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Alterations in the hypothalamic melanocortin pathway in amyotrophic lateral sclerosis

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Amyotrophic lateral sclerosis, the most common adult-onset motor neuron disease, leads to death within 3 to 5 years after onset. Beyond progressive motor impairment, patients with amyotrophic lateral sclerosis suffer from major defects in energy metabolism, such as weight loss, which are well correlated with survival. Indeed, nutritional intervention targeting weight loss might improve survival of patients. However, the neural mechanisms underlying metabolic impairment in patients with amyotrophic lateral sclerosis remain elusive, in particular due to the lack of longitudinal studies. Here we took advantage of samples collected during the clinical trial of pioglitazone (GERP-ALS), and characterized longitudinally energy metabolism of patients with amyotrophic lateral sclerosis in response to pioglitazone, a drug with well-characterized metabolic effects. As expected, pioglitazone decreased glycaemia, decreased liver enzymes and increased circulating adiponectin in patients with amyotrophic lateral sclerosis, showing its efficacy in the periphery. However, pioglitazone did not increase body weight of patients with amyotrophic lateral sclerosis independently of bulbar involvement. As pioglitazone increases body weight through a direct inhibition of the hypothalamic melanocortin system, we studied hypothalamic neurons producing proopiomelanocortin (POMC) and the endogenous melanocortin inhibitor agouti-related peptide (AGRP), in mice expressing amyotrophic lateral sclerosis-linked mutant SOD1(G86R). We observed lower *Pomc* but higher *Agrp* mRNA levels in the hypothalamus of presymptomatic SOD1(G86R) mice. Consistently, numbers of POMC-positive neurons were decreased, whereas AGRP fibre density was elevated in the hypothalamic arcuate nucleus of SOD1(G86R) mice. Consistent with a defect in the hypothalamic melanocortin system, food intake after short term fasting was increased in SOD1(G86R) mice. Importantly, these findings were replicated in two other amyotrophic lateral sclerosis mouse models based on TDP-43 (*Tardbp*) and FUS mutations. Finally, we demonstrate that the melanocortin defect is primarily caused by serotonin loss in mutant SOD1(G86R) mice. Altogether, the current study combined clinical evidence and experimental studies in rodents to provide a mechanistic explanation for abnormalities in food intake and weight control observed in patients with amyotrophic lateral sclerosis. Importantly, these results also show that amyotrophic lateral sclerosis progression impairs responsiveness to classical drugs leading to weight gain. This has important implications for pharmacological management of weight loss in amyotrophic lateral sclerosis.

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Abbreviations: ALS = amyotrophic lateral sclerosis; TZD = thiazolinedione

Introduction

Amyotrophic lateral sclerosis (ALS), the most common adult-onset motor neuron disease, is characterized by the simultaneous degeneration of upper and lower motor neurons, leading to muscle atrophy and paralysis, and death within 3 to 5 years after onset. A subset of ALS cases are of familial origin and five major genes are currently associated with familial ALS (*C9orf72*, *SOD1*, *FUS*, *TARDBP* and *TBK1*) (Leblond *et al.*, 2014; Cirulli *et al.*, 2015; Freischmidt *et al.*, 2015; Lattante *et al.*, 2015). The *SOD1* gene was the first associated with ALS and most ALS mouse models currently used are based upon overexpression of mutant forms of *SOD1* (Gurney *et al.*, 1994; Ripps *et al.*, 1995).

Beyond progressive motor impairment, patients with ALS suffer from major, yet incompletely characterized, defects in energy metabolism (Dupuis *et al.*, 2011). First, ALS is more likely to occur with lower premorbid body fat (Gallo *et al.*, 2013; O'Reilly *et al.*, 2013) or better cardiovascular or physical fitness (Turner *et al.*, 2012; Huisman *et al.*, 2013). Second, weight loss is negatively correlated with survival (Desport *et al.*, 1999; Marin *et al.*, 2011; Paganoni *et al.*, 2011). This weight loss is associated with, and likely caused by, intrinsic hypermetabolism (Desport *et al.*, 2001; Bouteloup *et al.*, 2009), and is exacerbated by dysphagia occurring with bulbar involvement. Third, ALS patients develop abnormalities in lipid (Dupuis *et al.*, 2008; Dorst *et al.*, 2011; Lindauer *et al.*, 2013) and glucose (Pradat *et al.*, 2010) metabolisms. Interestingly, these metabolic alterations are largely replicated in transgenic mice expressing mutant SOD1 (Dupuis *et al.*, 2004; Fergani *et al.*, 2007; Palamiuc *et al.*, 2015). Not much is known on the underlying mechanisms of energy metabolism impairment despite the fact that elucidation of such mechanisms would offer therapeutic strategies to treat weight loss pharmacologically. Furthermore, deciphering the mechanisms of energy metabolism impairment could identify disease-modifying interventions as a hypercaloric diet was recently found to increase survival of patients with ALS under gastrostomy (Wills *et al.*, 2014; Dorst *et al.*, 2015).

Most of the studies to date have characterized energy metabolism in patients with ALS in steady state, at one single time point. The dynamic nature of energy metabolism and its homeostatic regulation thus severely limit the interpretation of these studies. Interventions performed during randomized clinical trials often have metabolic effects, and such studies include long term follow-up of patients for many months. These clinical studies thus provide high-quality information useful to understand the metabolic defects of patients with ALS.

Here, we performed a *post hoc* analysis of samples obtained during the clinical trial of pioglitazone (Dupuis *et al.*, 2012) to characterize energy metabolism of ALS patients on a metabolic challenge. Indeed, pioglitazone, like other thiazolinediones (TZDs), has pleiotropic effects on energy metabolism that have been extremely well characterized in both mouse models and human patients. In the periphery, pioglitazone sensitizes to insulin, leading to decreased glycaemia, and decreases circulating levels of liver enzymes (Promrat *et al.*, 2004; Belfort *et al.*, 2006; Sanyal *et al.*, 2010; DeFronzo *et al.*, 2011). In the CNS, pioglitazone inhibits the hypothalamic melanocortin system to increase food intake (Diano *et al.*, 2011; Lu *et al.*, 2011; Ryan *et al.*, 2011; Long *et al.*, 2014). Specifically, pioglitazone decreases activity of the hypothalamic neurons producing proopiomelanocortin (POMC), the precursor of a number of anorexigenic peptides such as α-MSH (melanocyte stimulating hormone) (Diano *et al.*, 2011; Lu *et al.*, 2011; Ryan *et al.*, 2011; Long *et al.*, 2014). In humans, this leads to a robust (3–5 kg) weight gain that was repeatedly

observed in multiple clinical trials (Promrat *et al.*, 2004; Belfort *et al.*, 2006; Sanyal *et al.*, 2010; DeFronzo *et al.*, 2011). Here, we show that patients with ALS display normal peripheral action of pioglitazone, while they lack weight gain. In transgenic mice expressing mutant SOD1, pioglitazone failed to increase food intake. This was associated with prominent involvement of the hypothalamic melanocortin system, also observed in other mouse models of ALS, independent of mutant SOD1 overexpression. Last, we show that the melanocortin defect occurs downstream of the previously documented serotonin loss (Dentel *et al.*, 2013). Altogether, our analysis of the data from the pioglitazone trial disclosed a previously unanticipated defect in patients with ALS that could account for a subset of ALS-related metabolic defects.

Materials and methods

Patients and treatments

All the biological materials from human ALS patients were sampled as part of the GERP-ALS trial (clinicaltrials.gov reference: NCT00690118) (Dupuis *et al.*, 2012). Briefly, patients with possible, probable (clinically or laboratory-supported) or definite ALS according to the revised version of the El Escorial criteria were considered for enrolment into the study. Included patients displayed onset of progressive weakness within 36 months prior to study and had disease duration of > 6 months and < 3 years (inclusive) with disease onset defined as date of first muscle weakness, excluding fasciculation and cramps. They reached a best-sitting slow vital capacity between 50% and 95% of predicted normal. They were capable of thoroughly understanding the information provided and gave written informed consent. All included patients had been treated with 100 mg riluzole daily for at least 3 months prior to inclusion. Detailed exclusion and inclusion criteria have been described earlier (Dupuis *et al.*, 2012). The study protocol was approved by the ethics committee of the University of Ulm and all other participating centres.

The two treatment groups were 100 mg riluzole plus 45 mg pioglitazone (pioglitazone group) and 100 mg riluzole plus placebo (placebo group). Patients were randomly assigned to one of the two treatment groups and both groups were matched for age, gender and site of onset (Dupuis *et al.*, 2012).

Procedures and biochemical analysis of human samples

After inclusion, patients underwent a screening phase and a treatment phase (18 months), with stepwise increase in dosage (Dupuis *et al.*, 2012). Clinical and physical examinations, blood sampling, and drug compliance were recorded at on-site visits (1, 2, 6, 12 and 18 months after baseline visit). Body weight was recorded at on-site visits, except for 3-, 9- and 15-month time points (telephone contacts). There were no differences in results when excluding these three time points. Routine clinical laboratory tests were performed at each on-site visit (baseline and 1, 2, 6, 12 and 18 months after baseline). All tests were carried out according to standard laboratory procedures at each study centres' locally accredited laboratory, which defined the normal reference range for each analyte. The following laboratory tests were performed using standard methods: alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), fasting blood glucose. Adiponectin measurements were done in the neurochemical laboratory in Ulm (MSD assay).

Animals

Transgenic mice were housed in the animal facility of the medicine faculty of Strasbourg University, with 12 h/12 h light/dark and unrestricted access to food and water. In all experiments, littermates were used for comparison. Transgenic SOD1(G86R) were maintained in their initial FVB/N genetic background according to previous studies (Dentel *et al.*, 2013). Transgenic mice expressing TDP43(A315T) were previously described and were maintained as heterozygous in their initial C57Bl6/J background (Wegorzewska *et al.*, 2009). Heterozygous $Fus^{\Delta NLS/+}$ are knock-in mice expressing FUS protein deleted from its C-terminal nuclear localization signal (NLS) from one copy of the endogenous *Fus* gene. These mice were generated and maintained in C57Bl6/J background. The motor phenotype of these mice will be described elsewhere (Scekic-Zahirovic, submitted for publication). Tph2-YFP mice were purchased from Jackson laboratories (Bar Harbor; strain 014555) and maintained in their initial genetic background. Female Tph2-YFP mice were crossed with male SOD1(G86R) to generate compound transgenic mice.

For biochemical analysis, animals were sacrificed at the ages indicated at 2 pm, and tissues were quickly dissected, frozen in liquid nitrogen, and stored at -80°C until use. For histological analysis, animals were anaesthetized by intraperitoneal injection of ketamine (Imalgène 1000®, Merial; 90 mg/kg body weight) and xylazine (Rompun 2%®, Bayer; 10 mg/kg body weight) at the ages indicated at 2 pm. After perfusion of 4% paraformaldehyde (v/v PFA, Sigma), brains were removed, stored in the same fixative overnight at 4°C and stored in phosphate-buffered saline (PBS) until used. These experiments were authorized by the local ethical committee of Strasbourg University (CREMEAS).

Drugs and treatments

Pioglitazone (Actos®, Takeda) was dissolved in 10% (v/v) dimethyl sulphoxide (DMSO, Fisher Scientific) and a single oral administration was given by gavage at a dose of 40 mg/kg body weight. Fluoxetine (Sigma) was dissolved in 0.9% (w/v) NaCl (Sigma) and administrated intraperitoneally at a dose of 20 mg/kg body weight.

Measurements of food intake

For the pioglitazone experiment, mice were fasted from 9 am to 3 pm, and pioglitazone was administrated at 3 pm. Food was reintroduced 1 h after gavage and food intake was recorded for 24 h.

For short-term fasting experiments, mice were fasted from 8 am to 3 pm (7 h fasting conditions) or from 2 pm to 3 pm (1 h fasting conditions) and food was reintroduced after 7 h or 1 h

of fasting. Food intake was measured 1 h and 24 h after refeeding.

For fluoxetine experiment, mice were fasted from 1 pm to 2 pm, and fluoxetine was injected at 2 pm. Food was reintroduced 30 min after fluoxetine injection and food intake was measured for 1 h. For biochemical analysis, a second injection of fluoxetine was done after 1 h of feeding and mice were sacrificed 30 min later.

Histology

Fixed brains were included in 6% (w/v) agar (Sigma) and sectioned from Bregma 0.02mm to Bregma −2.90 mm into 40 µm coronal sections on a vibratome. Arcuate nucleus was identified according to Paxinos Brain Atlas. POMC immunohistochemistry was performed on half of the selected brain sections. AGRP and green fluorescent protein (GFP) immunohistochemistries were performed each on anatomically matched sections. Immunohistochemistry was performed on floating sections using standard histological techniques. Endogenous peroxidases were inactivated using 3% (v/v) H_2O_2. For POMC immunohistochemistry, permeabilization and saturation of non-specific sites were done with 0.25% (v/v) Triton™ (Sigma) and 50 mg/ml bovine serum albumin (BSA, Sigma). For GFP immunohistochemistry, permeabilization and saturation of non-specific sites were done with 0.5% (v/v) Triton™ (Sigma) and 5% (v/v) horse serum (Gibco, Life Technologies). For AGRP immunohistochemistry, antigen retrieval with citrate buffer was done before permeabilization and saturation of non-specific sites with 0.5% (v/v) Triton™ (Sigma) and 5%(v/v) horse serum (Gibco, Life Technologies). Rabbit anti-POMC primary antibody (Phoenix Peptide; 1:2000), rabbit anti-AgRP primary antibody (Phoenix Peptide; 1:2000) or rabbit anti-GFP primary antibody (Invitrogen, Life Technologies, 1:1000) were incubated overnight at room temperature. Biotinylated donkey anti-Rabbit secondary antibody (Jackson; 1:500) was incubated for 90 min at room temperature. Staining was performed using Vectastain Elite ABC kit (Vector). After revelation with 3,3'-diaminobenzidine (DAB, Sigma; 0.5 mg/ml), sections were mounted and images of all sections were taken.

For quantification, bright-field images of lower brain part for POMC and AgRP stainings or bright-field images of right arcuate nucleus part for GFP staining were acquired with a Nikon DS –Ri1 camera attached to a Nikon microscope (Nikon Eclipse E800) fitted with a Plan Apo 4× lens (N.A. = 0.20, Nikon) and a plan Apo 10× lens (N.A. = 0.45, Nikon), respectively. White balance, gain, exposure, and light settings were kept the same when acquiring all images of a given staining.

Image analysis

An operator blinded to the genotype quantified all experiments. Total numbers of POMC-positive cell bodies in the arcuate nucleus were determined for each animal and normalized per section. Quantification of GFP staining and AGRP staining was processed to binary images using NIH ImageJ as described previously (Grider *et al.*, 2006). Briefly, after changing tiff images to 8-bit images, images were inverted and processed with Feature J plugin for ImageJ (version 1.50d) to select the smallest Hessian values and « smoothing scale » of 1.0. The resulting images were transformed into binary images by thresholding. Threshold was determined on one cut and same threshold was applied to every image. Occupied area for AGRP or GFP staining was measured.

RNA extraction and quantitative reverse transcription-polymerase chain reaction

RNA was extracted from mouse hypothalami using TRIzol® reagent (Life Technologies). After reverse transcription with iScript™ reverse transcription Supermix for RT-qPCR (Bio-Rad), cDNA was obtained from 1 µg of RNA. Messenger RNA levels were obtained by quantitative PCR using Sso Advanced Universal SYBR Green Supermix (Bio-Rad) with corresponding sense and antisense primers. Standard curves were constructed by amplifying serial dilutions of cDNA. Starting quantities of samples were calculated with Bio-Rad software. Messenger RNA levels of genes of interest were normalized to expression levels of the 18S ribosomal, *Tbp* and *Pola2* RNA housekeeping genes using GeNorm (Vandesompele *et al.*, 2002). Primer sequences are shown in Supplementary Table 1.

Serotonin levels

Hypothalamic serotonin levels were measured using high performance liquid chromatography, as previously described (Dentel *et al.*, 2013).

Statistical analysis

For statistical analyses in ALS patients, group comparisons were performed using mixed effects regression model analysis by the Institute of Epidemiology and Medical Biometrics at the University of Ulm using the statistical software package SAS Version 9.2 under Windows.

For animal experiments, comparison of two groups was performed using unpaired Student's *t*-test, except for experiments with pioglitazone in which paired *t*-test was used. Comparison of three or four groups was performed using one-way ANOVA and Tukey *post hoc* test. Statistics in animal experiments were performed using Prism version 6.0.

All results from analysis were regarded as hypothesis generating only. All statistical tests were carried out two-sided at a significance level of 5%.

Results

Normal peripheral response to pioglitazone in patients with ALS

We took advantage of data collected during the pioglitazone GERP-ALS trial to investigate energy metabolism in ALS patients in response to pioglitazone.

In this clinical trial, 219 patients with ALS were enrolled and randomly allocated to either placebo ($n = 110$; bulbar: $n = 33$, spinal: $n = 77$), or pioglitazone ($n = 109$;

bulbar: $n = 32$, spinal: $n = 77$) treatment after stratification based on site of onset (bulbar or spinal) (Dupuis *et al.*, 2012).

The metabolic effects of TZDs, including pioglitazone are well understood in models and their effects are largely described in patients (Fig. 1A and Supplementary Table 2). Pioglitazone treatment is known to increase levels of adiponectin, an adipose-derived hormone through direct transcriptional activation of the adiponectin gene in adipocytes (Maeda *et al.*, 2001). Consistently, pioglitazone treatment increased the levels of circulating adiponectin 4-fold in patients with ALS after 6 months of treatment, and this was maintained after 12 months of treatment (Fig. 1B). Pioglitazone decreases glycaemia through hepatic and skeletal muscle PPARγ (encoded by *PPARG*). In patients with ALS, pioglitazone decreased glycaemia (Fig. 1C), although this effect was milder than observed in other populations, including non-diabetic patients (Belfort *et al.*, 2006; Sanyal *et al.*, 2010; DeFronzo *et al.*, 2011). Consistent with a direct action on liver, pioglitazone decreased levels of ASAT and more robustly levels of ALAT (Fig. 1D and E). In all, pioglitazone displayed the expected metabolic effects on adipocytic, muscular, and hepatic biomarkers, and was likely able to activate PPARγ in these tissues.

Pioglitazone does not lead to weight gain in patients with ALS

TZDs are also known to act in hypothalamic melanocortin neurons to promote feeding (Diano *et al.*, 2011; Long *et al.*, 2014), and this activation of PPARγ in melanocortin neurons is responsible for the robust weight gain associated with TZD treatment (Lu *et al.*, 2011; Ryan *et al.*, 2011; Long *et al.*, 2014). Thus, the evolution of body weight upon pioglitazone treatment represents a reliable proximal marker of PPARγ action in hypothalamic melanocortin neurons. In ALS patients, pioglitazone had no effect on weight loss (Fig. 2A), or body mass index (BMI) (Fig. 2B and Supplementary Table 2). Increased weight in response to pioglitazone is due to increased food intake, and a subset of ALS patients experience dysphagia. However, pioglitazone had also no effect on BMI and weight loss when considering spinal onset patients (Fig. 2C and D), or patients with preserved everyday life during at least 6 months (results from EuroQoL EQ-5D questionnaire, Fig. 2E and F). Lastly, patients with preserved bulbar function during at least 6 months after inclusion [as assessed using ALS functional rating scale-revised (FRS-R) bulbar subscale] did not lose weight, yet pioglitazone had no effect on their BMI (Fig. 2G and H). Importantly, there were no differences among groups for intake of drugs affecting body weight and food intake, in particular anti-epileptics, anti-diabetics, anti-psychotics or selective serotonin reuptake inhibitors (Supplementary Table 4). Thus, pioglitazone did not increase weight in ALS patients, and this was not related with dysphagia nor confounded by intake of other drugs.

Pioglitazone does not increase food intake in mutant SOD1 mice

We hypothesized that the lack of weight gain for patients with ALS under pioglitazone was due to defects in stimulating food intake. To test this hypothesis, we used transgenic mice expressing mutant SOD1(G86R) (SOD1m mice) as a model of ALS and examined food intake in response to pioglitazone. In rodents, pioglitazone has various effects on food intake depending on genetic background, dose, route and associated diet (Diano *et al.*, 2011; Ryan *et al.*, 2011; Long *et al.*, 2014). Using a protocol (Fig. 3A) adapted from Ryan *et al.* (2011), we observed that a single oral dose of pioglitazone (40 mg/kg) increased food intake by 10–15% in wild-type FVB/N mice (Fig. 3B and C). However, food intake was not increased in littermate SOD1m mice either 1 month before motor symptoms (Fig. 3 B) or at disease onset (Fig. 3C). Thus, pioglitazone was not able to increase food intake in SOD1m mice.

Defects in melanocortin neurons in mutant SOD1 mice

Hypothalamic melanocortin neurons constitute the primary target of TZDs to promote food intake (Diano *et al.*, 2011; Long *et al.*, 2014). The melanocortin system mainly comprises two antagonistic neuronal types located in the arcuate nucleus: POMC neurons, which secrete the anorexigenic peptide alpha melanocyte-stimulating hormone (αMSH), and AGRP neurons, which promote food intake, mostly through production of AGRP, an endogenous αMSH antagonist. *Pomc* mRNA levels were 2-fold lower in SOD1m mice at 75 days of age (Fig. 4A) or at onset (Fig. 4B), whereas *Agrp* mRNA levels were higher in non-symptomatic mice, but not at onset (Fig. 4A and B). This involvement of the melanocortin system was relatively selective, as we did not observe expression changes in multiple neuropeptides involved in energy homeostasis, in particular CART (*Cartpt*), NPY, CRF (*Crh*), AVP, TRH, galanin (GAL), somatostatin (SST) and BDNF. Importantly however, we observed decreased expression of MCH (*Pmch*) at both ages, and orexin, at onset (Fig. 4C). Consistent with decreased POMC expression, we observed ~30% fewer POMC-positive neurons in the arcuate nucleus of SOD1m mice as compared with their wild-type littermates at 75 days of age and almost 50% fewer at onset (Fig. 5). Furthermore, we observed increased density of AGRP-positive fibres in arcuate nucleus as well as in projection regions present on the same sections such as dorso-medial hypothalamus and lateral hypothalamus (Fig. 6). In all, the melanocortin system appears shifted towards decreased melanocortin tone in SOD1m mice.

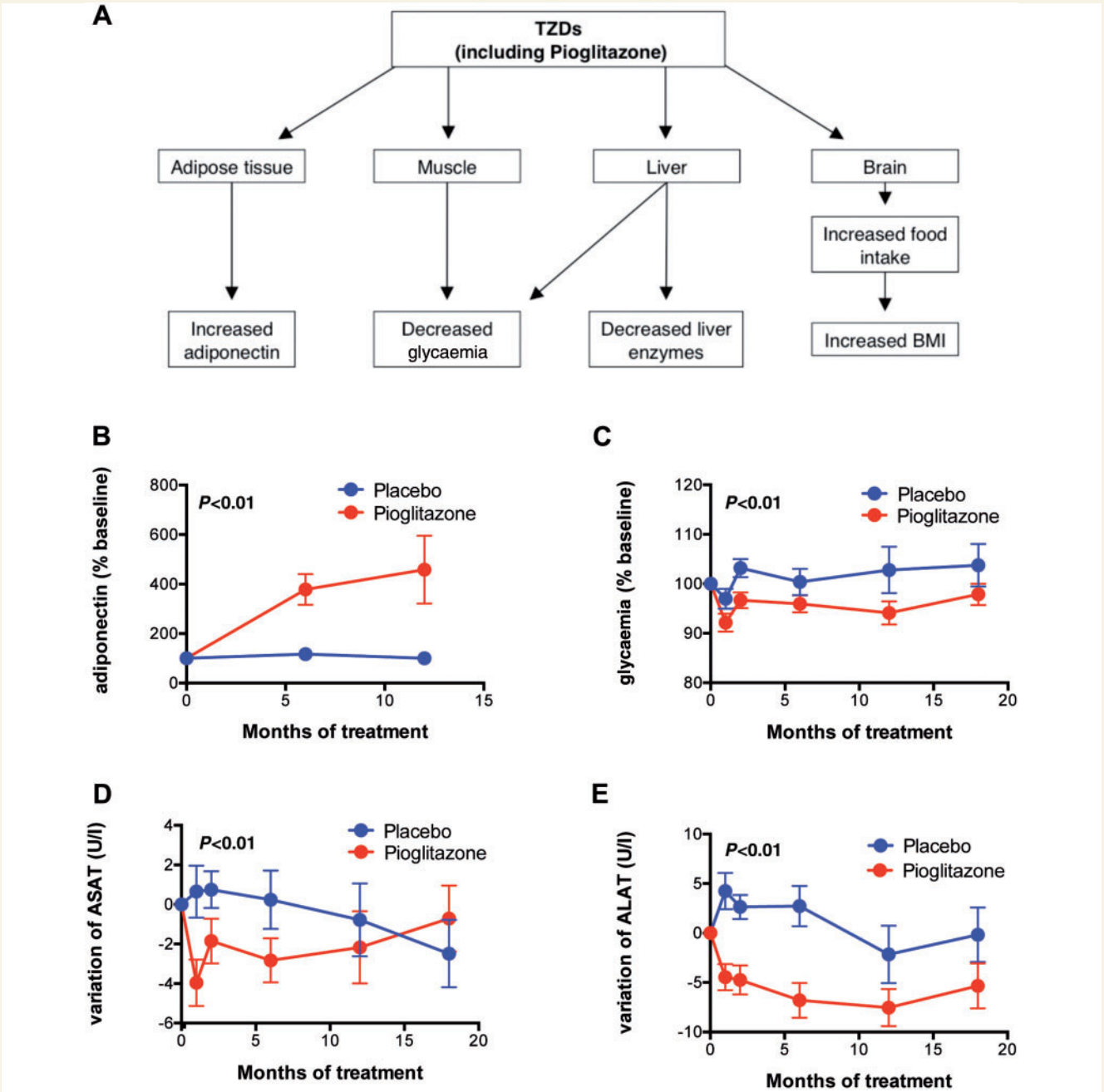

Figure 1 Effects of pioglitazone on peripheral biomarkers in ALS patients. (**A**) Summary of metabolic effects of pioglitazone (and TZDs) in humans. (**B**–**E**) Changes in plasma adiponectin (% from the baseline, **B**), glycaemia (% from the baseline, **C**), circulating aspartate amino-transferase (ASAT, changes in U/l from the baseline, **D**), and alanine amino-transferase (ALAT, changes in U/l from the baseline, **E**). Pioglitazone treated patients are significantly different from placebo treated patients for these items as assessed using a mixed effects regression model analysis. Data are presented as mean and standard error (SE).

Multiple ALS mouse models display functional and molecular alterations in hypothalamic melanocortin system

We then asked whether the observed melanocortin defects translated into functional abnormalities. Indeed, the combination of decreased POMC with increased AgRP is usually found in situations of promotion of food intake, such as during fasting or in the case of leptin deficiency (Mizuno *et al.*, 1998, 1999; Ziotopoulou *et al.*, 2000), and compromised melanocortin system is likely to affect food intake behaviour, in particular during refeeding (Perez-Tilve *et al.*, 2010). Thus, we measured food intake after short-term fasting in SOD1m mice. Consistent with decreased POMC levels, 1 h food intake of SOD1m mice was 2-fold higher than wild-type littermates after 7 h (Fig. 7A) or 1 h (Fig. 7B)

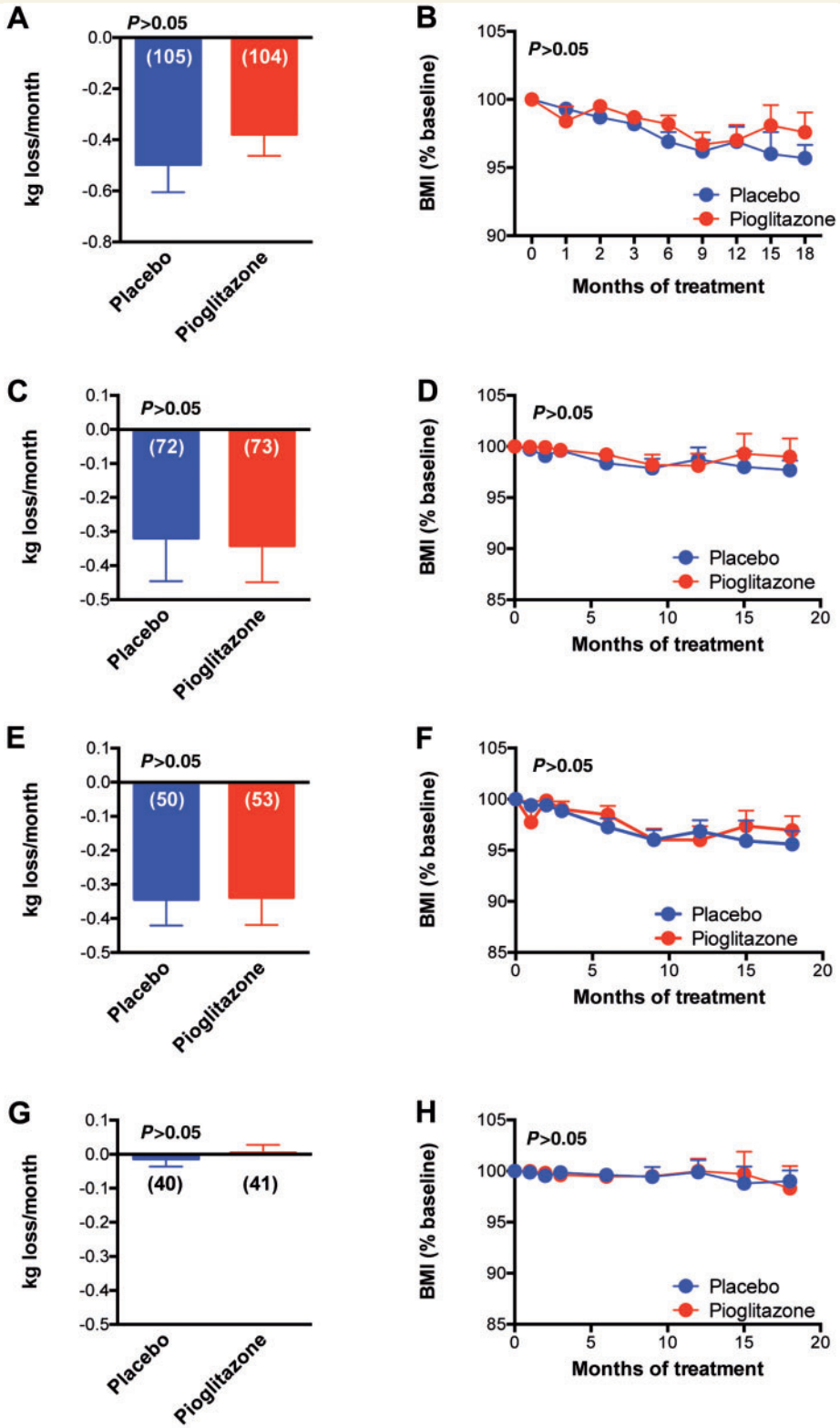

Figure 2 Effects of pioglitazone on weight loss in ALS patients. Weight loss (kg loss per month, **A**, **C**, **E** and **G**) and changes in BMI from the baseline (**B**, **D**, **F** and **H**) in the whole ALS population (**A** and **B**), spinal onset patients (**C** and **D**), in patients with relatively preserved quality of life (**E** and **F**) and in patients with preserved bulbar function (**G** and **H**). To select the patients with preserved quality of life, we used the results from the EuroQoL questionnaire to identify patients that had no or only few problems with their everyday life. Selected patients answered that they had either no or few problems in their everyday life during at least 6 months after their allocation to a group. To select the patients with preserved bulbar function, we used the results from ALS-FRS-R bulbar subscale and selected patients with a score equal or superior to 10 (maximum: 12) 6 months after inclusion. No significant difference is noted for these items. Data are presented as mean and SE.

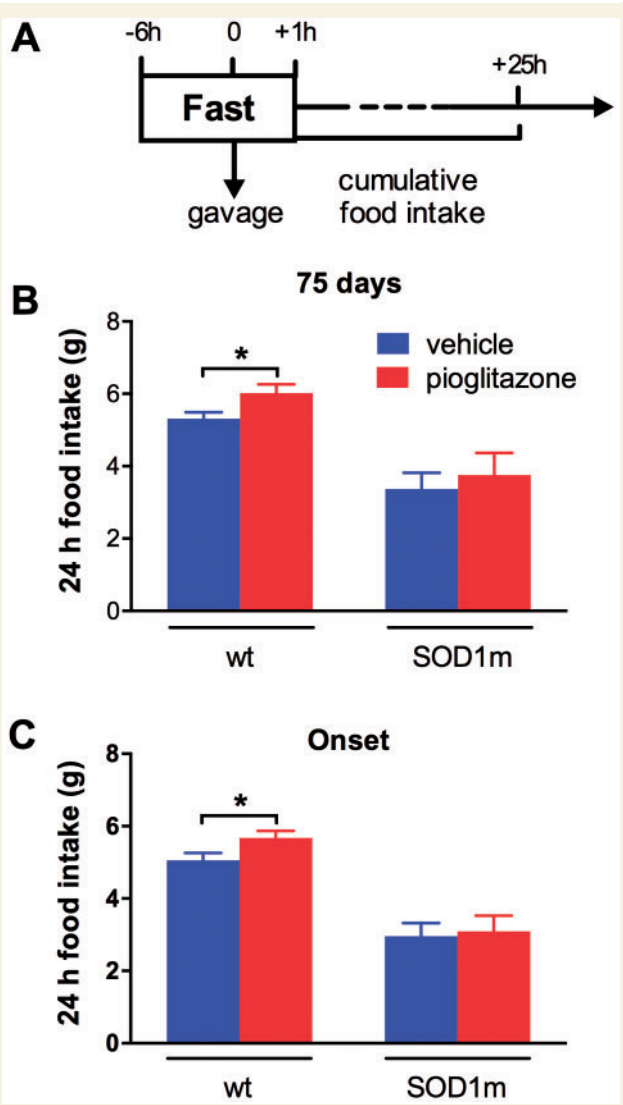

Figure 3 No effect of piogliotazone on SOD1m mice food intake. (**A**) Experimental scheme: pioglitazone was provided *per os* after 6 h of fasting. Food was reintroduced 1 h after gavage, and food intake recorded for the next 24 h. (**B** and **C**) Food intake after pioglitazone treatment in SOD1(G86R) mice (SOD1m) and control littermates (wt) at 75 days of age ($n = 12$, **B**) and symptom onset ($n = 13$, **C**), either treated with vehicle (blue) or pioglitazone 40 mg/kg body weight (red).$^{*}P < 0.05$, Paired Student's *t*-test for the drug. Data are presented as mean and SE.

of fasting. Mutations in *SOD1* only account for 20% of familial ALS cases, and alterations in hypothalamic melanocortin pathways could be SOD1-specific. However, *Pomc* mRNA levels were decreased in transgenic mice expressing A315T mutant *Tardbp* (Wegorzewska *et al.*, 2009) (Fig. 7C), and these mice also displayed transient hyperphagia in response to fasting (Fig. 7D). Last, *Pomc* mRNA levels tended to be lower in 10-month-old knock-in mice expressing a truncated FUS protein retained in the cytoplasm ($Fus^{\Delta NLS/+}$ mice) (Fig. 7E) (Scekic-Zahirovic, submitted for publication), and $Fus^{\Delta NLS/+}$ mice displayed increased food intake after short-term fasting as compared with their littermates (Fig. 7F). Thus, abnormal food intake behaviour and defects in the melanocortin system are hallmarks of ALS mouse models.

Involvement of serotonin loss in melanocortin defects associated with ALS

We then sought to understand the mechanisms underlying melanocortin defects in ALS. POMC neurons are affected by multiple stressors that could ultimately underlie the observed defects. We did not observe changes in expression of a series of genes related with oxidative or endoplasmic reticulum stress (e.g. gp47, splicing of *Xbp1* mRNA), peroxisome biogenesis [*Gpx1*, catalase (*Cat*)] or mitochondrial function (*Mfn2*; not shown) indirectly suggesting that neither overt oxidative stress, nor endoplasmic reticulum stress, nor defective peroxisomal biogenesis, nor mitochondrial abnormalities might account for decreased POMC neuronal counts. We hypothesized that the previously observed serotonin neuron degeneration (Turner *et al.*, 2005; Dentel *et al.*, 2013) could contribute to the observed melanocortin defects. Indeed, serotonin is known to promote POMC expression in arcuate nucleus through the 5-HT_{2C} receptor (encoded by *HTR2C*) (Heisler *et al.*, 2006; Lam *et al.*, 2008). Interestingly, serotonin levels tended to decrease at onset in the hypothalamus of SOD1m mice (Fig. 8A). To determine whether loss of serotonin axons occurred in the arcuate nucleus, we crossed SOD1m mice with Tph2-YFP mice, expressing yellow fluorescent protein (YFP) under the control of the *Tph2* promoter targeting expression in central serotonin neurons (Zhao *et al.*, 2011). We observed a sharp and profound decrease in the density of YFP-positive fibres in the arcuate nucleus of SOD1m mice, as compared with their littermates (Fig. 8B). Hypothalamic 5-HT_{2C} expression was increased suggesting that the hypothalamus sought to compensate for loss of serotonergic innervation (Fig. 8C). To probe for serotonergic involvement in defects of the melanocortin system, we used fluoxetine, a selective serotonin reuptake inhibitor to rescue serotonin signalling, using previously published doses and protocols (Kaur and Kulkarni, 2002; Liou *et al.*, 2012). Interestingly fluoxetine pretreatment completely abrogated the increased food intake in response to short-term fasting (Fig. 8D) and increased *Pomc* mRNA levels back to normal in SOD1m mice (Fig. 8E). Fluoxetine had no effect on *Agrp* upregulation (Fig. 8E). Thus, loss of serotonin innervation is contributing to the melanocortin defects observed in SOD1m mice.

Discussion

In this combined mouse and human study, we showed that ALS is associated with defects in the melanocortin system,

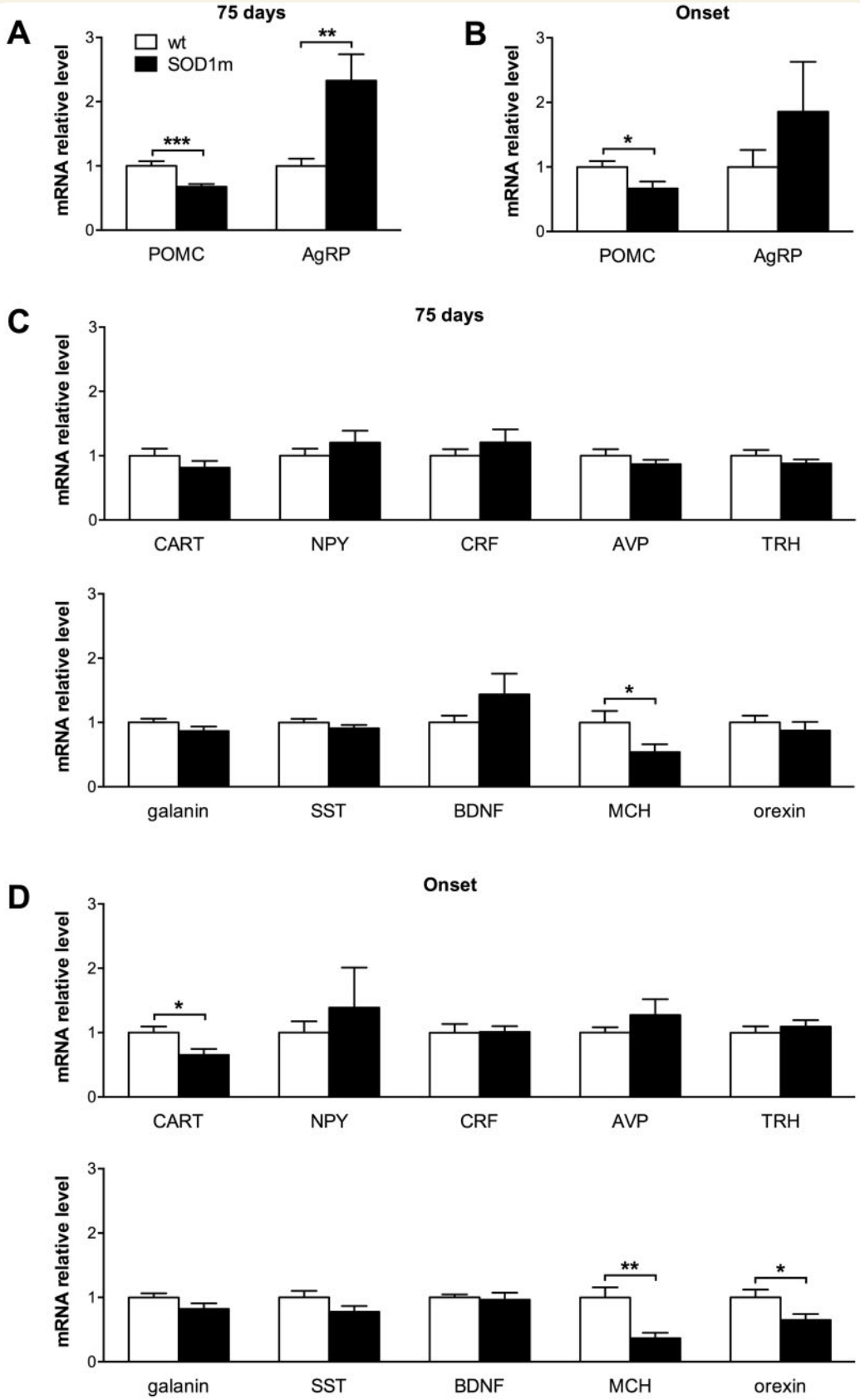

Figure 4 Altered melanocortin-related gene expression in SOD1m mice. (**A** and **B**) Messenger RNA levels of *Pomc* and *Agrp* in the hypothalamus of SOD1(G86R) mice (black, SOD1m) and control littermates (white, wt) at 75 days of age ($n = 15$, **A**) and symptom onset ($n = 11$, **B**). $^{*}P < 0.05$. $^{**}P < 0.005$. $^{***}P < 0.0005$, Student's *t*-test. Data are presented as mean and SE. (**C** and **D**) Messenger RNA levels of hypothalamic neuropeptides in the hypothalamus of SOD1(G86R) mice (black columns, SOD1m) and control littermates (white columns, wt) at 75 days of age ($n = 15$, **C**) and symptom onset ($n = 11$, **D**). $^{*}P < 0.05$; $^{**}P < 0.005$, Student's *t*-test. Data are presented as mean and SE.

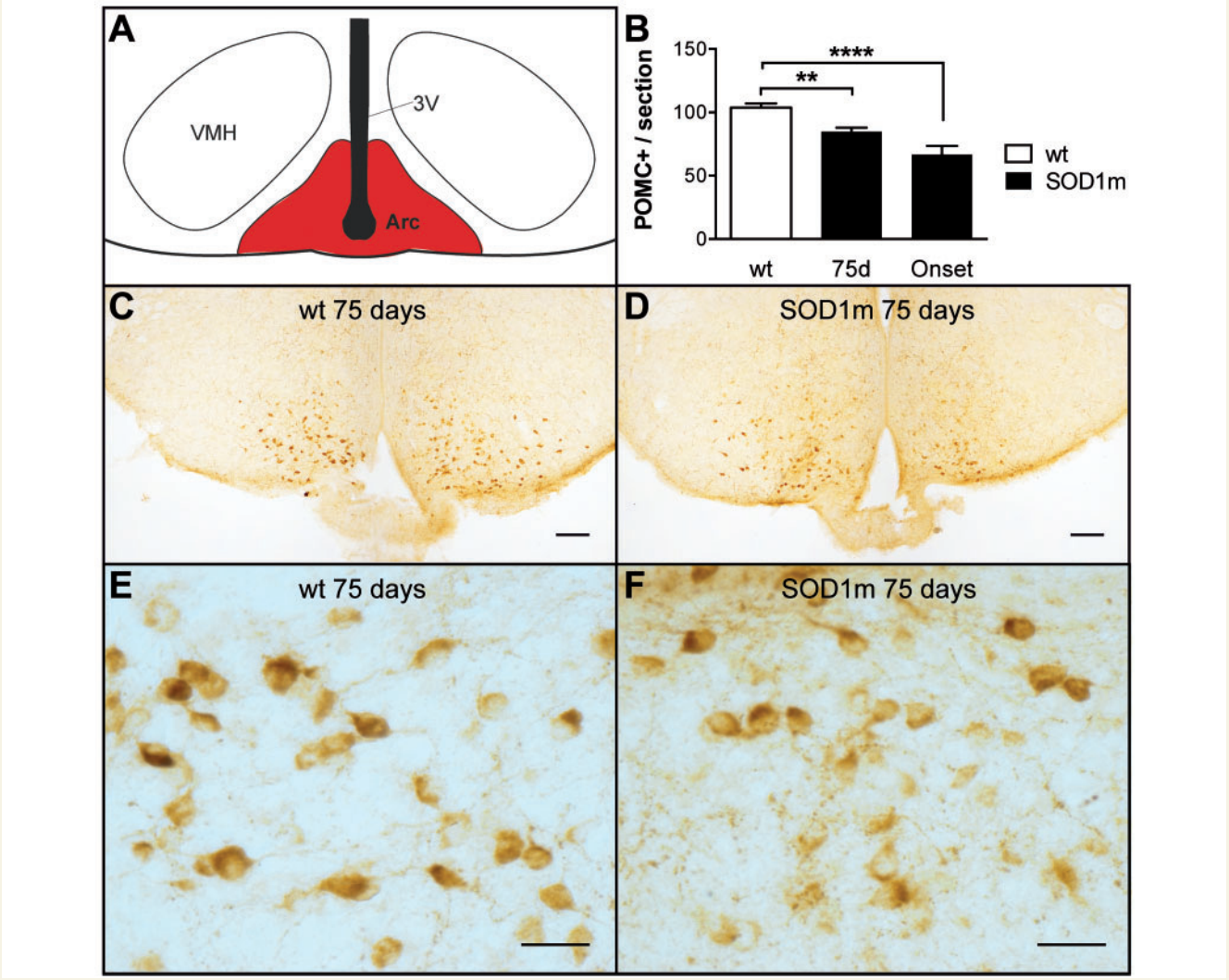

Figure 5 Decreased POMC-positive neurons in SOD1m mice. (**A** and **B**) Quantification of POMC neurons in the arcuate nucleus. The whole region was sectioned and half of these sections were stained for POMC immunohistochemistry after identification of arcuate nucleus according to Paxinos Brain Atlas (scheme of identified regions, in red, **A**). Total numbers of POMC-positive cell bodies in the arcuate nucleus were determined in SOD1m mice at 75 days of age ($n = 8$, **B–F**) and at symptom onset ($n = 7$, **B**) as compared with their wild-type littermates. $^{**}P < 0.005$, $^{****}P < 0.0001$, one-way ANOVA followed by Tukey *post hoc* test. Data are presented as mean and SE. (**C–F**) Representative images are shown for SOD1(G86R) mice (**D** and **F**) and control littermates (**C** and **E**) at 75 days of age at two magnifications. Scale bar = 200 μm (**C** and **D**); 20 μm (**E** and **F**).

the major hypothalamic circuit controlling food intake and energy expenditure. Pioglitazone did not increase weight in ALS patients, thus providing indirect evidence of altered hypothalamic melanocortin pathway. Pathological and functional deficits of melanocortin system were found in ALS mouse models directly demonstrating these defects. These findings further extend the spectrum of defects in ALS and provide a mechanistic explanation for a subset of metabolic signs observed in these patients. Our study also has important implications for the design of therapies to target weight loss in this disease.

We first observed that pioglitazone did not increase body weight or slow down weight loss in patients with ALS. This was a surprising observation as progressive weight gain has been repeatedly observed in all clinical trials of pioglitazone in multiple non-neurological diseases (Promrat *et al.*, 2004; Belfort *et al.*, 2006; Sanyal *et al.*, 2010; DeFronzo *et al.*, 2011). Despite this lack of weight gain, ALS patients under pioglitazone displayed all other biomarkers of efficacy, including decreased glycaemia, decreased circulating liver enzymes or increased adiponectin, thus ruling out that patients with ALS simply did not respond to the drug. Interestingly, a series of recent studies dissected out how TZDs lead to weight gain through activation of PPARγ in hypothalamic POMC neurons leading to increased food intake (Diano *et al.*, 2011; Lu *et al.*, 2011; Ryan *et al.*, 2011; Long *et al.*, 2014). Consistent with the notion that pioglitazone hypothalamic response was blunted in

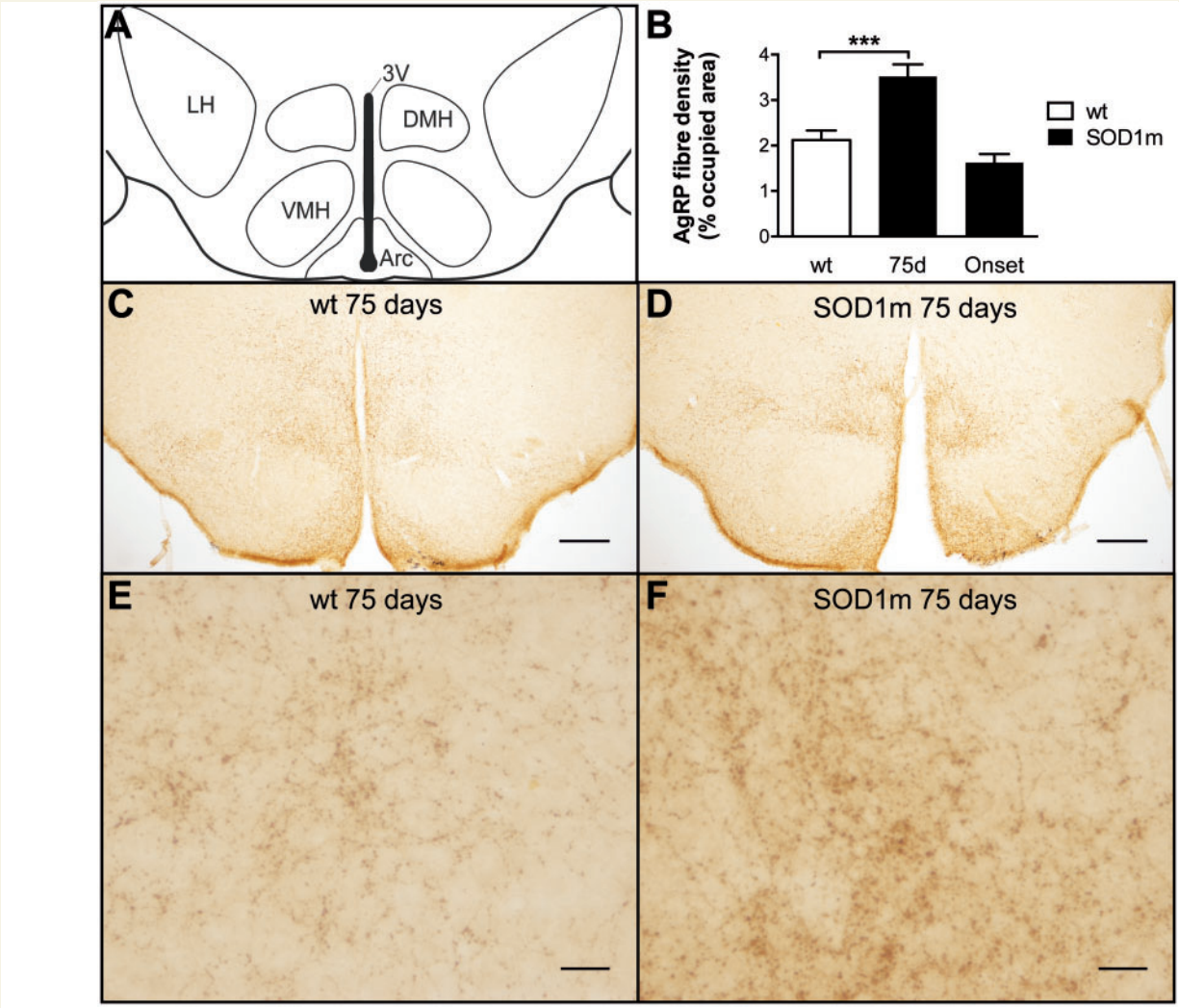

Figure 6 Defect in AGRP neurons in SOD1m mice. (**A** and **B**) Quantification of AGRP immunoreactive neurites in the hypothalamus. For each animal, one section (Bregma −1.58 mm) was identified according to Paxinos Brain Atlas (**A**, scheme of identified regions) and stained for AGRP immunohistochemistry. AGRP fibre density was determined in SOD1m mice at 75 days of age ($n = 7$, **B** and **F**) and at symptom onset ($n = 4$, **B**) as compared with their wild-type littermates. $^{***}P < 0.001$, one-way ANOVA followed by Tukey *post hoc* test. Data are presented as mean and SE. (**C–F**) Representative images are shown for SOD1(G86R) mice (**D** and **F**) and control littermates (**C** and **E**) at 75 days of age at two magnifications. Scale bar = 200 μm (**C** and **D**), 20 μm (**E** and **F**).

ALS patients, pioglitazone was not able to promote food intake in SOD1m mice. Indeed, the melanocortin system is dramatically affected in these mice, with decreased POMC expression and loss of POMC positive neurons; similar alterations were observed in TDP-43- and FUS-based mouse models pointing out that such defects are a general feature in ALS. As pioglitazone decreases the activity of POMC neurons thus increasing food intake (Diano *et al.*, 2011), we propose that the already decreased melanocortin tone in ALS prevents the silencing of POMC neurons by pioglitazone. Consistent with this, SOD1 mice displayed hyperphagia in response to short-term fasting, an orexigenic stimulus that also leads to decreased POMC neuronal activity (Perez-Tilve *et al.*, 2010; Diano *et al.*, 2011). Thus, our results point to a general decrease in melanocortin tone in ALS, leading to both a lack of response to TZDs, and abnormal food intake behaviour in response to fasting.

What is the contribution of impaired melanocortin system to the metabolic phenotypes associated with ALS? The melanocortin system exerts multiple actions on energy metabolism, either dependent on or independent of food intake. First, an expected consequence of decreased melanocortin tone is increased energy intake, especially in response to an orexigenic stimulus such as food deprivation. This is indeed what has been observed in multiple transgenic mouse models of ALS, suggesting that the defect in the melanocortin system translates into a functional deficit. Furthermore, consistent with the observed melanocortin defect, we previously observed slightly increased cumulative food intake in SOD1m mice (Dupuis *et al.*, 2004).

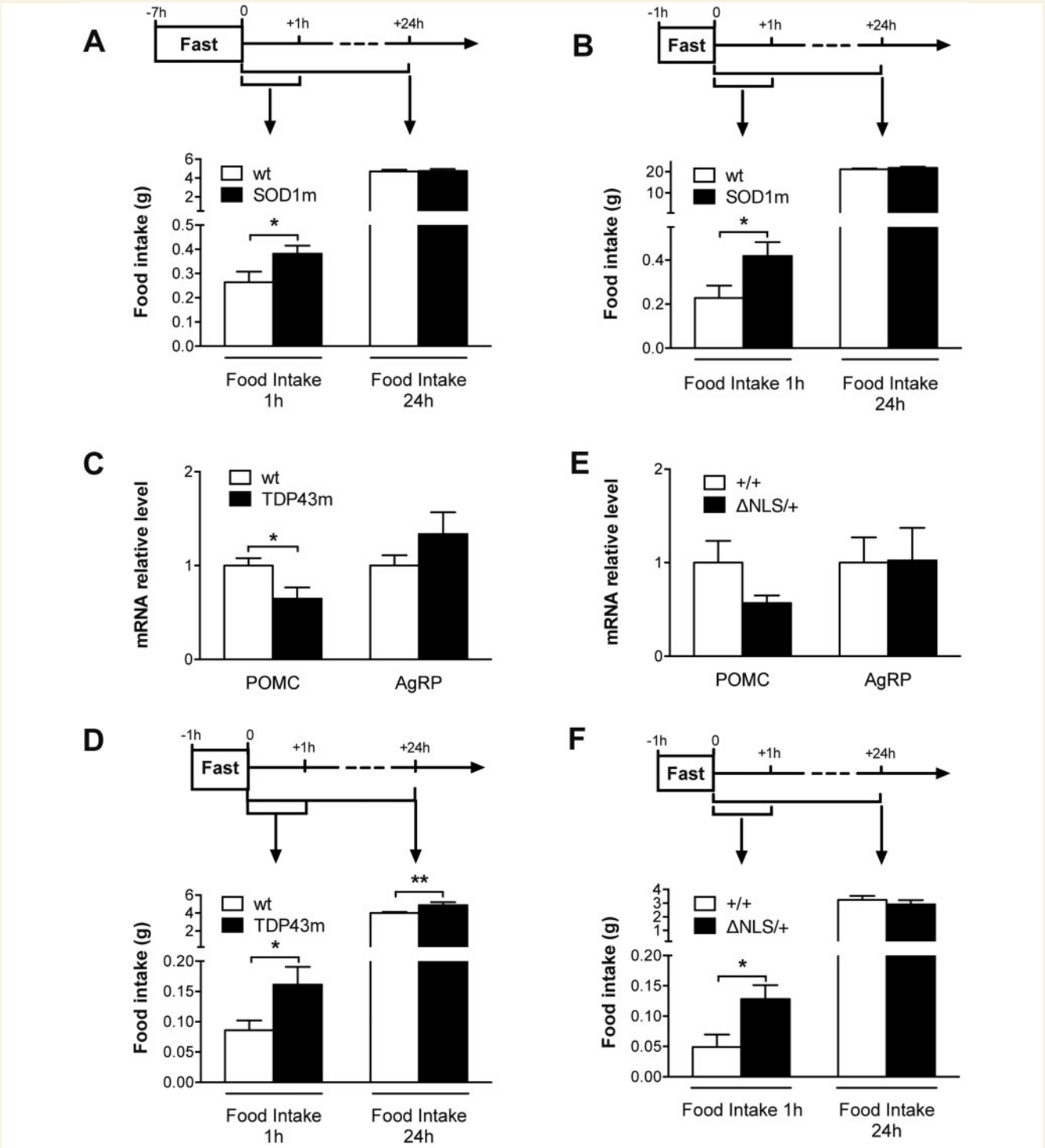

Figure 7 Multiple ALS mouse models display functional and molecular alterations in hypothalamic melanocortin system. (**A** and **B**) Food intake was measured for 1 h, after either 7 h (**A**) or 1 h (**B**) of fasting in SOD1(G86R) mice (black columns, SOD1m) and control littermates (white columns, wt) at 75 days of age ($n = 10$ and $n = 14$, respectively, for **A** and **B**). $^{*}P < 0.05$, Student's *t*-test. Data are presented as mean and SE. (**C**) Messenger RNA levels of *Pomc* and *Agrp* in the hypothalamus of transgenic mice expressing A315T TDP-43 mutation (black columns, TDP43m) and control littermates (white columns, wt) at non-symptomatic stage ($n = 6$). Unpaired *t*-test. $^{*}P < 0.05$. (**D**) Food intake was measured 1 h after refeeding in TDP43m mice ($n = 8$). $^{*}P < 0.05$, $^{**}P < 0.01$, Multiple *t*-test. Data are presented as mean and SE. (**E**) Messenger RNA levels of *Pomc* and *Agrp* in the hypothalamus of transgenic mice *Fus* ΔNLS/+ knock-in mice (black columns, ΔNLS/+) and control littermates (white columns, +/+) at 10 months of age ($n = 4$). Data are presented as mean and SE. (**F**) Food intake was measured 1 h after refeeding in ΔNLS/+ mice ($n = 10$) at 10 months of age. $^{*}P < 0.05$, Student's *t*-test. Data are presented as mean and SE.

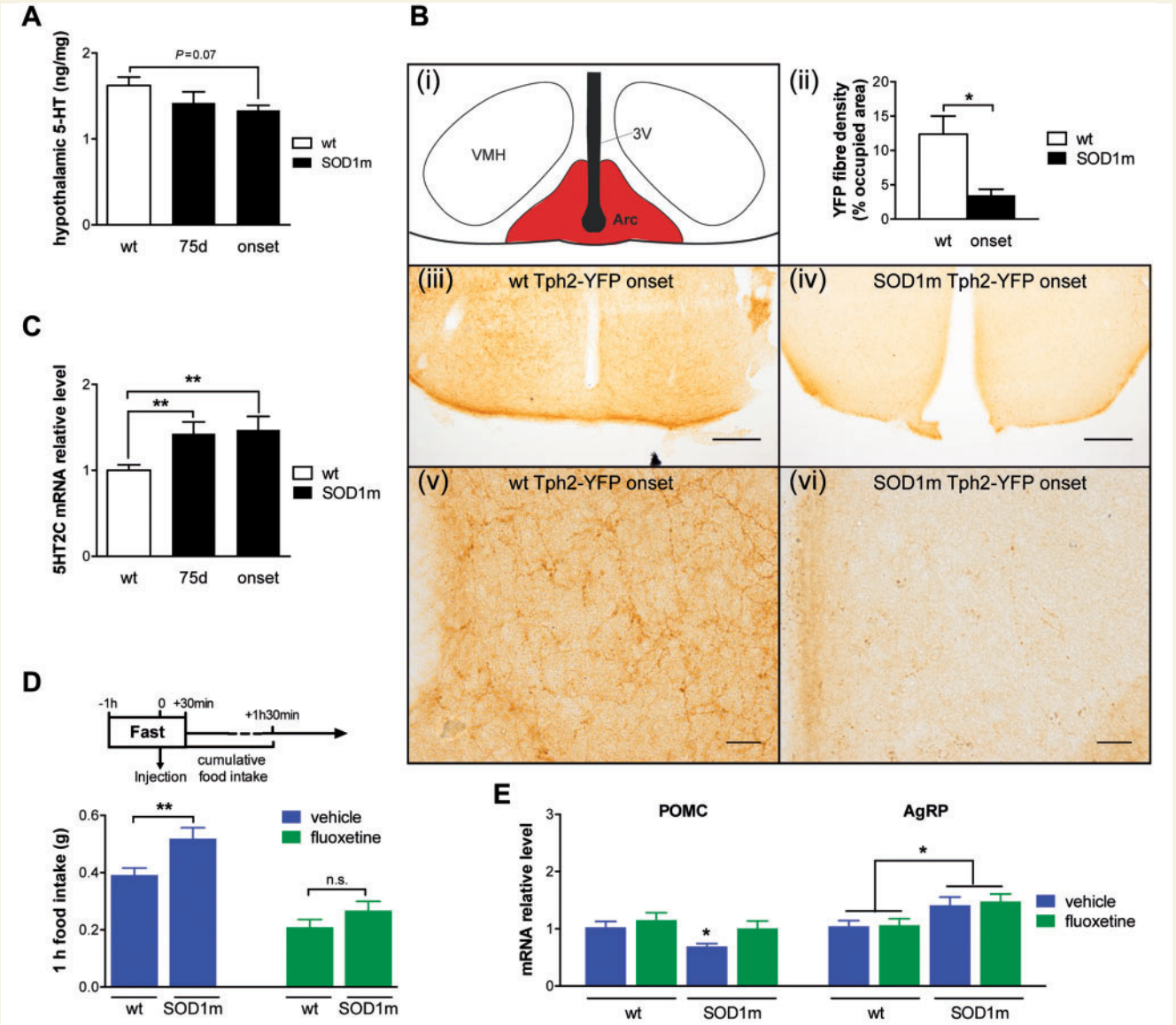

Figure 8 Involvement of serotonin loss in melanocortin defects of mutant SOD1 mice. (**A**) Serotonin levels in the hypothalamus of SOD1(G86R) mice (black columns, SOD1m) and control littermates (white columns, wt) ($n = 5$ at 75 days and $n = 5$ at onset). One-way ANOVA followed by Tukey *post hoc* test. Data are presented as mean and SE. (**B**) Serotonergic innervation in the arcuate nucleus. GFP immunohistochemistry was performed on Tph2-YFP mice on arcuate nucleus cuts after identification on Paxinos Brain Atlas (**i**, scheme of identified regions, in red, in the upper left column). Representative images are shown for SOD1(G86R) Tph2-YFP mice and control littermates at onset at two magnifications. Scale bar = 200 μm (**iii**, **iv**), 20 μm (**v** and **vi**). Serotonergic fibre densities in the arcuate nucleus were quantified by a blind observer as described previously (Grider *et al.*, 2006) in SOD1m mice at symptom onset (**ii**–**vi**, $n = 4$) as compared with their wild-type littermates. $^{*}P < 0.05$, Student's *t*-test. Data are presented as mean and SE. (**C**) Messenger RNA levels of *Htr2c* (5-HT_{2C} receptor) in the hypothalamus of SOD1(G86R) mice (black, SOD1m) at 75 days of age and onset and in control littermates (white, wt) ($n = 8$). $^{**}P < 0.01$, one-way ANOVA. Data are presented as mean and SE. (**D**) Food intake was measured 1 h after refeeding in SOD1(G86R) mice (SOD1m) at 80 days of age and control littermates (wt) after fluoxetine 20 mg/kg body weight (red) or vehicle (blue) injection ($n = 38$). $^{**}P < 0.005$, Multiple *t*-test. Data are presented as mean and SE. (**E**) Messenger RNA levels of *Pomc* and *Agrp* in the hypothalamus of SOD1(G86R) mice (SOD1m) and control littermates (wt) at 85 days of age after fluoxetine 20 mg/kg body weight (red) or vehicle (blue) injection ($n = 20$). $^{*}P < 0.05$, one-way ANOVA. Data are presented as mean and SE.

The situation in ALS patients is less clear with respect to energy intake due to a relative lack of studies reporting dietary intake accurately and to confounding effects of dysphagia in advanced ALS patients. However, two case-control studies reported that increased dietary fat intake was associated with ALS (Nelson *et al.*, 2000; Huisman *et al.*, 2015). Moreover, Huisman *et al.* (2015) and collaborators observed that presymptomatic daily energy intake was increased in ALS patients as compared with controls, and this would be consistent with both melanocortin

impairment and lack of weight gain under pioglitazone. Second, the melanocortin defect could be responsible for alterations in peripheral metabolic pathways. Indeed, the melanocortin system regulates peripheral lipid metabolism in rodents, by activating cholesterol reuptake by the liver (Perez-Tilve *et al.*, 2010) and reduces hepatic lipogenesis (Leckstrom *et al.*, 2011) independently of food intake. In the same line, the melanocortin system is controlling glucose metabolism and insulin response (Obici *et al.*, 2001). Interestingly, ALS patients have been reported to display increased circulating cholesterol levels (Dupuis *et al.*, 2008), and glucose intolerance (Pradat *et al.*, 2010). Third, the melanocortin defect could impair regulation of the autonomic nervous system (Sohn *et al.*, 2013), and autonomic abnormalities have sometimes been found in ALS patients (Baltadzhieva *et al.*, 2005). The relationships between these different phenotypes and melanocortin defects are unclear and will have to be clarified in further studies. Importantly, the observed melanocortin defect is unable to explain the weight loss associated with ALS, as, on the contrary, the increased orexigenic drive triggered by POMC deficiency likely compensates for weight loss by increasing food intake. Other mechanisms, either peripheral or central, have still to be identified to explain weight loss.

What causes the melanocortin defect in ALS? A first obvious potential mechanism is energy deficit. Indeed, SOD1m mice display weight loss due to hypermetabolism, leading to decreased fat mass. These mice also display decreased circulating insulin and leptin levels (Dupuis *et al.*, 2004). Ablation of leptin in *ob/ob* mice or fasting is indeed sufficient to cause decreased *Pomc* mRNA (Mizuno *et al.*, 1998, 1999; Ziotopoulou *et al.*, 2000). However, leptin levels are only decreased by 30% in 75-day-old mice (Dupuis *et al.*, 2004), an age at which we already observe strong *Pomc* mRNA decreases. We and others had previously observed serotonin loss in ALS (Turner *et al.*, 2005; Dentel *et al.*, 2013), and we hypothesized that this contributed to melanocortin impairment in SOD1m mice. Indeed, serotonin is a major activator of POMC neurons through the 5-HT_{2C} receptor, and this occurs through direct electrical stimulation (Heisler *et al.*, 2002) but also trough transcriptional activation (Zhou *et al.*, 2007; Lam *et al.*, 2008; Xu *et al.*, 2008). Consistently, loss of 5-HT_{2C} receptor leads to decreased *Pomc* mRNA in the hypothalamus (Wang and Chehab, 2006). Our observation of restoration of *Pomc* mRNA levels, as well as reversal of transient hyperphagia in SOD1m mice by fluoxetine argues for serotonin loss being a primary cause of melanocortin defect. This, however, does not exclude that direct modulation of electrical activity by either decreased leptin or other cues, either extrinsic or intrinsic, further exacerbate the observed defect. Brain serotonin system is itself affected by organismal energy status (Dwarkasing *et al.*, 2015; Zemdegs *et al.*, 2015) and our data do not exclude that defects in serotonin levels found in ALS patients and models is caused, or contributed by, weight loss and hypermetabolism. Consistent with this notion, decreasing leptin, whose major action is on the melanocortin system, was able to revert partial weight loss and increased energy expenditure in SOD1m mice (Lim *et al.*, 2014), suggesting that the melanocortin system can be further inhibited by leptin ablation.

What are the consequences of our current findings in ALS? There are at least three consequences of our finding for ALS research. First, melanocortin impairment seems to be a general event occuring in sporadic ALS patients, as well as in animal models caused by disparate mutations leading to ALS. Interestingly, a series of recent studies demonstrated the occurence of similar hypothalamic abnormalities in FTD (Piguet *et al.*, 2011; Ahmed *et al.*, 2014*a*, *b*, 2015), and in particular, increased AGRP (Ahmed *et al.*, 2015). Thus, melanocortin impairment appears associated with overall ALS/FTD continuum. It remains to be determined how melanocortin impairment relates with motor neuron degeneration in ALS. Second, this study reinforces the notion of systemic involvement in ALS. That melanocortin impairment appears downstream of serotonin loss, also brings about the notion that circuitry dysfunction might contribute to aspects of ALS phenotype. How these serotonin and melanocortin defects might be related to the spreading of TDP-43 aggregates (Brettschneider *et al.*, 2013) remains to be resolved. Second, these results have consequences for the design of pharmacological strategies to combat weight loss in ALS. Weight loss in ALS is likely to be multi-factorial, with primary causes such as hypermetabolism, and bulbar involvement, and could be exacerbated secondary to other symptoms such as deficit in upper limbs or depression. Treating weight loss could identify disease-modifying interventions as a hypercaloric diet was recently found to increase survival of ALS patients under gastrostomy (Wills *et al.*, 2014; Dorst *et al.*, 2015). Many drugs that could be used to prevent weight loss, including atypical antipsychotics (e.g. olanzapine) inhibit POMC neurons (Kirk *et al.*, 2009; Weston-Green *et al.*, 2012; Lian *et al.*, 2014). Drugs targeting the cannabinoid system increase body weight by increasing beta-endorphin release from POMC neurons (Koch *et al.*, 2015). As beta-endorphin is derived from POMC, which is decreased in ALS mice, it appears likely that cannabinoids might not be able to promote food intake through this mechanism in ALS. Thus, our current study provides a note of caution for the use of these drugs to counteract weight loss in the specific context of ALS patients, and suggest that disease progression might impair responsiveness of ALS patients to classical drugs leading to weight gain. A number of neural pathways controlling energy homeostasis have not yet been studied in the context of ALS and could be potential targets for treating weight loss (Morton *et al.*, 2014). First, pathways involved in the emergency response to glucose deprivation (glucopaenia) such as NPY might be useful, although the precise neurochemical pathways still need to be elucidated. Second, drugs affecting food reward might be of interest to improve the attractability of food during the disease. Last, the existence of emergency neuronal circuits involved in stress-

induced anorexia was recently elucidated. Inhibiting these pathways in ALS might also be a possible target for treating weight loss (Morton *et al.*, 2014). Alternatively, and besides pharmacological treatments, increasing caloric density of the diet is likely an efficient strategy to counteract weight loss (Wills *et al.*, 2014; Dorst *et al.*, 2015), although current results do not allow us to determine whether lipid enriched or carbohydrate enriched would be more efficient.

In all, our *post hoc* analysis of the pioglitazone trial revealed that the melanocortin system is profoundly altered in ALS, and that this might be important for understanding and preventing impairment of energy metabolism in ALS patients.

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Supplementary material

Supplementary material is available at *Brain* online.

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Appendix 1

Collaborators: GERP ALS Study Group

All centres were located in Germany. Investigators are listed by alphabetical order of centre and investigator:

Berlin (Department of Neurology, Charité University Hospital): Nadja Borisow; Theresa Holm; Andre Maier; Thomas Meyer; Bochum (Department of Neurology, University Hospital Bergmannsheil): Paula Budde; Torsten Grehl; Kai Gruhn; Bonn (Department of Neurology, University Hospital of Bonn): Malte Bewersdorff; Michael Heneka; Dresden (Department of Neurology, University Hospital Carl Gustav Carus, Technische Universität Dresden): Andreas Hermann; Alexander Storch; Göttingen (Department of Neurology, University Hospital of Göttingen): Tobias Frank; Bettina Göricke; Jochen Weishaupt; Halle (Department of Neurology, University Hospital of Halle/Saale): Katharina Eger; Frank Hanisch; Stephan Zierz; Hannover (Department of Neurology and Clinical Neurophysiology, Hannover Medical School (MHH), University Clinic): Anna-Lena Boeck; Reinhard Dengler; Sonja Koerner; Katja Kollewe; Susanne Petri; Jena (Department of Neurology, University Hospital Jena): Julian Grosskreutz; Tino Prell; Thomas Ringer; Jan Zinke; Munich (Department of Neurology, University of Munich): Johanna Anneser; Gian Domenico Borasio; Christine Chahli; Andrea S. Winkler; Muenster (Department of Neurology, University of Muenster): Matthias Boentert; Bianca Stubbe-Draeger; Peter Young; Regensburg (Department of Neurology, University of Regensburg): Ulrich Bogdahn; Steffen Franz; Verena Haringer; Norbert Weidner; Rostock (Department of Neurology, University of Rostock): Reiner Benecke; Stefanie Meister; Johannes Prudlo; Matthias Wittstock; Ulm (Department of Neurology, University of Ulm): Johannes Dorst; Corinna Hendrich; Albert C. Ludolph; Anne-Dorte Sperfeld; Ulrike Weiland; Wiesbaden (Department of Neurology, Neurological clinic, DKD): Sabine Neidhardt; Berthold Schrank; Wurzburg (Department of Neurology, University of Wurzburg): Marcus Beck; Peter Kraft; Klaus Toyka; Jochen Ulzheimer; Carsten Wessig.