Glucose clearance and uptake is increased in the SOD1$_{G93A}$ mouse model of amyotrophic lateral sclerosis in an insulin-independent mechanism

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Abstract

Metabolic disturbances are associated with the progression of the neurodegenerative disorder, amyotrophic lateral sclerosis (ALS). In this study we aimed to elucidate deficits in energy homeostasis in the SOD1$_{G93A}$ mouse model of ALS and tissue specific changes in the periphery that underlie these perturbations. We found that at the mid-symptomatic stage of disease, SOD1$_{G93A}$ mice had an increase in oxygen consumption, and faster exogenous glucose uptake, despite no change in insulin sensitivity. Fasting glucose homeostasis was disturbed, along with changed concentrations in the glucoregulatory hormones, insulin and glucagon. Finally, the metabolic gene expression profile in the liver of SOD1$_{G93A}$ mice, and increase in glycogen stores indicate that glucose cannot be utilised efficiently. Overall, we found that glucose homeostasis and uptake is altered in SOD1$_{G93A}$ mice at the mid-symptomatic stage, potentially due to the increase in insulin-independent glucose uptake and disturbance in glucagon sensitivity.

Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder which is characterised by the progressive loss of motor neurons from both the cortex and spinal cord [1]. This loss of motor neurons leads to symptoms that are associated with ALS including muscle weakness, reduced motor control and the denervation and atrophy of skeletal muscle [1, 2]. ALS is a heterogeneous disease with the site of symptom onset varying amongst patients, and is known to have a complex etiology, with the exact cause of disease onset still unknown [3, 4]. However, disturbances in several pathways including dysfunctions in energy metabolism have been associated with the progression of ALS [5, 6].

Both sporadic and familial ALS patients are unable to maintain their body weight, with a loss of both fat and muscle mass shown early in the disease progression [7-9]. Rapid weight loss in patients is associated with worse disease outcomes, whilst conversely, a higher body mass index tends to increase survival rate [10, 11]. Evidence also suggests that insulin resistance plays a role in disease progression in both patients and animal models of ALS. With studies finding increased risk of ALS in people with diabetes metillus, and several animal models displaying reduced sensitivity to exogenous insulin [12-16]. However, there are also conflicting reports that have shown that premorbid type II diabetes delays the onset of symptoms in ALS patients, and some studies in both patients and mouse models have shown glucose tolerance to be unchanged [13, 17-20].

Many studies have mainly focussed on assessing how changes in glucose tolerance and insulin resistance in ALS affect metabolism in skeletal muscle and spinal cord [2, 13, 14, 17, 21]. In this study we aimed to elucidate the changes in whole body glucose homeostasis and metabolism in ALS using the SOD1$_{G93A}$ mouse model, and identify some of the underlying disturbances in tissues such as the liver and pancreas, that are key drivers of regulating glucose

homeostasis. Overall, we aimed to find the underlying mechanisms that result in the metabolic phenotype observed during disease progression of ALS.

Results

SOD1$_{G93A}$ mice displayed loss of body weight and lean body mass with decreased activity and increased oxygen consumption at mid-symptomatic stage of disease.

We first aimed to delineate if the weight loss frequently observed in SOD1$_{G93A}$ mice was due to the reduction in food intake or increase in energy expenditure using indirect calorimetry. At disease onset, SOD1$_{G93A}$ mice showed no difference in body weight but a 10% loss in lean body mass when compared to wild-type (WT) littermates. Furthermore, at mid-symptomatic stage, SOD1$_{G93A}$ mice weighed significantly less than their WT littermates, with an 8 and 10% loss in total body weight and lean body mass respectively (Figure 1A and B). Despite this observed loss in body weight, total food intake was similar between WT and SOD1$_{G93A}$ mice during the light and dark cycles at both disease stages (Figure 1C). In contrast to the food intake, the average oxygen consumption in SOD1$_{G93A}$ mice at the mid-symptomatic stage was 121 and 113% higher than WTs during the light and dark cycles, respectively, while showing no differences at onset stage (Figure 1D – 1G). This increase in oxygen consumption at mid-symptomatic stage was not due to an increase in locomotor activity, as we showed a reduction of 18 and 25% in locomotor activity during the dark cycle at the onset and mid-symptomatic stages, respectively (Figure 1H – 1K). Interestingly, during the light phase, mid-symptomatic SOD1$_{G93A}$ mice were 126% more active than their WT littermates. Despite these biphasic changes, total locomotor activity over a 24-hour period was not significantly different at any time point (Figure 1I and 1K). Although we saw differences in the lean body mass between WT and SOD1$_{G93A}$ mice, no correlation was found between lean body mass and the average oxygen consumption over a 24-hour period (Figure 1L).

Exogenous glucose uptake is increased in SOD1$_{G93A}$ mice at mid-symptomatic stage of disease

We next aimed to determine if glucose handling was altered in the SOD1$_{G93A}$ mice by performing an intraperitoneal glucose tolerance test (ipGTT). At the onset of symptoms, SOD1$_{G93A}$ mice and their WT littermates responded similarly to exogenous glucose (Figure 2A-C). However, at mid-symptomatic stage of disease, SOD1$_{G93A}$ mice showed a faster rate of blood glucose clearance, with blood glucose levels being 33 to 50% lower in SOD1$_{G93A}$ mice at 15, 30, 60- and 120-minutes post glucose injection when compared to WT mice (Figure 2D). This difference is further demonstrated with a 46% drop in the area under the curve (AUC) of blood glucose levels (Figure 2D). Due to the reduction in body weight of SOD1$_{G93A}$ mice at mid-symptomatic stage, we showed that this reduction in the AUC of blood glucose levels did not correlate with the amount of glucose injected in these mice (Figure 2E and 2F). We also showed that females had a similar ipGTT response to males, where SOD1$_{G93A}$ mice at the mid-symptomatic stage had a 21% lower AUC of glucose concentration (Figure 2 – figure supplement 1). To further validate this increase in blood glucose clearance, we intraperitoneally injected WT and SOD1$_{G93A}$ mice with 2-deoxyglucose. Using LC-MS/MS, we found that 2-deoxyglucose uptake was 260 to 1040% higher in liver, brown and white adipose tissue of SOD1$_{G93A}$ mice (Figure 2G). To elucidate the changes in glucose clearance of SOD1$_{G93A}$ mice, we also measured plasma insulin and glucagon levels. Baseline plasma glucagon concentrations were 240% higher is SOD1$_{G93A}$ mice compared to their WT littermates at the mid-symptomatic stage (Figure 2H). Although baseline insulin concentrations were unchanged, the plasma insulin response to exogenous glucose was significantly lower in SOD1$_{G93A}$ mice, with a 44% reduction in AUC of insulin concentrations (Figure 2I). To confirm this reduction in insulin response, we also performed immunofluorescence of glucagon and insulin in the pancreas of WT and SOD1$_{G93A}$ mice. We showed 22% reduction in the

immunoreactive area of insulin-positive β-cells in the pancreas of SOD1$_{G93A}$ mice when compared to WT mice at mid-symptomatic stage (Figure 3C), while there was no difference in the immunoreactive area of glucagon-positive α-cells at onset and mid-symptomatic stage of disease (Figure 3D and E).

Insulin tolerance is unaffected in SOD1$_{G93A}$ mice, despite a loss in fasting blood glucose concentrations

After a week of recovery from ipGTT, an intraperitoneal insulin tolerance test (ipITT) was performed on the same mice to determine insulin tolerance. At onset of disease, the response to exogenous insulin in SOD1$_{G93A}$ mice was similar to that of their WT littermates (Figure 4A-C). Interestingly, SOD1$_{G93A}$ mice had lower baseline blood glucose concentrations compared to WT mice following a 5-hour fast at mid-symptomatic stage (Figure 4D). Despite this difference in baseline blood glucose concentrations both WT and SOD1$_{G93A}$ mice responded similarly to insulin, as shown by the inverse AUC of blood glucose levels (Figure 4D). We also found changes in the blood hormone profile during the ipITT, with plasma glucagon concentrations trending higher at every timepoint measured, resulting in an overall 210% increase in AUC of glucagon concentrations for the SOD1$_{G93A}$ mice (Figure 4E). Although a trend of decreased basal insulin concentrations was observed in the SOD1$_{G93A}$ mice, no significance was observed (Figure 4F). Similarly, female mice at mid-symptomatic stage also did not show any difference in their ipITT response, but unlike the males no change in fasting blood glucose concentrations was found (Figure 4 – figure supplement figure 1). Despite SOD1$_{G93A}$ mice weighing less at both the onset and mid-symptomatic stage, the amount of insulin injected had no correlation with the inverse AUC of blood glucose levels (Figure 4H).

Genes associated with glucose handling are altered in the liver of SOD1$_{G93A}$ mice

In addition to insulin and glucagon levels, we demonstrated that glycogen concentrations were 210 and 480% higher in the liver of SOD1$_{G93A}$ mice at both onset and mid-symptomatic stages of disease, respectively (Figure 5B). Following an overnight fast glycogen accumulation in the liver was still 400 to 500% higher in mid-symptomatic SOD1$_{G93A}$ mice in comparison to their WT littermates (Figure 5B). To understand if this increase in glycogen was due to changes in the metabolic pathways in the liver, we conducted a metabolic PCR array consisting of 84 genes associated with glucose metabolism on livers from WT and SOD1$_{G93A}$ mice at mid-symptomatic stage. The expression of twenty-four of these genes were significantly different between SOD1$_{G93A}$ and WT mice, and are involved in one of four distinct pathways of glucose metabolism (Figure 5A). Firstly, two isoforms of phosphoglucose mutase, a reversible enzyme that catalyses glucose 6-phosphate to glucose 1-phosphate are altered in SOD1$_{G93A}$ mice, where *Pgm1* expression is significantly reduced by 0.7-fold while *Pgm3* expression is increased by 1.3-fold when compared to WT mice. In the glycogenesis pathway, UDP-glucose pyrophosphorylase 2 (*Ugp2*, 0.74-fold) and glycogen branching enzyme (*Gbe1*, 0.7-fold) expression are reduced in SOD1$_{G93A}$ mice, whereas an isoform of glycogen synthase (*Gsk3a*, 1.6-fold), which inhibits glycogen synthase activity, is increased in SOD1$_{G93A}$ mice. Overall, this indicates that the glycogenetic pathway is decreased at the mid-symptomatic stage of disease. The expression of the liver isoform of glycogen phosphorylase (*Pygl*, 0.54-fold), which regulates glycogenolytic pathway is also reduced. However, the expression of three isoforms of phosphorylase kinase (*Phka1*, 1.9-fold; *Phkb*, 1.6-fold; *Phkg2*, 1.4-fold), that activate glycogen phosphorylase are higher in the liver of SOD1$_{G93A}$ mice (Figure 5C).

Genes associated with mitochondrial metabolism are also altered in the liver of SOD1$_{G93A}$ mice at the mid-symptomatic stage of disease. Two of the three enzymes that comprise the pyruvate dehydrogenase complex, pyruvate dehydrogenase E1 subunit β (*Pdhb*, 0.7-fold) and

dihydrolipoamide S-acetyltransferase (*Dlat*, 0.8-fold), were lower in the SOD1$_{G93A}$ mice when compared to WT mice. Conversely, the expression of an isoform of pyruvate dehydrogenase kinase (*Pdk2*, 0.55-fold) which inhibits pyruvate dehydrogenase was reduced in SOD1$_{G93A}$ mice, while pyruvate dehydrogenase phosphatase (*Pdpr*, 1.5-fold) that activates pyruvate dehydrogenase is increased in SOD1$_{G93A}$ mice (Figure 5D). In contrast to the other pathways, the genes associated with TCA cycle activity were elevated in the liver of SOD1$_{G93A}$ mice. Most of the enzymes in TCA cycle are upregulated in the liver of SOD1$_{G93A}$ mice, including aconitase (*Aco1*, 1.8-fold; *Aco2*, 1.9-fold), isocitrate dehydrogenase (*Idh2*, 1.6-fold), oxoglurate dehydrogenase (*Ogdh*, 1.9-fold), and an isoform of malate dehydrogenase (*Mdh2*, 2.4-fold). Only the two subunits of succinate dehydrogenase (*Sdha*, 0.8-fold; *Sdhb*, 0.78-fold) and another isoform of malate dehydrogenase (*Mdh1*, 0.65-fold) were reduced in the SOD1$_{G93A}$ mice (Figure 5E). Overall, this indicates that TCA cycle activity may be increased in the liver of SOD1$_{G93A}$ mice at mid-symptomatic stage of disease. Taken together, this suggests that glycogenesis/glycogenolysis and mitochondrial metabolism pathways are decreased, while TCA cycle is increased in the liver of SOD1$_{G93A}$ mice. In addition, we demonstrated that 4 main clusters with similar gene expression profiles were evident using 34 genes that showed changes in expression between WT and SOD1$_{G93A}$ mouse liver tissues with a p-value less than 0.1 (Figure 5-supplement figure 5A). These genes were analysed in the GEN2FUNC module of the Functional Mapping and Annotation of Genome-Wide Association (FUMA) software. The top 3 significant hallmark pathways (adjusted p-value $< 10^{-10}$) enriched for gene expression included “Oxidative Phosphorylation”, “Hypoxia” and “Glycolysis” (Figure 5-supplement figure 5B).

Discussion

The major finding of this current study is that there is a shift in energy balance in SOD1$_{G93A}$ mice, and this shift in energy expenditure is contributing to the increase in glucose clearance.

This change in glucose clearance could be due to the increase in glucose uptake in the liver and adipose tissue in an insulin-independent manner. We postulated that these changes are insulin-independent as we found no difference in glucose disposal in response to exogenous insulin. Furthermore, we have also demonstrated that SOD1$_{G93A}$ mice have a reduction in insulin-positive β-cell area, and an impairment in the release of insulin in response to exogenous glucose. SOD1$_{G93A}$ mice also showed an accumulation of glycogen in the liver, despite an increase in circulating glucagon concentrations and gene expression data, suggesting a decrease in both glycogen synthesis and breakdown. This indicated that glucagon signalling may be impaired in the liver of SOD1$_{G93A}$ mice. Finally, the gene expression profile of several metabolic enzymes suggested that the liver switches from using glucose to fatty acids as an energy source, which has previously been found in the skeletal muscle and CNS tissues in ALS.

Consistent with many other studies we found that SOD1$_{G93A}$ mice had reduced body weight, when compared to their WT littermates at the onset of disease symptoms. However, overall daily food intake, activity and oxygen consumption were similar between SOD1$_{G93A}$ and WT mice at onset stage, suggesting that the change in energy metabolism is not the driving factor in the initial loss of body weight. Instead at onset of disease symptoms it is likely that a loss in skeletal muscle mass is contributing to the weight loss in SOD1$_{G93A}$ mice, as we see a loss of lean body mass. This is similar to what is found in patients where sporadic and familial ALS patients are unable to maintain body weight of both muscle and fat mass early in disease [7-9]. In comparison at mid-symptomatic stage of disease, oxygen consumption is increased in SOD1$_{G93A}$ mice during both the light and dark phases. This is independent of changes in both food intake and activity, indicating that during disease progression a shift occurs, whereby energy expenditure increases in SOD1$_{G93A}$ mice. Indeed studies have shown that a high fat diet that supplies mice with higher calories increases survival, whilst calorie restriction accelerated

disease progression [22]. Although energy balance does not contribute to the initial loss of body weight, this change in energy balance at the later phase of the disease contribute to disease progression and survival rates.

To establish how alterations in whole-body metabolism contribute to the changes in energy expenditure during disease progression, we next assessed the response to both exogenous glucose and insulin using ipGTT and ipITT. We found that SOD1$_{G93A}$ mice showed a similar response to exogenous glucose as their WT littermates at the onset stage, while, blood glucose clearance was more efficient under fasting conditions in SOD1$_{G93A}$ mice at the mid-symptomatic stage. This was further validated in a separate cohort of mice, where 2-deoxyglucose uptake was higher in both liver and adipose tissues of SOD1$_{G93A}$ mice. These results support previous findings in SOD1$_{G86R}$ males, in which an increased rate of glucose clearance and higher 2-deoxyglucose uptake was observed in white adipose tissue [22]. However, the results of some recent studies using SOD1$_{G93A}$ mice contradict our findings. One study found no difference in glucose clearance across all stages of disease in a combination of males and females, whereas, another two studies found reduced glucose clearance in males [13, 17, 21]. This discrepancy in glucose clearance echoes what is found in ALS patients, with numerous studies showing both reduced and unchanged glucose tolerance [18, 23, 24]. The discrepancy in glucose homeostasis is also observed on tissue levels, where glucose uptake is increased in denervated forearm and skeletal muscle of ALS patients [25, 26]. In contrast, glucose metabolism was reduced in the cerebral cortex, and spinal cords of both patients and animal models [12, 13, 27-29]. This disparity in how glucose uptake and metabolism is changed in individual tissues may underlie the variety of responses we see in whole-body glucose clearance. Overall, changes in glucose tolerance are complex in ALS and still require further investigation.

In contrast to the ipGTT, we found the response to the ipITT was similar between SOD1$_{G93A}$ and WT mice at both stages of disease, indicating that insulin sensitivity is unaltered in these mice. This corroborates one study in ALS patients which found insulin sensitivity was unchanged when both low and high doses of insulin were infused during a euglycemic insulin clamp [30]. In an alternate study, they demonstrated that the glucose infusion rate required to maintain glycemia was significantly lower in ALS patients compared to healthy controls, and thus these patients were considered insulin resistant [12]. Furthermore, in isolated fast twitch skeletal muscles from both SOD1$_{G93A}$ and TDP43$_{A315T}$ mice, insulin-stimulated glucose uptake was decreased [13, 14]. However, at the later stages of disease basal glucose uptake, regulated by GLUT1 instead of GLUT4 was increased in the fast twitch muscle of SOD1$_{G93A}$ mice, suggesting that insulin resistant muscle fibres may be compensating by upregulating pathways involved in insulin-independent glucose uptake [13]. A limitation of our study is that we cannot determine which tissues are specifically transporting glucose in response to exogenous insulin. However, as we see no change in glucose response to insulin, but an increase in glucose clearance in response to exogenous glucose, it suggests that although insulin signalling is intact, insulin-independent glucose uptake is elevated in the SOD1$_{G93A}$ mice at the mid-symptomatic stage of disease.

To find the potential causes of the change in glucose disposal at the mid-symptomatic stage we analysed the concentrations of hormones responsible for maintaining blood glucose homeostasis, insulin and glucagon. At the mid-symptomatic stage of disease there was a reduced spike in plasma insulin concentrations in response to exogenous glucose. Potentially this loss in insulin may be due to the rapid uptake of glucose. Specifically, the change in blood glucose concentrations was not high enough or prolonged enough to stimulate insulin release

from β-cells. However, in other studies it has been shown that ALS patients had a reduced insulin response during an oral GTT, with these studies concluding that there is an impaired synthesis or release of insulin due to pancreatic islet cell damage in ALS [24]. This is further supported by our demonstration of reduced insulin-positive β-cells in the pancreas of SOD1$_{G93A}$ mice, suggesting that insulin synthesis and release is lower in SOD1$_{G93A}$ mice. Together this further indicates that the insulin-independent glucose uptake is upregulated in mid-symptomatic SOD1$_{G93A}$ mice.

In comparison to insulin, glucagon concentrations were both elevated in fasted SOD1$_{G93A}$ mice and in response to the falling blood glucose during the ipITT. In ALS patients it was found that an increase in disease severity correlated with higher circulating glucagon concentrations [31]. Despite increased circulating glucagon, we still saw reduced fasting glucose concentrations in the ipITT. This is similar to ALS patients where they observed an increase in both fasting and postprandial glucagon concentrations, coupled with reduced fasting glucose [32]. Significantly decreased blood glucose concentrations in the presence of higher circulating glucagon suggests that both ALS patients and SOD1$_{G93A}$ mice are glucagon insensitive. This could be caused by a decrease in glucagon uptake or activity in the liver. The primary role of glucagon in the liver is to stimulate glycogenolysis, the breakdown of glycogen to glucose, and the release of glucose into circulation [33]. Interestingly, we see increased glycogen accumulation in the liver even prior to a significant elevation of plasma glucagon. Also, shorter fasting periods are known to rely heavily on the glycogenolytic pathway to maintain blood glucose concentrations, whereas a prolonged fast of over 12 hours relies more on gluconeogenesis as glycogen stores get depleted [34]. This would help to explain why we see a difference in fasting glucose concentrations in the SOD1$_{G93A}$ mice after the 5-hour fast, but not the overnight fast. In this study we are unable to determine the cause of increased circulating glucagon, but it would be

of interest to follow up this avenue to understand how glucagon synthesis, secretion and signalling is changed in ALS.

The liver is crucial for maintaining glucose homeostasis, as it is responsible for both producing glucose during fasting and storing glucose following a meal. Since we see marked changes in glucose homeostasis in SOD1$_{G93A}$ mice we investigated the changes in the expression of genes involved in metabolic pathways in the liver. At the mid-symptomatic stage of disease the gene expression profile indicates that glycogenesis is reduced in the liver. We also found disturbances in glycogen breakdown, with glycogen phosphorylase expression decreased. However, three isoforms of phosphorylase kinase, which activate glycogen phosphorylase, were increased to potentially compensate for the loss of glycogen phosphorylase activity. Despite the activity of both pathways seemingly reduced we see an accumulation of glycogen in the liver from the onset of disease symptoms. Glycogen accumulation has previously been shown in the liver as well as skeletal muscle and spinal cord throughout the course of disease in SOD1$_{G93A}$ mice [35]. As mentioned above, this accumulation of glycogen may be due to a lack of glucagon signalling in the liver of SOD1$_{G93A}$ mice. Alternatively, glycogen may accumulate in the liver as glucose metabolism is impaired with pyruvate entry in the TCA cycle is reduced in the SOD1$_{G93A}$ mice, as the expression levels of two of the three enzymes that form the pyruvate dehydrogenase complex are lower in the liver. Further, the expression of several enzymes involved in the TCA cycle were upregulated in the SOD1$_{G93A}$ mice. This supports findings in other affected tissues that show a switch from using glucose to lipid as the primary fuel source for the TCA cycle [21, 36, 37]. Although the exact trigger that leads to this switch is unknown, it has been proposed that an increase in fatty acid metabolism occurs to compensate for the inability for tissues to use glucose and glycogen as energetic substrates. Chronic reliance on the metabolism of fatty acids via β-oxidation can lead to the accumulation

of toxic by-products, in particular, reactive oxygen species (ROS). An increased production in ROS has been shown to proceed muscle denervation in the tibialis anterior of SOD1$_{G86R}$ mice, however, it has yet to be investigated if elevated ROS production occurs in other peripheral tissues in ALS [37]. Overall, changes in energy substrate usage in the liver and a change in glucoregulatory hormone secretion and signalling may underlie the impairments observed in glucose homeostasis in SOD1$_{G93A}$ mice.

Conclusion

Our findings suggest that glucose homeostasis and whole-body metabolism was relatively unchanged at the onset of symptoms in SOD1$_{G93A}$ mice. However, throughout disease progression we found that glucose uptake was increased in SOD1$_{G93A}$ mice, likely through insulin-independent mechanisms. This increased glucose uptake however is stored as glycogen in tissues such as the liver rather than used as an energy source. Our results also indicate that SOD1$_{G93A}$ mice may develop glucagon resistance, which has been reported previously in ALS. Overall our study found that SOD1$_{G93A}$ mice develop imbalances in glucose homeostasis, and that the secretion and signalling pathways of both glucagon and insulin may be potential therapeutic targets for ALS and need to be further investigated.

Materials and Methods

Ethical Statement

All experimental procedures were approved by the University of Queensland Animal Ethics Committee and complied with the policies and regulations regarding animal experimentation. They were conducted in accordance with the Queensland Government Animal Research Act 2001, associated Animal Care and Protection Regulations (2002 and 2008), and the Australian

Code of Practice for the Care and Use of Animals for Scientific Purposes, 8th Edition. Animal studies are reported in compliance with the ARRIVE guidelines

Animals

Transgenic SOD1$_{G93A}$ mice (B6-Cg-Tg (SOD1-G93A) 1Gur/J) were obtained from the Jackson laboratory (Bar Harbor, ME, USA) and were bred on C57BL/6J background to produce SOD1$_{G93A}$ and wild-type (WT) mice. SOD1$_{G93A}$ mice at two predetermined stages of disease were used for this study; onset (OS, 70 days postnatal), the first sign of motor deficits as a significant decline in hind-limb grip strength and mid-symptomatic (MS, 130 days postnatal), where hind-limbs are markedly weaker and a tremor is observed when mouse is suspended by the tail [38]. The breeding colony was maintained at the University of Queensland Biological Resources Animal Facilities. Mice were housed under 12-hour light/dark cycle with free access to food and water unless otherwise stated.

Assessment of body weight, food intake, activity and metabolism

Mice were singly housed and acclimated to metabolic chamber (TSE systems, Bad Homburg Germany) for 1 week prior to recordings. Animals were continuously recorded for 48 hours, with food intake, locomotor activity (in X and Y axes) and gas exchange (O_2 and CO_2) measured every 30 minutes for using the Phenomaster open-circuit indirect calorimetry system. Our analysis was performed on the final 24 hours of recording to allow mice to acclimatise to the Phenomaster system for the first 24 hours. Mice were maintained on 12 hours light/ dark cycle (on at 0600 h and off at 1800 h) with free access to food and water. Lean body mass of WT and SOD1$_{G93A}$ mice were measured using Bruker Minispec LF50 Body Composition Analyser (7.5 MHz, Bruker Optics, Inc). Specifically, animals were placed in a clear plastic

cylinder and kept immobile by inserting a tight-fitting plunger into the cylinder. The tube was inserted into the sample chamber of the instrument and scanned for approximately 2 minutes.

Glucose/Insulin Tolerance Tests

For glucose/insulin tolerance tests, SOD1$_{G93A}$ mice were housed with WT littermate on purachip bedding. To reduce stress, mice were handled daily for a week prior to tests. Intraperitoneal glucose tolerance tests (ipGTT) were performed after fasting mice overnight for 16 hours. Basal blood glucose levels were measured from a tail bleed using a Freestyle Optium Neo glucometer. Blood was then sampled from tail bleeds 15, 30, 60 and 120 minutes after intraperitoneal injection of glucose (2 g/kg) in 0.9% saline. At each time point approximately 50 µL of tail blood was collected in lithium-heparin coated capillary tubes. The capillary tubes were centrifuged at 2000 x *g* for 10 minutes at 4°C, and plasma collected and stored at -80°C until used.

A week after the ipGTT, intraperitoneal insulin tolerance tests (ipITT) were performed following a similar protocol. Mice were fasted for 6 hours in the morning, with blood collections and blood glucose measurements conducted at the same time points as above, following an intraperitoneal injection of 0.75 IU/kg human insulin at onset, and 0.5 IU/kg at mid-symptomatic stage. Initially, we tried a dose of 0.5 IU/kg of insulin at onset stage, however, at this age mice unexpectedly responded with an initial spike in blood glucose levels. This was independent of both sex and genotype (*results not shown*). Hence, using a separate cohort of mice, we tested a higher dose of 0.75 IU/kg, and at this dose we observed the expected decline in blood glucose concentrations suggesting that mice at this age may be less sensitive to human insulin under the specific conditions of the ipITT. The higher dose was used for onset stage to analyse changes in blood hormone concentrations.

Enzyme-Linked Immunosorbent Assay (ELISA)

Plasma insulin and glucagon concentrations were measured using an Ultra-Sensitive Mouse Insulin ELISA Kit and Mouse Glucagon ELISA Kit (Crystal Chem, Illinois USA), respectively. Protocols were followed as per Manufacturer's instructions. The endpoint colorimetric assays were performed using a Tecan Spark multimode microplate reader (Tecan, Mannedorf Switzerland). Area under the curve (AUC) was calculated individually for each mouse on GraphPad Prism 8.4.2 (California USA) using the trapezoid rule. For ipITT blood concentrations over time, an inverse AUC (iAUC) was performed using basal blood glucose concentrations as baseline.

2-deoxyglucose uptake analysis

Mice were fasted overnight for 16 hours to replicate condition of ipGTT. Liver, intrascapular brown adipose tissue, inguinal white adipose tissue and epididymal white adipose tissue were collected 30 minutes after an intraperitoneal injection of 2-deoxyglucose (2 g/kg) in 0.9% saline. All tissues were store at -80°C until used.

Tissues were homogenised in a volume of milliQ water equivalent to tissue weight, spiked with [U-^{13}C]-glucose as an internal standard. Polar metabolites were extracted using a Bligh-Dyer water/methanol/chloroform extraction procedure previously optimised [39]. The upper polar phase was freeze dried using Labconco centrivap cold trap (Labconco, Missouri USA), and then analytes were derivatised using O - benzylhydroxylamine hydrochloride (O-BHA) and N-(3-dimethylaminopropyl) - N′ - ethylcarbodiimide hydrochloride (EDC) under the conditions previously optimised [40]. Following derivatisation, the lower organic layer was evaporated using a nitrogen dryer at 40°C, and reconstituted in 100 µl of 50% methanol. Standards were prepared similarly in matrix free milliQ water.

The LC-MS/MS system consisted of an API 3200 (AB SCIEX) triple quadrupole LC-MS/MS mass spectrometer containing Turbo V electrospray ionization (ESI) source system united with Genius AB-3G nitrogen gas generator (Peak Scientific). The mass spectrometer was coupled with Agilent 1200 series HPLC system (Agilent Technologies) equipped with a degasser, a column oven, a binary pump and temperature-controlled auto sampler. The auto sampler was set at 4°C and column oven temperature was maintained in the range of 45 ± 1°C. The system control and data acquisition were executed by Analyst software (AB SCIEX, Applied Biosystems Inc., USA, version 1.5.1). Chromatographic separation was implemented by injecting 5 µL of samples on a Kinetex EVO C18 analytical column (100 x 2.1 mm, 100 Å, 5µm, Phenomenex Inc., CA, USA) under binary gradient conditions using mobile phase A (milliQ water containing 0.1% formic acid) and mobile phase B (acetonitrile containing 0.1% formic acid) with 400 µl/min flow rate. Analytes were eluted using the water-acetonitrile gradient i.e. 2% acetonitrile from 0-1 minute with linear increase to 85% from 1 to 4.8 minutes followed by linear increase to 99% till 5 minutes, hold at 99% acetonitrile up till 10.5 minutes.

The mass spectrometer was operated in multiple reaction monitoring (MRM) scan mode for identification of fragments in positive ionization mode at unit resolution. The optimized compound dependent instrument parameters for analytes with their MRM transitions are summarized in Figure 2 – supplement table 1. For all analytes, the ion spray voltage and source temperature/auxiliary gas temperature were set at 5000 V and 550°C respectively. Nebulizer gas (ion source gas 1, GS1) and auxiliary gas (ion source gas 2, GS2) were set at 30 psi. The curtain and collision-activated dissociation gases were set at 35 and 7 on an arbitrary scale. Data was processed using Multiquant software (AB SCIEX, USA, version 2.0), Microsoft

Excel (Microsoft Inc., USA version 2013) and GraphPad Prism (LaJolla, CA, software version 8.4.2).

Glycogen concentrations

Liver glycogen content was analysed using a Glycogen Assay Kit (Cayman Chemical, Michigan USA). Tissues were homogenised in assay buffer provided in the kit with the addition of 5 µL of protease inhibitor cocktail (Roche, Basel Switzerland) and centrifuged at 800 x *g* for 10 minutes at 4°C. The resulting supernatant was used for the glycogen assay, following the protocol outlined in the kit. The endpoint fluorescence assays were measured using a Flexstation 3 multimode plate reader (Molecular Devices, California USA). Glycogen concentrations were normalised to the protein concentration, measured using the Pierce™ BCA Protein Assay kit (Thermo Fisher, Massachusetts USA).

Immunofluorescence

The pancreas was dissected from SOD1$_{G93A}$ and WT males at onset and mid-symptomatic stage of disease and incubated with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4, Sigma Aldrich, Missouri, USA) for 2 hours at room temperature, followed by 3 x 5 minutes washes in phosphate buffered saline (PBS, pH 7.4), and submersion in 15% and then 30% sucrose solution in PBS (pH 7.4). Pancreata were embedded in optimum cutting temperature (Sakura, California USA) and snap frozen in liquid nitrogen. Tissues were sectioned at 10 µm and dry mounted on Superfrost Plus slides (Menzel-Glaser, Braunschweig Germany) for quantification of α- and β- cell area.

Pancreas sections were rehydrated in PBS then blocked in PBS containing 5% bovine serum albumin (BSA) and 0.1% Triton X-100 for 1 hour at room temperature. Sections were

incubated overnight at 4°C with guinea pig anti-insulin (1:50, Abcam, Massachusetts USA) and mouse IgG1 anti-glucagon (1:1000, Abcam) antibodies, followed by a 2-hour incubation at room temperature with Alexa Fluor 488 dye-conjugated goat anti-guinea pig (1:500, Invitrogen, Oregan USA) and Alexa Fluor 555 dye-conjugated goat anti-mouse IgG1 (1:1000, Invitrogen). All antibodies were diluted in PBS containing 1% BSA and 0.1% Triton X-100. Sections were then incubated at room temperature for 10 minutes with 4, 6-diamidino-2-phenylindole (DAPI, 1:25000, Thermo Fisher) diluted in PBS and mounted with Prolong Gold Antifade Medium (Invitrogen). Quantification of insulin and glucagon positive area was measured in 50 pancreatic islets for each sample. Staining procedures and image exposures were all standardized between genotypes and between sections. The mouse genotype was not made available to the researchers until the completion of the study.

Real-time quantitative PCR

Total RNA was isolated from liver of WT and $SOD1_{G93A}$ mice using RNeasy Lipid Tissue extraction kit according to manufacturer's instructions (QIAGEN, CA, USA). The total RNA was purified from genomic DNA contamination then converted to cDNA using iScript™ gDNA Clear cDNA synthesis kit according to manufacturer's instructions (Bio-Rad, CA, USA). Commercially available SYBR® Green Glucose Metabolism Array consisting of 84 genes were used (Bio-Rad, CA, USA). Relative target gene expression to geometric mean of reference genes TATA-binding protein (Tbp), glyceraldehyde 3-phosphate dehydrogenase (Gapdh), and hypoxanthine-guanine phosphoribosyltransferase (Hprt) was determined using this formula: $2^{-\Delta CT}$ where $\Delta CT = (Ct_{(Target\ gene)} - Ct_{(Tbp,\ Gapdh\ and\ Hprt)})$. Final measures are presented as relative levels of gene expression in $SOD1_{G93A}$ mice compared with expression in WT mice. Pathway analysis was conduction using the "GENE2FUNC" function at FUMA GWAS web-based platform. Gene lists examined included those showed changes in expression

between WT and SOD1$_{G93A}$ mouse liver samples ($p < 0.1$). The p-values were adjusted using Benjamini-Hochberg (FDR) multiple correction method. A pathway was considered significant at the adjusted p-value < 0.05 threshold.

Statistical analysis

All analyses were performed using GaphPad Prism 8.4.2. All data generated from indirect calorimetry was analysed using a two-tailed student *t*-test with ages analysed separately. Blood glucose and hormone profiles over time for both ipGTT and ipITT were analysed using a two-way ANOVA followed by Bonferroni multiple comparison. For AUC, baseline hormone and glycogen concentrations and immunoreactive areas two-tailed student *t*-tests were used with different ages assessed separately. Two-tailed student *t*-test were also used for qPCR data, with all genes analysed individually. All correlations were assessed by Pearson correlation. All data is presented at mean ± SEM.

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Author's contributions

TSM and JDL conceived the project. TSM and JDL designed the study. TSM performed the experiments with the assistance from VK and JNTF. All authors contributed to the analyses and interpretation of the data. TSM wrote the paper with contribution from JDL. All authors read and approved the final manuscript.

Competing Interests

All authors declare they have no competing interests.

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Figure 1

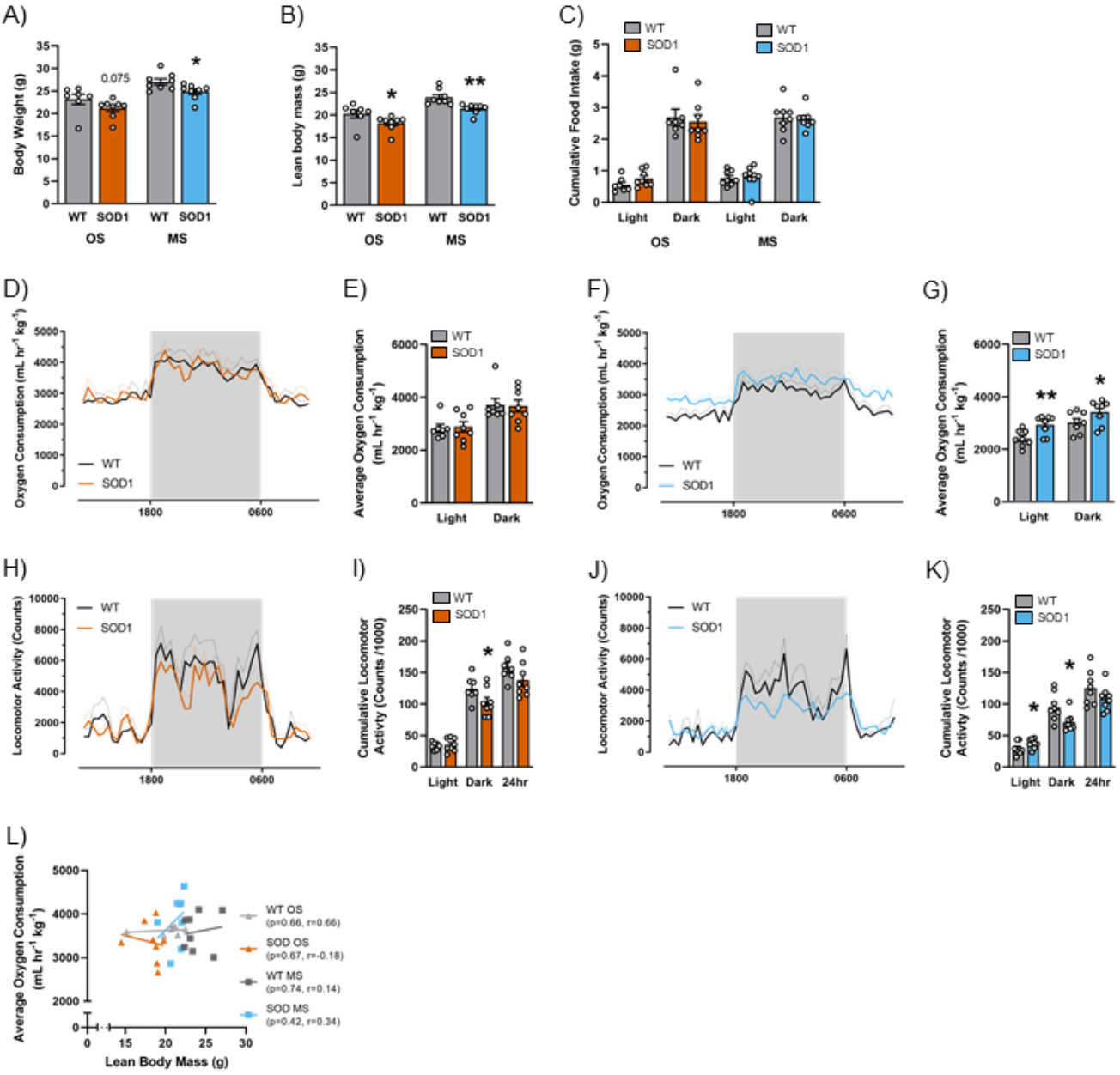


Figure 1. SOD1G93A mice displayed loss of body weight and lean body mass with decreased activity and increased oxygen consumption at mid-symptomatic stage of disease.

(**A, B**) Total body weight and lean mass of WT (grey bars) and SOD1G93A (onset stage (OS) - orange bars and mid-symptomatic stage (MS) - blue bars). (**C**) The cumulative food intake in both the light and dark phases, for WT and SOD1G93A mice at OS and MS. (**D-G**) Indirect calorimetry analysis was performed at both stages of disease using the Phenomaster TSE Metabolic Cage System. The 24-hour oxygen consumption profile in WT and SOD1G93A mice

at OS (**D**) and MS (**F**). From this profile the average oxygen consumption was calculated in both the light and dark cycle at OS (**E**) and MS (**G**). (**H-K**) The locomotor activity profile was also assessed in WT and SOD1$_{G93A}$ mice at OS (**H**) and MS (**J**). The cumulative locomotor activity in both SOD1$_{G93A}$ and WT littermates were calculated for both the light and dark phases at OS (**I**) and MS (**K**). (**L**) As lean body mass is predicted to correlate with energy expenditure we performed the correlation analysis between lean body mass and the average oxygen consumption across the 24-hour period. No significance was found across genotype or stage of disease. All data presented as mean ± SEM. n=8, for all measurements. All bar graphs analysed by two-tailed student t-test. * $p<0.05$, ** $p<0.01$.

Figure 2

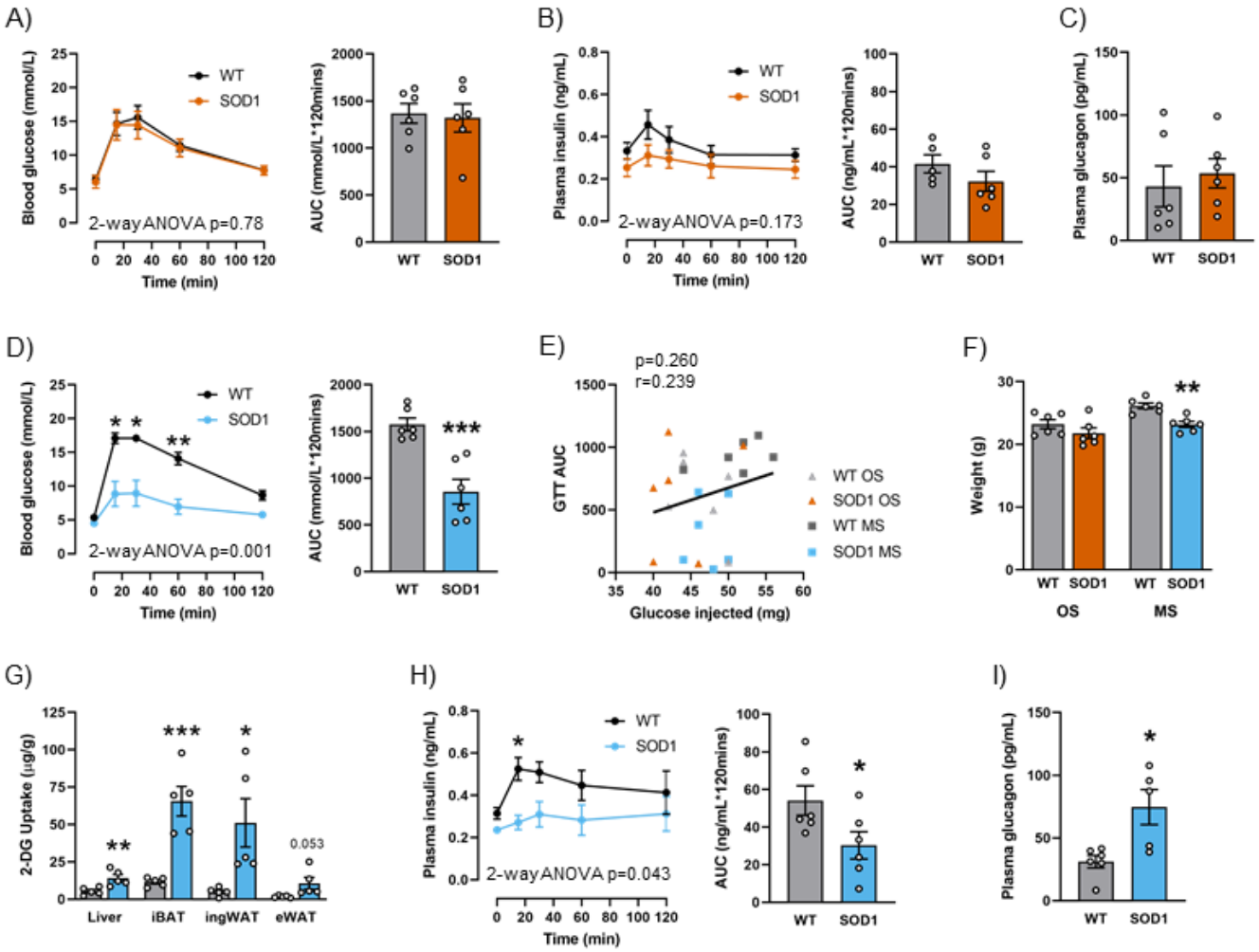


Figure 2. Glucose uptake is increased in SOD1G93A mice, despite a reduction in plasma insulin levels at mid-symptomatic stage of disease. Time course of blood glucose concentrations during a glucose tolerance test (ipGTT) following a 2 g/kg intraperitoneal injection of glucose at onset (OS; **A**) and mid-symptomatic (MS; **D**) stage of disease. The average area under the curve (AUC) calculated from the blood glucose time course for both WT (grey bars) and SOD1G93A (OS - orange bars and MS - blue bars). Time course of insulin concentrations measured in plasma collected from tail bleeds throughout ipGTT at both OS (**B**) and MS (**H**). The average AUC was calculated from the insulin time course as a measure of glucose-stimulated insulin release in WT (grey bars) and SOD1G93A (OS - orange bars and MS - blue bars). Plasma glucagon levels measured from baseline tail bleeds at OS (**C**) and MS (**I**).

(**E**) Correlation analysis between the amount of glucose injected and the AUC of blood glucose concentrations. Correlation analysis were performed on all WT and SOD1$_{G93A}$ mice from both disease stages. (**F**) Body weights of WT and SOD1$_{G93A}$ mice used for ipGTT at OS and MS. (**G**) 2-deoxglucose uptake was measured in the liver, intrascapular brown adipose tissue (iBAT), inguinal white adipose tissue (ingWAT) and epididymal white adipose tissue (eWAT) following a 2 g/kg intraperitoneal injection of 2-deoxyglucose in fasted WT and SOD1$_{G93A}$ mice. All data presented as mean ± SEM. n=6, for all measurements. Two-way ANOVA results listed on time course in panels **A**, **B**, **D** and **H** is the overall significance between genotypes, Bonferroni *post-hoc* test was used to determine significant changes at specific time points. All bar graphs analysed by two-tailed student t-test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 2 – supplemental figure 1

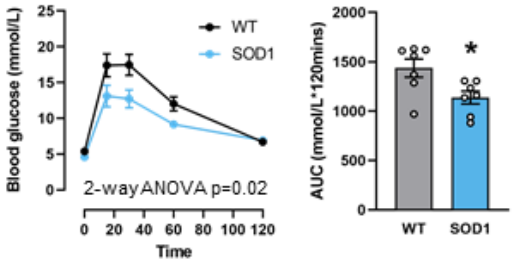


Figure 2 – supplement figure 1. Female SOD1G93A mice display a similar response to ipGTT in comparison to males. Time course of blood glucose concentrations during a glucose tolerance test (ipGTT) following a 2 g/kg intraperitoneal injection of glucose at mid-symptomatic stage of disease. The average area under the curve (AUC) calculated from the blood glucose time course for both WT (grey bars) and SOD1G93A (blue bars) to measure degree of glucose tolerance. All data presented as mean ± SEM. *n*=6, for all measurements. Two-way ANOVA results listed on time course graph is the overall significance between genotypes, Bonferroni *post-hoc* test was used to determine significant changes at specific time points. All bar graphs analysed by two-tailed student *t*-test. * $p<0.05$.

Figure 2 – supplement table 1. Optimized MRM transitions and instrument parameters for quantification

Analyte	Q1 Mass (Da)	Q3 Mass (Da)	DP (eV)	EP (eV)	CEP (eV)	CE (eV)	CXP (eV)	RT (min)
2dOG	270.2	91.1 124.1 55.1	41	6.5	16	31 25 77	4	5.7
2dOG6P	350.3	91.1 124.1 65	41	6.5	16	31 25 77	4	5.8
^{13}C-glucose (Internal standard)	292.07	91.1	36	9.5	24	35	4	6.4

Figure 3

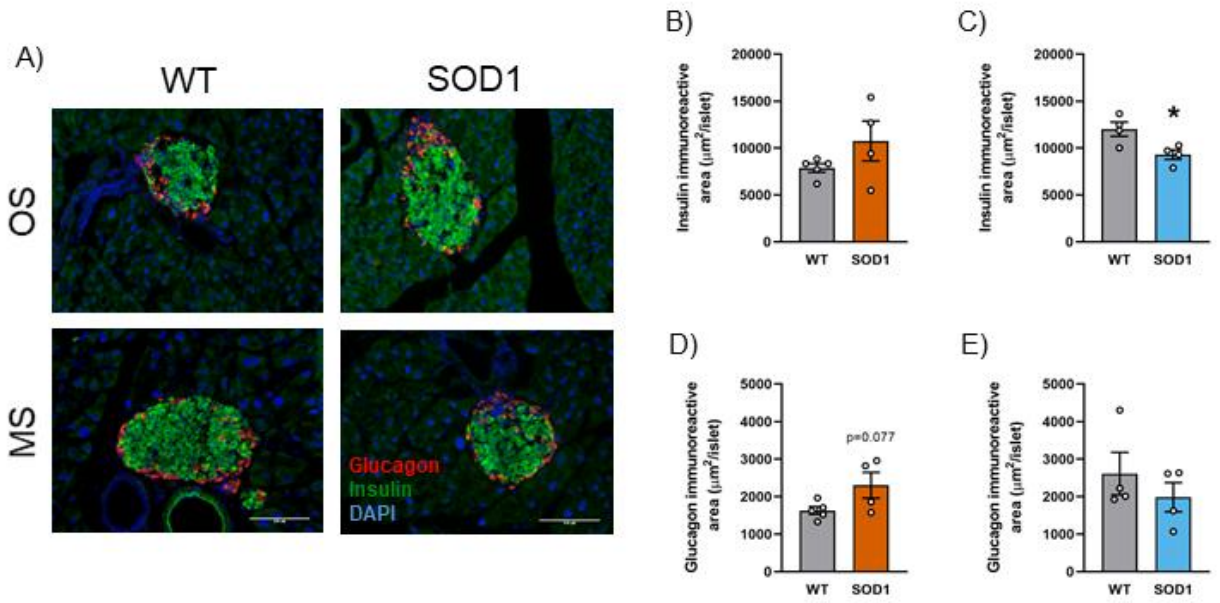


Figure 3. The area of β-cells in the pancreatic islets are reduced in SOD1$_{G93A}$ mice at mid-symptomatic stage of disease. (**A**) Representative images of glucagon-positive α-cells (red) and insulin-positive β-cells (green) in the pancreas of SOD1$_{G93A}$ mice at onset (OS) and mid-symptomatic (MS) stage of disease. The average immunoreactive area of insulin-positive β-cells in the pancreatic islet cells at OS (**B**) and MS (**C**). The average immunoreactive area of glucagon-positive α-cells in the pancreatic islet cells at OS (**D**) and MS (**E**) was also measured. Scale bar = 100 μM. All bar graphs analysed by two-tailed student *t*-test. * $p<0.05$.

Figure 4

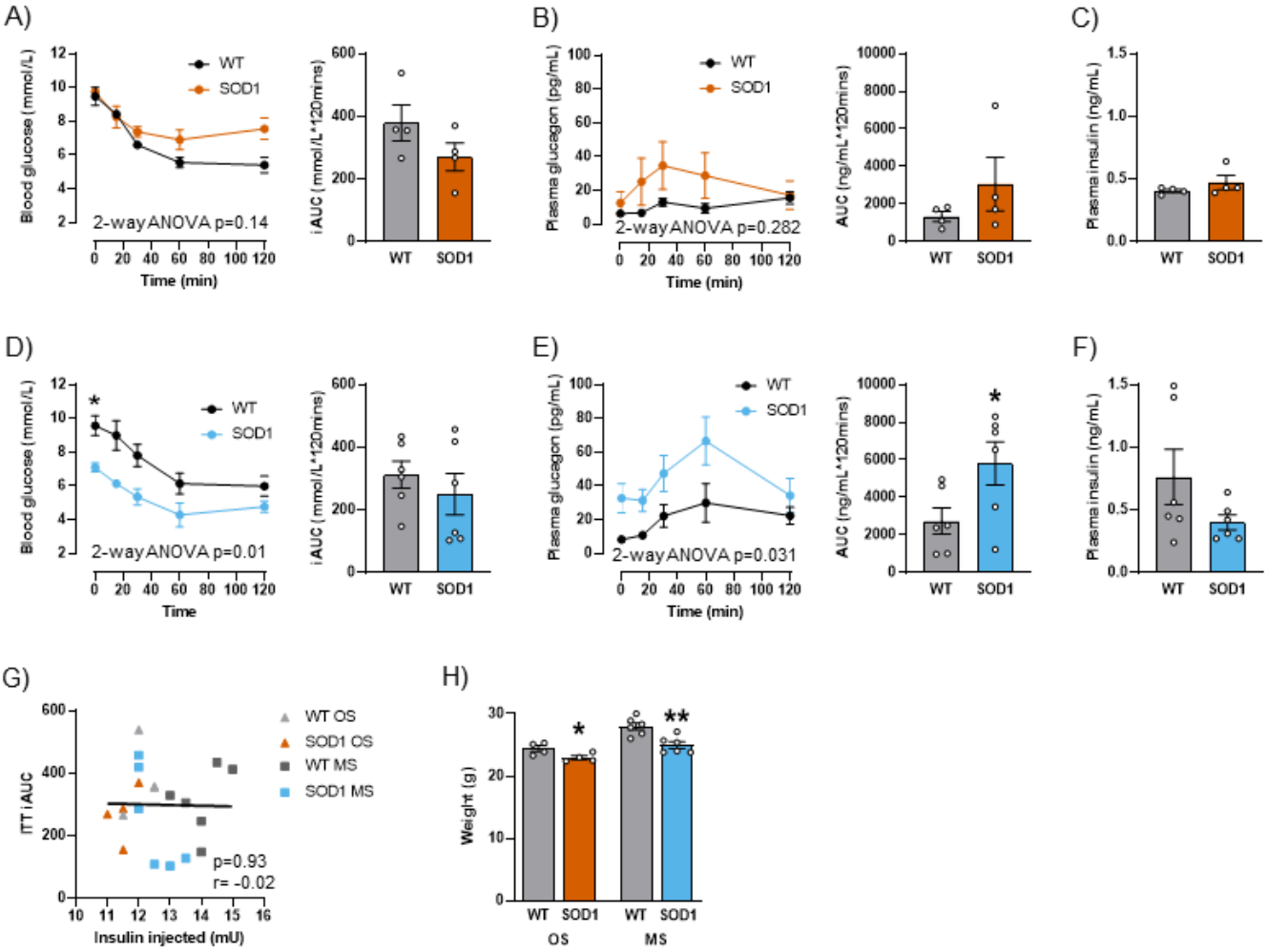


Figure 4. SOD1G93A mice show a similar response to exogenous insulin, despite lower fasting blood glucose levels. Time course of blood glucose concentrations during an insulin tolerance test (ipITT) at onset (OS; **A**) following a 0.75 IU/kg intraperitoneal injection of human insulin and at mid-symptomatic (MS; **D**) stage of disease following a 0.5 IU/kg dose of human insulin. The average inverse area under the curve (iAUC) was calculated from the blood glucose time course for both WT (grey bars) and SOD1G93A (OS - orange bars and MS - blue bars) to measure degree of insulin resistance. Time course of blood glucagon concentrations measured in plasma collected from tail bleeds throughout ipGTT at both OS (**B**) and MS (**E**). The average AUC was calculated from the glucagon time course as a measure of insulin-stimulated glucagon release for both WT (grey bars) and SOD1G93A (OS - orange bars and MS

- blue bars). Baseline plasma insulin levels were also measured from tail bleeds at OS (**C**) and MS (**F**). (**G**) Correlation analysis between the amount of insulin injected (mU) and the iAUC of blood glucose concentrations during ipITT. Correlation analysis were performed on all WT and SOD1$_{G93A}$ mice from both disease stages. (**H**) Body weights of WT and SOD1$_{G93A}$ mice used for ipITT at OS and MS. All data presented as mean ± SEM. *n*=4 at onset, and *n*=6 at mid stage, for all measurements. Two-way ANOVA results listed on time course in panels **A**, **B**, **D** and **E** is the overall significance between genotypes, Bonferroni *post-hoc* test was used to determine significant changes at specific time points. All bar graphs analysed by two-tailed student *t*-test. * $p<0.05$, ** $p<0.01$.

Figure 4 – supplemental figure 1

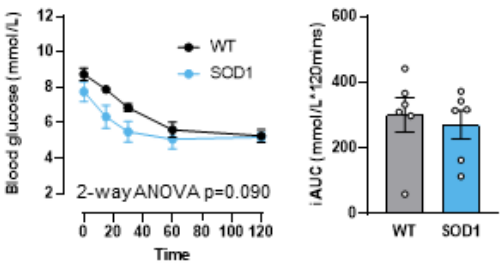


Figure 4 – supplement figure 1. Female SOD1G93A and WT littermates show a similar response to exogenous insulin. Time course of blood glucose concentrations during an insulin tolerance test (ipITT) following a 0.5 IU/kg intraperitoneal injection of insulin at mid-symptomatic stage of disease. The average inverse area under the curve (iAUC) calculated from the blood glucose time course for both WT (grey bars) and SOD1G93A (blue bars). All data presented as mean ± SEM. *n*=6, for all measurements. Two-way ANOVA results listed on time course graph is the overall significance between genotypes, Bonferroni *post-hoc* test was used to determine significant changes at specific time points. All bar graphs analysed by two-tailed student *t*-test.

Figure 5

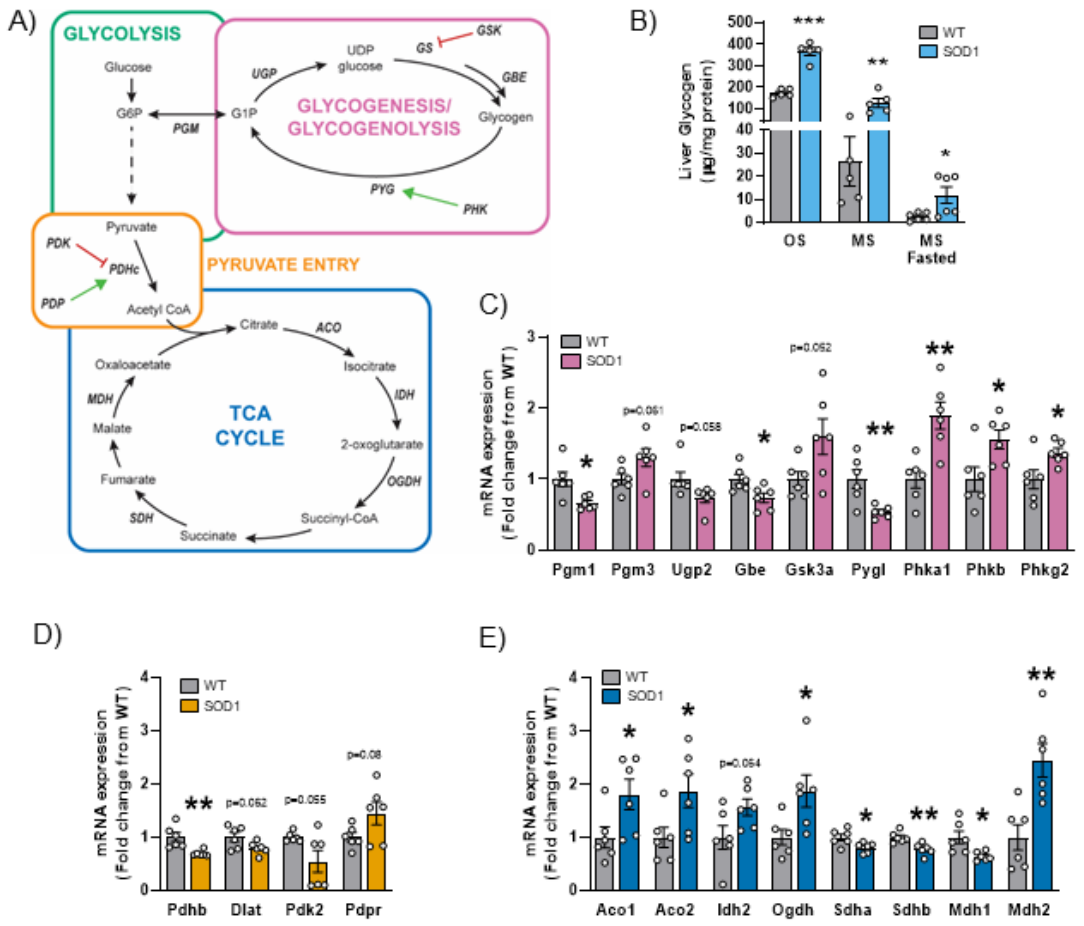
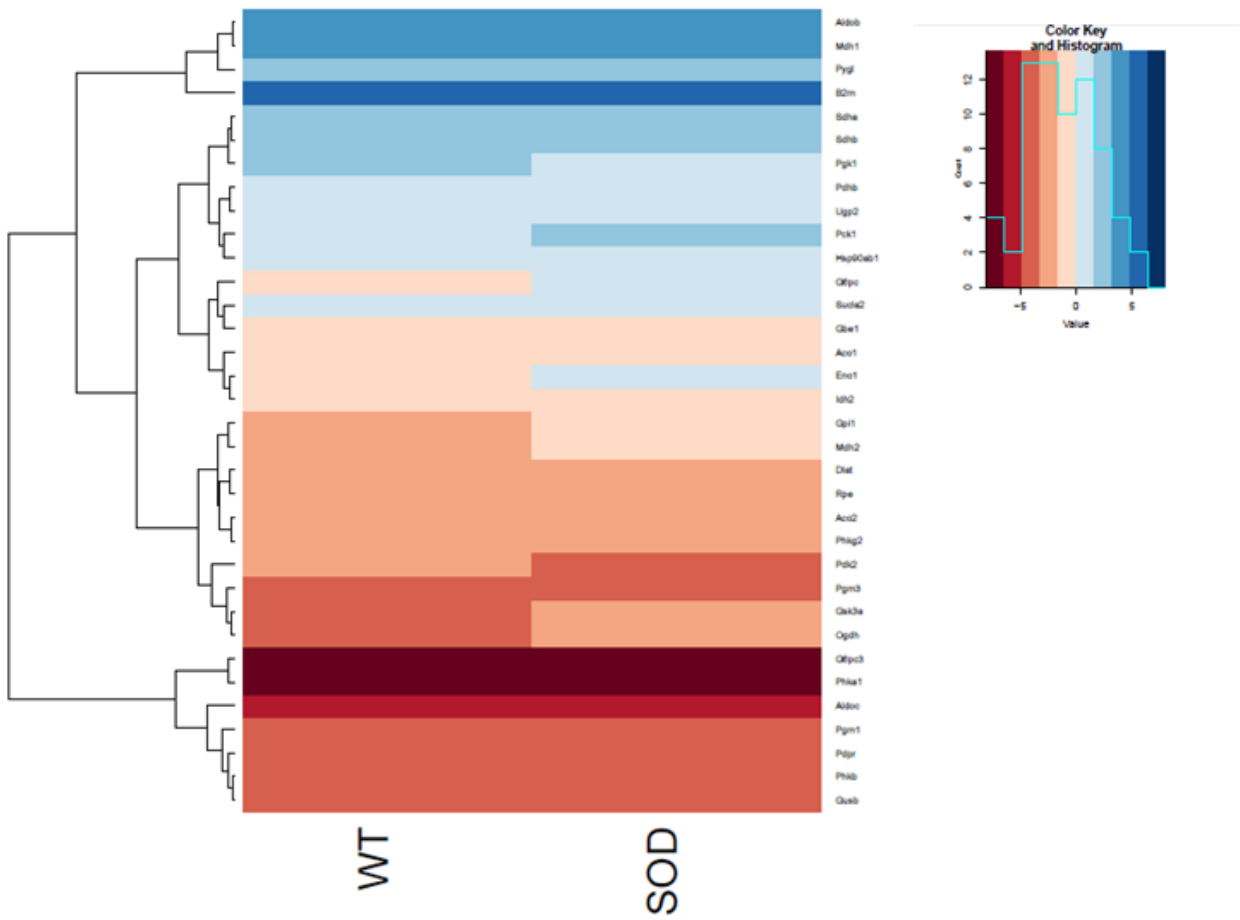


Figure 5. (**A**) Schematic of metabolic pathways that are changed in the liver of SOD1G93A mice at mid-symptomatic stage of disease. **(B)** Glycogen concentrations were measured in liver homogenates at onset (OS), mid-symptomatic (MS) stage, and overnight fasted MS. WTs represented by grey bars and SOD1G93A by light blue bars**. (C)** mRNA expression of genes involved in glycogenesis and glycogenolysis that are changed in SOD1G93A livers, including two isoforms of phosphoglucose mutase (Pgm1 and Pgm3), an isoform of UDP-glucose phosphorylase (Ugp2), glycogen branching enzyme (Gbe), and the liver isoform of glycogen phosphorylase. As well as several genes that regulate activities of enzymes involved in these pathways including glycogen synthase kinase 3-alpha (Gsk3a) and three isoforms of phosphorylase kinase (Phka1, Phkb, and Phkg2). WT represented by grey bars and SOD1G93A by pink bars. **(D)** mRNA expression of genes involved in pyruvate entry into the TCA cycle

that are modified in the SOD1$_{G93A}$ liver at MS, including two of the enzymes that form the pyruvate dehydrogenase complex (PDHc), pyruvate dehydrogenase E1 subunit β (Pdhb) and dihydrolipoamide S-Acetyltransferase (Dlat). We also saw a trend but not significant change in the expression two genes involved in regulating PDH activity, an isoform of both pyruvate dehydrogenase kinase (Pdk2) and pyruvate dehydrogenase phosphatase (Pdpr). WTs represented by grey bars and SOD1$_{G93A}$ by yellow bars **(E)** Several genes involved in TCA cycle metabolism were increased in the SOD1$_{G93A}$ liver, including two isoforms of aconitase (Aco1, Aco2), and isoform of isocitrate dehydrogenase (Idh2), oxoglutarate dehydrogenase (Ogdh), and an isoform of malate dehydrogenase (Mdh2). However, the expression of two subunits of succinate dehydrogenase (Sdha, Sdhb) and an alternate isoform of malate dehydrogenase (Mdh1) were lower in the SOD1$_{G93A}$ mice. WTs represented by grey bars and SOD1$_{G93A}$ by dark blue bars. All data presented as mean ± SEM. n=6, for all measurements. All bar graphs analysed by two-tailed student t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 5 – supplemental figure 1

A)



B)

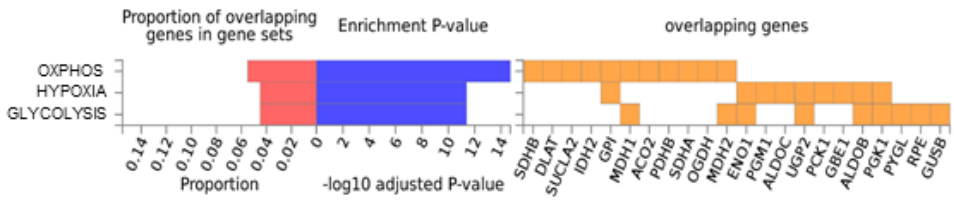


Figure 5 – supplement figure 1 (A) The heatmap showing the gene expression profile of 34 genes with marked changes between WT and SOD1$_{G93A}$ mouse liver samples ($p < 0.1$). The genes showing similar gene expression profiles are clustered. **(B)** Hallmark pathways enriched for genes with marked changes between WT and SOD1$_{G93A}$ mouse liver samples ($p < 0.1$) using FUMA.