

Degeneration of serotonergic neurons in amyotrophic lateral sclerosis: a link to spasticity

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Spasticity is a common and disabling symptom observed in patients with central nervous system diseases, including amyotrophic lateral sclerosis, a disease affecting both upper and lower motor neurons. In amyotrophic lateral sclerosis, spasticity is traditionally thought to be the result of degeneration of the upper motor neurons in the cerebral cortex, although degeneration of other neuronal types, in particular serotonergic neurons, might also represent a cause of spasticity. We performed a pathology study in seven patients with amyotrophic lateral sclerosis and six control subjects and observed that central serotonergic neurons suffer from a degenerative process with prominent neuritic degeneration, and sometimes loss of cell bodies in patients with amyotrophic lateral sclerosis. Moreover, distal serotonergic projections to spinal cord motor neurons and hippocampus systematically degenerated in patients with amyotrophic lateral sclerosis. In SOD1 (G86R) mice, a transgenic model of amyotrophic lateral sclerosis, serotonin levels were decreased in brainstem and spinal cord before onset of motor symptoms. Furthermore, there was noticeable atrophy of serotonin neuronal cell bodies along with neuritic degeneration at disease onset. We hypothesized that degeneration of serotonergic neurons could underlie spasticity in amyotrophic lateral sclerosis and investigated this hypothesis *in vivo* using tail muscle spastic-like contractions in response to mechanical stimulation as a measure of spasticity. In SOD1 (G86R) mice, tail muscle spastic-like contractions were observed at end-stage. Importantly, they were abolished by 5-hydroxytryptamine-2b/c receptors inverse agonists. In line with this, 5-hydroxytryptamine-2b receptor expression was strongly increased at disease onset. In all, we show that serotonergic neurons degenerate during amyotrophic lateral sclerosis, and that this might underlie spasticity in mice. Further research is needed to determine whether inverse agonists of 5-hydroxytryptamine-2b/c receptors could be of interest in treating spasticity in patients with amyotrophic lateral sclerosis.

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Abbreviations: ALS = amyotrophic lateral sclerosis; 5-HIAA = 5-hydroxyindoleacetic acid; 5-Ht = 5-hydroxytryptamine; Tph2 = tryptophan hydroxylase 2

Introduction

Spasticity is a symptom of many motor diseases that consists of velocity-dependent increase in muscle tone and exaggerated muscle responses to stretching. Spasticity develops either after trauma, in particular spinal cord injury, or in the course of degenerative diseases such as amyotrophic lateral sclerosis (ALS), a fatal neurodegenerative disorder affecting upper and lower motor neurons (Kiernan *et al.*, 2011). Spasticity represents the major phenotype of the upper motor neuron predominant subtype of ALS called primary lateral sclerosis and might be under recognized in other patients with ALS, as the physiological basis for detecting spasticity is disrupted by the degenerative process involving motor neurons of all classes (Swash, 2012). Spasticity is a painful and disabling symptom, and treatment options remain limited, especially in patients with ALS and those with primary lateral sclerosis (Ashworth *et al.*, 2012).

Mechanisms of spasticity have been mostly studied after spinal cord injury. In the current view, spinal cord injury-associated spasticity arises from several mechanisms, a major one being injury to serotonergic axons. Indeed, serotonergic axons, descending from several brainstem serotonergic nuclei, densely innervate lower motor neurons and maintain motor neuron excitability through increased persistent calcium current (Heckman *et al.*, 2009). After spinal cord injury, the transection of serotonergic axons leads to transient hypoexcitability of lower motor neurons. After a few weeks, lower motor neurons compensate for loss of serotonin input through the production of constitutively active 5-hydroxytryptamine (5-Ht)-2b and 5-Ht2c receptors, leading to an intrinsic hyperexcitability and subsequent spasticity (Murray *et al.*, 2010, 2011).

In ALS, degeneration of upper motor neurons, whose axons form the corticospinal tract, is traditionally thought to cause spasticity as part of the 'upper motor neuron syndrome' (Ivanhoe and Reistetter, 2004), but direct evidence linking upper motor neurons and spasticity in ALS is lacking. Other hypotheses, in particular the implication of serotonergic neurons, have not been explored so far. Indeed, studies on serotonergic involvement in ALS are scarce and limited. Early studies focusing on the quantification of serotonin and its metabolites yielded inconsistent results, most likely owing to the very limited numbers of post-mortem brain tissues included (Bertel *et al.*, 1991; Sofic *et al.*, 1991; Forrest *et al.*, 1996). More recent imaging studies have shown decreased binding of serotonin 1A (5-HT_{1A}) ligands in ALS raphe and cortex (Turner *et al.*, 2005, 2007). To address the potential involvement of serotonin in ALS, we recently measured levels of platelet serotonin in a cohort of 85 patients with ALS and a control group of 29 healthy subjects. We found that platelet serotonin levels were significantly decreased in patients with ALS, and that higher platelet serotonin levels were positively correlated with increased survival of the patients (Dupuis *et al.*, 2010), suggesting that

serotonin might influence the course of ALS disease. However, investigation of a direct involvement of central serotonin in ALS has not been performed until now. Here, we show that central serotonergic neurons degenerate during ALS. From a functional point of view, our animal studies also suggest that spasticity might arise from serotonergic loss, at least in animal models.

Materials and methods

Patient tissues

Autopsy samples from hippocampus, brainstem and spinal cord were obtained from seven patients with ALS and six control subjects. Patient 2 had familial history of ALS, but gene analysis demonstrated no pathogenic variations in the *SOD1* gene. Hippocampus and brainstem samples were available for all patients. Spinal cord specimens were available for all patients with ALS and control subjects. Patients and/or families had provided written informed consent. Clinical details are presented in Supplementary Tables 1 and 2. ALS diagnosis was obtained using El Escorial criteria (Brooks *et al.*, 2000) and was confirmed after autopsy. During autopsy, tissues were fixed in 4% formaldehyde and embedded in paraffin using standard protocols. Use of these tissues for research was declared at the French ministry for research and higher education (DC-2011-1433).

Transgenic mice

Transgenic mice carrying the *SOD1* (G86R) mutation (Ripps *et al.*, 1995; Dupuis *et al.*, 2000) and their non-transgenic littermates on a FVB/N background were housed in our animal facility with unrestricted access to food and water. Mice were sacrificed at different stages of the disease to perform the studies using the following clinical scale: asymptomatic mice show normal gait and no paralysis and were scored 4. EMG is typically normal in these mice. Animals with a score of 3 showed a mildly abnormal gait or one hindlimb with paralysis. Score 3 typically occurs between 90 and 100 days of age, and is associated with already detectable EMG abnormalities, i.e. spontaneous muscle electrical activity, but no loss of motor neuron cell bodies (Halter *et al.*, 2010). Frank paralysis of one limb is scored 2 and of both hindlimbs is scored 1. Profound weight loss and kyphosis are typical of score 0, and mice are euthanized at this stage. In this study, asymptomatic mice used were all scored 4, and were 75 days old. Mice at disease onset were mice with a score of 3. These mice were followed daily and were sacrificed the second day on which they showed a score of 3. End stage mice used in the EMG studies were scored 1 and thus showed frank paralysis of both hindlimbs. For ethical reasons, we did not use mice scored 0 in experiments but proceeded to their euthanasia.

For histology, brains were fixed by immersion in 4% formaldehyde in phosphate buffer 0.1M pH 7.4, and tissues were post-fixed 24 h before paraffin embedding. For molecular biology, brainstem and lumbar spinal cord tissues were snap frozen in liquid nitrogen. Animal experiments were performed under the supervision of

authorized investigators (L.D. and F.R.), and approved by the local ethical committee for animal experiments (CREMEAS, agreement N° AL/01/02/02/12).

Histology

Paraffin embedded tissues were cut in 4 µm sections using a HM 340E Microtome (Microm). Luxol Fast blue/Cresyl violet stain was performed using a standard histological technique. Immunohistochemistry was performed in a Benchmark XT automate slide system using the Ventana NexES® software and EZ Prep Ventana Roche® reagent. Sections were heated, and endogenous peroxidases were inactivated using H₂O₂ (Ventana Roche®). Primary and secondary antibodies were incubated for 2 h at 37°C. Staining was performed using ultraview DAB (Ventana Roche®). Human sections were counterstained with haematoxylin (Ventana Roche®). Primary antibodies were as follows: rabbit polyclonal anti-ubiquitin (Dakocytomation 1/200), rabbit polyclonal TDP-43 (Proteintech LTD, 1/800) and rabbit polyclonal tryptophan hydroxylase 2 (Tph2) [described in Gutknecht *et al.* (2009), 1/1000].

Quantification of tryptophan hydroxylase 2 positive neurons in human samples

The number of Tph2-positive cell bodies in various regions of interest was evaluated semi-quantitatively in at least two sections of the considered nuclei identified as shown in Supplementary Fig. 1. Number of neurons per section: negative = 0–10, + = 11–20, ++ = 21–30, +++ = >30. We systematically compared sections stained in parallel in matched regions. Regions of interest were identified in adjacent sections using Luxol Fast blue/Cresyl violet staining, and counting of neurons were performed at ×20 magnification in a blinded manner, on two sections of each region of interest.

Measurement of perikaryon size of tryptophan hydroxylase 2 positive neurons

Sagittal brain sections (4 µm) were cut in series starting from the midline. In each animal, one of every five serial sections was sampled for Tph2 immunostaining. Using the second edition of the mouse brain in stereotaxic co-ordinates atlas (Franklin and Paxinos, 1997), position of the dorsalis raphe nucleus was determined on each section (medio-lateral: 0 to +0.48 mm; antero-posterior: –4 to –5.3 mm from Bregma; dorso-ventral: +2.75 to +4 mm). Images of the dorsalis raphe nucleus were captured using a Nikon digital camera DXM1200 connected to a Nikon eclipse E800 microscope. Tph2-positive neurons were analysed in seven to nine sections per animal in each group. The cell body area of all Tph2-positive neurons with a visible nucleus in the dorsalis raphe nucleus was measured using the NIH Image analysis software (ImageJ, version 1.45r), and 200–800 neurons were measured per animal.

Real-time quantitative polymerase chain reaction

Total RNA was extracted using TRIzol® (Invitrogen) and standard procedures. Real-time quantitative PCR was performed as previously described (Braunstein *et al.*, 2010) using BIO-RAD iScript™ cDNA Synthesis Kit, iQ™ qPCR mix and a CFX95 thermocycler (BioRad).

Data were normalized with the GeNorm software (Vandesompele *et al.*, 2002) using geometric averaging of three internal standards (18S ribosomal RNA, Tata-box binding protein and RNA polymerase II subunit).

5-Hydroxytryptamine-2c receptor mRNA editing

We used the quantitative PCR method developed by Lanfranco *et al.* (2009, 2010) to measure 5-Ht2c messenger RNA editing. This method is based on the use of TaqMan® probes selective for the various edited isoforms. We used the DNA templates provided by Lanfranco *et al.* (2009, 2010) to check for selectivity and specificity of the measurements, and obtained quantitative PCR cycling conditions that discriminate fully between the different templates using the published TaqMan® probes.

High-performance liquid chromatography

Serotonin and 5-hydroxyindoleacetic acid (5-HIAA) were measured on tissue extracts using high-performance liquid chromatography with coulometric detection using a technique similar to Alvarez *et al.* (1999). Results were standardized to initial wet weight of tissue.

Electromyographical evaluation of spasticity

Spasticity in tail muscles was measured with percutaneous EMG wires inserted in segmental tail muscles at the midpoint of the tail, as described by Bennett *et al.* (2004) and adapted to mouse. During EMG recording, muscle spasms were evoked with mechanical stimulation of the tail skin, and the tail was free to move. EMG was sampled at 5 kHz, rectified and averaged for a 4-s interval starting 1 s after stimulation. EMG over 1 s before stimulation was averaged for measure of background signal.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software. For comparison between two groups, Student's *t*-test was used. For comparison between three or more groups, ANOVA followed by Newman–Keuls *post hoc* test was applied. Significance level was set at $P < 0.05$.

Results

Degeneration of serotonergic neurons in amyotrophic lateral sclerosis

We analysed autopsic brains from seven patients with ALS and six control subjects (Supplementary Tables 1 and 2). Three patients with ALS had a bulbar onset of symptoms, and four had spinal onset. We focused our studies on major serotonergic nuclei of the brainstem presented in Supplementary Fig. 1. Ubiquitin and TDP43 cytoplasmic aggregates, two pathological hallmarks of ALS (Neumann *et al.*, 2006; Kiernan *et al.*, 2011), were observed almost systematically in the raphe magnus and gigantocellular nuclei but more rarely in other nuclei studied (Supplementary Figs 2 and 3). One patient (Patient 5) showed extensive ubiquitin

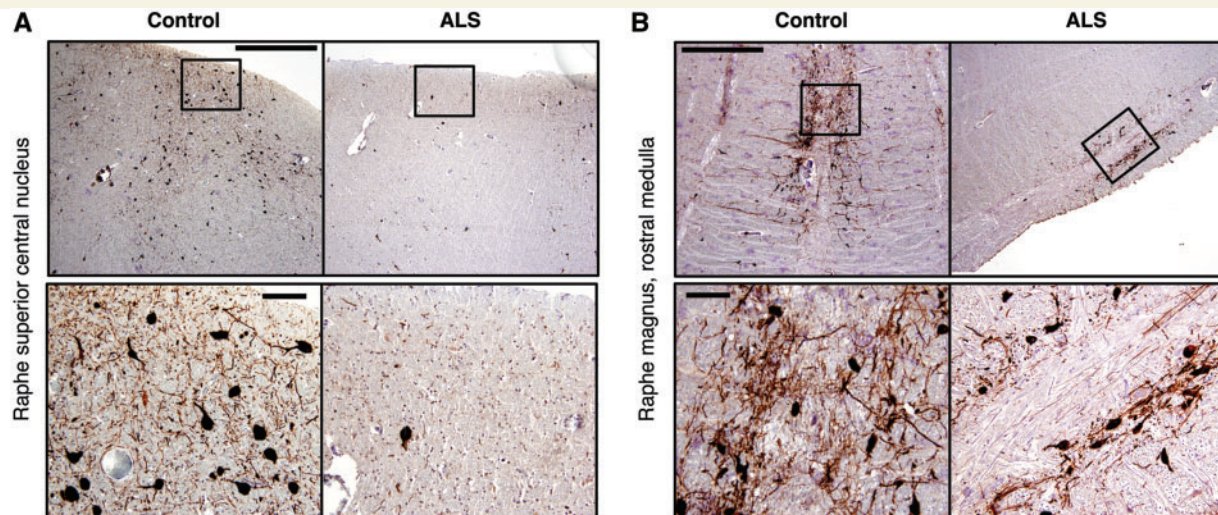


Figure 1 Serotonergic neurons degenerate in patients with ALS. (A) Representative TPH2 immunohistochemistry in the raphe superior central nucleus of one control subject (control subject 3, *left panels*) and one ALS (Patient 3, *right panel*). Upper pictures show low magnification. Lower pictures show a magnification of the rectangle in upper picture. This ALS case exhibits extensive degeneration of TPH2-specific cell bodies in this serotonergic nucleus. (B) Representative TPH2 immunohistochemistry in the raphe magnus nucleus (rostral medulla) of one control subject (control subject 6, *left panels*) and one ALS (Patient 7, *right panels*). Upper pictures show low magnification. Lower pictures show a magnification of the rectangle in upper picture. Neuronal density of TPH2-positive cell bodies are similar in ALS and control subject, but that intense degeneration of TPH2-positive neurites is visible in the ALS patient.

Table 1 Semi-quantitative analysis of Tph2-positive cell bodies in regions of interest in patients with ALS

	Case	Site of onset	Rostral pons		Caudal pons	Rostral medulla		Medulla (Inf. olive) RO
			RPF	RSCN	GCN	RM	RLN	
ALS	1	Bulbar	+++	+++	Neg	+++	+	
	2	Spinal	+++	+++	Neg	+++	++	
	3	Spinal	+++	Neg		+	Neg	+
	4	Spinal	+++	+++		+++	Neg	Neg
	5	Bulbar	+++	+++	+	+++	++	+
	6	Bulbar	+++	+	+	+	+	Neg
	7	Spinal	+++	+++		++	+++	+
Control	1				+			
	2		+++	+++		++	+++	+
	3		+++	+++		++	++	
	4				+			+
	5				+	++	++	
	6				+	+++	++	

Number of neurons per section: Negative (Neg) = 0–10, + = 11–20, ++ = 21–30, +++ = > 30.

Empty cells = tissue not available; Inf = inferior; RPF = reticular pontine formation; RSCN = raphe superior central nucleus; GCN = gigantocellular nucleus; RM = raphe magnus; RLN = reticular lateral nucleus. Inf. olive: inferior olive.

and TDP43 pathology in all serotonergic nuclei studied. Serotonergic neurons were easily detected in the pons and rostral medulla nuclei of control patients using an antibody directed against TPH2, the rate limiting enzyme in central serotonin synthesis. Patients with ALS showed loss of TPH2-positive cell bodies in serotonergic nuclei (Fig. 1A), although these nuclei were not uniformly affected in patients with ALS. In many cases, cell bodies were still present, but loss of TPH2-positive neurites was obvious (Fig. 1B). Semi-quantitative analysis of TPH2-positive cell bodies showed a heterogeneous decrease in cell density in the studied serotonergic nuclei, irrespective of the site of onset of disease,

gender or age (Table 1). Patients 3 and 6 showed widespread serotonergic degeneration, whereas degeneration of serotonin cell bodies was more localized in Patients 1, 2 and 4. Patient 5, although displaying prominent ubiquitin and TDP43 pathology in these nuclei, and Patient 7 appeared to show preserved neuronal counts. Analysis of serial sections revealed that the cells displaying TDP-43 or ubiquitin-positive inclusions were not serotonergic neurons (not shown). Thus, serotonergic neurons suffer from a degenerative process with prominent neuritic degeneration, and sometimes cell body loss in patients with ALS, but do not show typical ALS pathology.

Systematic degeneration of serotonergic projections in amyotrophic lateral sclerosis

We next sought to determine whether projections of serotonergic neurons degenerated in ALS. TPH2-labelled projections of serotonergic neurons were readily detectable in the hippocampus of control patients (Fig. 2A, left panel) but were almost absent in patients with ALS (Fig. 2A, right panel). In the spinal cord of Control 6, we observed spinal motor neurons densely innervated with TPH2-positive projections, as expected from the distribution of serotonin immunoreactivity in humans (Fig. 2B) (Perrin *et al.*, 2011). Contrasting with this, we occasionally observed isolated motor neurons with preserved serotonergic innervation, but neighbouring motor neurons were fully denervated (Fig. 2B). Even in Patients 5 and 7 with seemingly normal neuronal density, we barely observed motor neurons innervated by serotonergic axons. In all, we observed a massive and generalized reduction of TPH2-positive projections to spinal cord and hippocampus in patients with ALS.

Degeneration of serotonergic neurons and early serotonin depletion in SOD1 (G86R) mice

Studies in patients are hampered by the inaccessibility to presymptomatic period. To determine whether serotonergic neuron degeneration precedes motor symptoms, we studied SOD1 (G86R) mice, a mutant mouse strain that over-expresses an ALS-linked mutant form of SOD1. This mouse strain and other similar models have

been shown to demonstrate ALS-like disease, with both lower and upper motor neuron degeneration (Gurney *et al.*, 1994; Ripps *et al.*, 1995; Ozdinler *et al.*, 2011). At disease onset (score of 3), messenger RNA levels of cell-specific markers of serotonergic neurons (Tph2, serotonin transporter, 5-Ht1a receptor) were lower in the brainstem of SOD1 (G86R) mice, indirectly suggesting degeneration of these neurons (Fig. 3A). Direct visualization of serotonergic neurons in the dorsalis raphe nucleus using Tph2 immunohistochemistry revealed similar features in SOD1 (G86R) mice at symptom onset (score 3) to patients with ALS. Semi-quantitative analysis revealed that the area of the cell body of Tph2-positive neurons was decreased of about one-third in this nucleus (Fig. 3B and C). We further observed fragmentation of Tph2-positive neurites of SOD1 (G86R) mice (Fig. 3B). Most importantly, levels of serotonin itself were decreased when compared with wild-type mice, not only in symptomatic (score 3) but also in non-symptomatic (score 4) SOD1 (G86R) brainstem (Fig. 4A), spinal cord (Fig. 4B) and cortex (Fig. 4C). The ratio between 5-HIAA, the major serotonin metabolite depending of mono-amine oxidase A activity, and serotonin represents an indirect measurement of local serotonin turnover (Shannon *et al.*, 1986). In SOD1 (G86R) mice, the 5-HIAA/serotonin ratio was unchanged before symptoms in all three tissues tested (Fig. 4D–F) and increased at disease onset in brainstem and cortex. This shows that serotonin depletion precedes increase in serotonin turnover, suggesting that early loss of serotonin is due to decreased supply rather than to increased turnover. Thus, the development of ALS is associated with an early and general impairment of central serotonin function in an animal model of the disease.

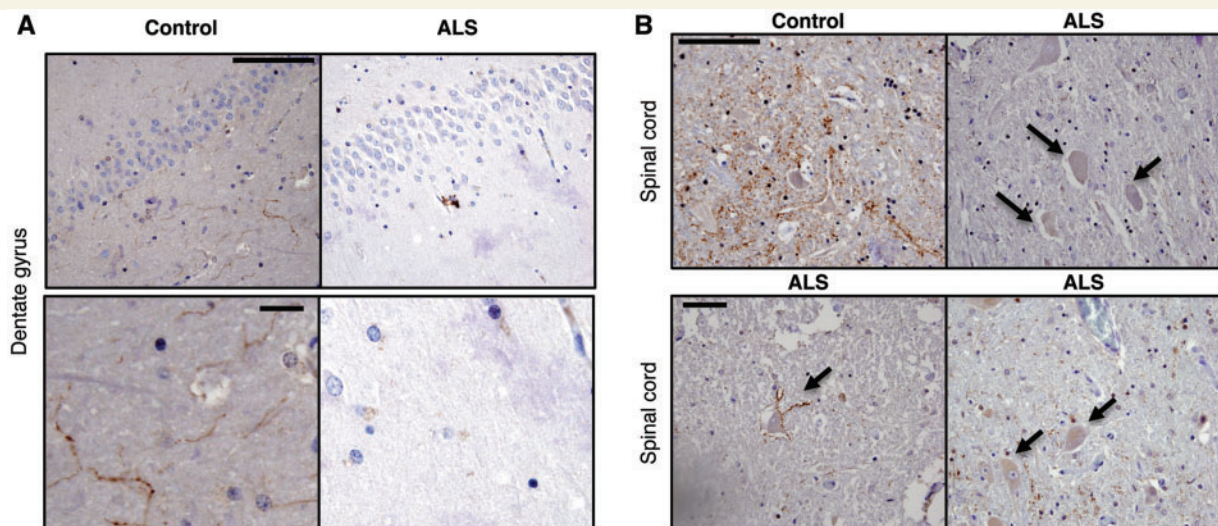


Figure 2 Serotonergic projections degenerate in spinal cord and hippocampus of patients with ALS. (A) Representative TPH2 immunohistochemistry in the dentate gyrus of one control subject (Control 3, left panels) and one ALS (Patient 4, right panels). Upper pictures show low magnification. Lower pictures show a magnification of upper pictures. Note the almost complete absence of TPH2 immunoreactivity in the dentate gyrus of the patient with ALS. (B) Representative TPH2 immunohistochemistry in cervical spinal cord of one control subject (Control 6, upper left panel) and three patients with ALS (Patients 1, 3 and 7). Note the punctate TPH2 immunoreactivity surrounding motor neurons (arrows) in the control subject, but not in patients with ALS. A single motor neuron innervated by TPH2-positive serotonergic neuron terminals is shown in a patient with ALS (Patient 7, lower left panel).

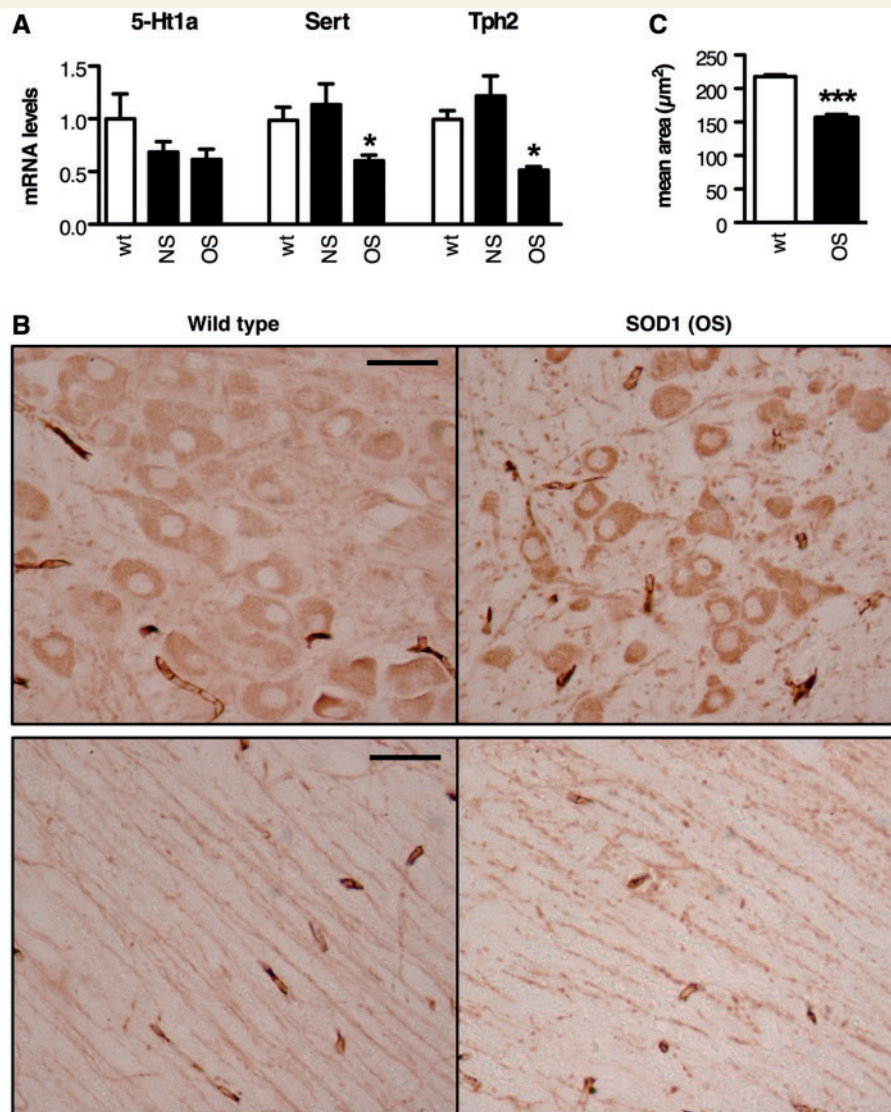


Figure 3 Serotonergic neurons degenerate in SOD1 (G86R) mice. (A) Messenger RNA levels of 5-Ht1a receptor (5-Ht1a), serotonin transporter (Sert) and Tph2 in SOD1 (G86R) mice at 75 days of age (asymptomatic, NS) or at symptom onset (~100 days of age, OS) and wild-type litter mates (Wt). Note that serotonin transporter and Tph2 gene expression levels are downregulated at symptom onset. $n = 7–10$ per group. $*P < 0.05$ versus wild type, ANOVA followed by Newman–Keuls *post hoc* test. (B) Representative Tph2 immunohistochemistry in SOD1 (G86R) mice at disease onset (SOD1; OS) and wild-type littermates. Note the shrunken Tph2-positive cell bodies in SOD1 (G86R) mice (*upper right*) and neuritic degeneration (*lower right*). $n = 3$ per group. (C) Mean area of Tph2-positive neurons in SOD1 (G86R) mice at disease onset (OS) and wild-type (Wt) littermates. The area of 200–800 neurons was measured per animal, with $n = 3$ per group. $***P < 0.0005$ versus wild type, Student's *t*-test.

Spasticity develops in SOD1 (G86R) mice, and is alleviated by 5-hydroxytryptamine 2 b/c receptors inverse agonists

We sought then to characterize whether serotonin depletion occurring early in SOD1 (G86R) had pathogenic consequences on motor neurons. Serotonin modulates excitability of motor neurons by allowing sustained entry of calcium (Heckman *et al.*, 2009). In animal models of spinal cord injury, it was recently shown that serotonin depletion due to transection of serotonergic

axons was over-compensated by motor neurons. More specifically, motor neurons produce constitutively active 5-Ht2b and 5-Ht2c receptors through still poorly defined mechanisms, decreased editing of the 5Ht2c messenger RNA being one of these (Murray *et al.*, 2010). This constitutive activity of 5-Ht2b/c receptors is responsible for the occurrence of spasticity on spinal cord injury (Murray *et al.*, 2010). Other serotonin receptors, including 5-HT1A, 2A, 3, 4, 5, 6 and 7 appear to not be involved in this event (Murray *et al.*, 2011). By analogy, we reasoned that the chronic loss of serotonergic innervation of lower motor neurons in patients with ALS and SOD1 (G86R) mice could lead to spasticity. To explore this hypothesis, we used an electromyographical

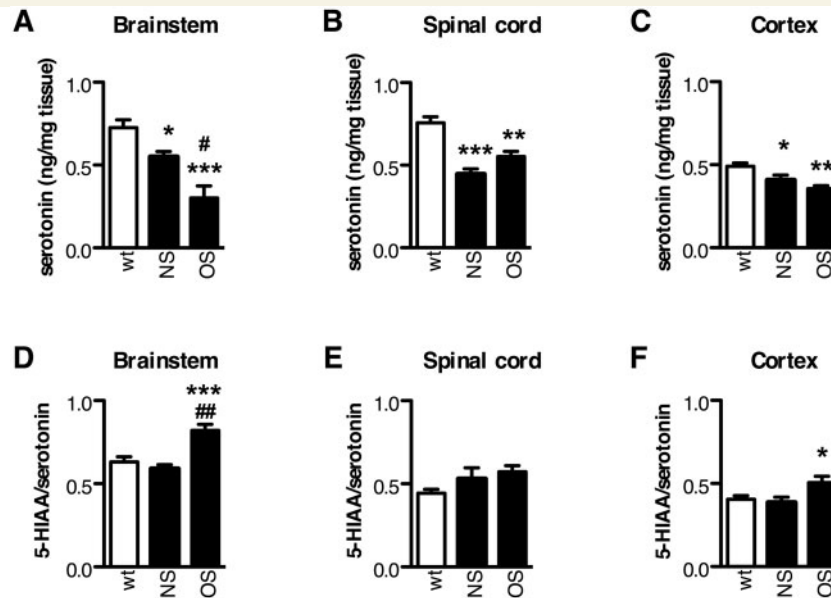


Figure 4 Decreased levels of serotonin in SOD1 (G86R) mice. Serotonin (ng/mg tissue, A–C) and 5-HIAA/serotonin (D–F) in the brainstem (A and D), the spinal cord (B and E) and the cerebral cortex (C and F) of SOD1 (G86R) mice at 75 days of age (asymptomatic, NS) or at symptom onset (~100 days of age, OS) and wild-type littermates (Wt) as measured using high-performance liquid chromatography. * $P < 0.05$ versus wild type, ** $P < 0.01$ versus wild type, *** $P < 0.001$ versus wild type, # $P < 0.05$ versus asymptomatic, ## $P < 0.01$ versus asymptomatic. ANOVA followed by Newman–Keuls *post hoc* test. $n = 5–10$ per group.

protocol adapted from Bennett *et al.* (2004). This objective and quantitative electromyographical method appears correlated with clinical evaluation of spasticity (Bennett *et al.*, 2004) and facilitates the study of the effect of classical anti-spastic drugs such as baclofen or clonidine (Li *et al.*, 2004; Rank *et al.*, 2011). We visually observed spasticity in the tail of some animals at disease onset (score 3), but this was difficult to distinguish from voluntary contractions in these mice that are not yet paralysed. When disease progresses, however, spasticity of the tail was systematically observed and strong in end-stage (stage 1) mice. Thus, the mechanisms for spasticity are already present at disease onset, and might help with maintaining motor function, but are blurred by voluntary movements. For this reason, we used end-stage SOD1 (G86R) mice to adapt the EMG protocol initially developed for rats with spinal cord injury. Under this experimental set-up, spastic-like contractions of tail muscles were easily recorded (Fig. 5A), providing objective evidence of spasticity as a component of ALS disease in this mouse model. We further studied involvement of constitutive activity of 5-Ht2b/c receptors in this measure of spasticity and found that spasticity was strongly alleviated by injection of 5-Ht2b/c inverse agonists SB206553 (Fig. 5B and D) and cyproheptadine (Fig. 5C and D). In rats with chronic spinal cord injury, spasticity is associated with increased production of the unedited isoform of the 5-Ht2c messenger RNA leading to increased expression of the constitutively active INI-5-Ht2c receptor. In SOD1 (G86R) mice, we observed, however, decreased production of this specific isoform and normal levels of total messenger RNA and of other various edited isoforms (Fig. 5E and F) in the lumbar and sacral spinal cords at disease onset (score 3), i.e. the disease stage at which spasticity arises. Contrasting with this, we observed

a 10-fold increased expression of the 5-Ht2b receptor in the same animals (Fig. 5E). In all, our results suggest that serotonin depletion leads to over-expression of 5Ht2b receptors and subsequent constitutive activity of this receptor during development of spasticity. In turn, this constitutive activity likely leads to spasticity.

Discussion

Here, we show that ALS is associated with degeneration of central serotonergic neurons, both in patients and animal model, and identify spasticity as a possible consequence of ALS-related serotonergic dysfunction.

The first major result of this study is that central serotonergic neurons degenerate in patients with ALS and in an animal model. We observed a major decrease in serotonergic innervation in target regions, such as the spinal cord and the hippocampus, along with obvious decreased density of serotonergic neurites in the serotonergic nuclei. In some nuclei, this was also accompanied by shrinkage and loss of cell bodies. We found limited ubiquitin and TDP-43 pathology in most serotonergic nuclei studied, but these inclusions were not in remaining cell bodies of serotonergic neurons. This might reflect either high intrinsic capacity in clearing protein aggregates in serotonergic neurons, or low production of aggregate-prone proteins in this neuronal type or, on the contrary, extreme sensitivity leading to degeneration of neurites, despite low levels of aggregates. Degeneration of serotonergic neurons could be either independent of lower motor neuron degeneration or be a secondary consequence of motor neuronal loss. However, we observed loss of serotonin in asymptomatic mice, as early as

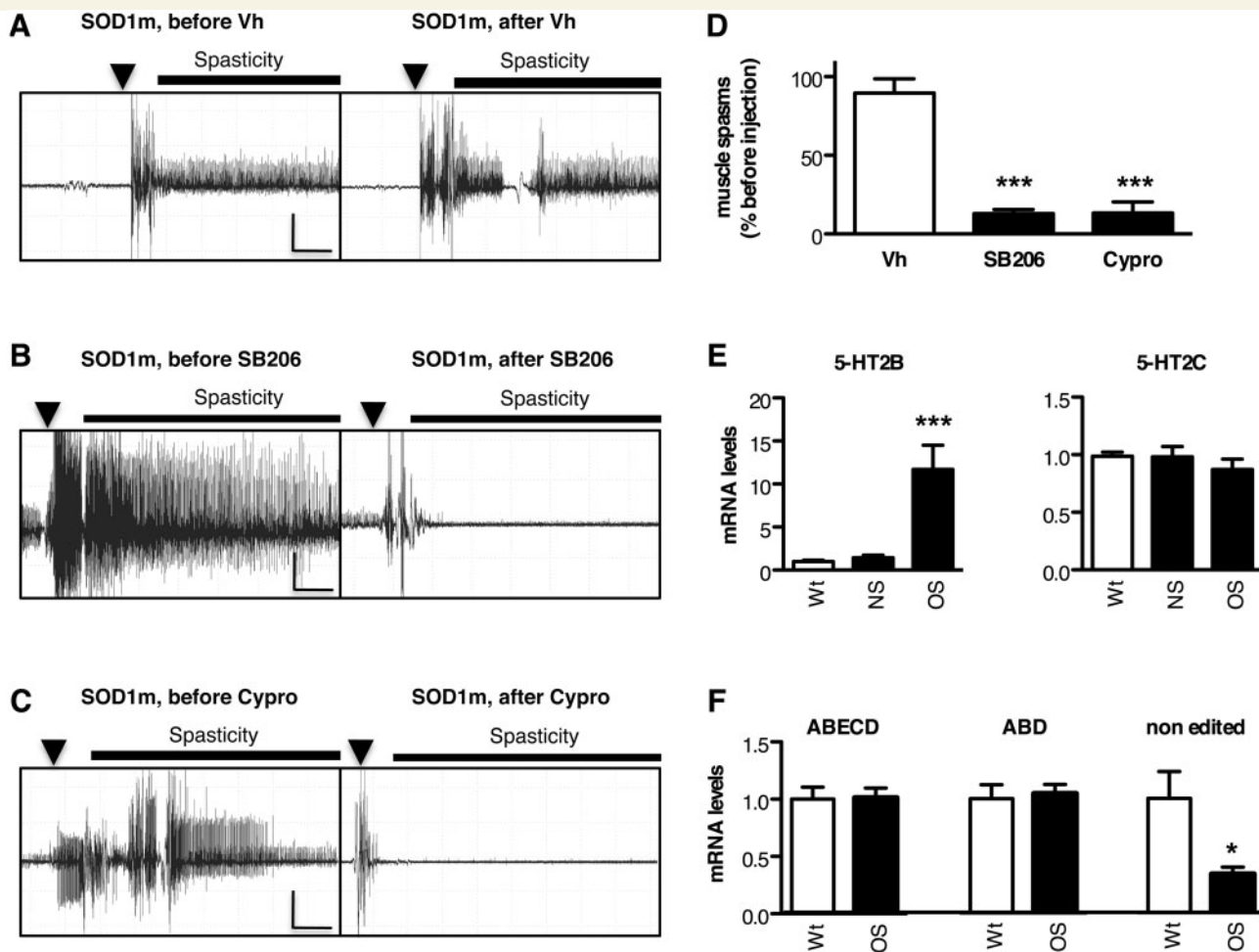


Figure 5 Spasticity in SOD1 (G86R) mice is alleviated by 5-Ht2b/c inverse agonists. (A–C) Representative recording of long-lasting reflexes using tail EMG in one diseased SOD1 (G86R) mouse before (*left*) and after injections of vehicle (Vh, A), SB206553 (SB206, B) or cyproheptadine (Cypro, C). Spasticity was considered to be electrical activity above the baseline recorded 1 s after mechanical stimulation (arrowhead). (D) Quantitative analysis. $n = 5–6$ mice per group and two to three EMGs were obtained before and after injection. We calculated a ratio between spasticity before and after injection and present the result as a percentage. $*P < 0.05$ versus before injection, ANOVA followed by Newman–Keuls *post hoc* test. (E) Messenger RNA levels of 5-Ht2b (5-Ht2b), and 5-Ht2c (5-Ht2c) receptors in SOD1 (G86R) mice at 75 days of age (asymptomatic, NS) or at symptom onset (~100 days of age, OS) and wild-type littermates (Wt). 5-Ht2b gene expression levels are heavily upregulated at symptom onset. $n = 12$ per group. $***P < 0.001$ versus wild type, ANOVA followed by Newman–Keuls *post hoc* test. (F) Levels of editing variants of the 5-Ht2c messenger RNA in the spinal cord of SOD1 (G86R) mice at onset (OS) and wild-type littermates (Wt). ABECD (messenger RNA variant with full editing of A, B, E, C and D sites), ABD (messenger RNA variant edited at A, B and D sites) and the non-edited messenger RNA, which leads to the production of the constitutively active IN1 receptor, were measured by TaqMan[®] assays. Levels of the non-edited messenger RNA are decreased. $n = 12$ per group. $*P < 0.05$ versus wild type, Student's *t*-test.

75 days of age, an age that precedes from several weeks the onset of motor neuron degeneration. This suggests that degeneration of serotonergic neurons occurs independently of motor neuron death, at least in animal models.

The loss of serotonergic neurons causes loss of serotonin itself in regions of projections. In our animal model, serotonin levels are decreased in the brainstem and the spinal cord long before motor symptoms arise. Previous studies on serotonin and 5-HIAA in patients with ALS yielded conflicting results. Bertel *et al.* (1991) observed normal levels of serotonin and decreased levels of 5-HIAA in autopsy samples, whereas Forrest *et al.* (1996)

observed normal serotonin levels but increased 5-HIAA levels. However, serotonin is a labile molecule that might be significantly altered in post-mortem human samples with hours of delay before autopsy (Yoshimoto *et al.*, 1993). Our study overcomes this problem by studying serotonergic neurons using TPH2 immunostaining in fixed tissues. In asymptomatic animals, the loss of serotonin was associated with normal serotonin turnover (unchanged 5-HIAA/serotonin ratio), strengthening the idea that decreased serotonin was owing to decreased supply in serotonin rather than to increased degradation. Contrastingly, in end-stage mice, we observed an increased serotonin turnover (increased 5-HIAA/

serotonin ratio). This late increased serotonin turnover is likely to be due to increased release of serotonin by remaining nerve terminals. Indeed, this increased mobilization of residual serotonin in end-stage mice coincides with the loss of serotonin transporter expression, an event expected to limit serotonin reuptake and increase its turnover. Our studies are consistent with imaging studies using the PET scan ligand WAY100635 (Turner *et al.*, 2005, 2007). This compound binds to the 5-HT_{1A} receptor, which is broadly expressed in serotonergic neurons, notably in brainstem and acts as an inhibitory somatodendritic autoreceptor in these neurons. The decreased binding potential of WAY100635 in the raphe of patients with ALS was hypothesized to be either owing to decreased sensitivity of 5-HT_{1A} receptors to WAY100635 or to loss of neurons that express this receptor. As 5-HT_{1A} receptor is strongly expressed in serotonergic neurons in the raphe, our current results argue for the latter view.

We next sought to delineate whether serotonin depletion had pathogenic consequences and focused on one of the potential consequences, the occurrence of spasticity. Spasticity had been previously shown to occur in spinal cord injury as a late consequence of transection of serotonergic axons (Murray *et al.*, 2010, 2011). Spasticity represents a symptom that is difficult to objectively measure in animals. We adapted an EMG technique measuring spastic-like contractions of tail muscles in response to a mechanical stimulation. Others had previously shown that this EMG method is clinically correlated with onset of spasticity in rats with spinal cord injury and sensitive to classical anti-spastic drugs (Bennett *et al.*, 2004; Li *et al.*, 2004; Rank *et al.*, 2011). This method thus represents a quantitative, observer-independent measurement of spasticity. In our model, we were able to almost abolish spasticity by the use of cyproheptadine, a broad 5-HT₂ inverse agonist, and SB206553, a much more selective compound known to target 5-HT_{2B} and C (Kennett *et al.*, 1996), arguing for the involvement of one of these two receptors in ALS spasticity. Murray *et al.* (2010) observed increased production of the unedited 5-HT_{2c} messenger RNA in rats with chronic spinal cord injury, whereas our result in mSOD1 mice was opposite. Recent work in another paradigm of spinal cord injury found unchanged levels of editing of the 5-HT_{2c} messenger RNA in rats (Navarrett *et al.*, 2012). This discrepancy might be due to species differences (rats versus mice), to the different kinetics of serotonin loss [abrupt in spinal cord injury but much slower in SOD1 (G86R) mice]. We found a strong increase in spinal 5-HT_{2b} receptor expression when spasticity was obvious, which could underlie the constitutive activity observed. Indeed, 5-HT_{2b} receptor has intrinsic constitutive activity, and the increase of concentration of a G-protein coupled receptor is on its own sufficient to further increase any constitutive activity (Seifert and Wenzel-Seifert, 2002). For instance, a 7-fold over-expression of the 5-HT_{2b} receptor in cardiomyocytes leads to a dramatic cardiac phenotype, suggesting that the over-expression of this receptor in the range we observed in SOD1 (G86R) mice is sufficient to induce strong constitutive activity (Nebigil *et al.*, 2003). Our mouse model of ALS is based on transgenic over-expression of mutant SOD1. Although these mouse models represent the only currently available model that display selective loss of both lower and upper motor neurons (Halter *et al.*, 2010; Ozdinler *et al.*, 2011), they only mimic the 20% of familial

cases that display mutations in the *SOD1* gene. Whether spasticity might also be alleviated by 5-HT₂ inverse agonists in other, non-SOD1 ALS cases represents an open question. In all, our animal study suggests that spasticity, at least in SOD1-linked ALS, is due to constitutive activity of the 5-HT_{2b} receptor rather than 5-HT_{2c}.

How far can these mechanistic results be compared with our pathology study in patients with ALS? Among the patients included in our study, only Patient 5 showed the complete picture of upper motor neuron signs, in particular spasticity, whereas the other patients exhibited either increased reflexes and/or Babinski signs, but not obvious spasticity (Supplementary Table 1). Patient 5, who displayed spasticity, showed strong loss of serotonergic terminals on motor neurons and thus appeared indistinguishable from the other patients with ALS in terms of loss of TPH2 projections. Interestingly, however, Patient 5 was the single case with ALS with widespread TDP43 pathology in serotonergic nuclei. Further work comparing autoptic material from patients with or without spasticity should be done to highlight potential correlations between serotonin loss and spasticity. Importantly, such a study could also investigate other phenotypes potentially related with serotonin such as weight loss, depression or dementia.

Our work has potential clinical implications for the management of spasticity in those patients presenting such a phenotype. This is especially true for patients with primary lateral sclerosis, a subtype of ALS with primary upper motor neuron involvement (Singer *et al.*, 2007; Gordon *et al.*, 2009). These patients develop prominent spasticity (Kuipers-Upmeijer *et al.*, 2001; Le Forestier *et al.*, 2001a, b) that is likely to be due to motor neuron hyperexcitability (Floeter *et al.*, 2005). Spasticity is also sometimes associated with ALS but difficult to detect clinically, as the tests used to assess spasticity rely on the integrity of alpha and gamma motor neurons, both degenerating during ALS (Swash, 2012). Spasticity in ALS and primary lateral sclerosis has been poorly studied, and few clinical trials have been performed to treat this symptom (Ashworth *et al.*, 2012). Only physical therapy was proven to be effective in a small trial (Drory *et al.*, 2001), and current guidelines of the European Federation of Neurological Societies state that other anti-spastic medications display class IV level of evidence of efficacy and 'may be tried' (Ashworth *et al.*, 2006). A rigorous clinical trial assessing cyproheptadine in ALS spasticity is thus needed, although treatment of spasticity might also lead to worsening of motor function as observed in spinal cord injury.

In conclusion, loss of serotonergic neurons is part of the degenerative process in ALS and may be involved in spasticity. Further research is needed to determine whether serotonergic degeneration has broader consequences on ALS pathophysiology.

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Supplementary material

Supplementary material is available at *Brain* online.

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