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Serotonin 2B receptor slows disease progression and prevents degeneration of spinal cord mononuclear phagocytes in amyotrophic lateral sclerosis

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Abstract:

Microglia are the resident phagocytes of the central nervous system and have been implicated in the pathogenesis of neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS). During neurodegeneration, microglial activation is accompanied by infiltration of circulating monocytes, leading to overall increased phagocytic activity and production of multiple inflammatory mediators in the spinal cord. Degenerative alterations in monocytes and microglia are commonly observed during neurodegenerative diseases, yet little is known concerning the mechanisms leading to their degeneration, or the consequences on disease progression. Here we observed that the serotonin 2B receptor (5-HT_{2B}), a serotonin receptor expressed in microglia, is upregulated in the spinal cord of three different transgenic mouse models of ALS. In mutant SOD1 mice, this upregulation was restricted to cells positive for CD11b, a marker of mononuclear phagocytes. Ablation of 5-HT_{2B} receptor in transgenic ALS mice expressing mutant SOD1 resulted in increased degeneration of mononuclear phagocytes, as evidenced by fragmentation of Iba1-positive cellular processes. This was accompanied by decreased expression of key neuroinflammatory genes but also loss of expression of homeostatic microglial genes. Importantly, the dramatic effect of 5-HT_{2B} receptor ablation on mononuclear phagocytes was associated with acceleration of disease progression. To determine the translational relevance of these results, we studied polymorphisms in the human *HTR2B* gene, which encodes the 5-HT_{2B} receptor, in a large cohort of ALS patients. In this cohort, the C allele of SNP rs10199752 in *HTR2B* was associated with longer survival. Moreover, patients carrying one copy of the C allele of SNP rs10199752 showed increased 5-HT_{2B} mRNA in spinal cord and displayed less pronounced degeneration of mononuclear phagocytes than patients carrying two copies of the more common A allele. Thus functional 5-HT_{2B} receptors limit degeneration of spinal cord macrophages (most likely microglia), and slow disease progression in ALS. Targeting this receptor might be therapeutically useful.

Keywords: amyotrophic lateral sclerosis; motor neuron; serotonin; microglia; SOD1

Introduction

Amyotrophic lateral sclerosis (ALS) is the major adult onset motor neuron disease with a lifetime risk of 1/400, and represents the third most frequent neurodegenerative disease after Alzheimer's and Parkinson's diseases. ALS is characterized by the selective degeneration of upper motor neurons in the cerebral cortex and lower motor neurons in spinal cord and brainstem, and leads to progressive paralysis and death within 3 to 5 years after onset [38]. A number of ALS cases are dominantly inherited and more than 20 genes have been associated with ALS, in particular *C9ORF72*, *TARDBP*, *FUS* and *SOD1* [44]. Most experimental research has involved expression of mutant SOD1 in transgenic mice, leading to development of typical ALS symptoms.

In recent years it has become increasingly clear that progression of ALS symptoms is not caused exclusively by intrinsic events within motoneurons, but rather involves many other cell types and in particular glial cells [4,53]. The role of mononuclear macrophages, either microglia that are resident macrophages of the central nervous system [37] or monocytes that infiltrate the CNS during disease, has been intensely studied. During neurological diseases, activation of macrophages is characterized by appearance of an amoeboid morphology, increased phagocytic activity and production of a number of cytokines and chemokines. Macrophage activation could either be protective by providing support to neurons and astrocytes in response to injury and by cleaning debris through phagocytosis, or deleterious by creating an inflammatory environment contributing to neuronal degeneration [53,47]. Importantly, decreased expression of mutant SOD1 in CD11b-positive cells, *i.e.* in all macrophages, prolongs disease progression suggesting that mutant SOD1 exerts toxic action in these cells thereby accelerating disease [5]. Furthermore, decreased activation of the pro-inflammatory transcription factor NF-kappaB in microglial cells potentially slowed down disease progression [25]. An open question is whether the toxic effect of macrophages is mediated by resident microglia and/or by infiltrating blood monocytes. The extent of monocyte infiltration during ALS in mouse models remains controversial, with one study observing major monocytic infiltration [9], and others only very little if any [12,42,1,46]. Recently, genome-wide characterization of microglial and monocyte expression patterns led to the identification of a molecular signature of homeostatic microglia. Specifically, microglia express genes not expressed in circulating monocytes or other resident tissue macrophages such as *P2ry12*, *Hexb*, *Olfml3*, *SiglecH*, *Tgfb1* or *Tmem119* [8,48,31,12,7]. Contrastingly, monocytes express surface markers not present on microglia, such as *Ly6c1* [9]. Importantly, microglia lose the expression of their homeostatic signature during disease progression [12,7], and develop unique expression patterns during experimental ALS distinct from inflammation-induced alteration [12,7]. This loss of typical microglial expression patterns could be directly correlated with degeneration of CNS macrophages, documented in different neurodegenerative diseases [58,57,69] including a transgenic model of ALS [24,58]. Whether macrophage degeneration is microglia specific or also occurs in infiltrating monocytes, and whether this degeneration might be involved in neurodegeneration is not known.

Recently, we observed that serotonin 2B receptor (5-HT_{2B}R) mRNA levels were strongly upregulated in the spinal cord of a mouse model of ALS - SOD1(G86R) mice, notably at later stages and coincident with development

of spasticity [15]. 5-HT_{2B}R is expressed in microglia in the CNS [40], with expression in other cell types including brain serotonin neurons [16]. In human peripheral macrophages, 5-HT_{2B}R mediates the response to serotonin by skewing macrophages to a M2, anti-inflammatory, phenotype [14]. More relevant to ALS, we showed that 5-HT_{2B}R is the major serotonin receptor expressed on perinatal microglia and regulates chemotaxis of microglial processes in response to serotonin [40]. These findings raise the possibility that the observed 5-HT_{2B}R upregulation could be linked to microglial activation and function in the pathogenesis of ALS.

Here, we show that the lack of 5-HT_{2B}R is associated with degeneration of spinal cord mononuclear phagocytes in ALS mouse models and human patients, and slows down disease progression of ALS. This has broad consequences for our understanding of serotonin function during disease, and provides a plausible pharmacological target to modulate neuroinflammation in ALS and other neurodegenerative diseases.

Materials and methods

Patients

Two independent populations of patients were studied here. In the human genetic study, cases from the Netherlands were diagnosed with probable or definite ALS according to the revised El-Escorial Criteria by neurologists specialized in motor neuron diseases [6]. Tertiary referral centers for ALS were University Medical Center Utrecht, Academic Medical Centre Amsterdam and Radboud University Medical Center Nijmegen. All participants gave written informed consent, and approval was obtained from the relevant local ethical committees for medical research. Characteristics of ALS patients included in this genetic study are presented in **Table S1**. Control individuals were free of any neuromuscular disease and matched for age, gender and ethnicity ascertained through a population based study on ALS in the Netherlands [32]. Patients included in the neuropathology study were from Ulm University. All autopsy brains were collected in accordance with local ethical committee guidelines and the federal law governing the use of human tissue for research in Germany. The characteristics of these patients are described in **Table S2**.

Animals

Transgenic mice were housed in the animal facility of the medicine faculty of Strasbourg University, with 12h/12h of light/dark and unrestricted access to food and water. In all experiments, littermates were used for comparison. Transgenic mice carrying SOD1 G86R mutation [18] and their non-transgenic littermates on a FVB/N background were genotyped and onset of symptoms was defined according to previous studies [15]. Dynactin mice expressing G59S mutation of P150^{Glued} have been previously described [43], and were used according to previous studies [67]. SOD1(G37R) mice were kindly provided by Dr Don W. Cleveland and have been described previously [5]. *Htr2b*^{-/-} mice in a 129S2/Sv PAS background have been described previously [49,45]. All animal experimentation was performed in accordance with institutional guidelines, and protocols were approved by the local ethical committee

from Strasbourg University (CREMEAS) under number AL/30/37/02/13 and AL/29/36/02/13, in accordance with European regulations.

Mouse breeding, survival and motor phenotyping

SOD1 (G86R) mice were crossed with *Htr2b*^{-/-} mice to obtain the F1 generation. Male *Htr2b*^{+/-} carrying SOD1 (G86R) transgene were then crossed with female *Htr2b*^{+/-} from the F1 generation to obtain the F2 generation with the genotypes of interest. *Htr2b*^{-/-} mice carrying SOD1 (G86R) transgene (n=16) were compared with littermate *Htr2b*^{+/-} mice carrying SOD1 (G86R) transgene (n=12), *Htr2b*^{+/-} mice carrying SOD1 (G86R) transgene (n=19), and *Htr2b*^{-/-} or *Htr2b*^{+/-} mice negative for SOD1 (G86R) transgene were used as control.

Mice were visually inspected daily and weekly monitored for body weight and motor symptoms from 4 weeks of age until end stage of the disease. To evaluate muscle strength, we used a gripmeter test (Bioseb, ALG01; France). The muscle force (in Newton) was measured three times per mouse. Results are the mean of three consecutive weekly sessions. Disease course and survival were assessed daily by visual inspection. Disease onset was calculated as time of peak of body weight. Disease duration was the time between the peak of body weight and death. After disease onset mice were followed daily and end stage was defined by full paralysis and when mice were unable to return after 10 seconds placed on the back. End stage mice were immediately euthanized.

Histological techniques

Muscle tissues and spinal cords were dissected and fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 overnight. The whole muscles were dissected into fiber bundles, stained using Alexa Fluor 594-conjugated α -Bungarotoxin (Sigma, T195; 1:500) for nicotinic acetylcholine receptors (AChRs), anti-synaptophysin (Millipore, 5258; 1:250) and anti-neurofilament (Abcam, Ab24574, 1/100) for pre-synaptic elements, followed by fluorescent secondary antibodies anti-mouse Alexa 488 (Life Technologies, A11001), and Draq 5 for nuclei (Cell Signaling, 4084; 1:1000). Single-layer images or Z-Stack images (1 μ m optical section) were acquired using a laser-scanning microscope (confocal Leica SP5 Leica Microsystems CMS GmbH) equipped with 63 \times oil objective (NA1.4). Excitation wavelengths were sequentially argon laser 488nm, diode 561nm, and helium neon laser 633nm. Emission bandwidths are 500-550nm for Alexa488, 570-620nm for Alexa594, and 650-750nm for draq5.

The lumbar parts of spinal cords were dissected and the L3-L5 region was identified according to previous studies [27]. Tissue was cryoprotected in 30% sucrose and snap frozen in melting isopropanol in TissueTek (O.C.T.Compound, SAKURA#4583). Cryosections (Leica CM 3050S) of 16 μ m were obtained for histological analysis of end stage mice (10 sections per animal). Spinal cord sections were incubated in phosphate buffered saline (PBS) 0.1% Triton with anti-choline acetyl transferase (ChAT) (Millipore, AB144-P; diluted 1:50) for motor neuron quantification and anti-Iba1 (Abcam ab5076, 1/100) antibody followed by biotinylated species-specific secondary antibody. The staining was revealed using the ABC kit (Vectastain), by the avidin-biotin complex

immunoperoxidase technique or immunofluorescence. Identical techniques and antibodies were used to stain paraffin sections of ALS patients for Iba1.

Quantification of histological results

Motoneuron counting was performed in L3-L5 ventral horn in every tenth section for ten sections in total per animal (160 μ m thick in total, spread over 1.6 mm). Only ChAT⁺ neurons located in a position congruent with that of motoneuron groups were counted [13]. All ChAT⁺ profiles located in the ventral horns of immunostained sections and clearly displayed in the plane of section were counted. Total estimated motoneuron numbers were obtained using a computer-assisted microscope (Nikon Eclipse E800) and associated software (Nis Elements version 4.0). Total numbers of motoneurons and the mean area of individual cells were obtained using ImageJ freeware (<http://rsbweb.nih.gov/ij/>) after image acquisition at 20X under the same exposition parameters with a digital camera (Nikon Digital Sight DS-U3) [56]. The observer was blinded to the genotype of studied mice.

To quantify ventral horn atrophy, the total surface of the ventral horn, as defined in Figure S4, was measured using Nis Elements version 4.0.

For staining of mononuclear phagocytes, measurement of Iba1 immunoreactivity was performed on images acquired from Iba1 immunostaining at 10x magnification. A standardized rectangle was drawn in the ventral horn and the surface of Iba1 staining relative to background was calculated using the Pixel classifier algorithm of Nikon Nis-element 3.10 SP3 software, using the intensity profile measurement function. The observer was blinded to the genotype of studied mice. >10 images per animal were quantified, with n=5 animals per genotype.

Mononuclear phagocyte morphology was defined according to previous work [58]: (i) resting phagocyte with small branched cellular processes and small cell soma, (ii) activated state with greater distal arborization, (iii) phagocytic state with increased cell soma size and (iv) dystrophic state with fragmented cytoplasm. Iba1-immunopositive cells were categorized according to their state in each ventral horn section of the spinal cord by an observer blinded to the genotype (HEO). Percentage of each state was then calculated for each genotype of interest.

To quantify degeneration of mononuclear phagocytes in ALS patients, an observer blinded to the genotype scored from 0 to 4 the occurrence of degeneration in 2-5 sections per patient, with a score of 0 for sections showing non-degenerating phagocytes. Features of microglial degeneration were according to Streit and collaborators [58]. Similar results were obtained by two independent blinded observers (HEO & LD).

Isolation of CD11b positive cells

Isolated spinal cords and brain stems from end stage SOD1 (G86R) mice were transferred into a 2 ml sterile tube with 1 ml of Hank's balanced salt solution (HBSS) (Invitrogen), then triturated to single-cell suspension using Miltenyi's Neural Tissue Dissociation Kit (P) (Miltenyi Biotec). After a final wash in HBSS containing CaCl₂ and MgCl₂, the cell suspension was incubated with anti-myelin magnetic microbeads using Myelin removal kit (Miltenyi Biotec). The cell suspension was passed onto LS columns (Miltenyi Biotec) exposed to a strong magnetic field. The flow through (demyelinated cells) was incubated with anti-CD11b magnetic microbeads using CD11b kit (Miltenyi Biotec). The cell suspension was passed onto LS columns (Miltenyi Biotec) exposed to a strong magnetic field. The

flow through (non-CD11b cells) was subsequently used as a negative control and the suspension obtained once the magnetic field was switched off (CD11b cells) used as a positive fraction. Both fractions were used for gene expression analysis.

Real-time quantitative polymerase chain reaction

Total RNA was extracted from the spinal cord and the brain stem of end stage mice using TRIzol (Invitrogen). RNA was reverse transcribed using 1µg of RNA and the iScript cDNA synthesis kit (BioRad). We performed real-time PCR using IQ SYBR green Supermix (BioRad) and data were normalized with GeNorm software [64] using two standard genes (Tata-box binding protein, and RNA polymerase 2 subunit). For microglia experiments total RNA was extracted by using RNeasy Micro Kit (Qiagen), RNA was reverse transcribed using 1µg of RNA and the iScript cDNA synthesis kit. Quantitative PCR was performed on a CFX96 Real-time System (BioRad) using iQ SYBR Green supermix (BioRad). Relative mRNA levels were calculated with BioRad CFX Manager 3.1 using $\Delta\Delta C_t$ method.

Primer sequences are provided in **Table S3**.

Echocardiography

Animals were analyzed for cardiac anatomy and function on a Sonos 5500 (Hewlett Packard, USA) with a 15MHz linear transducer (15L6). All the examinations were performed in mice anesthetized with 1-1.5% isoflurane. The heart was first imaged in the two-dimensional (2D) mode in the parasternal long-axis view to obtain the aortic root dimensions. The aortic flow velocity and the heart rate (HR) were measured with pulsed-wave Doppler on the same section. The cardiac output (CO) was calculated from the following equation: $CO = 0.785 \times D^2 \times VTI \times HR$ where D is the diameter of the aortic root and VTI is the velocity-time integral of the Doppler aortic spectrum. Left ventricular cross sectional internal diameters in end-diastole (EDLVD) and end-systole (ESLVD) were obtained by an M-mode analysis of a 2D-short axis view at the papillary muscle level. The shortening fraction was calculated as $SF = (EDLVD - ESLVD / EDLVD) \times 100$. From this view, the diastolic septum (SW) and posterior wall (PW) thicknesses were measured. The left ventricular mass (LVM) was calculated with the following formula: $LVM = 1.055 \times [(SW + PW + EDLVD)^3 - (EDLVD)^3]$. All the measurements were performed on at least three beats, according to the guidelines of the American Society of Echocardiography.

Microglial culture

Mouse microglial cells in primary culture were prepared as described previously [68]. Briefly, 1-5 day old C57Bl/6 mice were decapitated according to the guidelines of the animal research center of Ulm University, Ulm, Germany. Meninges were removed from the brains. Neopallia were dissected and dissociated by enzyme treatment (1% trypsin, Invitrogen, 0.05 % DNase, Worthington, 2 min) and then mechanically dissociated. The cells were centrifuged (200 x g, 10 min), suspended in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) (Invitrogen) and 10% heat-inactivated fetal bovine serum (FBS, PAA), and plated into 75-cm² flasks (BD Falcon) pre-coated with 1µg/ml poly-L-ornithine

(Sigma). Cells from the neopallia of two brains were plated at 10 ml per flask. After three days, adherent cells were washed three times with Dulbecco's phosphate buffered saline (DPBS) (Invitrogen) and incubated with serum-supplemented culture media. After 7-14 days in culture, floating and loosely attached microglial cells were manually shaken off, centrifuged (200 x g, 10 min) and seeded into 96-well plates or 6-well plates (PRIMARIA, BD Falcon) at a density of 4×10^4 or 6×10^5 cells/well respectively in DMEM without serum (Invitrogen) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) (Invitrogen) and Glutamax (Invitrogen). Cells in the flasks were reincubated with serum-supplemented media after shaking. Repopulating microglial cells were removed every 3-4 days for a total of 3 weeks until fewer microglial cells were observed.

LDH and WST-1 assays

The lactate dehydrogenase (LDH) assay and water soluble tetrazolium salt (WST-1) assay were performed as described in the manual for the LDH-Cytotoxicity Assay Kit (Bio Vision) and the WST-1 Assay Kit (Quick Cell Proliferation Assay Kit; BioVision). For a positive cell death control, microglia were treated with 1% Triton-X100 (Sigma) for 30 min. Results were expressed as a percentage of Triton treatment, with 0% being untreated control.

HTR2B Polymorphisms in human ALS

In total 1923 cases and 2881 controls were genotyped on two different platforms; IlluminaOmniExpress (n=3488) and Illumina2.5M (n=1316). Extensive quality control using standard procedures was performed as described [63]. Following this, 612666 SNPs and 3344 individuals (1207 cases and 2137 controls) were retained on the IlluminaOmniExpress chip, and the Illumina 2.5M chip yielded 1481461 single nucleotide polymorphisms (SNPs) and 1294 individuals (679 cases and 615 controls). After this extensive quality control, seven *HTR2B* SNPs were extracted and used for analyses.

Statistical analysis

All data are presented as means +/- standard error of the mean.

When the measured values were not detectable in some of the experimental points (Figure 1), these values were considered to be null. Comparison of discrete values for two groups (Figure 1, Figure 2d-e, Figure 8b-c) was performed using Student's t-test. Comparison of discrete values for more than two groups (Figure 3d-e; Figure 4a, c, d, Figure 5c; Figure 6a; Figure 7) was performed using One Way ANOVA followed by Tukey's post hoc test. Differences in survival or disease onset of animals (Figure 2 a, b, c) was evaluated using log rank test. These statistical analyses were performed using Prism 6 (GraphPad software).

All differences were considered significant at $p < 0.05$.

To investigate the association of these seven SNPs with ALS, a logistic regression was performed in PLINK, corrected for dataset and 2 principle components. These seven SNPs are strongly correlated ($D' = 1$, and five out of seven SNPs have an R^2 between 0.7 and 1, with a minor allele frequency > 0.4). Therefore the strongest correlated

SNP was tested for association with survival (rs10199752). A Cox proportional hazards (coxph) regression model in R was performed with survival and death as time and event respectively, genotype as predictor, and sex, age at onset, and site at onset as covariates. Patients with a maximum survival of 15 years were included in this analysis (n=1677). This coxph regression was conducted using a dominant model, comparing a wildtype homozygous reference group versus a variant group (combined homozygous variant and heterozygous).

Results

5-HT_{2B} receptor upregulation during murine ALS

We first asked whether 5-HT_{2B}R upregulation is a general feature of ALS mouse models. For this, we measured mRNA levels of the gene encoding 5-HT_{2B}R (*Htr2b*), in spinal cord and brainstem of three different mouse models of ALS at a late stage of disease progression *i.e.* when both hindlimbs were paralyzed. We used mice expressing the G86R SOD1 mutation in the murine gene (SOD1(G86R) mice), or the G37R mutation in the human gene [5]. Consistent with previous results[15], mRNA expression of *Htr2b* mRNA was strongly upregulated in spinal cord of SOD1(G86R) mice (**Fig. 1a**) but also of SOD1(G37R) mice (**Fig. 1a**). Importantly, *Htr2b* mRNA upregulation was also observed in non-SOD1 ALS mouse models, in particular in the spinal cord of mice expressing the G59S mutation in the p150 subunit of dynactin [43] (**Fig. 1a**). Last, *Htr2b* mRNA upregulation was also observed in the brainstem of SOD1(G86R) mice and p150(G59S) mice (**Fig. 1b**). Thus, 5-HT_{2B}R mRNA is upregulated in various ALS mouse models

The lack of 5-HT_{2B} receptor accelerates disease progression in mice

To determine the effect of 5-HT_{2B}R on motoneuron disease progression, we ablated the *Htr2b* gene in SOD1(G86R) mice. We crossed SOD1(G86R) mice with *Htr2b*^{-/-} mice generated previously [49]. After two breeding steps, we obtained SOD1(G86R); *Htr2b*^{+/+} mice, SOD1(G86R) *Htr2b*^{+/-} mice and SOD1(G86R); *Htr2b*^{-/-} mice, that were followed longitudinally until death. These mice were in a mixed FVB/N x 129S2/Sv PAS background. Inactivation of *Htr2b* led to reduction in body weight in SOD1(G86R) mice as compared to SOD1(G86R) mice expressing *Htr2b* (**Fig. S1**). Complete ablation of *Htr2b* shortened survival of SOD1(G86R) mice by 30% (**Fig. 2a-c**), while inactivation of only one allele of *Htr2b* yielded an intermediate effect (**Fig. S2**). Disease onset, as assessed either by peak of body weight [5] or by peak of grip strength, was not changed (**Fig. 2a-b**). Indeed, ablation of *Htr2b* did not modify the age of peak weight as a measure of disease onset (**Fig. 2d**), while drastically shortening disease duration after peak weight (**Fig. 2e**).

Since 5-HT_{2B}R has been implicated in cardiac development and function [49,51,52], we hypothesized that *Htr2b* ablation could affect cardiac function in SOD1(G86R) mice, leading to accelerated death. However, echocardiographic examinations did not show major reduction of cardiac contractility or output in SOD1(G86R); *Htr2b*^{-/-} mice. In particular, they did not exhibit echocardiographic signs of heart failure that could explain body weight loss (**Fig. S3**). To determine whether SOD1(G86R); *Htr2b*^{-/-} mice actually died of motoneuron disease, we examined neuromuscular junction histology in end-stage mice. All SOD1(G86R) mice examined at end-stage, irrespective of their *Htr2b* genotype, displayed heavily denervated neuromuscular junctions (**Fig. 3a-b**). Consistent with this, SOD1(G86R); *Htr2b*^{+/+} mice displayed motoneuron degeneration similar to SOD1(G86R); *Htr2b*^{-/-} mice (**Fig. 3c-d**), and ventral horn atrophy was not modified by the *Htr2b* genotype (**Fig. S4**). Motor neuron loss was similar in this mixed background as in previous studies in the original FVB/N background [20,19]. However, *Htr2b* ablation in SOD1(G86R) mice triggered atrophy of motoneuron cell bodies that was not present in SOD1(G86R);

Htr2b^{+/-} mice (**Fig. 3e**). Thus, consistent with its overexpression at symptom onset in SOD1(G86R) spinal cord [15], the lack of 5-HT_{2B}R potentially accelerates disease progression of SOD1(G86R) mice after onset.

5-HT_{2B} receptor is upregulated in CD11b-positive cells and is required for activation of mononuclear phagocytes during ALS

Microgliosis occurs at symptom onset in brainstem and spinal cord of transgenic models of ALS [53,4,5]. Since 5-HT_{2B}R is expressed in microglia and peripheral macrophages [14,40], increased 5-HT_{2B}R expression could reflect microglial activation and/or infiltrated monocyte activation. To address this question, we purified CD11b positive cells from late stage SOD1(G86R) mice using magnetic bead cell sorting, followed by RT-qPCR. To enable purification of CD11b positive cells from single individuals, we pooled brainstem and spinal cord areas that are associated with strong microgliosis in mutant SOD1 models [24]. Using this procedure, we obtained CD11b positive fractions with a >300 fold enrichment in Iba1 mRNA, and corresponding 5-10 fold decrease in GFAP and ChAT mRNA content (**Fig. S5**). Importantly, we observed that *Htr2b* mRNA upregulation was strictly limited to mRNA enriched from CD11b positive cells (**Fig. 4a**). Activation of CD11b+ cells was reduced by *Htr2b* ablation, with decreased Iba1 staining in the spinal cord (**Fig. 4b-c**). Consistently, the disease-elicited induction of multiple genes involved in neuroinflammatory responses was decreased in the brainstem of the same animals (**Fig. 4d**). Interestingly, genes involved in the pro-inflammatory, putatively neurotoxic action of microglia (**Fig. 4d**, *Nox2*, *Ccl4*, *Mhc2*), as well as genes involved in anti-inflammatory, potentially neuroprotective responses (**Fig. 4d** *Ym1*, *Dap12*) showed similar blunted expression upon *Htr2b* ablation. Notably, *Trem2*- a cell surface receptor associated with neurodegeneration - was especially upregulated in SOD1(G86R); *Htr2b*^{-/-} brainstem (**Fig. 4d**). Thus, 5-HT_{2B}R is required for the normal activation of mononuclear phagocytes during motoneuron disease.

5-HT_{2B} receptor improves microglial survival

Searching for a role for 5-HT_{2B}R in mononuclear phagocytes, we re-examined the phenotype of these cells in SOD1(G86R); *Htr2b*^{-/-} mice. We observed that spinal cord Iba1-positive cells of SOD1(G86R); *Htr2b*^{-/-} mice frequently displayed cytoplasmic fragmentation (also called cytorrhexia, **Fig. 5a**). This was further confirmed on z-stacks of Iba1-immunostained sections obtained using confocal microscopy (**Fig. 5b**). Quantitative analysis demonstrated that about half of Iba1-positive cells displayed cytorrhexia in SOD1(G86R); *Htr2b*^{-/-} mice (**Fig. 5c**), suggesting that the loss of *Htr2b* was associated with degeneration of mononuclear phagocytes in end stage SOD1(G86R) mice. To determine whether this degeneration of mononuclear phagocytes affected microglia and/or monocytes, we measured expression levels of genes known to be part of the homeostatic microglial signature [12,8,9,7], in particular *Cx3cr1*, *Hexb*, *Tmem119*, *P2ry12*, *Olflm3* and *SiglecH*. All these genes displayed lower expression in SOD1(G86R) mice lacking the *Htr2b* gene than in SOD1(G86R) mice with the *Htr2b* gene (**Fig. 6**). The establishment of the homeostatic microglial signature is dependent upon TGFβ and *Tgfb1*, the microglial receptor of TGFβ [8]. Consistent with previous work, we observed induction of TGFβ and its receptor in

SOD1(G86R) mice [23], and these two inductions were blunted in SOD1(G86R) mice lacking the *Htr2b* gene (**Fig 6b**). Importantly expression levels of *Ly6c1*, a cell surface marker expressed on monocytes, but not microglia [9], were unaltered in SOD1(G86R) mice, irrespective of the *Htr2b* genotype (**Fig. 6c**). To define the relationships between 5-HT_{2B}R and microglial survival, we stimulated primary microglial cultures with 5-HT_{2B}R-targeted drugs. Microglial viability was decreased after 48h in the presence of 1μM of SB204741, a fairly selective antagonist of the 5-HT_{2B}R (**Fig. 7a**), and increased in the presence of 3μM of BW723C86, a preferential 5-HT_{2B}R agonist. There was consistently more LDH release after 48h in the presence of SB206553, an inverse agonist of 5-HT_{2B}/5-HT_{2C}Rs, as compared with 3μM BW723C86 (**Fig. 7b**). To determine whether the 5-HT_{2B}R could also modulate microglial gene expression beyond survival, we measured mRNA levels of genes strongly modulated by *Htr2b* genotype in SOD1(G86R) mice (**Fig. 4d**) in primary microglia culture treated with 5-HT_{2B}R drugs and/or LPS as a neuroinflammatory stimulus. 5-HT_{2B}R pharmacological modulation was not sufficient to alter the expression levels of *Nox2*, *Ym1*, *Mhc2* or *Trem2* whether in the absence or presence of LPS for 48h (**Fig. 7c**). Thus, acute modulation of 5-HT_{2B}R modifies survival of primary microglia, but does not affect neuroinflammatory gene expression. Taken together, our results indicate that 5-HT_{2B}R actively promotes microglial survival during neurodegeneration.

HTR2B polymorphisms associated with differential levels of 5-HT_{2B}R mRNA affect morphology of mononuclear phagocytes and survival in ALS

Next, we studied whether the *HTR2B* gene was a modifier of ALS. To this end, we interrogated genome wide association study (GWAS) results of 1886 ALS cases and 2752 controls of Dutch origin. In these samples, we identified seven different intronic SNPs. None of these SNPs showed differential frequencies between cases and controls, showing that at least in this Dutch population *HTR2B* polymorphisms are not a risk factor for ALS. However, a significant association with survival was found for SNP rs10199752 as a prototypical example of these highly correlated SNPs. We isolated patients in whom all phenotype data were available (**Table S1**), and used a dominant model to compare the survival of the variant group (combined genotypes C/A and C/C, n=918) with the wild type homozygous reference group (A/A, n=759). This analysis revealed that patients with the variant genotypes have a longer survival time compared to the homozygous reference group (hazard ratio=0.88; confidence interval 0.79-0.98; p-value=0.019) (**Fig. 8a**). To determine whether *HTR2B* polymorphisms were associated with changes in *HTR2B* expression levels, we genotyped a cohort of autopsied ALS patients (**Table S2**). In this cohort, 6 patients carried an A/A genotype, and 10 patients carried a C/A genotype. There was no patient with the C/C genotype. In patients who carried the C/A genotype of rs10199752, we observed decreased spinal cord *HTR2B* mRNA levels as compared with patients carrying the A/A genotype of rs10199752 (**Fig. 8b**). We then performed Iba1 immunohistochemistry on lumbar spinal cord sections of these same patients and conducted a semi-quantitative evaluation of the degeneration of Iba1-positive cells (**Fig. 8c**). Notably, degenerating Iba1-positive cells - as evidenced by the presence of fragmented and/or beaded processes - were frequently observed in patients with the A/A genotype of rs10199752 (**Fig. 8d**, arrows in lower panels), but much less frequently in patients with the C/A genotype (**Fig. 8d**, upper panels). Quantification revealed more frequent and severe degeneration of spinal cord

Iba1-positive cells in patients with the A/A genotype than in patients with the C/A genotype (**Fig. 8c**). As a whole, *HTR2B* polymorphisms associated with higher spinal cord mRNA levels were also associated with increased survival and less pronounced degeneration of mononuclear phagocytes.

Discussion

Our current study provides multiple lines of evidence supporting that the 5-HT_{2B}R is a disease modifier of ALS progression by regulating survival and function of mononuclear phagocytes during neurodegeneration.

Our first important result is that *HTR2B* expression levels affect ALS progression, both in patients and in a transgenic mouse model. *Htr2b* ablation did not modify disease onset, but sharply accelerated disease progression in mutant SOD1 mice. The effect was observed despite the fact that double transgenic mice were generated in the mixed FVB/N x 129Sv background. The background was identical in all mice, as we used the F1 generation littermates to generate the F2 generation of interest. Interestingly, as illustrated in **Fig. 2c**, the mixed background increased overall survival of SOD1(G86R) mice and increased heterogeneity in survival. This effect of mixed genetic background has been previously reported, including by us [35,28]. Accelerated death of mutant SOD1 mice ablated of *Htr2b* was due to accelerated neurodegeneration, and not to potential confounding effects on cardiac patho-physiology. Consistently, ALS patients carrying common haplotypes associated with higher spinal cord *HTR2B* mRNA expression levels survived longer than patients with low expression haplotypes. The relationship between *HTR2B* polymorphisms and survival will have to be investigated in larger cohorts, and different genetic backgrounds, but constitutes an example of a common polymorphism modifying disease progression but not disease risk. An interesting open question is whether *HTR2B* might affect other phenotypes associated with ALS. It is conceivable that *HTR2B* modulates the development of frontal symptoms commonly associated with ALS [59]. Indeed, *Htr2b*^{-/-} mice were recently shown to display deficits in social interactions, as well as in learning and memory processes [54], and these processes require intact frontal lobe function. Along the same lines, the Q20* polymorphism, only found in the Finnish population, is associated with severe impulsivity [3], possibly caused by frontal lobe modulation. Further work is thus required to elucidate the extent of the contribution of *HTR2B* in ALS-associated phenotypes, in particular in relation with frontal symptoms.

The second major result is that 5-HT_{2B}R could regulate numbers, morphology and function of central nervous CD11b+ phagocytes, including microglia, in ALS. Here again we provide multiple complementary lines of evidence. Abnormalities of mononuclear phagocytes include (i) decreased overall Iba1 immunostaining, (ii) decreased induction of multiple pro- or anti-inflammatory genes, and (iii) fragmentation of Iba1-positive cells. A reasonable mechanism underlying all these different observations would be that 5-HT_{2B}R function is critical for survival of mononuclear phagocytes during prolonged neuroinflammation such as occurs in ALS. Survival of primary microglia was improved by 5-HT_{2B}R stimulation and decreased by its inhibition, consistent with previous work showing a protective function of 5-HT_{2B}R in newborn cardiomyocytes [50]. These studies will have to be extended to adult spinal cord microglia to determine the effect of aging, as well as the potential role of microglial location in their response to 5-HT_{2B}R stimulation. Our current work does not formally determine whether degenerating Iba1-positive cells are microglia and/or infiltrating monocytes. The extent of monocyte infiltration remains controversial in the field of ALS [9,12,42,1,46], contrary to Alzheimer's disease in which monocyte infiltration has been repeatedly observed [29,30]. Our gene expression results demonstrate consistent loss of

expression of multiple genes typical of homeostatic microglia but while the monocyte marker *Ly6c1* remained unaffected (**Fig 6**). Such loss of homeostatic microglial gene expression in whole tissue could be due to cell loss, leading to decreased proportion of microglial mRNAs. Alternatively, disease progression could also affect microglial phenotype, and a recent study performed on isolated microglia of mutant SOD1 mice observed that mutant SOD1 microglia lost their typical expression patterns with disease progression [7]. However, our *in vitro* experiments provide an initial proof of concept that microglial survival could be influenced by serotonergic stimulation of the 5-HT_{2B} R. Further work is required to determine the relative contributions of microglial cell death and altered microglial phenotypes in the observed degenerative phenotype *in vivo* as well as the importance of monocyte degeneration. Interestingly, degeneration of mononuclear phagocytes is commonly associated with human neurodegenerative diseases, and commonly referred to as microglial degeneration [58], whereas rodent models appear less affected by this phenotype. However, previous studies observed degeneration of mononuclear phagocytes in a transgenic mutant SOD1 rat [24], similar to our current results in mutant SOD1 mice carrying a wild type *Htr2b* gene. Loss of *Htr2b* almost doubled the proportion of fragmented Iba1-positive cells, showing the importance of this receptor in preventing phagocyte degeneration during disease, although detailed molecular pathways remain to be elucidated.

The processes underlying degeneration of mononuclear phagocytes during ALS remain elusive, and the relationships between microglial activation, for instance through inflammasomes [65], and microglial survival are not understood. Paradoxically, *Trem2* was found to be upregulated in SOD1(G86R) mice in the absence of *Htr2b*. Trem2 is a scavenger receptor found on the surface of myeloid cells, and *TREM2* mutations leading to impaired phagocytic activity [39] are strong risk factors for multiple neurodegenerative diseases, including ALS [26,36,10]. Whether increased expression of *Trem2* in SOD1(G86R); *Htr2b*^{-/-} mice is beneficial or deleterious remains unknown. However, recent work demonstrated that loss of *Trem2* leads either to microglial degeneration in different diseases of the central nervous system [55,66,11], or conversely to protection [34]. Thus, the exact mechanisms underlying 5-HT_{2B}R-mediated regulation of microglial survival and the role of scavenger receptors in this response will have to be investigated in further studies.

A major question arising from our work is whether the lack of 5-HT_{2B}R accelerates ALS progression through its effects on degeneration of mononuclear phagocytes. The hypothesis that degeneration of mononuclear phagocytes is directly linked to the lack of 5-HT_{2B}R in this cell type is plausible for several reasons. First, *Htr2b* expression has been detected in newborn brain microglia [40] although it remains low in basal conditions [12,8,7,31]. *Htr2b* is also highly expressed in peripheral macrophages [14]. *Htr2b* mRNA expression was restricted to CD11b-positive cells purified from spinal cord and brainstem, thus originating from mononuclear phagocytes, either microglia or infiltrating monocytes. Second, the late occurrence of *Htr2b* upregulation [15] coincides with activation of mononuclear phagocytes in SOD1(G86R) mice. Third, the effects of *Htr2b* ablation on ALS progression are strikingly similar to interventions performed on mononuclear phagocytes. For instance, knocking out mutant SOD1 or NF-kappaB signaling from CD11b-positive cells improved disease progression, but had no effect

on disease onset [5,25]. Despite these parallels, our current results cannot exclude that at least part of the effects of *Htr2b* ablation are caused by its expression in other cell types. Besides mononuclear phagocytes, *Htr2b* is expressed at detectable levels in serotonergic neurons themselves [16], and is known to regulate serotonin release [2,17]. It is therefore possible that the observed decrease in weight in SOD1(G86R) mice devoid of the *Htr2b* gene was due to serotonergic neurons indirectly affecting hypothalamic circuitry, consistent with our recent results (Vercruysse et al., Brain, in press). To date, no conditional knock-out model of *Htr2b* gene has been characterized. Thus, the current knowledge of the function of *Htr2b* in microglial cells remains limited to *in vitro* studies (this study (**Fig. 7**) or previous results [40]). The generation and characterization of knock-out mice in which *Htr2b* deletion is restricted to microglia will yield invaluable insights into this question.

To our knowledge, these results provide the first genetic evidence for an involvement of the serotonergic system in ALS. We previously showed that platelet serotonin content correlated with survival [22]. Brain-derived serotonin was decreased in ALS patients and mice [15], and imaging studies consistently report dramatic reduction in the binding of 5-HT_{1A} ligand [62,61]. Pharmacological interventions targeting serotonin provided some evidence for its involvement. For instance, perinatally administered fluoxetine accelerated weight loss and disease onset in ALS rats [41], while 5-hydroxytryptophan, the serotonin precursor, delayed disease in mutant SOD1 mice [60]. Furthermore, spasticity appears linked to serotonergic abnormalities [15]. Here we provide genetic evidence that the serotonin system is involved in disease progression and microglial function. The observation that lack of *Htr2b* accelerated weight loss in ALS mice raises questions on the role of serotonin in weight loss during ALS progression [21], and our most recent results show that loss of serotonin is involved in the development of melanocortin defects (Vercruysse et al., Brain, in press).

Collectively, our results suggest that the 5-HT_{2B}R might be an interesting target to prolong survival of ALS patients after onset. However this should be tempered by the known cardiovascular side effects of 5-HT_{2B}R agonists. Chronic use of 5-HT_{2B}R agonists has been associated with cardiac valvulopathies and pulmonary hypertension [33], and a therapeutic strategy targeting 5-HT_{2B}R should consider the balance between potential benefits and cardiovascular risks in a currently intractable and rapidly progressive disease like ALS, in which patients are most often in good cardiovascular health. Since we identify common *HTR2B* polymorphisms as differentially associated with *HTR2B* expression in the CNS, eventual clinical studies should also take into account *HTR2B* genetic variations.

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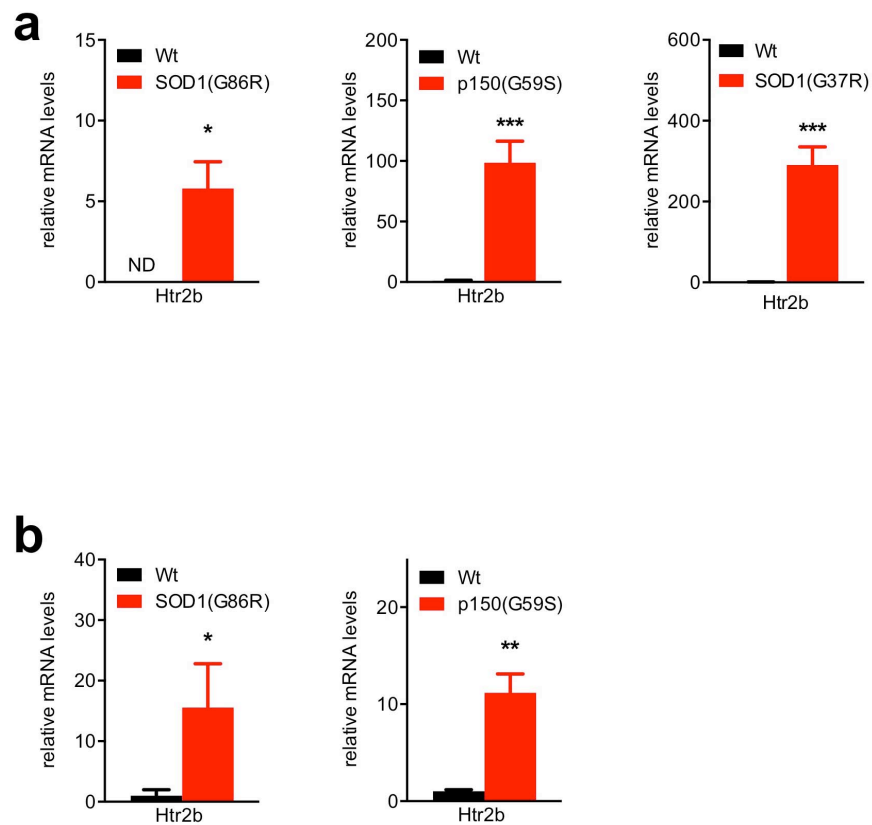


Fig. 1: Increased mRNA levels of 5-HT_{2B} receptor in ALS mice.

mRNA levels of 5-HT_{2B}R (Htr2b) in the spinal cord (**a**) and brainstem (**b**) of end stage SOD1 (G86R) mice, p150(G59S) mice, SOD1 (G37R) relative to their respective wildtype (Wt) littermates. N= 7. * P<0.05 versus corresponding wild type, by Student's t-test.

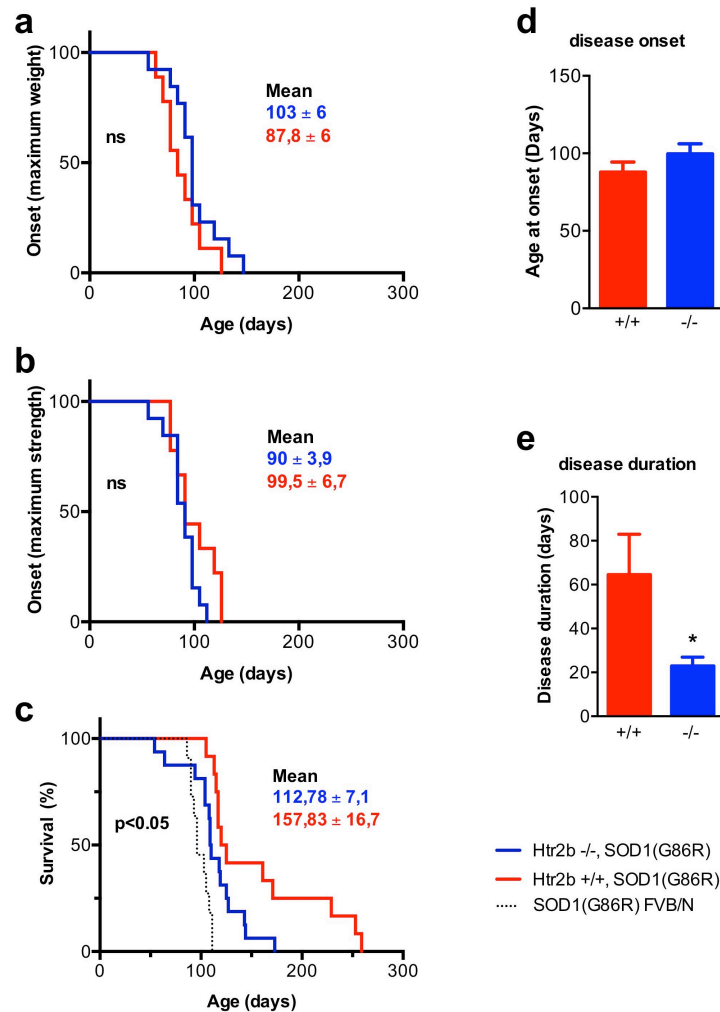


Fig. 2: The ablation of *Htr2b* in SOD1 (G86R) mice decreases life span and accelerates disease progression.

a-c: Kaplan Meier plot of disease onset, as determined by the peak weight (**a**) or the peak of grip strength (**b**), and of survival (**c**) for SOD1(G86R) mice wild type for *Htr2b* (red) or knockout for *Htr2b* (blue). The mean of each group \pm SEM is indicated on each panel. $P < 0.05$, log rank (Mantel – Cox) for panel **c**. The survival curve of SOD1 (G86R) mice in the original FVB/N background is indicated in dashed line.

d-e: duration of early disease (**d**, from birth to peak weight) and late disease (**e**, from peak weight to death) for SOD1(G86R) mice wild type for *Htr2b* (+/+, red) or knock-out for *Htr2b* (-/-, blue). $p < 0.05$, unpaired Student's t-test.

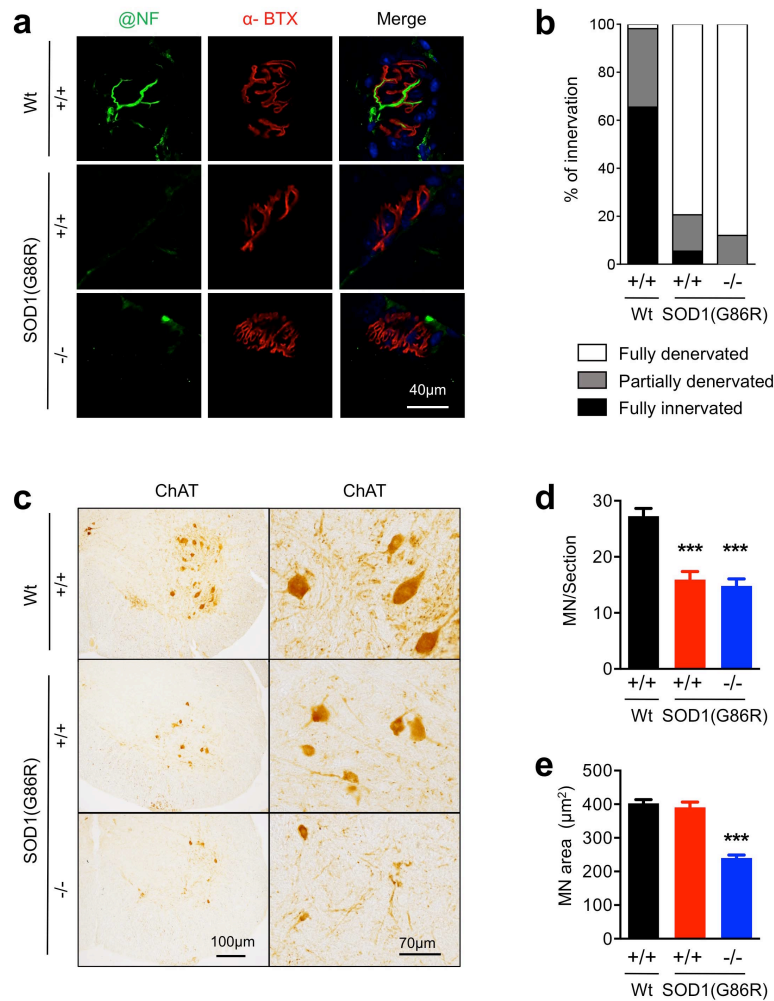


Fig. 3: The ablation of *Htr2b* in SOD1 (G86R) mice exacerbates neurodegeneration

- a:** Representative confocal images of post and pre-synaptic apparatus of the neuromuscular junction in Tibialis muscle of end stage mice. Axons were labeled with anti-neurofilament (@NF, green), acetylcholine receptors with fluorescently labeled bungarotoxin (α -BTX, red) and nuclear with Draq 5 (blue) in wild type mice (+/+), and SOD1(G86R) mice wild type for *Htr2b* (+/+) or knock-out for *Htr2b* (-/-) at end stage. N= 5.
- b:** distribution of innervation profiles in the tibialis anterior of wild type mice (+/+), and SOD1(G86R) mice wild type for *Htr2b* (+/+) or knockout for *Htr2b* (-/-) at end stage.
- c:** Representative ChAT immunohistochemistry images in wild type mice (+/+), and SOD1(G86R) mice wild type for *Htr2b* (+/+) or knockout for *Htr2b* (-/-). Two magnifications are shown. N= 5
- d-e:** motor neuron numbers (**d**) and area (**e**) in wild type mice (black columns), and SOD1(G86R) mice wild type for *Htr2b* (+/+ red columns) or knockout for *Htr2b* (-/- blue columns). ***, $p < 0.001$ vs. wild type, One way ANOVA followed by Tukey *post hoc* test.

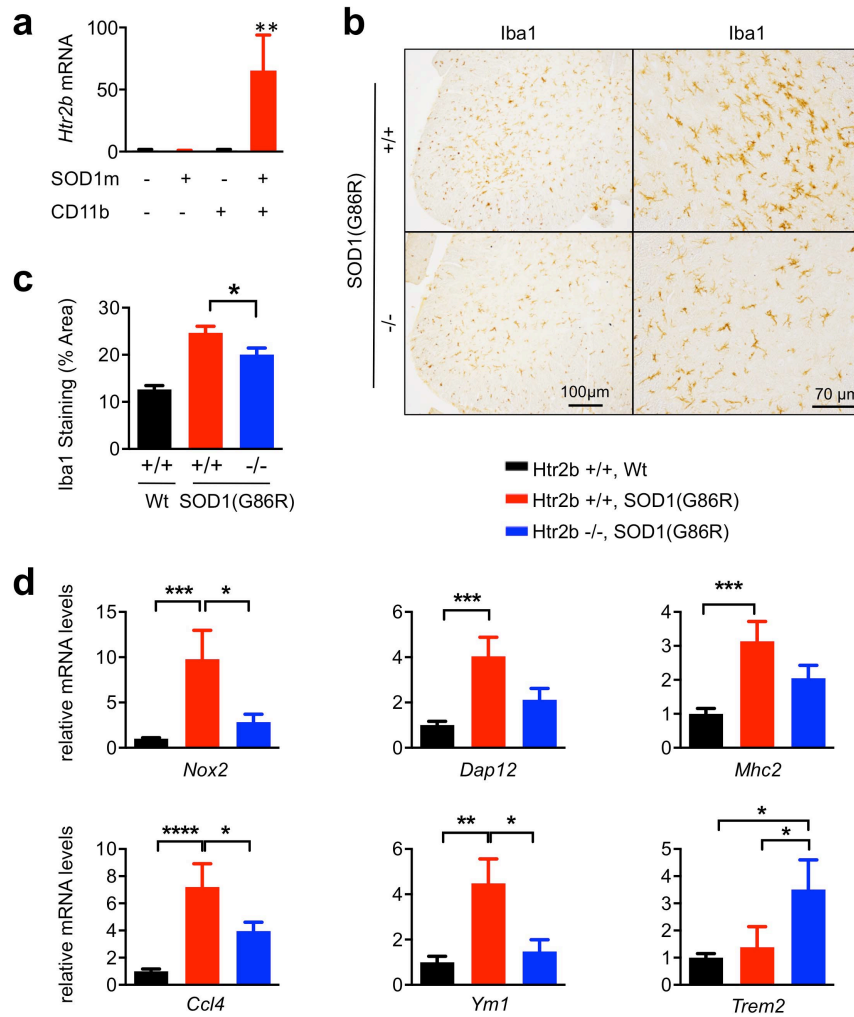


Fig. 4: The ablation of *Htr2b* in SOD1 (G86R) mice compromises activation of mononuclear phagocytes

a: *Htr2b* mRNA levels in CD11b-positive cells (CD11b^{Pos}) and CD11b-negative cells (CD11b^{Neg}) in brainstem and spinal cord of end stage SOD1(G86R) mice (SOD1) relative to control littermates (Wt). N= 5 **, p<0.01, Student's t-test.

b: Representative Iba1 immunohistochemical images in ventral spinal cord of SOD1(G86R) mice wild type for *Htr2b* (+/+) or knockout for *Htr2b* (-/-).

c: quantification of Iba1 staining coverage in lumbar spinal cord of wild type mice (black columns), and SOD1(G86R) mice wild type for *Htr2b* (+/+ red columns) or knock-out for *Htr2b* (-/- blue columns). *, p<0.05, One way ANOVA followed by Tukey *post hoc* test. N= 5.

d: mRNA levels of the indicated genes in brainstem of SOD1(G86R) mice wild type for *Htr2b* (+/+ red columns) or knock-out for *Htr2b* (-/- blue columns) relative to wild type mice (black columns). *, p<0.05, **, p<0.01; ***, p<0.001 One way ANOVA followed by Tukey *post hoc* test. N= 7.

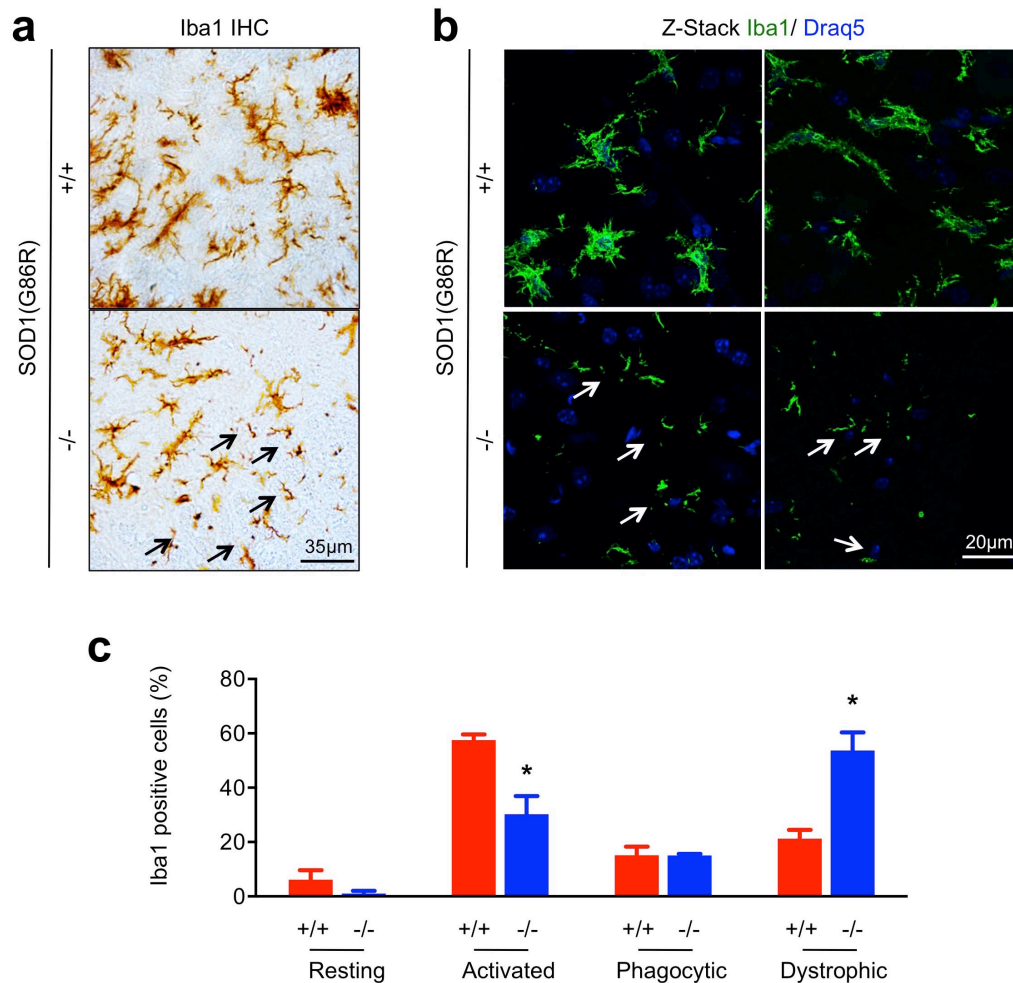


Fig. 5: The ablation of *Htr2b* in SOD1 (G86R) mice leads to degeneration of mononuclear phagocytes

a: representative Iba 1 immunostaining in spinal cord of SOD1(G86R) mice wild type for *Htr2b* ($+/+$) or knock-out for *Htr2b* ($-/-$) showing cytorrhexia of Iba1+ cells (arrows).

b: Z-stack confocal images of Iba1 immunofluorescence (green) counterstained with nuclear DRAQ5 staining in SOD1(G86R) mice wild type for *Htr2b* ($+/+$) or knockout for *Htr2b* ($-/-$). Microglial cytorrhexia is shown with arrows.

c: relative proportions of resting, activated, phagocytic and dystrophic (cytorrhexia) Iba1+ cells in SOD1(G86R) mice wild type for *Htr2b* ($+/+$ red columns) or knockout for *Htr2b* ($-/-$ blue columns). *, $p < 0.05$, One way ANOVA followed by Tukey *post hoc* test. N= ???

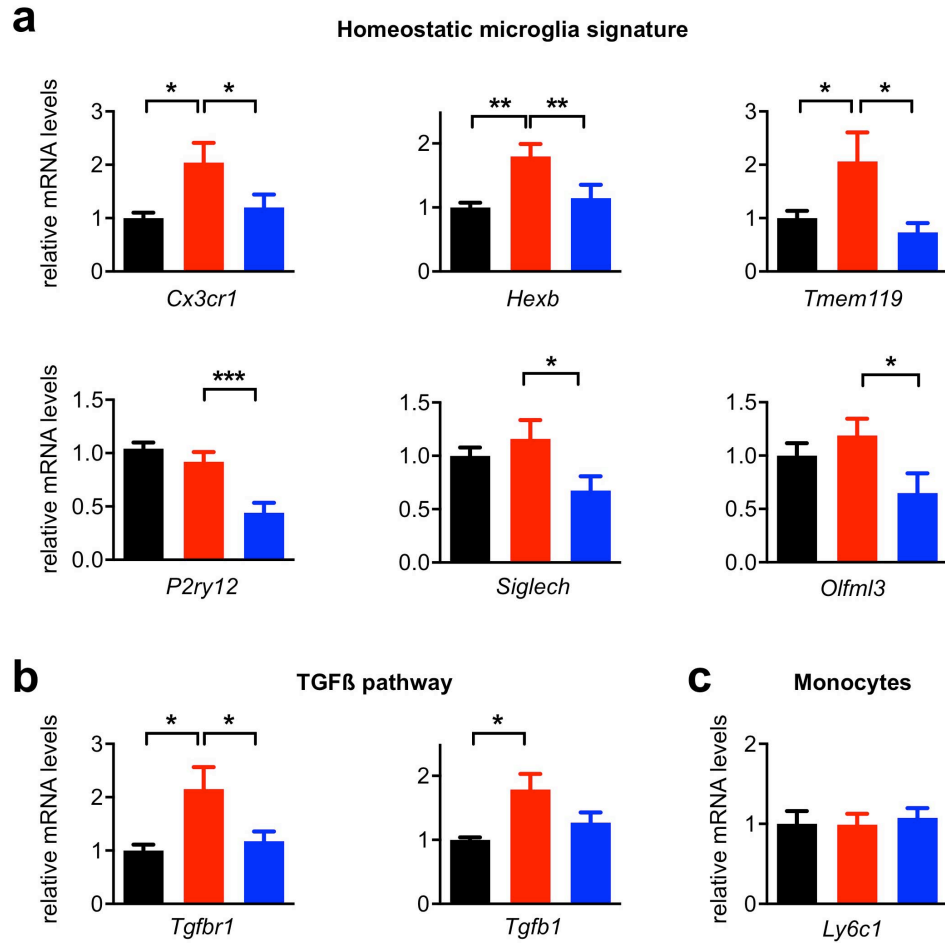


Fig. 6: The ablation of *Htr2b* in SOD1 (G86R) mice decreases expression of homeostatic microglia genes, while not affecting monocyte marker *Ly6c1*

a-c: mRNA levels of the genes typical of the homeostatic microglia signature (**a**), TGF β pathway (**b**), and of *Ly6c1*, a monocyte marker, in brainstem of SOD1(G86R) mice wild type for *Htr2b* (+/+ red columns) or knock-out for *Htr2b* (-/- blue columns) relative to wild type mice (black columns). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. One way ANOVA followed by Tukey *post hoc* test. N= 7.

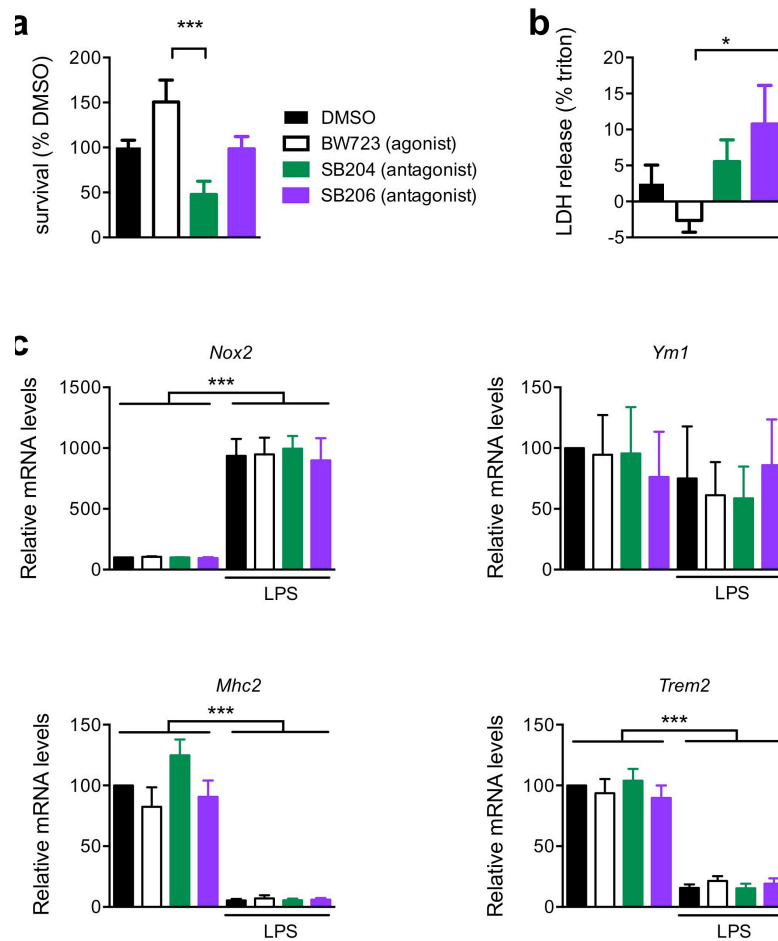


Fig. 7: Pharmacological modulation of the 5-HT_{2B} receptor modulates microglial survival in culture but not gene expression

a-b: WST-1 survival (a) and LDH release (b) assays of primary microglial cells treated with 1μM SB204741 (5-HT_{2B} antagonist), 1μM SB206553 (5-HT_{2B} inverse agonist), or 1μM BW723C86 (5-HT_{2B} agonist). The WST-1 values are relative to vehicle (DMSO). LDH release is expressed as a percentage of positive control (triton treatment), that releases all LDH contained in cells after subtracting the value of untreated cells. Note that treatment with BW723C86 (5-HT_{2B} agonist) leads to decrease in basal LDH release, leading to negative values. ***, p<0.001; *, p<0.05, One way ANOVA followed by Tukey *post hoc* test. The experiments were performed in triplicate in three independent experiments.

c- mRNA levels of the indicated genes in primary mouse microglia incubated with 3μM of BW723C86 (5-HT_{2B} agonist), 1μM of SB204741 (5-HT_{2B} antagonist), 3μM of SB206553 (5-HT_{2B} inverse agonist) relative to vehicle (DMSO) with or without 1μg/ml LPS for 48 hours. mRNA levels are given in % of control. Data are presented as mean ± SEM of n=3-5 experiments ***p<0.001 significantly different (One-way ANOVA followed by Tukey *post hoc* test).

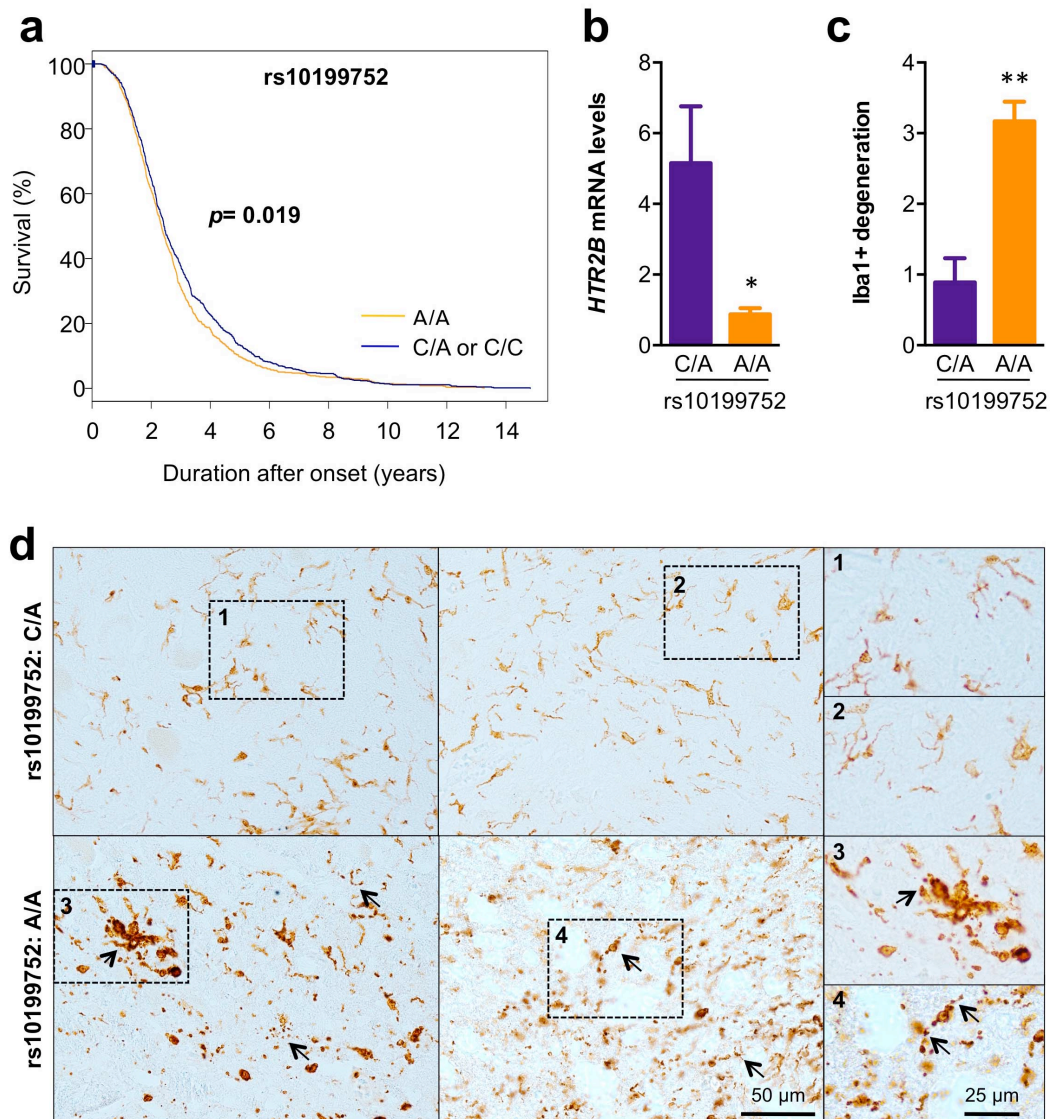


Fig. 8: *HTR2B* polymorphisms in gene expression and degeneration of mononuclear phagocytes.

a: Kaplan Meier plot for a dominant SNP model associated with patient survival. Patients with the variant genotypes (C/A and C/C, n=918) have a longer survival compared to the wildtype homozygous reference group (AA, n=759), hazard ratio=0.88 (confidence interval 0.79-0.98), p-value=0.019.

b: *HTR2B* mRNA levels in the spinal cord of ALS patients with *HTR2B* C/A relative to patients with *HTR2B* A/A genotype for the rs10199752 SNP. * p<0.05, Student's t-test. No patient with the C/C genotype was present in this cohort.

c: quantification of degeneration of Iba1+ mononuclear phagocytes. Degeneration was semi-quantitatively scored

(from 0 to 4) by an observer blinded to the genotype in Iba1-stained spinal cord sections of ALS patients with *HTR2B* A/A relative to C/A genotype for the rs10199752.. **, $p < 0.01$, Student's t-test.

d : representative Iba1 immunostaining in 4 ALS patients with *HTR2B* C/A (upper row) or A/A genotype (lower row) for the rs10199752 SNP. Magnifications of rectangles 1-4 is shown on the right. Note that microglial cells in *HTR2B* C/A carriers show the typical pattern of resident microglial cells with ramified processes (rectangles 1-2) whereas the A/A carriers frequently exhibited degenerating microglial cells (arrows), with fragmented and beaded processes (rectangles 3-4, arrows).