Original Paper

Combined Treatment of an Amyotrophic Lateral Sclerosis Rat Model with Recombinant GOT1 and Oxaloacetic Acid: A Novel Neuroprotective Treatment

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Key Words

Sporadic amyotrophic lateral sclerosis · Excitotoxicity · Glutamate · Neuroprotection · Blood glutamate scavenger

Abstract

Background/Aim: The sporadic form of the disease affects the majority of amyotrophic lateral sclerosis (ALS) patients. The role of glutamate (Glu) excitotoxicity in ALS has been extensively documented and remains one of the prominent hypotheses of ALS pathogenesis. In light of this evidence, the availability of a method to remove excess Glu from brain and spinal cord extracellular fluids without the need to deliver drugs across the blood-brain barrier and with minimal or no adverse effects may provide a major therapeutic asset, which is the primary aim of this study. **Methods:** The therapeutic efficacy of the combined treatment with recombinant Glu-oxaloacetate-transaminase (rGOT) and its co-factor oxaloacetic acid (OxAc) has been tested in an animal model of sporadic ALS. **Results:** We found that OxAc/rGOT treatment provides significant neuroprotection to spinal cord motor neurons. It also slows down the development of motor weakness and prolongs survival. **Conclusion:** In this study we bring evidence that the administration of Glu scavengers to rats with sporadic ALS inhibited the massive death of spinal cord motor neurons, slowed the onset of motor weakness and prolonged survival. This treatment may be of high clinical significance for the future treatment of chronic neurodegenerative diseases.

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Introduction

Amyotrophic lateral sclerosis (ALS) is a common adult-onset neurodegenerative disease of unknown origin. It is characterized by progressive loss of motor neurons of the anterior horns in the spinal cord bulb and cerebral cortex that leads to paralysis and death typically 2–5 years after diagnosis [1]. Pathophysiological investigations of chronic neurodegenerative diseases such as ALS [1–5], glioma [6–11], acute brain insults such as stroke [12–16], and traumatic brain injury [17–21] have revealed the existence of a common pathogenic denominator that is the presence of excess glutamate (Glu) in brain/spinal cord fluids. The role of Glu excitotoxicity in ALS has been extensively documented and remains one of the prominent hypotheses of ALS pathogenesis. It suggests that the brain major excitatory neurotransmitter Glu applies an

L.B. passed away in 2009; V.I.T. passed away in 2011.

excessive excitation of motor neurons and therefore leads to their death. Four basic sets of observations have been made on human ALS patients and animal models that support this notion. These include the following: (1) Glu levels are abnormally high in ALS patients: studies of the cerebrospinal fluid (CSF) Glu levels in ALS patients have demonstrated an increase of mean Glu levels compared to age-matched controls in at least 40% of cases [4, 22]. In addition, Spreux-Varoquaux et al. [22] showed a correlation between high Glu concentrations in the CSF and the onset of the disease. The low values of limb functional parameters and the higher rate of muscle deterioration suggest that the Glu values could reflect the intensity of the motor neuronal insult. At least one report describes elevated levels of blood plasma Glu in ALS patients [23]. (2) Glu transport is abnormal in ALS: Glu transport was found to be selectively decreased in the motor cortex and spinal cord of patients with ALS compared to neurologically normal individuals [24], and the abnormality of Glu transport was attributed to the selective loss of the astrocyte GLT-1 (EAAT2) subtype of the Glu transporter [25]. In fact, about 60–70% of sporadic ALS patients display a 30–95% loss of the GLT-1 transporter [25]. However, it remains unclear whether the alterations in the expression and function of GLT-1 represent a primary defect or are part of an escalating cycle of motor neuronal injury [26]. (3) The expression levels of the Glu receptors from the GluR2 subtype are reduced in ALS: deficient RNA editing of the Glu receptor subunit GluR2, at the Q/R site, is a primary cause of neuronal death and has been reported to be etiologically tightly linked to motor neuronal death in sporadic ALS [5, 26]. (4) The first drug for ALS approved by the FDA, riluzole, is a Glu release inhibitor [2]. Although having a very modest activity (increasing survival by approximately 2 months), riluzole was found to be more effective in patients with high serum or CSF and Glu levels. Moreover, following riluzole regimen, the level of Glu in patients was decreased [3].

So far, several attempts to develop a successful treatment to prevent Glu excitotoxicity in humans using Glu receptor antagonists have failed [27, 28]. Therefore, we developed an alternative strategy in which excess Glu is removed from the brain interstitial fluid into the systemic blood circulation following the administration of a blood Glu scavenger (BGS) such as oxaloacetic acid (OxAc) [29, 30]. BGS activate the blood resident enzyme Glu-oxaloacetate transaminase (GOT), thereby decreasing the levels of blood Glu and increasing the driving force for the efflux of excess and deleterious Glu from the brain interstitial fluid into the blood. We have previously proven this using a rat model, where only animals whose blood Glu levels were reduced as a result of treatment with the oxaloacetate showed an efflux of radiolabeled Glu from the brain into the blood [31]. Similarly, in our recent studies that used a paraoxon intoxication model, and in an ischemic stroke model, the levels of Glu in the blood have been shown to be substantially reduced upon treatment with BGS [16, 29].

This approach turned out to be extremely effective in providing neuroprotection in animal models of acute excitotoxic conditions such as traumatic brain injury [30], stroke [13] and paraoxon intoxication [29, 30]. The neuroprotection was detected in terms of neurological and behavioral tests, as well as in histological sections. These promising results encouraged us to investigate the applicability of the blood Glu scavenging approach to chronic ALS.

In our study we used the protocol of Sun et al. [5], in which the use of kainic acid (KA)/cyclothiazide (CTZ) produces a strong excitotoxic activation of neurons. KA is a well-known excitotoxin and its diffusion within the spinal cord interstitial fluid causes neuronal death, which is accompanied by the release of Glu from the large metabolic pool of the dying neurons. The released Glu then produces an excitotoxic effect due to the amplification of the depolarizing action on AMPA receptors by the presence of CTZ, a drug that prevents the desensitization of AMPA receptors. Thus, excess Glu that is released from dying neurons is likely to cause amplification in terms of time and space of Glu-mediated neuronal death. Based on these findings, it is likely to expect that BGS provoking an increased efflux of excess Glu from CNS interstitial fluid into the blood will also exert a neuroprotective effect. In this study we bring evidence that the administration of Glu scavengers to rats with sporadic ALS inhibited the massive death of spinal cord motor neurons, slowed the onset of the motor weakness and prolonged survival.

Materials and Methods

Chemicals and Equipment
OxAc and KA were obtained from Sigma. CTZ was purchased from Tocris. His-tagged version of the recombinant human GOT (rGOT) cDNA was cloned from the human hepatoma cell line hepG2, expressed in *Escherichia coli*, and purified by Ni-agarose chromatography. Each test compound was dissolved in artificial CSF pH 7.2 (122 mM NaCl, 3.1 mM KCl, 5 mM NaHCO$_3$, 0.4 mM KH$_2$PO$_4$, 1.3 mM CaCl$_2$, 1.0 mM MgSO$_4$, and 10 mM D-glucose, pH 7.4). The resulting solution was used to fill an ALZET pump.

Osmotic minipumps, ALZET Model 2001 (1 μl/h) were purchased from Alza Scientific Products (Mountain View, Calif.,

USA). Adhesive was purchased from Araldite Rapid, Ciba-Geigy. A surgical microscope (Olympus SZH, Japan; magnification 6.5–70×) was used. Precision operating instruments necessary for the procedures in this study included the following: (1) microsurgical forceps, 11 cm in length with a very fine straight shank (Fine Science Tools, No. 11255-20) (used for the isolation of the dura mater); (2) straight spring scissors, 8.5 cm in length (Fine Science Tools, No. 15003-08) (used for the incision of the dura mater); (3) a small retractor, maximum spread 1.8 cm (Fine Science Tools, No. 17003-3), and (4) a Friedman-Pearson micro-rongeur with extra-fine tips (Fine Science Tools, No. 16121-14) curved for the nibbling of the vertebral bone. Polyethylene tubing, Intramedic PE 60 (i.d. 0.76 mm, o.d. 1.22 mm) and PE 10 (i.d. 0.28 mm, o.d. 0.61 mm; Becton Dickinson), was used for the intrathecal infusion of the experimental solution.

Pharmacokinetic Experiment

A total of 6 naïve healthy rats were used for the pharmacokinetic experiment. The tail artery and vein of the rats were cannulated on the same day of the experiment using Venflon sterile tube. The animals were anesthetized with isoflurane 2%; respiration rates were monitored and animal reflexes such as pinch and eyelid movements were tested. The tail artery and vein were catheterized using sterile Venflon tubes, and the catheters were fixed to the tail with surgical sutures. Upon completion of the catheterization, the animals were awakened and injected 1 h later with 0.39 mg rGOT bolus/rat, either intravenously through the tail vein catheter or subcutaneously (rGOT was dissolved in 200 μl of 0.9% saline). Blood samples (50 μl) were collected at different time points (0, 2, 10, and 30 min and 1, 2, 4, 10, 16, 24, 32, and 38 h) from the tail artery catheter using a sterile syringe conditioned with heparin. After each blood collection the animals were injected intravenously with 100 μl of 0.9% saline. The blood was centrifuged at 10,000 g for 5 min, and rGOT plasma levels were determined immediately using a Reflotron Plus apparatus and Reflotron sticks.

Animals and Sporadic ALS Model

All experiments were conducted according to the Guidelines for the Use of Experimental Animals of the European Community approved by the Animal Care Committees of the Weizmann Institute of Science. In this study, we used a rat model of sporadic ALS that was developed and characterized by Sun et al. [5] based on a long-term activation of AMPA receptors through the continuous co-infusion into the spinal subarachnoid space of KA, an AMPA receptor agonist, and CTZ, an allosteric modulator of AMPA receptors that prevents their desensitization. These rats displayed a progressive motor-selective behavioral deficit with delayed loss of spinal motor neurons, mimicking the clinical pathological features of ALS. A total of 38 Wistar male rats (8–9 weeks old, 240–250 g; Harlan Laboratories, Israel) were used in this study (6 naïve rats for the pharmacokinetic study and rotarod test control, 14 rats in the histology experiment and 18 rats in the survival experiment) and let to acclimate for at least 5 days before starting the experiment. Throughout the whole study, the animals were housed in a limited access rodent facility, keeping 2 rats in each cage, and after osmotic pump implantation, the animals were housed individually in polypropylene cages (37.5521218 cm). The animals were provided with ad libitum diet and free access to drinkable water. Environmental conditions were automatically controlled and set in order to maintain the temperature at 20–24°C with relative humidity of 30–70%, a 12:12 light:dark cycle and 15–30 air changes/hour within the study room. On the first day of each experiment the animals were randomly divided into two groups: control and treated.

Preparation of Osmotic Minipump

In detail, 0.375 mM KA plus 0.375 mM CTZ dissolved in artificial CSF solution were administered using an osmotic minipump (see below). An infusion cannula was prepared by connecting a 1-cm-long polyethylene tube (PE 60) to a 5-cm-long small caliber tube (PE 10) using an adhesive. An osmotic minipump was weighed before and after being filled with the KA/CTZ solution in order to calculate the volume of the stored solution. The PE 60 end of the infusion cannula was connected to the shorter end of a flow moderator of the pump. The longer end of the flow moderator was inserted into the pump. The pumps were filled with 200 μl of KA and CTZ and administered intrathecally at a rate of 1 μl/h chronically. The solution-filled pump was incubated in sterile saline at 37°C overnight at least 6 h before the implantation.

Surgery

Pentobarbital (60 mg/kg) was administered intraperitoneally to the rats, and the adequate depth of anesthesia was confirmed by the absence of corneal reflexes. The skin over the fifth lumbar spinal process was incised, and the paravertebral muscles were separated from the vertebral lamina with scissors and retracted with a retractor. The lamina of the fifth lumbar vertebra (L5) was removed with a micro-rongeur under the binocular view of a surgical microscope. The dura mater was exposed for the insertion of an infusion cannula (approx. 2–3 mm in diameter). A small subcutaneous pocket was made over the sacral vertebrae caudal to the incision, and an ALZET osmotic minipump was placed in the pocket with the cannula end facing the incision. The infusion cannula was cut to an appropriate length to allow its insertion into the subarachnoid space 1–1.5 cm rostral. The dura mater was cut with fine spring scissors, and the infusion cannula was inserted into the subarachnoid space under the binocular view of a surgical microscope. The infusion cannula was then attached to the fascia over the paravertebral muscles with a nylon string, a drop of adhesive for surgery was applied and the muscles and the skin were sutured.

Study Design

The treated and control groups were infused with KA/CTZ in artificial CSF for 14 days for histology analysis or up to 36 days for survival experiments. The day after the implantation, the treated group was injected subcutaneously with a priming dose of 0.39 mg/rat of rGOT in 200 μl saline followed by a daily maintenance dose of 0.135 mg/rat of rGOT in 200 μl saline. The control group was injected subcutaneously with 200 μl saline administrated daily until the animals were sacrificed or died. Starting on day 1 after the implantation until the termination of the experiment, the rats had access to drinkable water consisting of 0.2 M OxAc in water. The pH of OxAc was adjusted to 7.0–7.4 using 0.5 M NaOH solution. Since the OxAc solution included Na$^+$ molecules originated from the neutralizing NaOH, the control group was provided with NaCl (0.3 M) solution in the drinkable water and injected subcutaneously daily with 200 μl saline until the end of the experiment. The minipump was replaced every week with freshly prepared KA/CTZ or artificial CSF solutions. At day 14 of the treatment, the animals for histology analysis were perfused, and the spinal cords were used for immunohistochemistry. Animals that did not sur-

vive the first 24 h after pump implantation were excluded from the study. At the end of each experiment the catheter location was visually examined, and animals with a dislocated infusion catheter were also excluded from the study.

Evaluation of Movement Behavior

Throughout the study, the movement behavior of the rats in both experiments was measured twice a week by using an Ugo Basile 7650 accelerating rotarod (20 rpm) that automatically recorded the time that the rats could stay on the rotarod (Linton Instruments, UK). Rotarod pretraining for 10 days was performed before the implantation in order to obtain a baseline for rotarod performance. At the end of the training period all rats remained at least 180 s on the rotarod. Starting from the second day after the implantation or the osmotic pump replacement, the motor performance of the rats was measured in a blinded manner. The time that each rat remained on the rotarod was recorded (latency to fall). The maximum latency was arbitrarily set to 180 s. Each test was repeated 3 times, and the longest time spent on the rotarod data/results was used for the statistical analysis. For the experiments described in figure 4b, the group of 6 naïve rats served as a standard. This group was also pretrained for 10 days and tested twice each week for a period of about 2 months.

Assessment of Life Span

The age of death was set to be the time in which the animal was unable to right itself by 30 s after being placed on its back.

Histology

The animals were anesthetized and perfused transcardially with cold phosphate-buffered saline followed by cold 4% paraformaldehyde. Each spinal cord and the associated nerve roots were dissected en bloc, fixed in 4% paraformaldehyde overnight and processed for paraffin embedding. Twenty serial sections at the levels L1–L5 (6 μm thick) from each spinal cord were stained with Nissl and the modified Bielschowsky methods [32] and examined using a Nikon Eclipse E800 microscope with an attached Nikon DMX 1200 digital camera. ImagePro Plus software (Media Cybernetics, Silver Spring, Md., USA) was used for quantitative analysis. Five sections in each series were used for histological analysis after each staining. Motor neurons were defined as cells present within the gray matter, ventral to the central canal and having a cell body >25 μm in diameter. All sections were collected at the same level across animals. The number of neurons per 100 mm² was calculated in 10 microscopic fields of each sample (objective magnification 40×). A total of 70–100 motor neurons were measured for each spinal cord specimen. Only motor neurons having a distinct nucleus were counted, and their area was measured using ImagePro Plus software. All morphometric analyses were performed in a blinded manner. Data are presented as the mean ± SE of the number of motor neurons per group.

Results

A total of 49 animals were used for the current study, from which 38 animals were included; 6 rats (12.2%) died within the first 24 h after surgery, and 5 rats (10.2%) – 3
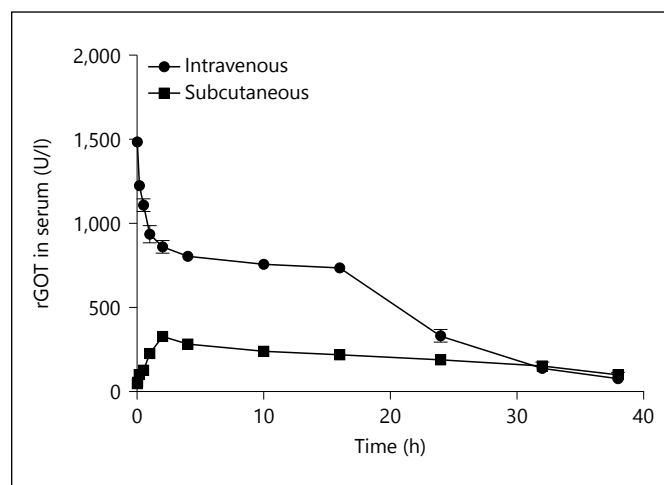

Fig. 1. Pharmacokinetic properties of rGOT. rGOT was injected intravenously or subcutaneously, 0.39 mg rGOT/rat, as described in Methods. Blood aliquots were collected at different time points and the level of blood GOT was measured using a Reflotron Plus apparatus and Reflotron sticks. Each time point represents the mean value ± SD of 3 rats.

in the treated group and 2 in the control group – were excluded due to incorrect location of the infusion cannula. A total of 6 naïve rats were used for the rotarod test control and afterwards for the short pharmacokinetic experiment of rGOT.

OxAc/rGOT Treatment Rescues Spinal Motor Neurons and Improves Motor Performance in an ALS Rat Model

Sporadic ALS was induced using the basic model of Sun et al. [5]. Accordingly, a kainite and AMPA/NMDA receptor agonist KA was combined with CTZ (0.375 mm each), which is an allosteric modulator of AMPA receptors that prevents their desensitization. The treatment with rGOT and OxAc started 1 day after the ALZET minipump implantation. In order to achieve chronically elevated levels of rGOT in the blood, a pharmacokinetic study aimed to determine the half-life time of rGOT in the blood after either intravenous or subcutaneous administration. In this experiment, 6 naïve rats, 3 in each group, were injected intravenously or subcutaneously with 0.39 mg rGOT/rat in 200 μl saline. Blood aliquots were collected starting at time 0 and up to 38 h, and blood rGOT levels were determined. Analysis of the data (fig. 1) revealed that the intravenous administration of rGOT increased blood rGOT levels within 2 min to a maximal concentration of 1,485 U/l and quickly de-

creased, showing a half-life time of 16 h. In contrast, the subcutaneous administration of rGOT increased blood rGOT levels to a maximum of 333 U/l (3-fold higher than the baseline level of the enzyme in the blood) after 240 min and gradually reduced, showing a half-life of 31.5 h. On the basis of the latter data, we decided to use subcutaneous administration, which results in a moderate but stable increased level of rGOT in the blood. Given that the half-life of rGOT in the blood after subcutaneous administration was greater than 24 h, we were able to perform daily injections throughout the experiment. Also, the long half-life prompted us to use a maintenance dose that was one third of the bolus injection, with the aim of maintaining a constant 3-fold level of rGOT over basal blood levels. The doses of the enzyme and the treatment regimen were designed according to our previous study in glioma animal models [10]. In this regard we took into account the enzymatic activity of rGOT in our protein preparation and combined it with our preliminary in vitro experiments where we have shown the effectiveness of the combined administration of OxAc with rGOT on the level of Glu in the blood (see online suppl. material; for all online suppl. material, see www.karger.com/doi/10.1159/000382034). To this end, the single priming dose of 0.39 mg rGOT/rat was followed by a daily maintenance dose of 0.135 mg rGOT/ rat in 200 µl saline.

To evaluate the potential beneficial effect of OxAc/ rGOT treatment in ALS our experimental setup included two groups of 7 rats. Both groups were provided with daily prepared drinking water containing either 0.2 M OxAc in combination with subcutaneous injection of rGOT (treated group) or drinking water containing 0.3 M NaCl in combination with subcutaneous injection of saline (control group) for 14 days. Motor performance was monitored on days 4, 7 and 12 after cannulation using a rotarod test. The animals were sacrificed after 14 days, and their spinal cords were harvested for histology analysis. We found that OxAc/ rGOT treatment prevented the deterioration in motor performances of the rats (fig. 2). At day 7 the average score of motor performance was 40 ± 6 s in the control group compared to 117 ± 5 s in the treated group, and at day 12 the average was 21 ± 3 s in the control group compared to 100 ± 5 s in the treated group. The slightly better motor performance at day 4 compared to day 7 in the treated group is probably a result of recovery from surgery.

The improved motor performance of the treated group was confirmed by histology. We showed that in-
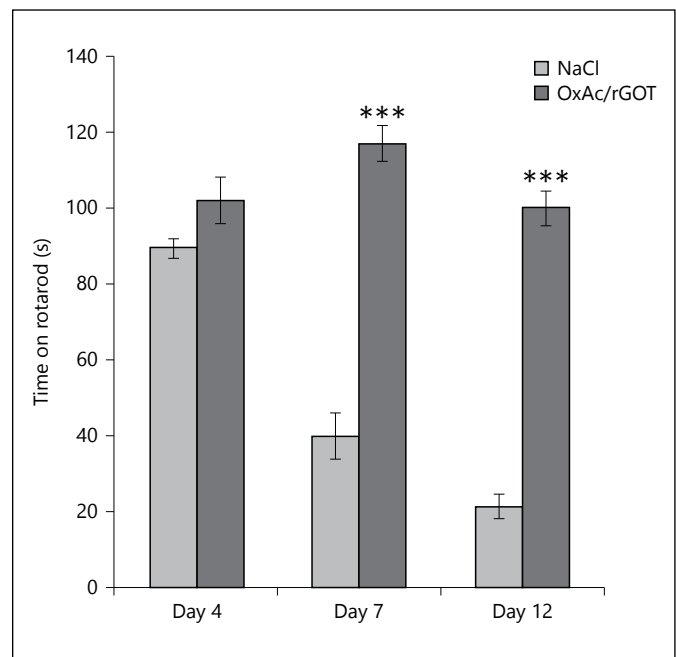

Fig. 2. Motor performance was assessed on the rotarod. Control animals were given 0.3 M NaCl as drinking water (n = 7). The water of the treated group was supplemented with 0.2 M OxAc, and the rats were injected with 0.39 mg/rat of rGOT for bolus followed by a daily maintenance dose of 0.135 mg/rat (n = 7). The statistical analysis was performed using ANOVA and Dunn's multiple comparison tests. Data show the average time spent on the rotarod ± SEM of the rats at 4, 7 or 12 days after sporadic ALS stimulation. *** p < 0.001.

trathecal infusion of KA/CTZ (0.375 mM each) induced selective death of spinal motor neurons as expected (fig. 3a; Silver stain, control group). Treatment with OxAc/rGOT induced protection of the motor neurons, as shown by the significant difference in the size and number of neurons; in the control group motor neurons were significantly smaller (38%) and fewer (88 vs. 141) compared to the treated group (fig. 3b, c). In addition, the control group that was injected with saline showed axonal damage, as revealed by Bielschowsky staining (fig. 3a; Silver staining). Interestingly, the motor neurons from the control rats were pathologically similar to those in animals treated with OxAc/rGOT (fig. 3a: the two pictures in the right panel compared to those at the top). However, it should be mentioned that no statistical analysis was done on the naïve group because of the small number of animals in this group. Thus, OxAc/rGOT produced a significant protection of the spinal cord motor neurons and provided increased resistance to the loss of motor ability.
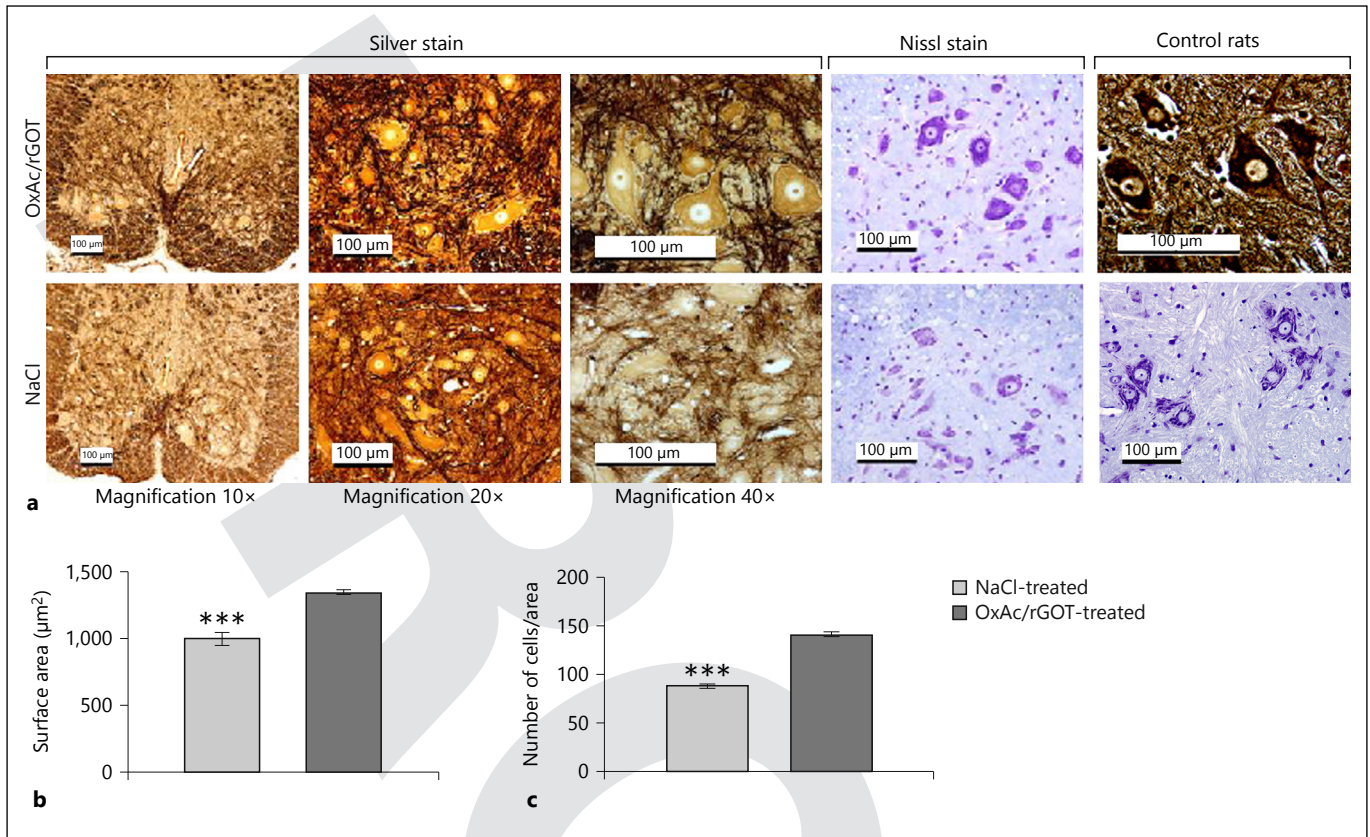
Fig. 3. Histology and morphology of motor neurons in the spinal cord of the KA/CTZ-treated rats. **a** Silver and Nissl stains of spinal cord sections at levels L1–L5 from rats that had access to water prepared as 0.2 M OxAc solution in combination with subcutaneous injection of rGOT (treated group, upper panel) or as 0.3 M NaCl solution in combination with subcutaneous injection of saline (control group, lower panel). In the upper panel, motor neurons are clearly identified by the presence of a white nucleus (Nissl and Silver staining) and black axons (Silver staining), which are almost absent in the lower panel. Silver and Nissl staining of naïve animals is shown in the 2 panels on the right. **b** Motor neuron av- erage area of 5 sections/rat expressed in micrometers squared is shown in NaCl- and OxAc/rGOT-treated rats, respectively. **c** Numbers of intact motor neurons are shown in NaCl- and OxAc/ rGOT-treated rats, respectively. Quantification of the motor neurons from Nissl-stained sections. In the panel only motor neurons having a distinct nucleus were counted and their area was mea- sured using ImagePro Plus software (n = 7 rats/group, unpaired Student's t test). *** p < 0.001. For each sample the number of neu- rons per 100 mm² was calculated in 10 microscopic fields. Objec- tive magnification. ×40.

OxAc/rGOT Treatment Improves Motor Performances and Survival of Sporadic ALS Rats

In order to evaluate the neuroprotective effect of long-term OxAc/rGOT treatment, 18 rats (9 in each group) were treated daily for up to 36 days, and their motor performance and survival were monitored. Figure 4a illustrates the time-dependent decline of motor performance of the rats treated with either NaCl or OxAc/rGOT, as assessed by the rotarod sensitive motor task. Data showed that the rats treated with OxAc/rGOT performed better than those treated with NaCl at different time points (p < 0.01). In order to quantify the differences between the two treatments presented in figure 4a, we measured, using the trapezoidal method, the area under the curve (AUC) for each individual rat and calculated the average (fig. 4b). The AUC is a function of the decline of motor performance over time and was normalized to the AUC of 6 rats in the naïve group. This group was trained and analyzed in parallel to the treated animals and stayed on the rotarod for at least 180 s. Figure 4b demonstrates that the rats treated with OxAc/rGOT lost only about 20% of their motor skills compared to the loss of 62% in the NaCl-treated rats.

In this model of sporadic ALS the rats treated with NaCl displayed not only a significant loss of motor ability but also a reduced survival. Figure 5 shows the survival
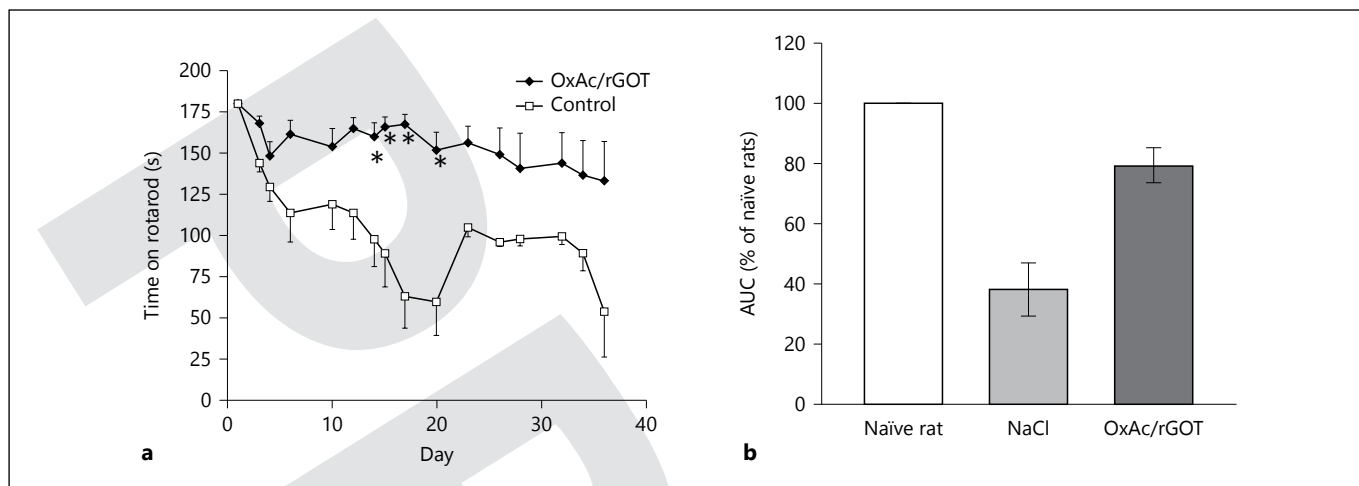
Ruban/Cohen-Kashi Malina/Cooper/
Graubardt/Babakin/Jona/Teichberg

Fig. 4. Motor performance of chronic treated animals vs. control. **a** Motor performance was assessed on the rotarod. Control animals were given 0.3 M NaCl as drinking water (n = 9). The water of the treated group was supplemented with 0.2 M OxAc, and rats were injected with 0.39 mg/rat of rGOT for bolus followed by a daily maintenance dose of 0.135 mg/rat (n = 9). Data show the average time spent on the rotarod. An unpaired Student t test was used to assess the significance of the differences between the 2 groups of animals (p < 0.01). **b** Quantification of the shown data by measurement of AUC. For each individual rat tested on the rotarod the AUC was calculated; all AUCs were averaged and normalized to the AUC for naïve rats that ran on the rotarod for time periods which exceeded 180 s (which we set as 100%).
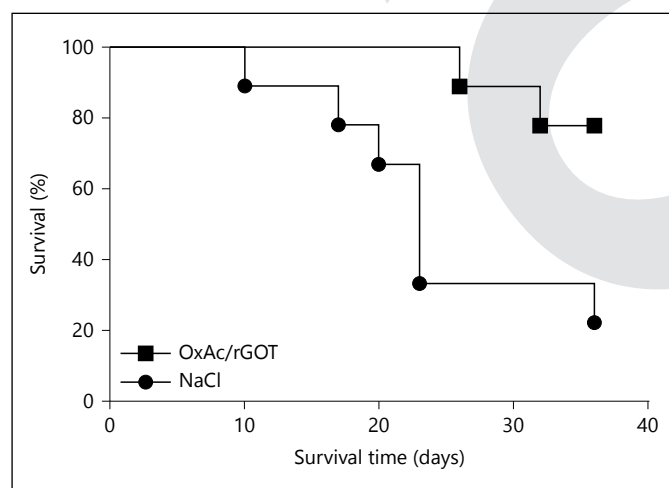

Fig. 5. Kaplan-Meier survival curve for control rats vs. rats treated with OxAc/rGOT. Control animals were given 0.3 M NaCl as drinking water (n = 9). The water of the treated group was supplemented with 0.2 M OxAc, and the rats were injected with 0.39 mg/rat of rGOT for bolus followed by a daily maintenance dose of 0.135 mg/rat (n = 9). The log-rank test shows a p value <0.008.

curve calculated using the Kaplan-Meier method (log-rank test p value <0.008). In the control group only 22% of the animals survived the whole length of the experiment compared to 78% of the treated rats. Whereas most of the rats in the control-treated group (6/9) died by day 23 after ALS induction, in the OxAc/rGOT-treated group the first animal died only on day 26. Moreover, whereas more than half of the OxAc/rGOT animals were able to run on the rotarod close to 180 s, the 2 surviving control animals were able to run for less than 90 s.

Discussion

In the past years we have developed the novel neuroprotective treatment termed BGS, which has been shown to reduce the levels of the Glu in the blood, which in turn results in an efflux of excess Glu from the CNS into the blood [10, 16, 29, 30]. The mechanism of this process has been shown both in vitro as well as in various animal models where Glu levels were reduced in CNS upon BSG treatment.

Based on the increasing evidence that there is an elevation in the level of CSF Glu in ALS patients, which was also shown to correlate with the onset and progression of the disease [4, 22–25], we sought to test the efficiency of the BSG technology. Specifically, our aims were to test whether we could inhibit the onset and the progression of disease in rats with induced sporadic ALS, delay the deterioration of the motor performance and increase their life expectancy. To this end we chose to use the protocol described by Sun et al. [5]. The selective motor behavioral

and neuropathological changes induced in this sporadic ALS rat model are hallmarks of the clinicopathological changes seen in ALS patients. In addition, the delayed and progressive nature of these changes mimics ALS and thus suggests that the KA/CTZ-infused rat is an appropriate model for sporadic ALS.

Although 90% of ALS patients suffer from sporadic ALS, only limited literature exists about the etiology and pathogenesis of such form of the disease. However, a set of clinical observations suggested that Glu excitotoxicity contributes to the progress of the disease. Based on these studies, in vivo models of progressive spinal motor neurons death caused by overactivation of Glu/AMPA receptors have been developed as a model of sporadic ALS. The findings about the abnormal Glu homeostasis and Glu transporter dysfunction in humans with ALS are, to some degree, confirmed in animal models of the disease. In all SOD1 mouse transgenic models of ALS, including the G85R, G37R and G93A mutants, a large reduction in the EAAT2 (GLT-1) Glu transporter was observed compared to control animals [33]. In fact, EAAT2 has been shown to be a target of mutant SOD1-mediated oxidation [34], which causes a reduction of its Glu transporter capacity. These results suggest that the induction of Glu excitotoxicity is sufficient to cause ALS-like symptoms. If Glu excitotoxicity is indeed involved in human sporadic ALS, it might be of high therapeutic relevance when aiming to eliminate this negative contribution to the disease process. The modest success of riluzole [3], a Glu release inhibitor (among other functions, to delay the death of ALS patients) supports the concept that an efficient elimination of excess Glu, present in the brain and spinal cord interstitial fluids of ALS patients, can be clinically significant.

The results presented in figure 3b and c confirmed that the protocol of Sun et al. [5] indeed leads to a motor neuronal disease. We found that both the size of the motor neurons as well as their number was significantly reduced in the control animals. Based on the excitotoxicity theory described above, the dying neurons release their cell content, including Glu, into the CSF, thereby increasing its concentration to the neurotoxic level. Given the complexity of the ALS model used in this study, we avoided measuring the levels of Glu in the blood and the CNS, which would have forced us to perform additional surgical intervention and anesthesia. Moreover, the animals displayed a significant motor deficit and decreased survival as shown in figures 4 and 5. In contrast, the animals treated with BGS displayed a highly significant neuroprotection that was manifested by the preservation of motor neuronal axons, size and number of cells, relative conser-vation of motor performance, and increased survival. Interestingly, whereas in the treated animals 4 of the 7 surviving rats had a motor performance close to naïve animals, the 2 surviving untreated rats showed less than 50% motor performance, which supports our conclusion that the treatment has a strong neuroprotective outcome in this ALS model. It has previously been proven by Perez-Mato et al. [16] that OxAc in combination with rGOT significantly reduced the blood and brain Glu levels. Thus, it is reasonable to conclude that the elimination of the Glu component in the experimental ALS model, achieved by the treatment with BGS, can contribute in a significant and positive manner to the disease outcome. Our data are in line with the suggestion that excess Glu has a significant role in the contribution to motor decline and eventual death in these ALS models. Even though we demonstrated in this study that the treatment of rats with human GOT resulted in enhanced neuroprotection, we cannot rule out a rat anti-human immune response towards the human GOT that was injected. Nonetheless, if an immune response indeed took place, we could predict that the effectivity of the human GOT should be reduced. Hence, it is reasonable to postulate that the use of the rat GOT protein in a rat model may elicit a stronger neuroprotection effect and/or needs lower quantities of the GOT enzyme to provide a similar neuroprotection effect, which is a hypothesis that we plan to test.

Corresponding to our results, Park et al. [35] treated ALS G93A mice with weekly injections of pyruvate, another BGS. However, in this study the authors did not investigate the neuroprotective mechanism of pyruvate but proposed that pyruvate antioxidative effect offers a plausible explanation. We suggest that a possible neuroprotective mechanism of pyruvate could be explained by blood Glu scavenging activity, which causes the elimination of excessive Glu from the brain. Intravenous pyruvate has been shown to activate the blood plasma resident enzyme Glu-pyruvate transaminase, to cause a decrease of blood and brain Glu levels (though not as effectively as OxAc) and to display neuroprotective properties in animal models of traumatic brain injury [30, 36] and permanent ischemia [37].

It is relevant to point out that the blood basal GOT levels in the rats were in the range of 80–120 U/l, whereas the range in humans is 7–35 U/l (http://www.bloodbook. com/ranges.html). The reduced levels of GOT in human blood highlight the compulsory need for a supplementation of rGOT in order to effectively treat any human disease in the future with BGS. Another important clinical consideration is that in the current study the treatment regimen may not be optimal since oral OxAc is subjected

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to a first-pass metabolism in the liver as well as to an absorption into the gut wall on its way to the portal vein, with a consequent significant reduction in concentration.

The clear advantage when using the BSG technology that we developed is that both the administration and the site of its action are the peripheral rather than the central CNS. The subcutaneous or intravenous administration of OxAc/rGOT only transiently affects the Glu levels in the blood but helps to create a gradient between the levels of Glu in the blood and in the brain, with a consequent rapid efflux of excess Glu from the CNS to the blood without affecting any other CNS function [38]. As plasma Glu levels normally fluctuate by approximately 50% during the circadian cycle, it is likely to speculate that in the transition of the BGS technology into clinical trials no severe side effects are expected [39].

To conclude, BGS is a treatment that avoids the injection of materials into the brain or the nervous system, yet exploits the presence of a balance of Glu in the blood stream and the brain. By neutralizing the excess of Glu in the blood a gradient of Glu between the blood and parts of the nervous system is formed, and Glu moves from the nervous system into the blood. Although Glu scavenger treatment is not expected to cure ALS, it might contribute to the alleviation of some of the symptoms and decrease the severity and rapid evolution of the disease by preventing the catastrophic cycle of events that lead to the massive destruction and death of neuronal cells.

Disclosure Statement

There is no duality of interest to declare.

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