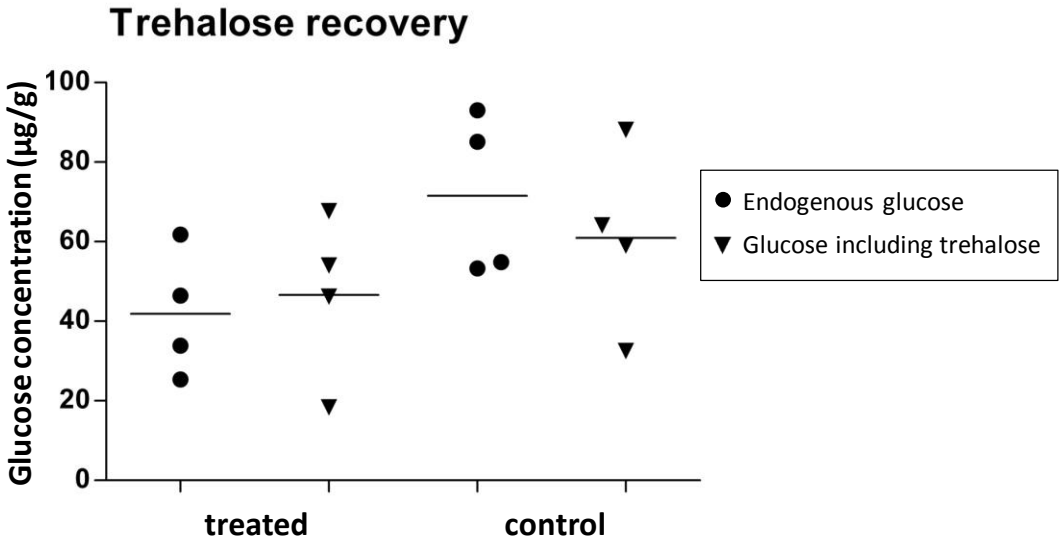


Figure 5. Trehalose level in the CNS of 5% trehalose treated or control female SOD1^{G93A} mice at 90 days of age.

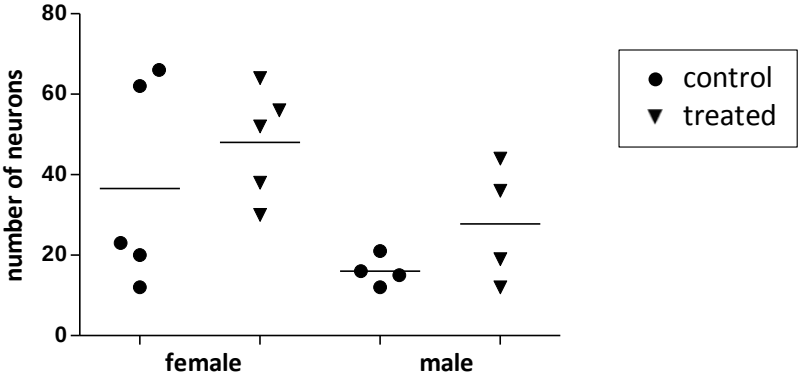
Scatter plot showing the glucose level in the CNS of female SOD1^{G93A} mice treated with 5% trehalose or female SOD1^{G93A} control mice as found by enzymatic assay. The endogenous glucose level is defined as the glucose concentration before digestion of trehalose into glucose, whereas the glucose level including trehalose is the glucose concentration after trehalase digestion. There is no significant difference between the concentrations before and after trehalase digestion in the trehalose treated group (n=4) or the control group (n=4). Significance was defined as $p<0.05$ in analysis using the Kruskal-Wallis one-way analysis of variance.

Histology

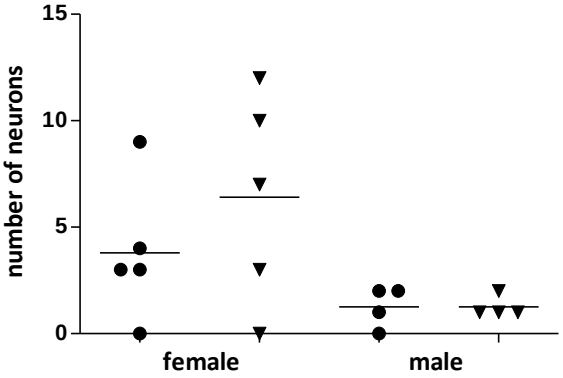
There was generally more staining in the motor neurons of the trehalose treated mice (Figure 6 A), especially in the more intense staining categories, except for intense inclusions in female mice (Figures 6 F and H). However, faint inclusions in male mice showed an opposite pattern (Figure 6 D), whereas diffuse staining in male mice and faint inclusions in female mice showed very similar distributions between treated and untreated animals (Figures 6 B and D). However, none of the differences reached significance of $p<0.05$ in the Kruskal-Wallis one-way analysis of variance.

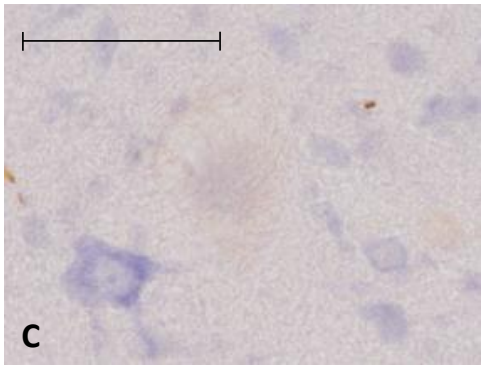
The extracellular staining showed no apparent differences between trehalose treated and untreated male mice (Figure 7 B) and did not reach significance by analysis using generalized estimating equations (OR = 1.298, p = 0.813). The same test for the extracellular staining in female mice (Figure 7 A) did however show significance (OR = 6.099, p = 0.031) and there was an obvious trend where treated females had many sections classified as having extensive extracellular staining and none in the category of no extracellular staining.

A. Total number of USOD stained neurons

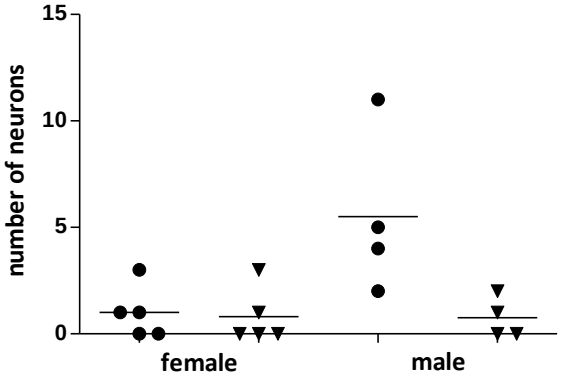


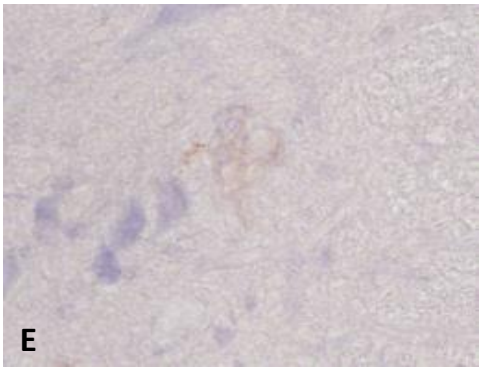
B. Diffuse USOD staining





D. Faint inclusions of USOD staining





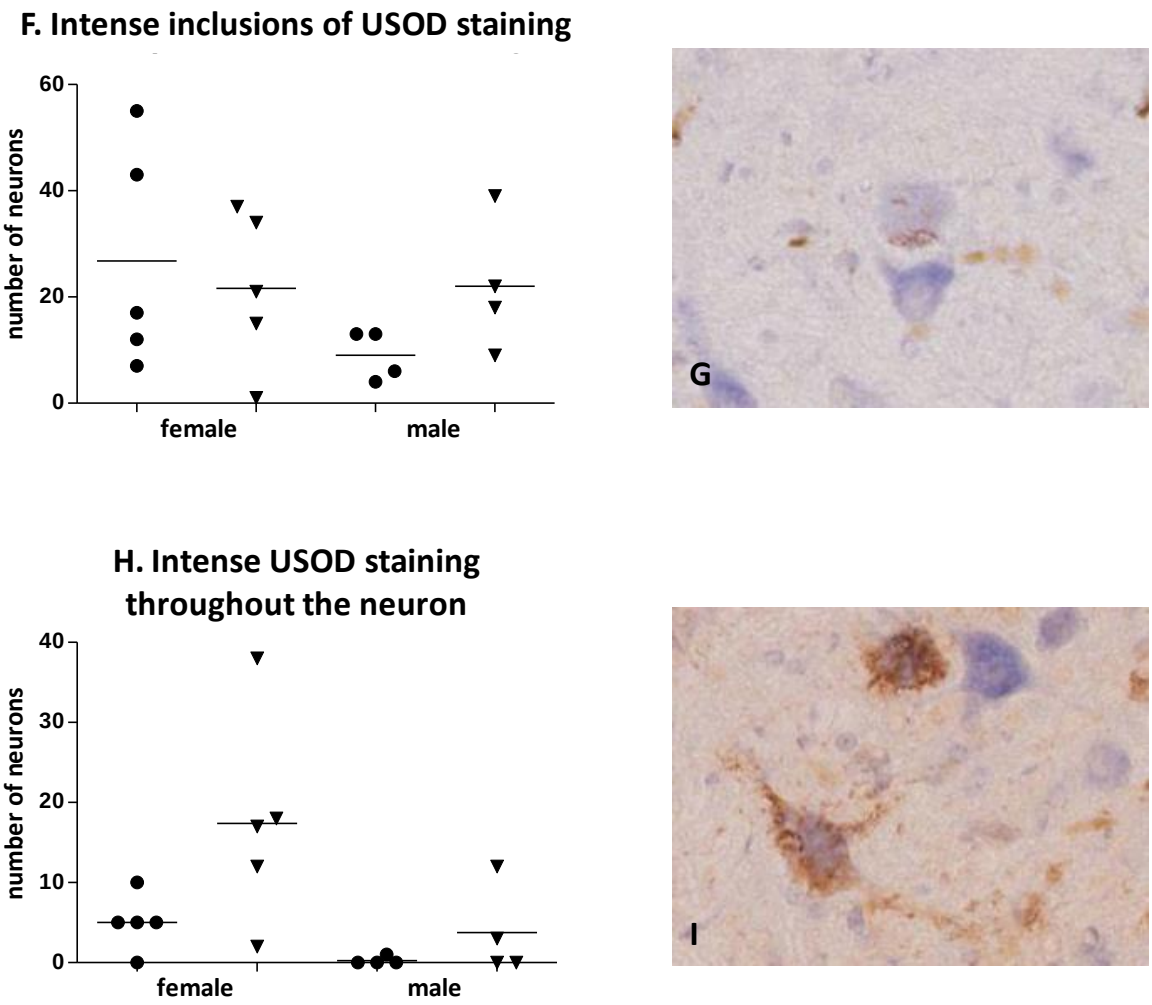


Figure 6. Intracellular USOD-staining in 5% trehalose treated and control SOD1^{G93A} mice at 75 days of age

The scatter plots show the number of USOD-positive motor neurons in the spinal cord of 5% trehalose treated or control SOD1^{G93A} mice, displayed for each animal and the mean of the groups (n=5 female and n=4 male mice per group). The graphs show the sum of all USOD-positive motor neurons (A), the number of diffusely stained motor neurons (B), the number of motor neurons containing faint inclusions (D), the number of motor neurons containing intense inclusions (F), and the number of motor neurons dominated by intense USOD staining (H). To the right hand side of each scatter plot is a corresponding picture of the degree of USOD staining (brown), from a faint, diffuse staining (C), to distinct but not intense inclusions (E), intense inclusions in a small portion of the neuron (G), and intense USOD-staining throughout a large area of the neuron (I). Observe the clear difference in staining intensity between (E) and (G), and the fact that the aggregates seem to surround vacuolar structures to a high degree in (G) and (I).

Seven matching sections from the lumbar region (L2 – L5) of 75 day old animals were stained with the primary antibody USOD, and a biotinylated secondary antibody using a peroxide reaction to visualise the detection of SOD1 (Kaneb, 2012). Nissl staining was used to visualise nuclei and somas of the cells. The scale bar indicates 200 µm and is applicable to all pictures (C, E, G, I). No significant differences were found between treated and control animals using the Kruskal-Wallis one-way analysis of variance with a significance level of $p<0.05$

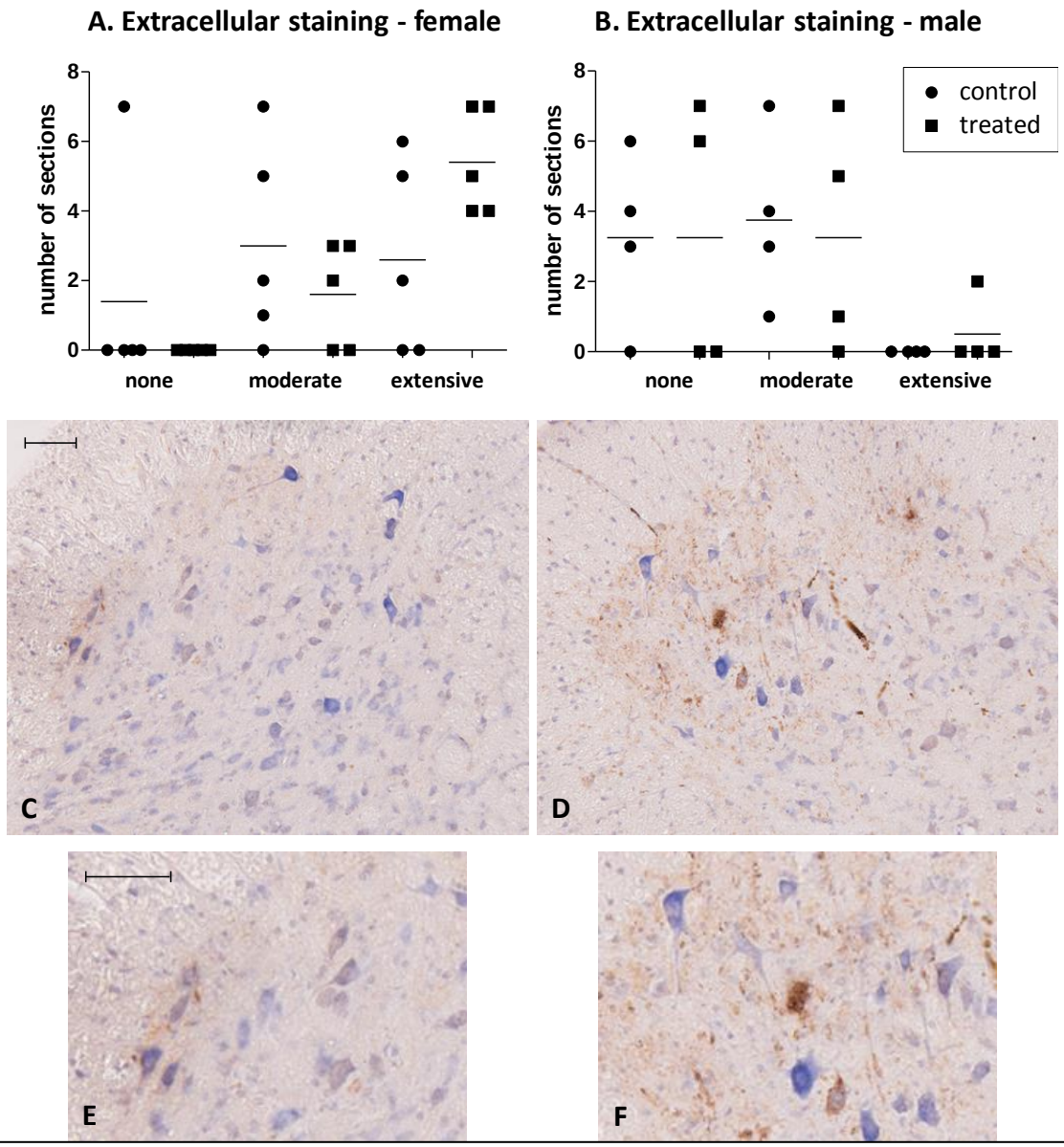


Figure 7. Extracellular USOD-staining in 5% trehalose treated and control SOD1^{G93A} mice at 75 days of age

(A-B) Scatter plots showing the number of transverse sections in the spinal cord of 5% trehalose treated or control female (A) and male (B) SOD1^{G93A} mice that showed no extracellular USOD-staining (none), mild-moderate extracellular USOD-staining (moderate), or extensive extracellular USOD-staining (extensive), displayed for each animal and the mean of the groups (n=5 female and n=4 male mice per group). A total of seven equivalent sections from the lumbar region (L2 – L5) of each animal were included. A significant difference (p=0.031) was observed between treated and control females, but not between treated and control male animals (p=0.813). Results were analysed using generalized estimating equations, odds ratio = 6.099 for females and odds ratio = 1.298 for males.

(C-D) Representation of the degree of USOD-staining (brown) in the categories mild-moderate extracellular staining (C) and extensive extracellular staining (D). The categories are defined by the staining intensity, density and area covered by the extracellular aggregates. The sections were stained with USOD as primary antibody, and a biotinylated secondary antibody using a peroxide reaction to visualise the detection of SOD1 (Kaneb, 2012). Nissl staining was used to visualise nuclei and somas of the cells. Scale bar indicates 200 µm and is applicable to both pictures (C) and (D).

(E-F) Enlarged sections of pictures (C) and (D), respectively. Scale bar in (E) indicates 200 µm and also applies to (F).

DISCUSSION

The main hypothesis of this study was that trehalose does not enter the CNS in the SOD1^{G93A} mouse model of ALS and it is over all supported by the experiments performed. Significant levels of trehalose were not found in the brains of trehalose treated SOD1^{G93A} mice, which is in accordance with the hypothesis. The histology only showed statistically significant differences in one of six categories (extracellular USOD staining), and only in one gender.

Trehalose assay

The fact that no trehalose was detected in the samples is in direct contradiction to what Tanaka *et al.* (2004) found. They reported trehalose levels of 0.11 ± 0.02 nmol per μg of protein in the brain of R6/2 transgenic mice, which corresponds to about 3.77 mg of trehalose per gram of brain tissue (see Appendix). This is over twice the fasting blood glucose reported for the same animals (Tanaka *et al.*, 2004), and 40 times the highest endogenous glucose concentration of the brain of SOD1^{G93A} mice as measured in this experiment. This concentration seems extremely high and it is questionable whether it could reside inside the brain without affecting its delicate chemical balance.

Theoretically, trehalose is also unlikely to be absorbed in large quantities. Most mammals, including mice and humans, express intestinal trehalase (Murray *et al.*, 2000; Kamiya *et al.*, 2003) and it has been shown that rats do not absorb significant amounts of trehalose without hydrolysing it to glucose (Dahlqvist and Thompson, 1963). In addition, no mechanism for trehalose uptake from the intestines has been shown. To assume that mice metabolise and absorb trehalase in the way that has been shown for rats seems reasonable but has not been proven. Possible strain differences in the metabolism or absorption of trehalose have not been investigated, and could theoretically exist between the SOD1^{G93A} mouse investigated in this study and the mouse strains showing positive results of trehalose administration, including the R6/2 mouse used by Tanaka *et al.* (2004).

Apart from the study by Tanaka *et al.* (2004), there are two studies in which trehalose ingestion has been reported to cause significant changes in the progression of neuro-degenerative diseases, although the trehalose level of the CNS or other tissues were not measured. These are the investigation of the A17-1 mouse model of oculopharyngeal muscular dystrophy (Davies *et al.*, 2006) and parkin-deleted/tau overexpressing mice that model Parkinson's disease (Rodríguez-Navarro *et al.*, 2010). It is interesting to find a decrease in the effect of trehalose in the latter study, which also reported a significant weight loss in trehalose treated mice. The weight loss could be interpreted as failure to alleviate a clinical sign, and the effects of body mass on the disease course could possibly be discussed. Overall the positive results from these studies suggest that trehalose does have a positive effect on certain conditions, despite the fact that high concentrations might not reach the CNS. It is possible that small quantities have a significant effect on these mouse strains, as the mechanism of action has not been explained. Tanaka *et al.* (2004) also stated that the possibility that positive effects of trehalose were exerted from outside the CNS could not be excluded. Such mechanisms remain to be elucidated.

It has been suggested that the net addition in glucose intake that should follow after the intestinal digestion of trehalose could be responsible for certain effects (Tanaka *et al.*, 2004; Kaneb, 2012). This has been investigated by administration of 2 % glucose in the drinking water of R6/2 mice, but this did not alleviate the Huntington-like pathology of the strain (Tanaka *et al.*, 2004). It has also been shown that trehalose does not cause a significant increase in blood glucose in R6/2 or wild-type mice (Tanaka *et al.*, 2004), or in the mouse model of Parkinson's disease (parkin-deleted/tau overexpressing) (Rodríguez-Navarro, 2010). No corresponding investigations have been reported for the A17-1 mouse (Davies *et al.*, 2006).

The assay procedure used to measure the trehalose concentration in small volumes of brain lysates was thoroughly validated to accurately detect relatively slight increases in trehalose concentration. The glucose assay and the trehalase used were validated for use in 40 µl of the standardised brain lysate diluted 1:2, i.e. 20 µl of original lysate, which was used in the final assay. This concentration was chosen because it showed the best fit to the standard curve while avoiding excessive dilution. In measuring the glucose content of such a small volume, there is a risk of false negative answers if the actual concentration is very small, or false positive answers since all answers are substantially magnified when calculated as µg per g of tissue. All the differences between the treated and control groups (average values as presented in Figure 5) are within the margin of error and, as stated previously, show no statistical significance. Therefore there is no question about whether large amounts of trehalose were present in the brain lysates.

It has not been verified that trehalose moves freely in tissue lysates produced using this method, so there is a possibility that the concentration of trehalose is higher in the pellet. It is however believed that this would require a mechanism for "trapping" the molecule, since trehalose is expected to behave similarly to glucose, which moves freely in this kind of solution. The molecule should not be limited by the cell membrane, since this should be properly disrupted by the pulverisation of the frozen tissue and repeated freeze-thaw. Again comparing to the previous study of trehalose concentrations, a similar method was used by Tanaka *et al.* (2004) to treat the brain tissue and there is no reason to believe that the small differences in method should account for the very different results.

There is a big discrepancy between the endogenous glucose content of the brain of the $SOD1^{G93A}$ mice in the present study and the normal blood glucose level of mice, which is approximately 100 mg/dl, i.e. 1000 µg/ml (Takeda et al, 2009). Part of the reason for this might be the transport of glucose across the blood-brain barrier and the high glucose consumption of the brain. The glucose concentration of the mouse brain has been reported to be 10-20 % of the concentration in serum (Nilsen *et al.*, 2011), which would give a normal glucose concentration in the range of 100-200 µg per gram of brain. The endogenous glucose concentrations as measured in the $SOD1^{G93A}$ mice ranged from 25 to 95 µg/g, which implies that glucose was broken down before the assay had been carried out. This is entirely possible, since the tissue has been collected and processed at room temperature, and a rapid

consumption of glucose is expected immediately post-mortem. However, a breakdown of glucose is no reason for concern in this type of study, since it has been established that the glucose concentration is stable after the heat treatment, and no trehalose is expected to be degraded before the addition of trehalase. This is the only enzyme known to digest trehalose and has never been reported to be present in the brain of any mammal.

Since trehalose has been shown to have positive effects in cell cultures transfected with G93A-mutant SOD1 (Gomes *et al.*, 2010), it is still an interesting molecule to investigate and alternative ways of administration should be explored. Intramuscular or intraperitoneal injection seems like an obvious way of circumventing the enteric digestion of trehalose and would make an interesting study of the effects of trehalose in $SOD1^{G93A}$ mice. Also, more studies on the absorption, distribution, metabolism and mechanism of effect of ingested trehalose could be required in the mouse models where it has been reported to alleviate disease.

Histology

The results of the histological study showed no significant differences between treated and control animals, except in the extracellular USOD staining of female mice. The fact that there was a bigger difference in the female animals is worth considering, since female $SOD1^{G93A}$ mice show less pronounced pathology than do their male counterparts at the same age (Alves *et al.*, 2011). Additionally, trehalose treatment has not resulted in any alleviation of the disease clinically or as shown by other measurements in female $SOD1^{G93A}$ mice, although treated males have shown a delayed onset of weight loss (Kaneb, 2012). Taking into consideration the small n-values and the great range of the number of USOD positive neurons between the samples, there is a possibility that the significant change displayed in extracellular staining could be caused by chance as opposed to trehalose treatment.

On the other hand, some of the negative results might have reached significance in a larger sample size. If the results are an indication of a statistically significant effect, it is particularly interesting to see that the number of intensely stained neurons was generally higher in female mice (Figures 6 F, H and Figure 7) – both in treated and untreated mice – whereas male mice exhibit a more aggressive pathology (Alves *et al.*, 2011). Since it has often been assumed that SOD1 positive aggregates are causing the pathology, significant results showing a higher degree of aggregation in females would challenge this assumption. There are already diverging theories about the role of protein aggregates, ranging from major cause of pathology to a way of reducing the toxic effects of the mutated protein (Shaw, 2005), and future research will hopefully reveal what significance the aggregation of protein has. The original assumption of protein aggregation causing ALS pathology would however further suggest that the differences found in the female animals of this study (both significant and non-significant) would be coincidental.

In working with the histology, an important and sometimes difficult task was drawing the line between the categories of staining. The difference between moderate and extensive

extracellular staining was especially difficult to define, and this might affect the validity of these categories. However, the variability in the assessment was controlled by making the final ruling on all the sections during the same day without any interference. A combined score of area, intensity and size of the extracellular aggregates seemed to be the most stable method as there were a number of variables between the different sections. The extracellular staining did not appear to be background staining, since it was very specific to the ventral horn and generally seemed to extend from heavily stained motor neurons and structures which appeared to be disintegrated neurons, which implies that it might originate from these cells. This was also noted by Kaneb (2012). Furthermore, all sections from mice of the same gender were stained at the same time and in an identical fashion (Kaneb, 2012). There was another type of extracellular staining, which was distinctly different to the one assessed in that it seemed to be situated in another plane, above the histological sections. Therefore, all staining that could be separated by changing the focus of the microscope was excluded from the study, but it is possible that positive staining could have been obscured and that false negatives were thereby created. However, it is estimated that such a margin of error should be small, since not many cases occurred in the ventral horn. A larger cause of error could be the sections which were folded upon themselves. It did seem like both "levels" of these folded sections were correctly stained, so in the end many of these cases were included – unless neurons were piled onto each other and this made the distinction of how many cells were stained difficult.

Another possible source of error was the fact that the samples had been contaminated by fungus during the sucrose cryoprotection stage. This meant that most sections contained large tree-like, USOD-positive structures which could have been broken down into pieces that could be mistaken for extracellular, and maybe intracellular, staining. However, this was unlikely as intermediates in this potential breakdown were not observed.

The USOD antibody has been verified by Kerman *et al.* (2010) to correctly stain misfolded SOD1, and Kaneb (2012) has reported that it only stained motor neurons in the $SOD1^{G93A}$ mouse. Kaneb (2012) also reported that there is no significant difference within each gender, between the trehalose treated and control mice in the number of *surviving* motor neurons, which in itself is an indicator of the inability of trehalose to alleviate the pathology induced by the $SOD1^{G93A}$ mutation. It also verifies the method of comparing the absolute number of stained neurons, as opposed to calculating the ratio of existing neurons, as these should thereby share the same relationship.

The conclusion of this study is that trehalose does not reach the CNS in significant levels when ingested by the $SOD1^{G93A}$ mouse model of ALS and that other ways of administration should be investigated.

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APPENDIX

The level of trehalose in the brain of treated mice was 0.11 ± 0.02 nmol/μg protein according to Tanaka *et al.* (2004).

The protein content in different areas of the rat brain is 10-13 % of fresh tissue weight, and the corresponding figure for the human brain varies between 9-10 % (M. Banay-Schwartz *et al.*, 1992). It seems reasonable to assume that the mouse brain has a similar protein concentration, and for simplicity 10 % will be used for the calculation. Provided that the molecular weight of trehalose is 342.31 (Richards *et al.*, 2002), the conversion to mg/g of brain tissue should follow:

$$\frac{1\ nmol\ trehalose}{1\ \mu g\ protein} = \frac{342.31\ ng\ trehalose}{10\ \mu g\ brain\ tissue} = \frac{34.231\ mg\ trehalose}{1\ g\ brain\ tissue}$$

This means that the concentration of trehalose reported in the study by Tanaka *et al.* (2004) would be:

$$\frac{0.11\ nmol\ trehalose}{1\ \mu g\ protein} = \frac{34.231 \times 0.11\ mg\ trehalose}{1\ g\ brain\ tissue}$$
$$= 3.76541\ mg\ trehalose\ per\ g\ brain\ tissue$$