Universitat Autònoma de Barcelona

CHARACTERIZATION OF ALTERATIONS IN SENSORY NEURONAL POPULATIONS IN AMYOTROPHIC LATERAL SCLEROSIS

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ACADEMIC DISSERTATION

To obtain the PhD in Neuroscience by the

Universitat Autònoma de Barcelona (2021)

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“If you trust in yourself. . .and believe in your dreams. . .and follow your star. . . you'll still get beaten by people who spent their time working hard and learning things and weren't so lazy.”

— **Terry Pratchett,** The Wee Free Men

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ABBREVIATIONS

[18F] FDG-PET: 18 F-fluorodeoxyglucose positron emission tomography/computed tomography

AD: Alzheimer's disease

ALS: amyotrophic lateral sclerosis

ALSFR-R: *Revised Amyotrophic Lateral Sclerosis Functional Scale*

AOA2: ataxia-oculomotor apraxia type 2

AUC: area under the curve

BSA: bovine serum albumin

CGRP: calcitonin gene related peptide

ChAT: choline acetyltransferase

CHEPS: contact heat-evoked potentials

CMT: Charcot-Marie-Tooth hereditary neuropathy

CNS: central nervous system

DAPI: 4′,6-diamidino-2-phenylindole

DRG: dorsal root ganglion

DTI: diffusion tensor imaging

fMRI: functional magnetic resonance imaging

FOSMN: facial onset sensory motor neuropathy

FTD: frontotemporal dementia

FTLD: frontotemporal lobar degeneration

GABA: *gamma*-aminobutyric acid

GCL: ganglion cell layer

GDNF: glial cell line-derived neurotrophic factor

HC: healthy control

IB4: isolectin B4

IENF: intraepidermal nerve fiber

IML: intermediate lateral column

INL: inner nuclear layer

MRI: magnetic resonance imaging

MS: Multiple sclerosis

MSNA: muscle sympathetic nerve activity

NAA: N-acetyl aspartate

NC: neurological control

NGF: nerve growth factor

OCT: optical coherence tomography

OPL: outer plexiform layer

PAS: Periodic Acid-Schiff stain

PB: phosphate buffer

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PD: Parkinson's disease

PGP 9.5: protein gene product 9.5

PLS: primary lateral sclerosis

PMA: progressive muscular atrophy

pTDP-43: phosphorylated TDP-43

PV: Parvalbumin

Q-SART: quantitative sudomotor axon reflex test

RNFL: retinal nerve fiber layer

ROC: receiver operating characteristic

SBMA: spinal-bulbar muscle atrophy

SCA2: spinocerebellar ataxia type 2

SEM: standard error of the mean

SENP: subepidermal nerve plexus

SEP: somatosensory evoked potentials

SGNFD: sweat gland nerve fiber density

SMA: spinal muscular atrophy

SPG: hereditary spastic paraplegia

SSNA: skin sympathetic neural activity

SSR: sympathetic skin response

TBS: tris buffered saline

TH: tyrosine hydroxylase

UPSIT: University of Pennsylvania Smell Identification Test

VIP: vasoactive intestinal peptide

SUMMARY

Although amyotrophic lateral sclerosis (ALS) is eminently a motor disease, the existence of non-motor manifestations, including sensory involvement, has been described in the last years. The aim of this thesis is the characterization of sensory involvement in this disease.

To meet this objective, we first considered whether the most used animal model of the disease (SOD1^{G93A} mouse) also presents some of the sensory alterations already known in humans with ALS, and therefore if it was a valid model to deepen these studies. Subsequently, we studied the different sensory populations from the most peripheral part (skin) to the body of neurons (dorsal root ganglion; DRG) to have a map of the temporal and spatial evolution of this involvement. Next, we investigated whether ALS patients, in addition to the already known loss of sensory axons at cutaneous level, also presented other pathological alterations typical of the disease such as cytoplasmic translocation of TDP-43.

In a first study we found that indeed in the murine model there is a loss of intraepidermal axons already at a presymptomatic stage. Furthermore, there is not only a loss of unmyelinated/thin myelinated fibers but also of large fibers such in the case of Meissner corpuscles in dermal papillae. We also found a gradient of involvement from the most superficial to the deepest part, with greater loss in the epidermis with respect to the dermis, suggesting the existence of a distal sensory axonopathy.

Then, a characterization of the different affected sensory populations was performed, not only through the follow-up in the different evolutionary stages but also their progression from the axon to the body of the neurons. At the peripheral (cutaneous) level, peptidergic (CGRP) and non-peptidergic (IB4) populations were studied, as well as the cholinergic sympathetic innervation of sweat glands (VIP). At the level of the DRG we distinguished 3 groups of neurons: CGRP, IB4, and parvalbumin (PV), the latter corresponding to mechanoreceptors and especially proprioceptors. In the cutaneous analysis we observed a loss of intraepidermal axons of both sensory populations in the SOD1 mice, already at the presymptomatic motor stage, but with an earlier involvement of the non-peptidergic fibers. In addition, a loss of sudomotor innervation was also detected. When analyzing the group of neurons in the DRG, no loss of their somas was found in any neural population studied.

The involvement of the distal portion of the axons together with the preservation of the body of the neurons leads us to consider that the underlying sensory involvement in this disease follows a pattern of distal sensory axonopathy.

Finally, skin samples from ALS patients were studied to determine whether there was accumulation of TDP-43 in the sensory axons. We obtained skin biopsy samples from ALS patients and compared them with a group of healthy controls and another group of neurological controls (patients with multiple sclerosis and Parkinson's disease). We did not find colocalization of TDP-43 signal in the cutaneous axons of either group studied. However, we did observe cytoplasmic deposition of TDP-43 in the keratinocytes of the epidermis, and in fibroblasts of the dermis. Such cytoplasmic translocation in fibroblasts was greater and more frequent in patients with ALS.

In conclusion, in the murine model of ALS ($SOD1^{G93A}$) there is a distal predominant involvement of intraepidermal sensory fibers, globally, although with a greater and earlier predominance of non-peptidergic fibers. Such involvement progresses over time, precedes motor symptomatology, and is not accompanied by neuronal body degeneration, suggesting a model of distal sensory axonopathy, similar to what has been reported at the motor level, where in the last years has been described a denervation of skeletal muscle with a loss of synapses long before the onset of symptoms, and more importantly, long before the loss of anterior horn motor neurons in the spinal cord. Furthermore, in human with ALS we did not find an accumulation of TDP-43 in cutaneous sensory axons but found it significantly in the cytoplasm of fibroblasts.

ARTICLES PRODUCED FROM THE WORK OF THIS THESIS

Peer reviewed publications:

Rubio MA, Herrando-Grabulosa M, Vilches JJ, Navarro X. Involvement of sensory innervation in the skin of SOD1(G93A) ALS mice. J Peripher Nerv Syst. 2016 Jun;21(2):88-95. doi: 10.1111/jns.12164. PMID: 26880731.

In preparation:

Rubio MA, Herrando-Grabulosa M, Gaja-Capdevila N, Vilches JJ, Navarro X. Sensory involvement characterization in SOD1 G93A ALS mouse model

Rubio MA, Herrando-Grabulosa M, Velasco R, Blasco I, Povedano M, Navarro X. TDP-43 cytoplasmic translocation in the skin fibroblasts of ALS patients

INTRODUCTION

1. Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder that involves both upper and lower motor neuron. The signs and symptoms derived from the involvement of both motor pathways are the ones that take the patient to consult for the first time, to its functional decline and finally, to its death, with a median survival of 3-5 years. Based on the clinical picture, it is not surprising that it has been classically considered as an exclusively motor pathology. However, in recent years there is growing evidence about non-motor symptoms, including cognitive, extrapyramidal, and sensory impairment (McCombe et al., 2017; Tao et al., 2018).

Hereafter we review the evidence about the involvement of the different sensory pathways in ALS, based on animal models, series of clinical cases, neurophysiological studies, neuroimaging tests and neuropathological studies. These include the somatosensory, visual, olfactory, gustatory, and auditory systems, as well as the autonomic nervous system.

1.1. ALS as a purely motor disease

The traditional concept of purely motor disease starts with the first descriptions of the disease. Despite not being the first to describe a motor neuron disease, Jean-Martin Charcot (Paris, 1825-1893) was the first to use the term “amyotrophic lateral sclerosis” and to provide a very accurate description. Already in his first publication on the disease, he clearly described the absence of sensory symptoms in arms and legs, as well as the absence of apparent pathological alterations in the posterior columns of the spinal cord or in the sensitive peripheral nervous trunks.

This concept is also evident in the different diagnostic criteria used to date (Brooks et al.,1994; Brooks BR, et al., 2000; de Carvalho et al., 2008). In these, it is

discussed the role of sensory neurography to rule out other pathologies, pointing out that for an ALS diagnosis, both amplitude and conduction velocity of sensory nerve potentials should be normal, although a slight impairment is accepted (in both amplitude and conduction velocity) only in case of an entrapment neuropathy or for another known cause. Coexistence of a marked unexplained sensory neuropathy is therefore considered a red flag in ALS diagnosis. Beyond this assumption, the severe motor symptoms can easily overshadow other mild neurological manifestations such as sensory or autonomic disturbances.

1.2. TDP-43, a pathological hallmark of ALS

Throughout all the histopathological changes described in the disease, such as loss of Nissl staining of motoneurons, the presence of Bunina bodies, and reactive astrogliosis, the cytoplasmic translocation and aggregation of TDP-43 is considered nowadays the pathological hallmark of ALS (Kwong et al., 2007; Neumann et al., 2006; Lomen-Hoerth et al., 2004; Hasegawa et al., 2008). Cytoplasmic TDP-43 is not only found in genetic forms related to the *TARDBP* gene, but also in many other genetic, and most importantly in sporadic ALS. Almost 97% of ALS patients present pathological inclusions of TDP-43, with the rare exceptions of cases due to *SOD1* and *FUS* mutations.

TDP-43 is a nuclear and ubiquitously expressed 43kDa DNA binding protein, composed of 414 aminoacids, with multiple functions, encoded by *TARDBP* gene. Its physiological functions have been related to exon skipping, splicing inhibitory activity (Buratti et al., 2008; Lagier-Tourenne et al., 2010), microRNA processing (Buratti et al., 2010), apoptosis, cell division, stabilization of messenger RNA (Kraemer et al., 2010; Wu et al., 2010; Sephton et al., 2010), regulation of neuronal plasticity, maintenance of dendritic integrity (Moisse et al., 2009; Sato et al., 2009; Lu et al., 2009) and transport of ribosomal proteins mRNAs through the axon and local translation (Nagano et al., 2020).

It is hypothesized that different upstream events (gene mutations, inflammation, environmental stress, toxicity) could induce TDP-43 post-translational modifications (hyperphosphorylation, ubiquitination, protein cleavage, nuclear export) and lead to its cytoplasmic aggregation and subsequently to impairment of the ubiquitin proteasome system (UPS) and other autophagic pathways (Palomo et al., 2019).

TDP-43 is related to an autosomal dominant form of ALS and frontotemporal dementia (FTD) (Ou et al., 1995; Mackenzie et al., 2010), narrowing the limits between these two entities and merging them into a continuous spectrum of disease (Neumann et al., 2006). The clinical phenotype overlap between ALS and frontotemporal lobar degeneration (FTLD) was confirmed later by the correspondence with the most common genetic abnormality of both sporadic and familial ALS, the hexanucleotide expansion in *C9ORF72 (*DeJesus-Hernandez et al., 2011; Renton et al., Neuron 2011*)*. Additionally, different genetic forms of FTDL are associated with certain neuropathological subtypes of TDP-43 proteinopathy. These subtypes, categorized from type 1 to type 4, are based on three descriptors: amount of neuronal cytoplasmic inclusions, presence of dystrophic neurites, and intranuclear inclusions (Mackenzie et al., 2010; Kwong et al., 2007, Mackenzie et al., 2020). Although these correlations between genetic background and TDP-43 phenotype are limited to FTLD and not in ALS forms, it implies that these deposits are much more than a bystander in the neurodegenerative process.

Beyond the different subtypes of TDP-43 neuropathology, there is a variability of its cytoplasmic aggregation in ALS through the nervous system. In spinal cord, both motor neuron and glial cells show a mixture of phosphorylated and ubiquitinated with phosphorylated, ubiquitinated and acetylated TDP-43 deposits (**Table 1**). Interestingly, in the brain, Betz cells, which are the largest upper motor neurons located in the fifth layer of the primary motor cortex, present only sparse cytoplasmic translocated normal form of TDP-43. Other brain neurons rather than Betz cells and glial cells present deposits of the native form of the protein, a phosphorylated and ubiquitinated form and C-terminal fragments (Feneberg et al., 2018; Braak et al., 2017). These C-terminal fragments are formed through protein cleavage as part of post-translational modifications. TDP-43 is typically cleaved into 35kDa and 25KDa fragments. The 25KDa fragments are frequently detected in the brain of ALS patients whereas 35KDa portions are rarely observed. However, SOD1 mouse models present low levels of both fragments in brain and spinal cord (Berning et al., 2019).

Interestingly, a staging system in ALS and FTLD based on TDP-43 proteinopathy has been proposed. It distinguishes four different stages, through agranular frontal cortex, prefrontal neocortex, and sensory neocortex, suggesting on the one hand a gradient of affectation and in addition a model of propagation (Braak et al., 2013;

Brettscheneider et al., 2013). **Table 1** lists the different typifications of TDP-43 proteinopathy at molecular, cellular, and structural levels.

Table 1. TDP-43 proteinopathy and different molecular aggregations, cellular subtypes, and staging system. ALS: amyotrophic lateral sclerosis. FTD: frontotemporal dementia. FTLD: frontotemporal lobar degeneration. bvFTD: Behavioural variant of FTD. PNFA: progressive non-fluent aphasia. SD: semantic dementia. IBMPFD: Inclusion body myopathy associated with Paget disease and FTD.

TDP-43 proteinopathy – molecular level typification			
	Brain		**Spinal cord**
	Betz cells	**Other neurons and glia**	**Motor neurons and glia**
Native form	+/- (very few)	+	-
Ubiquitinated-Phosphorylated	-	+	+
Ubiquitinated-Phosphorylated-Acetylated	-	-	+
C-terminal fragments	-	+	-

TDP-43 proteinopathy – classification by cellular involvement					
	Neuronal cytoplasmic inclusions	**Dystrophic neurites**	**Neuronal intranuclear inclusions**	**Major clinical phenotypes**	**Associated gene defects**
Type 1	+++	+++	-/++	FTLD-bvFTD and PNFA	*GRN, C9ORF72*
Type 2	+	+++	Generally absent	FTLD - SD	*C9ORF72*
Type 3	++	+	Generally absent	FTLD- bvFTD and FTD with ALS	-
Type 4	+	+++	+++	FTLD - IBMPFD	*VCP*

TDP-43 proteinopathy –staging by areas involved		
	Cortical structures	**Subcortical structures**
Stage 1	Primary Motor Cortex	Alpha-motor neurons
Stage 2	Premotor areas	Parvocellular red nucleus Reticular formation Inferior olivary complex Ventrolateral posterior thalamic nucleus Ventral anterior thalamic nucleus Mediodorsal thalamic nucleus Precerebellar nuclei
Stage 3	Prefrontal fields/Sensory associated areas (temporal and occipital lobes)	Caudate nucleus and putamen Dorsal striatum Striatopallidal axons
Stage 4		Trans entorhinal region Entorhinal region Hippocampal formation Ventral striatum

However, attempts to correlate the amount of cytoplasmic deposits of TDP43 throughout the entire nervous system and different clinical parameters (age, survival, disease duration, rate of progression, site of onset) have failed (Cykowski et al., 2017). Only cognitive impairment seems to be correlated with the overall burden of TDP-43 cytoplasmic aggregation, but this statement should be taken with caution due to a larger number of brain areas analyzed compared to other regions. In the same direction, analysis of the TDP-43 pathology of the motor neurons in the different levels of spinal cord did not show correlation with the onset of symptoms, whereas the extent of neuronal loss in the different spinal levels did show an excellent correlation.

Another aspect that raises concern about the precise role of TDP-43 in the neurodegeneration process is the discrepancy of certain regions between its aggregation and neuronal loss. In some areas TDP-43 aggregation is far more extensive than neuron loss, in others are equivalent, and in certain regions neuronal loss surpasses the amount of TDP-43 deposits (Nishihira et al., 2008; Brettschneider et al., 2013; Takeda et al., 2009, Braak et al., 2017; Takeda et al., 2019) (**Table 2**).

Table 2. Nervous system areas classified according to the ratio between prevalence of neuronal loss and TDP-43 aggregation in ALS patients. CA1: *Cornu Ammonis* Area 1.

Prevalence of neuron loss > TDP-43 aggregation	Prevalence of neuron loss = TDP-43 aggregation	TDP-43 aggregation > prevalence of neuronal loss
Betz cells	Motor neurons in anterior horn of spinal cord	Inferior olivary nucleus
Subiculum	Hypoglossal neurons	Reticular formation
	Amygdala	Thalamus
	CA1 hippocampal area	Striatum
	Substantia nigra	Red nucleus
	Temporal cortex	Dentate granular cells

2. Non-motor and non-sensory alterations in ALS

Despite the marked and severe involvement of the motor nervous system, with loss of muscle strength as the main symptom, abnormalities have been also reported in extra-motor functions, defined as abnormalities that are not due to dysfunction of the upper or lower motoneurons or the corticospinal tract. It is unclear whether these extra-motor abnormalities occur in all patients, such that ALS can be considered a multisystem disorder, although with a core set of motor symptoms required for diagnosis (Turner et al., 2015; McCluskey et al., 2014). It is also unclear whether extra-motor abnormalities are associated with specific pathology or specific causal mutations.

The spectrum of extra-motor alterations is wide and reinforces the concept that the disease is far from being motor-centric (McCombe, et al., 2017). Already in the first observations from Charcot (Charcot and Joffroy, 1869) it was noticed some special features in the skin of ALS patients, that almost one century later were histologically confirmed and better characterized (Fullmer et al., 1960; Watanabe et al., 1987; Ono et al., 1988). ALS patients are usually immobilized and confined to bed or a wheelchair, but they rarely develop bedsores, even in its terminal stages (Furukawa and Toyokura, 1976). This particular cutaneous feature can also be tested clinically stretching the skin of the patient and observing what it is known as a ‘delayed relaxation phase’. Skin collagen alterations are thought to be responsible of this feature. Collagen bundles in the dermis, especially in the papillary layer, have a small diameter, are disoriented, fragmented, and separated by hyaluronic acid accumulations, and less dense (Ono et al., 1986; Ono et al., 2000). These observations are more marked as disease advances. Moreover, there is an altered expression of several proteins (angiogenin, cystatin C, insulin growth factor 1, galectin 1, hepatocyte growth factor, progranulin, interleukin-6, laminin 1, TNF-alpha, ubiquitin, valosin-containing protein, TDP-43, and vascular endothelial growth factor) in the skin of ALS patients (Paré et al., 2017).

However, the most clinically relevant non-motor feature is cognitive impairment, present in approximately 30-50% of patients. Furthermore 5-15% of ALS patients meet the diagnostic criteria of FTLD, predominantly the behavioral variant (bvFTD) (Beeldman et al., 2016; Phukan et al., 2012; Montuschi et al., 2015; Xu et al., 2017; Lomen-Hoertz et al., 2004; Rascovsky et al., 2011). This comorbidity has been consistently linked to a worse prognosis (Elamim et al., 2011; Westeneng et al., 2018). Neuroimaging studies have shown involvement of extra-motor brain regions like hypothalamus (Gorges et al., 2017), frontotemporal areas, cerebellum, and basal ganglia (Christidi et al., 2018; Senda et al., 2017; Tu et al., 2018; Bede et al., 2018).

In addition, extrapyramidal features have been described in some patients. Almost 70% of patients present rigidity in the lower limbs (Pradat et al., 2009). This association was well-known in the original description of the ALS-Parkinsonism-Dementia complex (also known as Lytico-Bodig disease) in indigenous residents in Guam island (Waring et al., 2004). ALS patients harboring pathological hexanucleotide expansion in *C9ORF72* gene can also present atypical Parkinsonian features, Huntington-like symptoms and hemiballism (Floris et al., 2012; Wilke et al., 2016; Lindquist et al., 2013). Extrapyramidal symptoms have been described also in combination with ALS features in patients with the mutation A382T in the *TARDBP* gene (Borghero et al., 2011). Another interesting link between ALS and extrapyramidal symptoms is through the role of *ATXN2*. *ATXN2* pathological expansion trinucleotides is linked to familial spinocerebellar ataxia type 2 (SCA2) but it is also a risk factor for ALS (Lattante et al., 2014; Sproviero et al., 2017), and it is linked to TDP-43 proteinopathy (Watanabe et al., 2020; Becker et al., 2017; Hart et al., 2012).

Other non-motor symptoms like sleep disorders (Lo Coco et al., 2016; Ebben et al., 2012) and vestibular deficits (Sanjak et al., 2014) have been described.

3. Involvement of the somatosensory system in ALS

Alterations in the somatosensory system are not unusual in ALS patients, although as indicated above, abnormalities in sensory nerve conduction are noted as uncertain condition for the diagnosis. Thus, the recognition and characterization of sensory signs and symptoms are of clear importance in the knowledge of etiopathogenic and clinical features of ALS. It should be noted that there are common links between sensory afferents and the motor aspects of the disease, since fasciculations, a characteristic and main feature in ALS, can be modulated by sensory nerve stimulation (de Carvalho et al., Muscle Nerve 2019). This peculiarity reinforces the fact that sensory pathways play a role in the clinical manifestations of the disease, and therefore should not be considered as an isolated compartment, away from the pathophysiological mechanisms occurring in motor pathways.

3.1. Somatosensory cortex

Parallel to the loss of pyramidal neurons in layer V of the motor cortex, alterations of the somatosensory cortex have been described, most based on neuroimaging and pathological studies. MRI studies have shown a thinning not only of the primary motor cortex, but also of non-motor areas such as the frontotemporal and parietal cortices (Mezzapesa et al., 2013). This finding has been seen in both ALS patients with and without dementia, although cortical thinning is more pronounced in the presence of cognitive impairment (Mioshi et al., 2013; Schuster et al., 2014). As expected, sensory cortical involvement is more severe in classical ALS patients than in lower motor neuron-predominant patients (Spinelli et al., 2016). Interestingly, decreased grey matter density of parietal lobe (and other extra-motor regions) is associated with an increased central motor conduction time, a neurophysiological parameter that indicates worse motor outcome (Christidi et al., 2018).

Non-invasive *in vivo* indicators of neuronal loss, as the reduction of N-acetylaspartate (NAA) and NAA:creatine assessed through MRI spectroscopy, are present in primary sensory cortex, thalamus, basal ganglia, and other frontal and parietal regions (Han et al., 2010; Chiò et al., 2014).

Reactive gliosis is a usual pathological feature accompanying neuronal loss in ALS. The use of certain radiotracers (11C(R)K119574 and 18F-DPA-71475) allowed to detect increased activated microglia in sensory cortex of ALS patients, as well as in motor cortex and in midbrain (Turner et al., 2004; Corcia et al., 2012).

Studies with functional MRI (fMRI) have also shown alterations in the form of increased functional connectivity in somatosensory, prefrontal and thalamus regions (Agosta et al., 2011; Agosta et al., 2014). This increase in connectivity, which is also more pronounced in patients with primary lateral sclerosis (PLS), is attributed to a compensatory effect in the event of a deterioration in the function of these pathways. Despite this increased connectivity, [18F] FDG-PET studies have shown hypo-metabolism in sensorimotor cortex that correlated with the severity of motor symptoms in spinal-onset patients (Sala et al., 2019).

Neurophysiological studies have shown interesting similarities between motor cortical and somatosensory projections. Cortical motor hyperexcitability is a well-known feature of ALS, that it is furthermore associated with survival (Menon et al., 2015; Shibuya et al., 2016). Using neurophysiological techniques, it has been found also an increased somatosensory cortex excitability in ALS. There is an increased amplitude in cortical components of somatosensory evoked potentials (SEP) evoked by stimulation of the median nerve (N25p-P25p; activity derived from the thalamo-cortical fibers to the primary somatosensory area, and subsequent projections through thalamus to motor and premotor areas) that correlated also with a worse prognosis (Shimizu et al., 2018). Also, an increased sensory cortical excitatory activity has been suggested by the finding of loss of activity of cortical inhibitory interneurons, as assessed by high-frequency somatosensory potentials (Nardone et al., 2020).

Moreover, in some patients it has been described an initially increased SEP amplitude (enlarged N20 response) followed a progressive reduction until its total loss, as the disease advance to a locked-in state (Shimizu et al., 2020). In line with such dynamic changes, other authors found a decrease in sensory cortical excitability (reduction of early and late SEP cortical components: N20, P25, N30, N60, P100) but in this study the peripheral component of SEP (N9) were also diminished (Sangari et al., 2018).

Pathological studies have described neuronal loss in the somatosensory cortex (Mochizuki et al., 2011; Oyanagi et al., 2015). Based on these findings, question raised whether impairment of sensory cortex occurs as a consequence or as an extension of the motor cortex impairment or if there are specific and independent pathologic changes in the sensory cortex. On this regard, clues from TDP-43

proteinopathy indicates that there is a sequential propagation of pTDP-43 pathology, disseminating from agranular neocortex and bulbar motor neurons, to finally reaching sensory neocortical areas of the parietal, temporal, and occipital lobes in advanced stages (stage 3 from TDP-43 staging system) with a corticofugal spreading pattern (Braak et al., 2013).

3.2. Spinal ascending sensory pathways

Parallel to the pallor of the corticospinal tract in the spinal cord that gives its name to the disease, sensory ascending tracts that integrate different sensory modalities are also affected in ALS. In fact, combining neuroimaging and neurophysiological data, subclinical sensory alterations of spinal sensory ascendant pathways are found in up to 85% of patients (Iglesias et al., 2015). Aside from the above-mentioned application in the study of cortical component in sensory integration, SEPs have been used mostly to assess integrity of the dorsal column pathways of the spinal cord in ALS, by stimulating both the tibial and median nerves (Georgesco et al., 1994; Constantinovici et al., 1993; Radtke et al.,1986; Dasheiff et al., 1985). Alterations in amplitude and/or latency were consistently found in 33% to 69% of patients, more in SEPs derived from stimulation of lower extremity nerves. However, most patients with altered SEPs remain asymptomatic, suggesting that this impairment does not have enough magnitude to elicit clinical manifestations.

Focusing specifically on dorsal columns, diffusion tensor imaging (DTI) showed the same alterations in cervical proprioceptive pathways as in the lateral corticospinal tract, in both sporadic and *SOD1* familial cases. No volumetric data of these regions are available up to date, due to technical issues derived from the size of the region of interest. DTI analysis shows a decrease in the anisotropy fraction and increase in radial diffusion, and a decrease (although not significant) in the magnetization transfer sequences (which reflects a lower myelin content) of dorsal columns of the cervical segment (Cohen-Adad et al., 2013). DTI metrics of ascending sensory pathways correlates with SEP amplitude alterations in N9 component (Iglesias et al., 2015). Use of this non-invasive and *in vivo* studies confirmed that these alterations are more marked with disease progression, suggesting that its involvement advances in parallel to motor neuron degeneration. Regarding its clinical impact, it is estimated that between 18% and 50% of ALS patients may present some alterations of the vibratory sensitivity on physical examination, not

attributable to another cause (Mulder et al., 1983; Dyck et al., 1975; Hammad et al., 2007), and in rare cases causing a clinical sensory ataxia.

Pathological studies confirmed that degeneration of the dorsal columns in up to half of the patients, especially in lumbar regions, including both genetic (*SOD1*) and sporadic forms (Ince et al., 2003; Hirano et al., 1967; Lawyer et al., 1953; Sasaki et al., 1992; Hammad et al., 2007).

Predominant affection of lumbar spinal cord segments in both functional and pathological examinations suggest an axonal length-dependent degeneration, taking as a starting point cortical and subcortical brain structures, as suggested by the corticofugal propagation proposed by Braak (Braak et al., 2013). Supporting this hypothesis of cortical influence in degeneration of spinal sensory ascending axons is the fact that dorsal columns degeneration is less frequent (8%) in pure progressive muscular atrophy (PMA) and in those patients that progressed to ALS (30%).

3.3. Dorsal Root Ganglion and Dorsal root

Dorsal root and dorsal root ganglion (DRG) have been studied mostly in animal models of the disease. However, descriptions of necropsies have also reported axonal loss in the dorsal root and DRG, especially of larger fibers (Kawamura et al., 1981). In the familial form of ALS secondary to the p.A382P mutation of the *TARBPD* gene, a clinical sensory neuronopathy was confirmed by histopathology (Camdessanché et al., 2011).

Studies related to the sensory pathways of SOD1 transgenic mice, the most used murine model of ALS, have demonstrated pathological changes in the axons of the dorsal columns, dorsal horn of spinal cord, dorsal root, and in the soma of DRG neurons (Fischer et al., 2005; Guo et al., 2009), similar to those observed in Wallerian degeneration. In addition, an anomalous accumulation of SOD1 protein in sensory axons and in the soma of DRG neurons has been reported (Sábado et al., 2014). These alterations were mostly seen in large axons and were already evident at an early stage of the mice (60 days of life), when they are still asymptomatic from the motor point of view. Also *in vitro* studies of DRG neurons of SOD1G93A mice showed accumulation of peripherin56, a splice variant of peripherin that disassembles light and medium neurofilaments, resembling axonal stress features that appear in motor neurons (Sassone et al., 2016). In cultured sensory neurons

harbouring known ALS causing-mutations, SOD1G93A and TDP43A315T, neurites growth was slower and showed an increased sensitivity to cell stressors like vincristine. Although both neuronal cultures showed abnormalities, in TDP43A315T neurons neurites grew significantly slower and were more sensitive to vincristine than SOD1G93A cells (Vaughan et al., 2018).

In addition to the sensory features of the SOD1G93A mouse, a non-transgenic animal model of motor neuron disease, a neurodegenerative disease called canine degenerative myelopathy, produces also sensory symptoms in addition to the motor impairment. This disorder, that occurs in several adult dog breeds, is characterized by the development of proprioceptive ataxia and spastic paraparesis of the hind legs, which over time progresses to flaccid tetraplegia and the apparition of dysphagia. In 12 months, the animal becomes quadriplegic, although this time can vary from 6 to 36 months. Pathological studies showed degeneration of motor neurons at the anterior horn as in ALS disease, but also loss of somas at the DRG and axons in the sensory roots. Interestingly a missense mutation in the *SOD1* gene has been described as responsible of the disease (Nardone et al., 2016; Awano et al., 2009).

3.4. Peripheral nerve trunk

Although previous diagnostic criteria only admitted very narrow alterations in sensory nerve conduction studies, there are abundant data indicating impairment of sensory peripheral nerve in ALS. Up to 32% of patients may have subjective sensory symptoms in the form of glove and sock hypoesthesia, paraesthesia, or dysesthesia, resembling the typical manifestations of length-dependent axonal neuropathies (Hammad et al., 2007). The results of a multicenter study showed that 12.5% of patients met electrophysiological criteria for sensitive polyneuropathy of unknown origin (Pugdahl et al., 2007). In addition, up to 10.3% of patients may have some alteration in the sensory conduction studies of some nerves without reaching the criteria of a polyneuropathy, and without a proven aetiology. Other studies estimate that neurophysiological sensory alterations are present in between 13% and 22% (Gregory et al., 1993; Mondelli et al., 1993; Shefner et al., 1991; Theys et al., 1999), being also relatively frequent (38%) in *C9ORF72* ALS patients (Pegat et al., 2019). Such alterations in sensory neurography can result from both amplitude and/or conduction velocity. As an example, sural nerve, which is the most studied nerve,

shows a reduction of conduction velocity in 14-27% of cases (Mondelli et al.,1993; Kothari et al., 1996), a reduction in amplitude in 6-27% (Hammad et al., 2007), and in 17% a reduction in both amplitude and velocity (Pugdahl et al., 2008). Longest axons seem to present more commonly such abnormalities, since exploration of distal sensory nerves (dorsal sural and medial plantar nerves) has shown alterations in conduction nerve studies in up to 66.7% ALS cases (Isak et al., 2016). With a different approach, based on the use of near nerve electrodes, slow sensory conduction velocity has been reported in 50% of ALS patients (Shefner et al., 1991). SOD1G93A mice have also shown a slowing of sensory conduction velocity (Mancuso et al., 2011). ALS sensory myelinated axons present normal membrane properties and excitability within normal values, as assessed by threshold tracking techniques (Matamala et al., 2019), in contrast with motor axons.

Although in most cases, these alterations are mild or even subclinical, in certain genetic forms sensory peripheral involvement is characteristic and clinically relevant. Familial ALS cases associated with the Gly93Ser mutation of the *SOD1* gene may suffer urinary alterations and a sensory neuropathy (Kawata et al., 1997). In the FOSMN syndrome (facial onset sensory motor neuropathy) patients present trigeminal neuropathy, facial and bulbar involvement. Although FOSMN is considered a motor neuron disease apart of ALS, mutations in heterozygosis of the *SOD1* gene (D90A mutation) suggest a link to classical ALS (Dalla Bella et al., 2014).

Pathological studies have confirmed that sensory nerves, as both sural and superficial peroneal nerves, present axonal alterations in ALS, with up to 91% of the sural nerves having significant loss of axons (Hammad et al., 2007; Ben Hamida et al., 1987; Luigetti et al., 2012; Isaacs et al., 2007). This loss involves predominantly large fibers (73%), although small, myelinated axons are also affected (23%). Similar results have been obtained in sural nerves of pseudopolyneuritic forms of ALS (Patrikios' syndrome), showing also Wallerian-like degeneration, and an increased number of mitochondrial and neurofilaments (Di Trapani et al., 1987; Di Trapani et al., 1986).

Some demyelinating changes were shown in teased fibers, but clearly in lesser amount when compared to axonal degeneration. The myelin lesion was suggested to be consequence of alterations in interactions between Schwann cells and axons

(Dayan et al., 1969; Dyck et al., 1975) and as a result of the dynamic physiological changes of the axon injury and its repair (Arnold et al., 1970). Another detailed pathological study of sural biopsies from ALS patients showed an increase of clustered remyelinated internodes in parallel to disease duration (Heads et al., 1991). In the same study, although an increased number of myelin lamellae was found, the diameter of the nerve fibers (axon and myelin sheath) was significantly smaller. Disturbances of the large and therefore fastest axons could explain slow conduction velocities in sensory neurography, rather than a primary myelin or membrane problem. Additionally, major involvement of the longest nerves, as suggested by both pathological and neurophysiological studies, could suggest axon transport deficiencies, and agrees with the "dying-back" process of ALS (Dadon-Nachum et al., 2011).

In mixed nerves, cutaneous sensory and muscular motor fascicles are in proximity, only separated by the perineurium, but even muscular fascicles contain motor and sensory axons innervating the skeletal muscle. ALS patients and animal models present a low expression of occludin (Winkler et al., 2014; Garbuzova-Davis et al., 2012), a tight-junction protein present in perineurium that contributes to the blood-nerve barrier maintenance, as well as in blood-spinal cord barrier in the CNS (Alanne et al., 2009). This suggests that blood-nerve barrier could be compromised and therefore allowing leakage between motor and sensory fascicles in peripheral nerves.

3.5. Proprioceptive afferences

Up to 25% of ALS patients have clinically relevant proprioceptive deficits (Simmatis et al., 2019) with postural abnormalities and problems with axial control (Krieg et al., 2019). A study combining posturography and neurophysiological examinations found that stance control in ALS patients was not affected by paresis or reflex hyperexcitability (Nardone et al., 2001). Although manifestations of this nature could be attributed, at least in part, to the aforementioned involvement of neurons in the DRG, there are data supporting the involvement of proprioceptive afferents. However, a high percentage, up to 68%, of patients present a diminished laryngeal adduction reflex, that is caused by a loss of small sensory fibers of the epiglottis (Ruoppolo et al., 2016).

Degeneration of sensory fibers Ia/II of the muscle spindles has been observed in SOD1G93A and TDP43A315T mutated mice at a presymptomatic stage (Vaughan et al., 2015) but with preservation of the neuron soma in the DRG. Moreover, the sensory denervation of intrafusal muscles was more marked as the disease progressed. In the case of TDP43A315T mouse this finding was independent of alpha motor neuron degeneration, while in the SOD1 mice these changes were observed alongside motor neuron loss. Overall, these findings suggest that degeneration of proprioceptive endings occurs independently from the defects in the motor system. Interestingly, the authors reported muscle spindles innervated by gamma-motor neurons without Ia/II fibers, near to alpha-motor endings retracting from neuromuscular junctions in extrafusal muscle fibers. At the symptomatic phase of the SOD1G93A mouse they found loss of proprioceptive synapses in the anterior horn in the spinal cord, but not in the TDP43A315T mouse. This loss of central synapses appeared later than the alterations in the peripheral endings at muscle spindles. On the other hand, Ib fibers (the afferents from the Golgi tendon organs) were mostly preserved.

Moreover, a detailed electrophysiological study analyzed Ia afferents of jaw proprioceptive sensory neurons (jaw reflex) of SOD1G93A mouse and showed electrical abnormalities with an impaired excitability of these fibers and a reduction of the voltage-gated Na+ currents (Nav1.6 channel) reflecting the vulnerability of the proprioceptive-reflex circuit (Seki et al., 2019). Similar evidence has been found in the Drosophila SOD1G85R model, (Held et al., 2019) with an impairment of the sensory feedback even before motor neuron degeneration is stablished.

Other neurons that participate in the spinal circuitry and proprioceptive integration are also known to degenerate in ALS; that is the case of Renshaw cells (Wootz et al., 2013; Siembab et al., 2016), commissural neurons (important for bilateral coordination and locomotion) (Nagao et al., 1998; Casas et al., 2013), intermediate neurons of Clarke's column (lamina VII), neurons of the lateral cuneate nucleus and spinal border cells (Murayama et al., 1989; Williams et al., 1990; Kokubo et al., 1999; Fujita et al., 2011).

The close interaction between proprioceptive afferences and alpha-motor neurons, in addition of the loss of interneurons between them, suggests that neurodegenerative mechanisms could mutually influence reciprocally. On this

regard, the SOD1 mice that carries also dominant mutations in cytoplasmic dynein (a protector factor for motor neuron loss) significantly present a slow degeneration with a practically absence of alpha-motor neuron loss at 18 months and with a marked loss of proprioceptive sensory axons (Ilieva et al., 2008). This finding opens the question whether the proprioceptive fibers could play a role in neurodegeneration, mediated by glutamatergic excitatory inputs to motor neurons. In line with this, gamma-motor neurons have proven to be resistant in ALS, and the lack of synaptic contact from primary Ia afferent fibers is one interesting distinction with the most vulnerable alpha-motor neurons. In addition, functional reduction of Ia activation delays symptoms onset and prolongs lifespan in the $SOD1^{G93A}$ mouse (Lalancette-Hebert et al., 2016).

In other motor neuron disorders, such as in the spinal muscular atrophy (SMA) mouse, there is a reduction, also at early stages, of the synapses between primary afferent proprioceptive neurons and spinal motor neurons (Mentis et al., 2011). This loss of proprioceptive synapses is more severe in motor neurons projecting to proximal muscles, the ones most affected in the disease, and is the earliest and most pronounced pathological feature of the SMA mice. Although it is hypothesized that loss of these synapses is due to a failure in postnatal maturation, similarities with ALS point to other shared mechanisms involved in impairment of sensory-motor connectivity.

3.6. Small sensory nerve fibers

In humans, although there are no specific studies on involvement of small nerve fibers, published data shows that 50-78% of ALS patients experience pain (Chio et al., 2012; Pagnini et al., 2012; Pizzimenti et al., 2013; Hanisch et al., 2015), and 20-30% described it as "electric" or "burning", which may correspond to symptoms secondary to nociceptive fiber involvement. However, although these characteristics could resemble small fiber neuropathy symptoms, those sensations have been described mostly in lumbar and proximal regions of upper limbs, opposite to the distal limb pattern expected in classical length-dependent small fiber neuropathies.

Studies based on neurophysiological techniques have shown controversial results. While no changes have been detected using contact heat-evoked potentials (CHEPS) (Xu et al., 2009), amplitude alterations were obtained using laser evoked potentials (Simone et al., 2010). Thermal quantitative sensory testing (QST) studies

(another technique for the study of pain thresholds and thermal sensations of cold and heat), have shown also contradictory results (Deepika et al., 2006; Lopes et al., 2018), finding in some cases alterations in the threshold of the heating stimulus, especially in spinal forms (Truini et al., 2015). Nevertheless, the differences in results between studies might suggest technical issues. It must point out that nociceptive-evoked potentials are based on the stimulation of A-Delta type II fibers (Treede et al., 1998) and no of C-fibers, while thermal studies are based on the assessment of both A-Delta and C axons.

More consistent are the results of the study of unmyelinated fibers in skin biopsies. ALS patients have lower IENF density, with up to 79% of patients meeting criteria for small fiber neuropathy (Weis et al., 2011; Nolano et al., 2017). These alterations are not only in number, but also in morphology, with axonal swellings, suggesting an alteration in axonal transport, and with an insufficient growth of terminal branches (Isak et al., 2017). This finding is not only seen in sporadic ALS patients, but also in genetic forms including *SOD1*, *C9ORF72* and *SQSTM1*. Some authors have found differences between bulbar and spinal onset forms, with lower IENF density in the latter (Truini et al., 2015), but other groups have not found differences between onset type, phenotype, clinical course, and severity (Dalla Bella et al., 2016). TDP-43 deposition or its phosphorylated form has not been found in IENF, and only in autonomic dermal fibers (Ren et al., 2018). Similar results have been found in the $SOD1^{G93A}$ mouse (Genç et al., 2015; Sassone et al., 2016).

The loss of small fibers is not restricted to the skin. The use of corneal confocal microscopy has allowed the study of small fibers *in vivo,* with a non-invasive, repeatable, and quantitative analysis of small fibers from trigeminal nerve. ALS patients have a decreased number of corneal nerve fibers and morphological alterations with increased tortuosity and decreased size. The degree of bulbar involvement (quantified according to the ALSFR-R bulbar subscale) was inversely related to the length of these fibers (Ferrari et al., 2014). However, similar as found in skin biopsies, there were not relationship with the duration of the disease, age of the patient, or with the degree of impairment of upper or lower motor neurons.

Interestingly also a diminished sensitivity of the larynx has been reported in up to 54% of ALS patients (Amin et al., 2006). One study in 114 patients found a laryngeal sensory deficit exploring the laryngeal adduction reflex (with preserved functionality

of the adductor muscles), being more frequent the impairment in bulbar-onset patients. Three of these patients underwent a biopsy of epiglottis mucosa; in one there were total absence of intraepithelial innervation and in the other two there were pathological changes of remaining fibers: large axonal swellings, chaotic nerve branching and branches not crossing the entire epithelium (Ruoppolo et al., 2016).

While many of these findings could be explained by a longitudinal dependent pattern and a distal sensory axonopathy, it is worth noting that small fiber impairment is not always limited to the most distal regions, and in some cases, similar loss of IENF has been found between proximal and distal regions of the limbs (Nolano et al., 2017). Nevertheless, the existence of a sensory ganglionopathy could explain such pattern (Gwathmey et al., 2016).

4. Involvement of the Visual system in ALS

Although some studies have shown the presence of abnormal eye movements (Averbuch-Heller et al., 1998; Gizzi et al., 1992; Puligheddu et al., 2016; Münte et al., 1998), oculomotor function is classically considered to be preserved in ALS. In contrast there is abundant evidence of involvement of the visual sensory pathway.

A substantial part of this evidence comes from histopathological studies or imaging tests since the results of functional studies, as expected from clinical observations, are minimal or non-existent, and often give contradictory conclusions (Rojas et al., 2020). Visual field studies seem to be unreliable due to motor difficulties inherent to the disease (fixation losses, false positives and negatives) and only one study have described an impairment in the visual field in a cohort of ALS patients (Liu et al., 2018). Visual acuity (high and low contrast) is preserved (Volpe et al., 2015; Moss et al., 2016; Rojas et al., 2019), although one study showed lower visual acuity in both high and low contrast using Sloan charts (Moss et al., 2012) and another described an impaired contrast sensitivity in some *C9ORF72* patients (Fawzi et al., 2014). Visual evoked potentials have shown normal values of amplitude and latency (Ghezzi et al., 1989; Palma et al., 1993) but mild increased latency or other minimal abnormalities have also been reported (Matheson et al., 1986; Gonzalez Diaz et al., 2004). fMRI neuroimaging has found a reduction in the response to visual stimulation in secondary visual areas (Lule et al., 2010).

Histopathological examinations of visual pathway have revealed a remarkable difference between the involvement of retrochiasmatic structures and the anterior segment of the visual pathway. Tractus opticus, corpus geniculatum laterale, colliculus superior, radiation opticus and primary visual cortex are preserved in advanced ALS patients (Oyanagi et al., 2015) and in the SOD1G93A mice (Ringer et al., 2017), although reactive astrogliosis has been shown in occipital areas (Kushner et al.,1991).

On the contrary, ganglion cells in the retina seem to be the most vulnerable neuron type of the visual system in ALS. Assessment of the thickness of different retinal layers, including the ganglion cell layer (GCL) and their long axons, the retinal nerve fiber layer (RNFL) can be easily done with optical coherence tomography (OCT). OCT is a non-invasive and objective technique, reproducible that provide a high-resolution of retina, and have been shown to be useful in the assessment and monitoring optic nerve damage of various neuroinflammatory and neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's Disease (AD) and multiple sclerosis (MS) (Albrecht et al., 2012; Siger et al., 2008).

Using OCT, most of studies have consistently reported a thinning of the RNFL in ALS compared to controls (Mukherjee et al., 2017; Rohani et al., 2017; Hübers et al., 2016; Simonett et al., 2016). Only a few studies have not found differences in the OCT measurements. These contradictory results could be explained by methodological aspects, such as heterogeneity in the technology used (Cervero et al., 2019), but also by the different profile of ALS patients (Roth et al., 2013) and controls selection (Liu et al., 2018). Additionally, an early retinal thinning also occurs in individuals with frontotemporal dementia (FTD) due to progranulin (*GRN*) mutations, a gene that is involved in dominant forms of ALS and ALS-FTD (Ward et al., 2014).

Interestingly one study also showed an increased thickness in certain areas of the retina (Rojas et al., 2019), suggesting alterations in axonal transport, protein aggregation and neuroinflammation as a possible explanation. Supporting this finding, in post-mortem studies a significantly greater number of PAS positive spheroids have been found in RNFL, especially in the peripapillary region (Sharma et al., 2020). The increased presence of phosphorylated neurofilaments in those

spheroids could be explained by a special vulnerability of the long axons to axonal transport deficits.

In addition of the apparent vulnerability of ganglion cells in the disease, it has been elucidated the critical role of Ranbp2 in the signalling between the microglia and the retinal ganglion cells in the immune response in ALS (Cho et al., 2019), a protein already involved in nucleo-cytoplasmic transport whose regulation is impaired in both sporadic and familial ALS (Ferreira et al., 2019).

Other retinal cells, rather than ganglion cells, and layers have been found impaired, although findings are less constant as in RNFL. In an animal model of an X-linked form of ALS (*UBQLN2*), ubiquitin2 positive aggregates are found predominantly in the inner nuclear layer (INL), but fewer are found also in the outer plexiform layer (OPL) and in the GCL (Volpe et al., 2015). In the $SOD1^{G93A}$ mouse model, vacuolization occurs in the excitatory dendrites of retinal neurons of the inner plexiform layer, but also in the GCL and INL (Ringer et al., 2017).

Findings in other genetic forms of ALS support the involvement of other retinal cell subtypes, suggesting that those impairments could be gene-specific. P62 positive and pTDP43 negative perinuclear inclusions have been described in *C9ORF72* ALS patients in bipolar cells (located in INL), GCL, amacrine and horizontal cells (Fawzi et al., 2014), with colocalization of poly-GA dipeptide and ubiquitin in such inclusions. Moreover, *senataxin* (*SETX*) ALS could involve more profoundly photoreceptors. *SETX* mutations are involved either in recessive forms of ataxia-oculomotor apraxia 2 (AOA2) and a dominant juvenile onset form of ALS (ALS4). In the ALS4 phenotype, it is hypothesized that neurodegeneration occurs as a result of a partial gain-of-function of senataxin protein, which functions are related to nucleic acid processing. Although senataxin is ubiquitously expressed in many tissues (including lens and retina), its levels are cell-specific and it is highly expressed in inner and outer segments of photoreceptors and outer plexiform layers, but poorly presented in inner plexiform and ganglion cell layers (Chen et al., 2016).

Those findings in the retina raise the question whether they are secondary to retrograde trans-synaptic degeneration or if are due to a primary retinal damage. Consistent with the latter is the fact that pathological examinations found no substantial impairment in the rest of the visual pathway, in contrast to the described

in other cortical non-motor structures. Also, in favour of a primary retinal involvement, vascular changes in the retina of ALS patients have been found (Abdelhak et al., 2018), as in other anatomical regions, suggesting shared mechanisms of degeneration. In fact, there are several similarities in the pathways involved in ALS neurodegeneration and certain optic nerve disorders, such as increased levels of oxidative stress, axonal transport deficits and mitochondrial damage. Several genes associated with familial forms of ALS (*optineurin*, *TBK1* and *ataxin2*) are also associated with chronic primary open angle glaucoma, suggesting common mechanisms, as in the case of *optineurin*, that is involved in neuroinflammation, vesicular trafficking and autophagy.

5. Involvement of the Olfactory system in ALS

Olfactory dysfunction appears with normal aging (Boyce et al., 2006; Landis et al., 2004) with a prevalence between 6 and 16% in elderly healthy people (Rouby et al., 2011). It is also a common feature in neurodegenerative disorders and constituting part of the prodromal manifestations in Alzheimer's and Parkinson's disease (Growdon et al.,2015; Jennings et al., 2014). Between 46% and 75% of ALS patients present a decreased ability to identify odours, and 11% have a total anosmia, based on psychophysical tests, mainly the University of Pennsylvania Smell Identification Test (UPSIT) (Elian et al., 1991; Ahlskog et al., 1998; Hawkes et al., 1998; Takeda et al., 2015; Viguera et al., 2018, Masuda et al., 2021). This feature seems especially common in the subset of patients with behavioural and cognitive impairment (Pilotto et al., 2016). Although those figures have been found in respiratory spared ALS patients, it has been observed that smell detection threshold in these patients are influenced by respiratory impairment (Günther et al., 2018), probably because a reduced inspiratory flow. This differs from the results of olfactory evoked potentials where no pathological results were obtained, although one third of patients could not be tested due to technical difficulties related to the disease (Hawkes et al., 1998). Other authors using different functional tests did not find differences of smell identification between ALS and healthy controls either (Lang et al., 2011).

Despite the similarity with other neurodegenerative diseases, histopathological studies have shown a very different pattern from that observed, for instance, in

Parkinson's disease. Although no loss of neurons has been found in the olfactory pathway (Oyanagi et al., 2015) except for a lower volume of the amygdala (Machts et al., 2015; Pinkhardt et al., 2006), central structures of olfactory conduction have shown pTDP-43 inclusions in dentate granular cells, transentorhinal cortices, corticomedial and basolateral part of the amygdala, the anterior olfactory nucleus, and the piriform cortex (Takeda et al., 2014; Takeda et al., 2015). Interestingly, quantitative analysis showed a gradient of p-TDP-43 inclusions, which are most frequent in the hippocampus and least in the olfactory bulb, with an intermediate density in the olfactory cortex. Based on this observation, it has been proposed that in the fourth and final stage of the TDP-43 neuropathological staging (Eisen et al., 2017), cortical pathology progresses in the temporal lobe and reaches the allocortical entorhinal region as well as the hippocampal formation. Then spread centrifugally from the hippocampus to the primary olfactory center, and later to the olfactory bulb (Takeda et al., 2015), which also presents lipofuscin deposit accumulations (Hawkes et al 1998). This differs from Parkinson's disease, in which alpha-synuclein pathology involves predominantly the olfactory bulb with a centripetal spreading (Rey et al., 2018).

Interestingly, the SOD1G93A mice show vacuolization without inflammation in the excitatory dendrites of the granule cell layer of the olfactory bulb. This is considered a sign of neurodegeneration, present at the presymptomatic stage, that increased overtime until end-stage, but with preservation of the anterior olfactory nucleus olfactory tract or olfactory tubercle (Ringer et al., 2017).

Olfactory neurons (bipolar cells with modified non-motility sensory cilia) located in olfactory bulbs of healthy humans express proteins involved in several neurodegenerative diseases, such as alpha synuclein, beta amyloid, and TDP-43 (Brozzetti et al., 2020). Moreover, a proteomic analysis of olfactory bulb of ALS patients showed an aberrant expression of proteins involved in autophagy, axon development, and vesicle-transport (Lachén-Montes et al., 2020). In line with this, cells of the olfactory mucosa of ALS patients can induce disease specific changes and decrease neuronal survival when co cultured with human spinal cord neurons, as well as induce a glial inflammatory response (García-Escudero et al., 2015).

The widespread impairments of olfactory pathways imply shared mechanisms with classical motor neuron degeneration beyond TDP-43 pathology. For example,

senataxin, a protein associated with a dominant juvenile form of ALS (ALS4), through a gain-of-function mechanism, have its highest expression in hippocampus and olfactory bulb (Chen et al., 2006). Also, a transcription factor, Runx1, that is expressed in somatic motor neurons in the murine brainstem and cervical spinal cord, plays a significant role in proliferation and development of olfactory ensheathing cells and in the transition between undifferentiated neural progenitor cells and neurons in the olfactory epithelium (Wang et al., 2017).

6. Involvement of the Gustatory system in ALS

Taste perception problems are present in several central nervous system disorders, such as Alzheimer's disease and mild cognitive impairment (Steinbach et al., 2010), Parkinson's disease (Shah M et al., 2009), stroke (Heckmann et al., 2005; Kim et al., 2009), and major depression (Scinska et al., 2004), but also are existing in 1% of general elder population (Imoscopi et al 2012). In ALS perceived reduction of taste may be present, more frequently in patients with enteral tube (Tarlarini et al., 2018), and in some cases are restricted to bitter and sweet tastes (Ashary et al., 2020). However, some authors did not find gustatory impairment in ALS population based on functional tests with taste strips (Lang C et al., 2011).

Limited histological studies focused on central gustatory pathways showed consistent data of its involvement. The fact that patients on enteral nutrition have more hypogeusia/ageusia could suggest that there is also a component of peripheral involvement, but to date scarce histological data on this regard have not reported abnormalities (Ashary et al., 2020). However, severe neuronal loss has been described in the nucleus parabrachialis medialis (region that carries gustatory information from solitary nucleus to ventral posteromedial nucleus of the thalamus) and the tractus trigeminothalamicus dorsalis (Oyanagi et al., 2015). Neuronal loss, reactive astrocytosis and TDP-43 deposition can also be found in the motor nuclei of the trigeminal and facial nerves, dorsal motor nucleus of vagal nerve and nucleus of the hypoglossal nerve. TDP-43 pathology in the solitary nucleus is rare (Nishihira et al., 2008), and neuronal loss in this region has been reported in *FUS* ALS patients (Tateishi et al., 2010).

TDP-43 pathology on gustatory neural pathways have been also explored in facial onset sensory and motor neuronopathy (FOSMN), another degenerative motor

neuron disorder related to ALS, which can also present taste disorders (Ohashi et al., 2020). On these cases, TDP-43 inclusions are present in motor and sensory nuclei of the trigeminal and facial nerves, dorsal motor nucleus of the vagal nerve, nucleus ambiguous, nucleus of the solitary tract and in the hypoglossal nerve (Sonoda et al., 2013; Ziso et al., 2015; Rossor et al., 2019).

7. Involvement of the Auditory system in ALS

Information on involvement of the auditory pathway in ALS is scarce, and no functional data are available. However, histopathological examinations have shown a significant neural loss at brainstem regions involved in hearing, including nucleus olivaris superior, lemniscus lateralis and colliculus inferior. On the contrary, superior structures such as corpus geniculatum medialis and gyrus temporalis transversus are relatively well preserved. Although this could point to a more peripheral involvement, dorsal cochlear nucleus, the immediate relay structure of auditory nerve in the brainstem, are preserved in ALS (Oyanagi et al., 2015).

8. Involvement of the Autonomic Nervous System in ALS

Autonomic disturbances are rarely described in classical forms of ALS; however, observations from composed functional tests and self-reported questionnaires indicated that autonomic manifestations, although mostly mild, are not so uncommon (Low et al., 1994; Piccione et al., 2015). Several studies investigating the subclinical occurrence of dysautonomia have reported dysfunctions of cardiovascular, sudomotor, urinary, gastrointestinal, and salivary visceral regulation, even in early ALS cases, causing symptoms that may further deteriorate the health state. Autonomic disturbances may even lead to arrhythmia, circulatory collapse, or sudden death in advanced stage ALS patients (Shimizu et al., 1994; Baltadzhieva et al., 2005).

Cardiovagal function was found impaired in up to 50% of ALS patients (Piccione et al., 2015), being more frequent in those with low respiratory capacity (Pimentel et al., 2019; Pimentel et al., 2021). Some studies have found an abnormally augmented cardiovascular sympathetic tone in ALS patients (Sachs et al., 1986), and in the SOD1G93A mouse (Kandinov et al., 2011; Kandinov et al., 2012),

especially regarding alpha-sympathetic hyperactivity rather than beta-sympathetic hyperactivity. Additionally, there is an increased activity of norepinephrine transporter in cardiac sympathetic nerve terminals, that is indeed associated with a shorter survival (Tanaka et al., 2013). ALS patients present significantly increased plasma levels of norepinephrine, especially those bedridden (Beck et al., 2005). Preganglionic sympathetic denervation may result in inappropriate levels of norepinephrine, but also those increased levels can be caused by the impaired reuptake secondary to nerve terminals degeneration. Moreover, as opposed to the resting sympathetic hyperactivity in ALS, it also exists a down-regulation of alpha-adrenoceptors, providing a 'ceiling effect'.

Nevertheless, this increased cardiovascular sympathetic drive is not described in all patients at the same stage (Dalla Vechia et al., 2015) and seems to be more related to the flail arm/leg phenotype (Tutaj et al., 2017). This is in line with the concept that muscle atrophy can lead to a diminished number of arterioles and vessels involved in vascular resistance and therefore an insufficient capacity to accommodate temporary increases in blood flow (Tutaj et al., 2017). In this regard, although rarely, some patients can present even hypertensive crisis, alternating with nocturnal hypotension without tachycardia, occasionally leading to circulatory collapse and sudden cardiac arrest, especially those respiratory-dependent (Shimizu et al., 1996).

Importantly, cardiovascular sympathetic activity decreases as disease advances (Asai et al., 2007; Beck et al., 2005; Druschky et al., 1999), a deterioration thought to be due to impairment of centrally sympathetic mechanisms instead of locally altered responses (Karlsborg et al., 2003). Nevertheless, vessel axons show degeneration and ultrastructural abnormalities (swelling and vesicle accumulations) (Provinciali et al., 1994), and sympathetic fibers present a decreased firing rate in the control of vascular resistance in ALS patients (Oey et al., 2001).

In addition, there is a cardiovascular parasympathetic hypoactivity with a decreased vagal tone (Pavlovic et al., 2010; Merico et al., 2011; De Maria et al., 2015; Pisano et al., 1995; Linden et al., 1998), also during sleep (Congiu et al., 2019). Although autonomic cardiovascular response is often impaired, orthostatic hypotension is rarely present in ALS (Piccione et al., 2015). Absence of symptomatic orthostatic hypotension contrast with other neurodegenerative disease such as Parkinson's

disease and multisystem atrophy, but this could be explained by the severe motor impairment in ALS, that limits physical demand.

Microneurographic studies revealed abnormalities of the spontaneous and reflex activity of sympathetic efferents in ALS patients, suggestive of ANS involvement and neurodegeneration (Donadio et al., 2015). The muscle sympathetic nerve activity (MSNA) at rest was found higher than in healthy subjects and in patients with other neuromuscular disorders, at early but not at advanced stages of ALS (Shindo et al., 2009). However, this elevated resting MSNA responded weaker to activating maneuvers in ALS patients compared to controls (Oey et al., 2001; Nygren et al., 2011). On the other hand, the resting sudomotor and vasoconstrictive skin sympathetic neural activity (SSNA) was also found significantly greater in ALS patients than in healthy controls, but again with smaller responses to activating maneuvers such as mental arithmetic test (Shindo et al., 2011). ALS patients also exhibited a slight prolongation of SSNA reflex latencies. All these observations support that sympathetic activity is abnormal in ALS patients, mainly showing resting hyperactivity that may be induced by central autonomic drive.

Sudomotor function has been studied and up to 33% of ALS patients showed sweating abnormalities (Ren et al. 2018). Quantitative studies of sudomotor regulation in different stages of the disease have shown that there is a higher activity with hyperhidrosis (specially in thenar and hypothenar region) at early stages. But as disease progresses, there is a significant decrease of about 40% in sweat production (Beck et al., 2002; Beck et al., 2005) and in 25% of cases with a marked asymmetry (Santos-Bento et al., 2001). Higher sweat rate near onset of the disease could be explained by a hypersensitivity to partial denervation of sweat glands. On this regard, a higher sympathetic firing rate was found by microneurographic measurements in mildly disabled ALS patients, compared with equally disabled patients with other neuromuscular disorders (Shindo et al., 1995; Shindo et al., 2011). On the other hand, decreased sweat production could also be explained by the degeneration of postganglionic sympathetic fibers and with the atrophy of the sweat glands. Sympathetic skin response (SSR) testing has consistently confirmed this impairment by a diminished or even absent response, especially in the lower limbs, and independently of possible influencing factors such as muscle wasting, inactivity or skin temperature (Hu et al., 2016; Oey et al., 2001; Dettmers et al., 1993; Barron et al., 1987; Masur et al., 1995), although some authors did not find

SSR impairment in the palmar region (Miscio et al., 1998). Similar impairment in sweat function has been described using thermoregulatory sweat test and quantitative sudomotor axon reflex test (Q-SART) (Kihara et al., 1994). Interestingly, Q-SART showed that sweat impairment can present in different forms; most frequently in a distal or length-dependent pattern (54%), but also as patchy (32%) or more diffuse affection (14%) (Piccione et al., 2015). A closer look at sweat glands shows that, innervating axons presents swellings, degeneration, TDP-43 accumulation (Ren et al., 2018) and synaptic vesicle accumulations, as well as the sweat glands present increased intracytoplasmic lipofuscin granules (Provinciali et al., 1994). Pilomotor nerve fiber density is also reduced, as well as there is profound denervation of arrector pili muscles in ALS. This finding is significantly more severe in PLS patients, raising the question if this is phenotype dependent or if only translates a higher disease duration (Nolano et al., 2016). In contrast to what it is observed in the cardiovascular system, all these observations suggest an involvement of unmyelinated postganglionic sympathetic fibers.

Urinary autonomic disturbances are also described, with a mild degree of neurogenic bladder in up to 20% of cases (Shindo et al., 2004), most frequently in SOD1 ALS and in PLS (50%) (Takeda et al., 2019). Urinary incontinence frequency ranges between 4% and 33%, especially in the form of urge incontinence, where is especially frequent in patients older than 60 years (Nübling et al., 2014). Aside from age, predominance of upper motor neurons is also associated with urge incontinence, but whether this is directly related with the pathophysiology of the disease or with the higher use of anticholinergic drugs or muscle relaxants for this particular phenotype, is not clear. Erectile dysfunction in male ALS patients has also been reported (Piccione et al., 2015).

Sialorrhea is a common feature affecting up to 20% of ALS patients, and aside from the dysphagia, there is evidence that autonomic disturbances play a role in this disabling symptom. Although excess of saliva in mouth may impair salivary function, primary secretory function of submandibular and parotid glands seems to be preserved. However, the inability to elicit a good response through indirect stimulation (Carchaflie et al., 1976), and salivary gland function assessed by quantitative scintigraphy with 99mTc-pertechnetate (Giess et al., 2002), suggested alterations in the neuroendocrine mechanisms that regulate secretory activity, even at early stages of the disease, independently of the severity of motor symptoms.

Lacrimal function has been described also to be decreased in ALS (Copperman et al., 1974).

Regarding intestinal dysfunction, constipation has a high prevalence (about half) among ALS patients, but other factors beyond autonomic gastrointestinal dysfunction could play an important role, such is the case of lack of activity, inadequate content of fiber in the diet or poor fluid intake (secondary to dysphagia). However, functional explorations have found delayed gastric emptying (Toepfer et al., 1999), delayed colonic transit time (Toepfer et al.,1997), as well as other disturbances in gastric and esophageal motility (Lawyer et al., 1953; Smith et al., 1957), all suggesting an autonomic gastrointestinal involvement. On this regard, it is worth mentioning that these gastrointestinal abnormalities are found even in patients without dysphagia. Also, in some patients with profound autonomic manifestations, beyond typical ALS neuropathological features, alpha-synuclein-positive intracytoplasmic inclusions have been found in ganglion cells of the esophageal nerve plexus (Yamada et al., 2014) suggesting that in selected cases, alpha-synucleopathy, and therefore an added early/presymptomatic Parkinson's disease, may be related, at least in part, with autonomic dysfunction.

Although there is large evidence of different impairment of autonomic control in different target organs, some are characteristically spared, as such is the case of pupil responses (Bogucki et al., 1987; Kandinov et al., 2011). In impaired organs, both sympathetic and parasympathetic systems are affected, resulting mostly in a hypofunction that worsens overtime. Sweat and cardiovascular features present an increased activity during early stages of the disease, probably reflecting the complexity of interactions during autonomic control dysfunction. Sympathetic over function could be explained also by the involvement of limbic motor system and other central autonomic network structures (insular cortex, anterior cingulated gyrus and basolateral nuclei of amigdala) (Ohno et al., 2001; Nagai et al., 2010).

Pathological evidence points to preganglionic autonomic neurons as the main group of cells involved in autonomic dysfunction. Primary autonomic control centers, dorsal vagal and solitary nuclei, are considered to be spared in ALS (Hayashi et al., 1989; Kato et al., 1993). Exceptionally in SOD1 V118L and C146R ALS, there is evidence of marked neuronal loss in dorsal vagal, solitary tract nuclei and ambiguous nuclei (Shimizu et al., 2000; Hayashi et al., 2016), and in some cases,

TDP-43 inclusions are present in the nucleus ambiguous which drives parasympathetic heart control (Yamada et al., 2014). Morphometrically assessment of vagus nerve showed preserved density of myelinated and unmyelinated fibers, supporting the hypothesis that parasympathetic autonomic disturbances are not explained by vagal deafferentation (Shimizu et al., 2011).

In the sympathetic system, loss of neurons in the intermediate lateral column (IML) of the spinal cord has been reported in ALS patients (Konno et al., 1986; Kennedy et al., 1985), and alterations in protein metabolism of this group of neurons, but with preservation of the sympathetic ganglion cells (Itoh et al., 1992). IML neuronal loss begins typically in the upper spinal cord and progress caudally towards lower thoracic segments (Takahashi et al., 1993). No cytoplasmic aggregates or Bunina bodies have been described in IML neurons (Kandinov et al., 2013). In SOD1G93A mice, down-expression of choline acetyltransferase (ChAT) in superior cervical ganglia and tyrosine hydroxylase (TH) in adrenal gland, imply also a preganglionic sympathetic denervation, that can cause trans-synaptic postganglionic dysfunction.

Histopathology studies have also shown neuronal loss in Onuf's nucleus in the ventral horns of the spinal cord, indicating an alteration in bowel and bladder innervation (Pullen et al., 1995; Carvalho et al., 1995; Sasaki et al., 1993). Only in sweat gland dysautonomia there are evidence of postganglionic impairment. This is of special interest, since postganglionic axons innervating eccrine sweat glands, although sympathetic, are also cholinergic, like all preganglionic autonomic neurons and somatic motor neurons.

Despite the above-mentioned neuronal loss of autonomic structures in central nervous system, it is clear that its functional repercussion is mild, in contrast to the motor manifestations. This is similar to other motor neuron disorders, such as Kennedy's disease, where although patients do not usually show autonomic symptoms, there is pathological evidence of subclinical impairment of parasympathetic and sympathetic neurons (Manzano et al., 2018). On the contrary, other motor neuron diseases, related to ALS, such as SMA (spinal muscular atrophy) simply do not show histological data of autonomic nervous system involvement (Powis et al., 2016). Disparities between functional and tissue involvement in ALS can be explained indirectly by reduced physical activity and muscular atrophy, but different expression of glutamate receptor profiles between

somatic and autonomic motor neurons could also be, in part, responsible. While ionotropic glutamate receptor may trigger excitotoxicity, group I metabotropic glutamate receptors (mGluRs1 and 5) exert neuroprotective effects. Parasympathetic Onuf's nucleus and thoracic autonomic motor neurons express higher levels of this neuroprotective receptors (mGluR5), contrasting with the absence in somatic motor neurons (Anneser et al., 1999).

HYPOTHESIS AND OBJECTIVES

Peripheral sensory involvement is present alongside the neurodegenerative process in ALS, as described in human patients and in different animal models. Sensory involvement may share some similarities with the motor counterpart and knowing the impact of extra-motor structures can help to increase our knowledge about the physiopathology of ALS.

The main objective of this thesis is to characterize the alterations in the peripheral somatosensory system in ALS, its evolution overtime, and to detect differences in sensory neuron susceptibility.

To achieve this aim we have divided the thesis in three different sections with the following specific objectives:

Chapter 1. Involvement of sensory innervation in the skin of SOD1^{G93A} ALS mice.

- To assess if SOD1^{G93A} mice have reduced cutaneous innervation as in humans with ALS.
- To study epidermal and dermal sensory innervation in the SOD1^{G93A} mouse: intraepidermal fibers, Meissner's corpuscles, subepidermal nerve plexus, dermal nerve fiber density.
- To study cutaneous sensory impairment along the course of the disease.

Chapter 2. Characterization of somatosensory neurons involvement in the SOD1^{G93A} mouse model

- To characterize loss of different sensory nerve populations in the SOD1^{G93A} mouse.
- To study cutaneous autonomic fibers, as an example of non-somatic innervation.
- To assess neuronal loss of different sensory populations in the dorsal root ganglia (DRG), in parallel with the skin innervation.
- To study sensory involvement evolution along the course of the disease.

Chapter 3. TDP-43 cytoplasmic translocation in intraepidermal axons and skin fibroblasts of ALS patients

- To study the TDP-43 expression and localization in the skin of ALS patients.
- To assess TDP-43 cytoplasmic translocation in skin structures.
- To analyze possible correlation of cytoplasmic TDP-43 in skin with clinical features.

MATERIALS AND METHODS

Transgenic mice

The most commonly used animal model of ALS is the related to the mutation of Cu/Zn superoxide dismutase 1 (SOD1) gene. The SOD1 mice G93A, G37R, G85R develop motor neuron disease with pathological changes reminiscent of the human, with a massive loss of motor axons in the final stages, through a toxic gain of SOD1 function (Gurney et al., 1994; Yim et al., 1996). For our procedures we used $SOD1^{G93A}$ and wild type mice. $SOD1^{G93A}$ mice remain asymptomatic until about 12 weeks, when they start developing early motor difficulties with progressive paralysis starting in the hind limbs to a gradual loss of motor function in the four limbs at the end-stage of the disease at 16-20 weeks of age.

Transgenic mice with the G93A human SOD1 mutation [B6SJL-Tg(SOD1-G93A)1Gur] were obtained from Jackson Laboratories, and maintained at the Animal Service of the Universitat Autònoma de Barcelona. The offspring was identified by PCR amplification of DNA extracted from the tail tissue. All experimental procedures were approved by the Ethics Committee of the Universitat Autònoma de Barcelona, where the animal experiments were performed, and followed the guidelines of the European Commission on Animal Care.

Animals were euthanized under anesthesia with pentobarbital (50 mg/kg i.p.) and transcardially perfused with 4% paraformaldehyde in PBS. The plantar pads of the hindfoot of $SOD1^{G93A}$ and wild type mice at 4, 8, 12 and 16 weeks were collected. Footpads were carefully harvested and kept frozen in a cryoprotective solution (PBS with sucrose at 30% and sodium azide). 60 µm-thick sections were serially cut with a cryotome (Leica) and collected free-floating in PBS medium. L4 and L5 dorsal root ganglia were also extracted from wild type and $SOD1^{G93A}$ mice at 4, 8, 12 and 16 weeks. DRGs were maintained at 4ºC in a cryoprotective solution and then were

embedded in optimal cutting temperature compound (OCT) and cut by cryotome into 20-µm thick sections collecting the slices in gelatinized slides.

Human samples

Subjects with definite ALS and healthy controls (HC) were prospectively recruited by the ALS units from Hospital del Mar and Hospital de Bellvitge, Barcelona. A third group of neurological controls (NC) was also selected. Healthy subjects did not have any signs, symptoms, or history of neurological diseases. Neurological controls were selected among a cohort of patients with Parkinson's disease and relapsing-remitting multiple sclerosis. ALS patients fulfilled the diagnostic criteria (Brooks et al., 2000) and were selected by experienced neurologists specialized in motor neuron diseases. There were no statistically significant differences in sociodemographic variables between the groups. All ALS patients were tested for the hexanucleotide expansion in the *C9ORF72* gene, and in all but one, no pathological expansion was detected. The study was approved by the local ethics committee of both participating hospitals. Written informed consent for skin biopsy was obtained from all subjects.

Three-millimeter skin punch biopsies were obtained from the distal leg after local anesthesia with mepivacaine. Skin biopsies were fixed in paraformaldehyde solution at 4% and maintained at 4ºC in PB with sucrose. Then, biopsies were cut by cryotome into 60-µm thick sections collecting the slices in PBS.

Immunohistochemistry

Sections were blocked with PBS-Triton 0.3%-normal donkey serum 1.5% and incubated with primary antibodies overnight. A summary of the antibodies used is shown in the table below (**Table 3**). Those samples processed with antibodies against *Griffonia Simplicifolia* isolectin B4 (IB4), were blocked also with normal goat serum (10%) with 1% bovine serum albumin (BSA) and additionally incubated with lectin (unconjugated *Griffonia Simplicifolia* Lectin I, L-1104, Vector) at 4º overnight. In those samples incubated with anti-TDP-43 antibodies, sections were previously blocked with endogenous Biotin-Blocking Kit (Invitrogen) and TBS-Triton 0.3%-normal donkey serum 10%.

After washes, sections were incubated overnight at 4ªC with Alexa Fluor 488 (1:200, ThermoFischer), donkey anti-rabbit Cy5 (1:200, Swant), donkey anti-mouse Alexa Fluor 594 (1:200, Thermofischer) or cyanine 3 (1:200, Jackson Immunoresearch) as secondary antibodies. Those samples processed with anti-TDP43 antibodies were incubated with horse anti-rabbit biotinylated antibody (1:200, Vector Laboratories) and conjugated streptavidin Alexa Fluor 488 (1:200, ThermoFischer). DAPI staining was also used to ensure correct recognition of tissue structures. After immunohistochemical processing, sections were adhered to slides with agar and mounted with Fluoromount G (Sourthern Biotech). To assess antibody specificity, control samples were processed in parallel as described but without primary antisera. Immunofluorescence method was used in order to improve the contrast of the biomarker.

Table 3. Primary and secondary antibodies used in the studies.

PRIMARY ANTIBODIES			
Antigen	**Host**	**Dilution**	**Producer/cat.number**
PGP 9.5	Rabbit	1:800	Ultraclone
PGP 9.5	Mouse	1:500	Ab8189
PGP 9.5	Rabbit	1:50	Cedarlane
CGRP	Rabbit	1:200	PC205L Millipore
VIP	Rabbit	1:500	20077, Immunostar
***Griffonia Simplicifolia* Lectin I – unconjugated**	Goat	1:500	AS2104, Vectorlab
Parvalbumin	Rabbit	1:1000	Swant
TDP-43	Rabbit	1:200	Proteintech, cat#12892-1-AP
Vimentin	Mouse	1:200	Sigma
SECONDARY ANTIBODIES			
Fluorophore/Conjugate	**Antigen**	**Host**	**Dilution**	**Producer/cat.number**
Cyanine3	Rabbit	Donkey	1:200	Jackson Immunoresearch
Alexa Fluor488	Goat	Donkey	1:200	Thermofisher
Cyanine5	Rabbit	Donkey	1:200	Swant
Alexa Fluor594	Mouse	Donkey	1:200	Thermofisher
Streptavidin Alexa Fluor488	-	-	1:200	S11223, Invitrogen
Streptavidin Alexa Fluor594	-	-	1:200	S32356, Invitrogen
DAPI	-	-	1:2000	D9564-10MG, Sigma

Protein gene product 9.5 **(PGP 9.5).** Also known as Ubiquitin C-terminal hydrolase L1 (UCHL1), is a protein ubiquitously expressed in neuronal (Thompson et al., 1983) and non-neuronal tissue (melanocytes, dermal fibroblasts, Merkel cells, distal renal tubular epithelium, mammary epithelial cells, Leydig cells, spermatogonia, epididymis) (Campbell et al., 2003). It is expressed in neurons of both peripheral and central nervous system, as well as in neuroendocrine cells. Neurons and axons strongly express PGP 9.5, despite the particular type (motor, sensory, autonomic) (Wilson et al., 1988) and irrespective from the neurotransmitter involved. However, there are exceptions of neurons with poor expression of PGP 9.5: a few neurons in the trigeminal ganglion and in the myenteric plexus (M Day et al., 2010).

CGRP **(calcitonin gene related peptide).** CGRP is a 37 aminoacid neuropeptide that is expressed in central and peripheral nervous system neurons (Benarroch et al., 2012; Eftekhari and Edvinsson 2011). CGRP positive neurons are mainly located in DRG, trigeminal and vagal ganglia and are regulated by NGF (nerve growth factor) (Iyengar et al., 2017). These neurons are small myelinated (Aδ type) and unmyelinated (C type) sensory and use glutamate as neurotransmitter (van Rossum et al., 1997). Somatic CGRP sensory fibers terminate in free endings and act as polymodal nociceptors, responding to high-threshold mechanical, thermal and chemical inputs (McCarthy and Lawson 1990; Lawson et al., 1996; 1997), while autonomic CGRP fibers modulate cutaneous vasodilation, playing an important role in neurogenic inflammation. The central axons of unmyelinated CGRP fibers innervate predominantly laminae I and II (outer layer) in the spinal cord, whereas small myelinated CGRP fibers innervate I and III/IV Rexed laminae.

IB4 **(*Griffonia Simplicifolia* isolectin B4).** IB4 is present in unmyelinated axons (C fibers) from non-peptidergic neurons located in the DRG that mediate nociception (Stucky et al., 1999) but also in the enteric nervous system. Those neurons are regulated by the glial cell line derived neurotrophic factor (GDNF) and their central projections synapse into the II (inner layer) Rexed lamina (Silverman and Kruger 1990; Alvarez et al., 1991).

Vasoactive intestinal peptide **(VIP).** VIP is a 28-aminoacid peptide involved in vasodilation, myocardial contractility, glycogenolysis and smooth muscle relaxation. VIP is located in sympathetic and somatosensory C fibers (Gibbons et al., 2009; Gibbons et al., 2010; Bjorklund et al. 1986). In the skin, autonomic VIP fibers are

sympathetic axons reaching blood vessels, eccrine sweat glands and arrector pili muscles (Donadio et al., 2019). Sweat gland sympathetic innervation fibers are cholinergic.

***Parvalbumin* (PV).** PV is a calcium binding protein with functional and structural similarities with calmodulin and troponin C, involved in buffering of intracellular calcium and therefore regulating calcium homeostasis. In the nervous system is found in autonomic neurons in Onuf's and intermediolateral nuclei, in the motor nuclei of cranial nerves in brainstem, in pyramidal neurons in sensory cortex, and in large neurons in DRG (Sasaki et al., 2006; Alexianu et al., 1994; de la Cruz et al., 1998). In DRG, parvalbumin neurons are most of them (≥90%) proprioceptors, while the rest correspond to mechanoreceptors (Walters et al., 2019; de Nooij et al., 2013; Antal et al., 1990). Their synapses are located in the II inner and III layers of Rexed in the dorsal horn of the spinal cord (Hughes et al., 2012; Yamamoto et al., 1989) and are mainly inhibitory (Hughes et al., 2015), using GABA and glycine as neurotransmitters. PV positive neurons are regulated by brain-derived neurotrophic factor (BDNF).

***TDP-43*.** The anti-TDP43 antibody used in this study recognizes the C-terminal cleavage product (20-30 KDa) and the native and phosphorylated forms of TDP-43. Antibodies against the non-phosphorylated form have been widely used to determine the TDP-43 translocation to the cytoplasm without the risk of missing different post-translational modifications and non-phosphorylated cytosolic distribution of TDP-43 (Braak et al., 2017; Paré et al., 2015; William et al., 2017). In the studies published to date on skin of ALS patients, only two have used antibodies against p-TDP-43, finding no labeling in one of them (Codron et al., 2018) and labeling only one third of the cases in the other (Ren et al., 2018). The rest of the studies have successfully used antibodies against the non-phosphorylated form (Suzuki et al., 2010; Wang et al., 2015; Paré et al., 2015; Sabatelli et al., 2015; Yang et al., 2015; Abe et al., 2017; Riancho et al., 2020; Romano et al., 2020).

***Vimentin*.** Vimentin is an intermediate filament protein found in cells with mesenchymal origin. It is expressed in the cytoplasm where, along with actin microfilaments and microtubules, make up the cytoskeleton. It is responsible for cell shape and integrity maintenance as well as intracellular transport. In the skin,

vimentin is expressed in melanocytes and Langerhans cells (epidermis), and in fibroblasts (dermis), but not in keratinocytes.

Immunohistochemical analysis

Samples were viewed under an Olympus BX-51 microscope equipped for epifluorescence using appropriate filters.

Intraepidermal nerve fiber quantification. For the quantitative analyses of intraepidermal nerve fibers (IENF), individual fibers were counted as they pass through the basement membrane. Branching occurring within the epidermis did not increase the number of the IENFs counted. IENF were counted at the lateral side of the footpad, in the animal model, and in the center area of the sample in the human. IENF density was expressed as number of fibers per 1 mm length of the epidermis. The average density of IENF per animal was then derived. The lateral side of the footpad was selected for IENF counting because in this site the epidermis is less interrupted by dermal papillae and the unmyelinated endings have a more homogeneous distribution.

Subepidermal nerve plexus and dermal nerve fiber density. To quantify the subepidermal nerve plexus (SENP) and dermal nerves fibers immunoreactivity, microphotographs of the skin of the hind footpad were taken at x200, and after defining the threshold for background correction, the percentage of area above the threshold of PGP 9.5 labeling was measured using ImageJ software. A mean of 3 measures per section of a predetermined area centered in the SENP (12x120μm^2) and of an area of the dermis just below the SENP (158x158μm^2), respectively, were analyzed.

Meissner corpuscles were identified by visual inspection with the PGP9.5 reactivity and the total number of labeled corpuscles in the tip of the footpad (animal model) was counted. Meissner's corpuscles were easily spotted as nerve fibers within the dermis (dermal papillae) with a skein shaped terminal ending.

Other dermal sensory structures. Digital images were taken of the sweat glands of the footpad of each animal, and nerve fibers in the gland were selected using computer assisted image analysis. Sweat gland nerve fiber density (SGNFD) was estimated as the percent area of nerve fibers within the area of interest (Gibbons et

al., 2010). Area of the gland (area of interest) was manually selected, and nerve fibers were quantified subtracting background based on the pixels above threshold, using ImageJ software.

DRG sensory neurons quantification. Immunolabeled sections were first viewed under a Nikon microscope equipped for epifluorescence using appropriate filters. Photographs of the entire area of the DRG were taken using a confocal microscope. Every second serial section from the DRG was analyzed (with a distance of 20 µm between sections), and a total of 6 sections were analyzed for each DRG (comprising a total thickness of 320 µm, as representative area of the ganglia). The number of immune-positive DRG neurons was determined counting neurons that contained a nucleus and showed a strong signal intensity in the cytoplasm. The total number of neurons were counted, as well as the number of CGRP+, PV+, IB4+ and double IB4+/CGRP+ neurons. Percentage of each group was then derived.

Intraepidermal TDP-43 nerve colocalization. To study the possible colocalization of TDP-43 in the cutaneous innervation, we performed immunohistochemical co-labeling for PGP9.5 and TDP-43. Confocal images were taken with a confocal microscope for counting IENF and for TDP-43 colocalization assessment. IENF were measured in a subset of ALS and healthy control subjects as described above. Colocalization was studied searching TDP-43 immunoreactivity within intraepidermal PGP9.5 positive fibers.

Non-neural cytoplasmic TDP-43. Epidermal TDP-43 amount was measured in microphotographs taken from two representative areas of epidermis (101.61x101.61 μm^2 each) of each case, using ImageJ software. The mean of the two measures of the percentage of area with TDP-43 labeling was calculated after defining the threshold background correction. The two layers of the dermis (papillary and reticular dermis) were evaluated separately. This distinction was made given that papillary and reticular cells have different pattern of protein synthesis and expression (Ono et al., 2000; Uhlen et al., 2010). Differentiation of both layers was based on visual recognition of different density of connective tissue, and the superficial vascular plexus at the boundaries between both layers. Images of two representative areas (101.61x101.61 μm^2 each) of papillary and reticular dermis were taken and analyzed. Dermal cells containing TDP-43 were fibroblasts as identified by co-labeling against vimentin. The levels of cytoplasmic TDP-43 were

quantified as: 1) percentage of cytoplasmic TDP-43 immunoreactivity, and 2) percentage of cells with positive TDP-43 into the cytoplasm in each defined area. For calculating the percentage of cytoplasmic TDP43 immunoreactivity, first a threshold was defined for background correction, then the percentage of pixels in the area above threshold of TDP-43 labeling was measured using ImageJ software. Additionally, the mean percentage of cells with TDP-43 positivity within their cytoplasm was calculated in the analyzed confocal images. Results are expressed as the mean of the two measures per subject and layer.

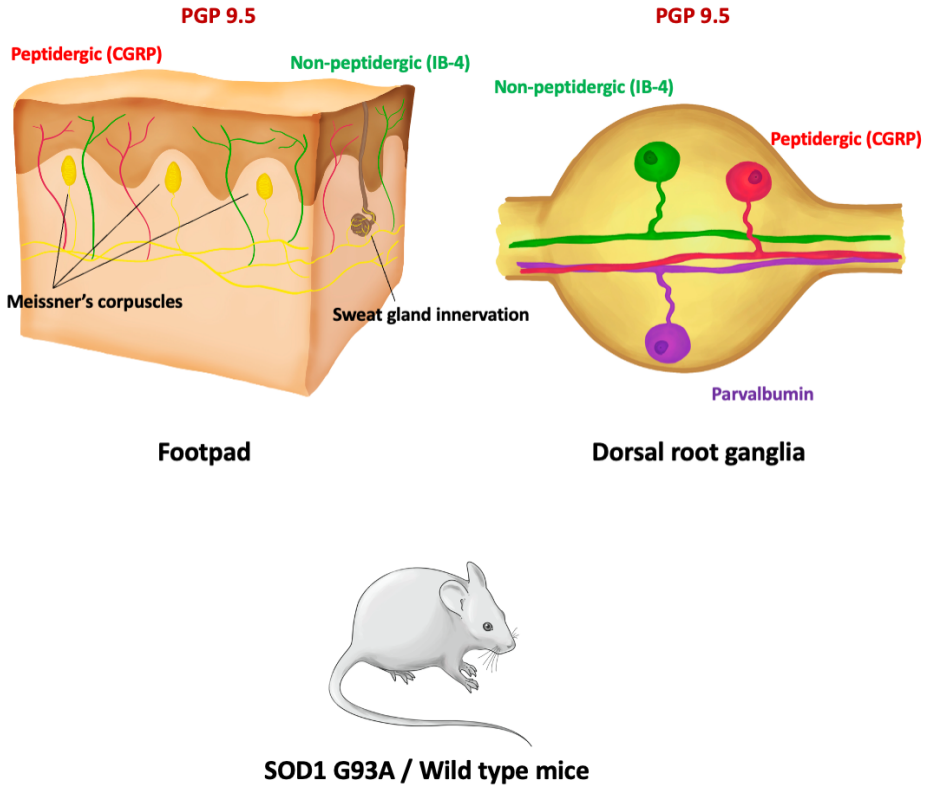

Figure 1. Schematic representation of the studies performed in the SOD1^{G93A} and wild type mice. Footpads from the hindlimbs of mice at different ages were extracted and immunolabeled with PGP 9.5 as a panneuronal marker, IB-4 (non-peptidergic; green), CGRP (peptidergic; red), and VIP (sympathetic innervation of the sweat glands). DRG were extracted at the same age and sensory populations studied were identified using IB-4, CGRP and parvalbumin.

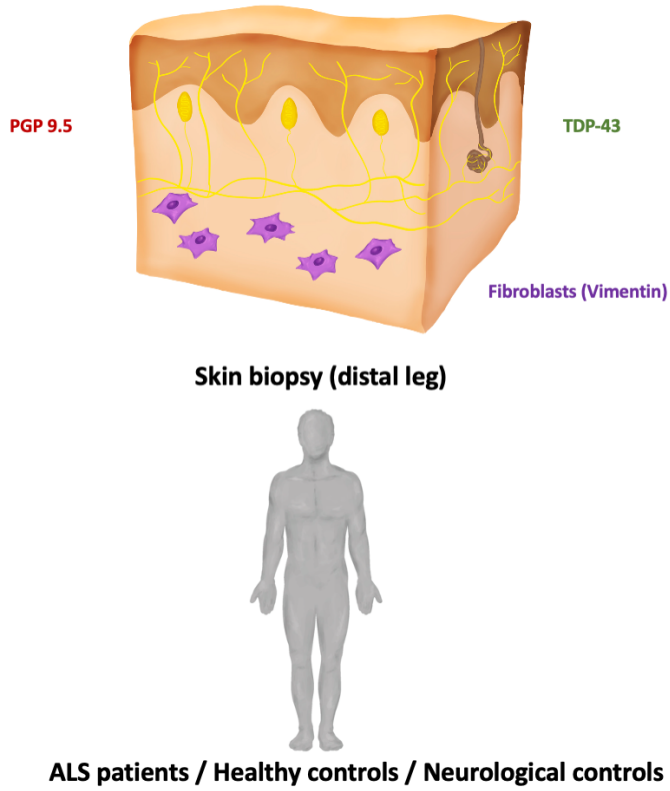

Figure 2. Schematic representation of the studies performed in ALS patients, healthy and neurological controls. IENF density from skin biopsies (distal leg) were studied using PGP 9.5. Anti-TDP-43 antibody was used to assess its localization in neural and non-neural structures in the skin. Vimentin was used to identify fibroblasts in the dermis.

Statistical analysis

Data are expressed as mean ± SEM. Means were compared by t-test and ANOVA applying Tukey's post hoc test when necessary (SPSS statistics 19 software). The level of significance was set at $p < 0.05$. Pearson's correlation coefficient was used to assess possible linear association between two continuous quantitative variables. When necessary, ROC curves and area under the curve (AUC) were calculated and optimal cut-off values of certain variables were selected using Youden's index.

RESULTS

CHAPTER 1

Involvement of sensory innervation in the skin of SOD1^{G93A} ALS mice

Journal of the Peripheral Nervous System 21:88–95 (2016)

RESEARCH REPORT

Involvement of sensory innervation in the skin of SOD1^{G93A} ALS mice

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Abstract Sensory alterations have been described in both amyotrophic lateral sclerosis (ALS) patients and mouse models. While involvement of intraepidermal and subepidermal axons has been shown in skin biopsies of ALS patients, it is unclear if the SOD1^{G93A} mouse presents similar alterations. We analyzed the epidermal and dermal innervation, based on PGP9.5 immunostaining, of SOD1^{G93A} mice at different stages. The results showed a marked reduction of intraepidermal nerve fibers, Meissner's corpuscles, and subepidermal nerve density already at 4 weeks. This loss of innervation progressed over time. Dermal axonal density decreased at a later stage of the disease. There was a gradient of axonal loss, with a more severe decline in the epidermis compared with deeper structures, indicating a distal axonal neuropathy as the mechanism of degeneration. These findings suggest that the analysis of the cutaneous sensory innervation may be an accessible and useful tool to assess the neurodegeneration process in motoneuron diseases.

Key words: amyotrophic lateral sclerosis, PGP9.5, sensory innervation, SOD1^{G93A} mouse

Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder characterized by the degeneration of upper and lower motor neurons. Several transgenic animal models of ALS have been developed, being the most widely used a transgenic mouse that over-expresses the human mutated form of the *sod1* gene *(Gurney et al., 1994)*. This mouse model has proven useful for examining the pathophysiology of motor neuron degeneration in ALS *(Mancuso and Navarro, 2015)*.

Non-motor manifestations may occur in both ALS patients and animal models, including sensory and autonomic dysfunctions *(Pugdahl et al., 2007)*. About one-third of ALS patients have sensory abnormalities *(Hammad et al., 2007)*. Morphometric and biochemical studies of peripheral nerves from ALS patients revealed a loss of large sensory axons, and preferential vulnerability of large dorsal root ganglia neurons *(Kawamura et al., 1981; Ben Hamida et al., 1987)*. Using skin biopsies, reduction of intraepidermal innervation as well as of the subepidermal and sweat gland innervation have been found in patients with sporadic and familiar ALS *(Weis et al., 2011; Dalla Bella et al., 2016)*.

Nevertheless, although in the SOD1^{G93A} mice abnormalities have been shown in dorsal root, dorsal root ganglia, posterior horn, and in dorsal funiculus *(Fischer et al., 2005; Guo et al., 2009)*, it is unknown if there is a loss of innervation of cutaneous sensory fibers as seen in ALS patients. Thus, we performed a systematic analysis of the axonal loss of different types of fibers in the skin of the paw of SOD1^{G93A} mice along the course of the disease.

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Materials and Methods

Transgenic SOD1^{G93A} mice

Transgenic mice with the G93A human SOD1 mutation [B6SJL-Tg(SOD1-G93A)1Gur] were obtained from the Jackson Laboratory (Bar Harbor, ME, USA), and maintained at the Animal Service of the Universidad de Zaragoza. Hemizygotes B6SJL SOD1^{G93A} males were obtained by crossing with B6SJL from the CBATEG (Bellaterra, Spain). The offspring was identified by polymerase chain reaction (PCR) amplification of DNA extracted from the tail tissue. All experimental procedures were approved by the Ethics Committee of the Universitat Autònoma de Barcelona, where the animal experiments were performed, and followed the guidelines of the European Commission on Animal Care.

Immunohistochemical processing and analysis

Under anesthesia with pentobarbital (50 mg/kg i.p.), plantar pads of the hindfoot of SOD1^{G93A} mice at 4, 8, 12, and 16 weeks were collected (five mice per age). A group of 7 wild-type mice of 12 weeks of age was also used as control. Footpads were harvested and fixed in paraformaldehyde 4% for 24 h at 4°C, and then kept frozen in a cryoprotective solution (PBS with sucrose at 30% and sodium azide). For immunohistochemistry, samples were processed as previously described *(Navarro et al., 1995; 1997)*.

Intraepidermal nerve fibers (IENFs) were counted as they crossed the basement membrane. IENFs were counted at the lateral side of the footpad, where the epidermis is not interrupted by dermal papillae and the unmyelinated endings have a more homogeneous distribution, and expressed as number of fibers per 1 mm length of the epidermis. Confocal images were taken with a Leica microscope for IENFs counting. Meissner's corpuscles were identified as nerve fibers within the dermal papillae with a skein shaped terminal ending, and the total number of labeled corpuscles in the tip of the footpad was counted. To quantify the subepidermal nerve plexus (SENP) and dermal nerves fibers immunoreactivity, microphotographs of the footpad were taken at ×200, and after defining the threshold for background correction, the percentage of area above threshold of PGP9.5 labeling was measured using ImageJ software. A mean of 3 measures per section of a predetermined area centered in the SENP ($12 \times 120\ \mu m^2$) and of an area of the dermis just below the SENP ($158 \times 158\ \mu m^2$) (Fig. 1A), respectively, were taken.

Data analysis

Data are expressed as mean ± SEM. Means were compared by ANOVA applying Tukey's post hoc test when necessary (SPSS statistics 19 software). The level of significance was set at $p < 0.05$.

Results

The general distribution of immunoreactive nerve fibers in footpad samples of the wild-type and SOD1^{G93A} mice corresponded to the pattern previously described for the normal mouse footpad *(Navarro et al., 1995; 1997)*. Intense immunoreactivity to PGP9.5 was seen in nerves throughout the pad, in a dense SENP that gives rise to small nerve branches that penetrate the epidermis, and in a dense network that innervates the sweat glands (Fig. 1).

IENF density

In the control wild-type mice, the mean number of IENFs labeled against PGP9.5 in the footpad was 64.4 ± 3.9/mm epidermal length. We found a lower IENF density in the SOD1^{G93A} mice at all stages when compared with the wild-type mice ($p < 0.001$). The reduction of IENFs was detected as early as at 4 weeks (42.3 ± 2.2) and 8 weeks (38.5 ± 2.0) of age, in the pre-symptomatic stage of the mice. The axonal loss progressively increased over time (at 12 weeks 33.7 ± 1.8; at 16 weeks 33.0 ± 0.6), although no statistical significance was found between values at the different stages (Fig. 2).

Meissner's corpuscles

In the wild-type mice, we found a mean of 6.5 ± 0.6 corpuscles in the tip of the footpad. In the SOD1^{G93A} mice, there was a loss of Meissner's corpuscles at an early stage, similar at 4 (4.0 ± 0.7) and 8 weeks (4.0 ± 0.5) ($p < 0.02$). This loss was more pronounced at week 12 (2.2 ± 0.4) and at week 16 (2.2 ± 0.4) ($p < 0.001$) of age (Fig. 3).

SENP density

The PGP9.5 immunoreactivity of the SENP measured as percentage of a predefined area averaged 54.1 ± 1.9% in the wild-type mice. We found a significant reduction of the density of innervation in the SENP at 4 (41.7 ± 2.3%) and 8 weeks (42.5 ± 1.9%) compared with controls ($p = 0.001$). This finding was significantly more marked at 12 (32.2 ± 0.7%) and 16 weeks (31.6 ± 2.9%) ($p < 0.001$ vs. wild-type, $p < 0.05$ vs. 4w and 8w) (Fig. 4).

Dermal nerve fiber immunoreactivity

The density of dermal fibers measuring the percentage of PGP9.5 immunoreactivity in a determined area just below the SENP. In the wild-type mice the mean density of PGP9.5 immunoreactivity was

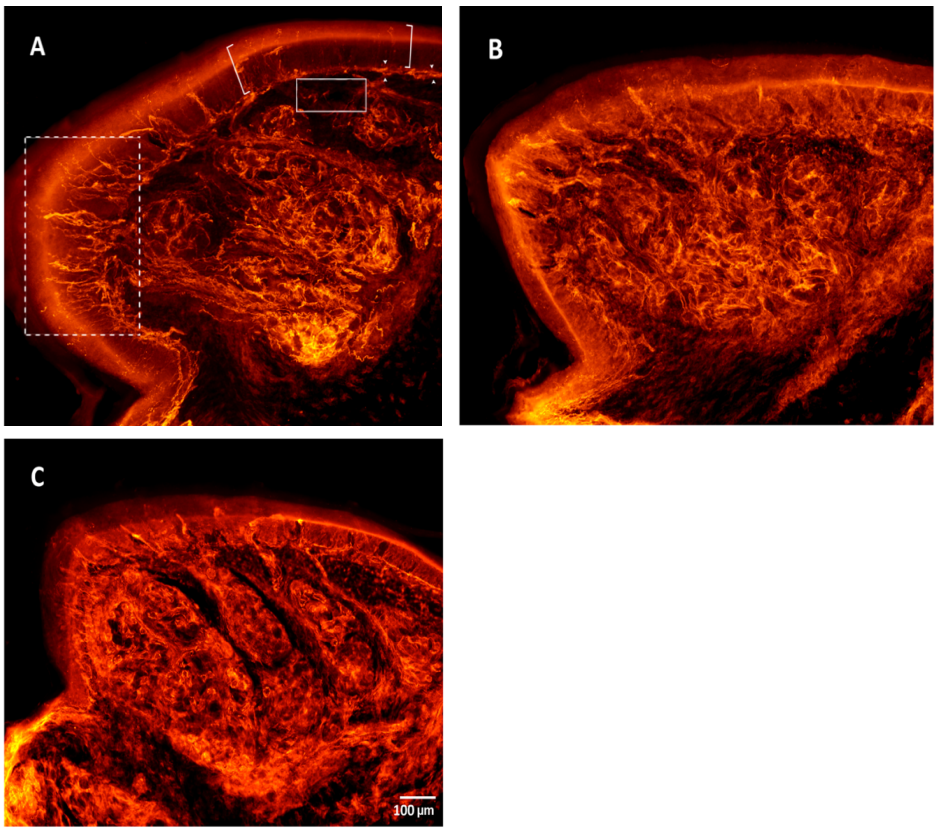

Figure 1. PGP9.5 immunoreactive nerve fibers in footpads from wild-type (A) and SOD1^{G93A} mice of 8 (B) and 16 weeks (C). Intraepidermal nerve fiber density was calculated in the lateral side of the footpad (brackets in A). Meissner's corpuscles were counted in the tip of the footpad (dotted rectangular area). Subepidermal nerve plexus (arrows) and dermal (square area) immunoreactivity were also calculated.

77.0 ± 2.5%. In the SOD1 mice, dermal innervation was preserved at 4 (74.6 ± 4.5%), 8 (79.9 ± 3.1%), and 12 weeks (70.2 ± 4.8%), without statistical differences compared to controls. Only at the later stage of the disease we found a decreased density of innervation in the dermis (49.4 ± 2.8%), compared with the wild-type group ($p < 0.001$) and the rest of the stages of SOD1^{G93A} mice ($p < 0.05$).

Sensory distal axonopathy

In order to assess the progression of the sensory axonal degeneration, we analyzed the differences of the mean axonal loss between different locations: epidermis, SENP, and below SENP (dermal fibers). The sensory axonal loss was estimated as a percentage normalized with respect to wild-type mice results. We found a distal to proximal gradient of axonal degeneration, with a more severe loss of innervation in the epidermis compared with dermis at 4 weeks (34.3 ± 3.4% vs. 3.2 ± 5.8%; $p < 0.05$), 8 weeks (40.2 ± 3.1% vs. −3.8 ± 4.1%; $p < 0.001$), and 12 weeks (47.7 ± 2.8% vs. 8.8 ± 6.2%; $p < 0.001$). There was also a difference of axonal loss between SENP and dermis at 8 (40.2 ± 3.1%; $p < 0.02$) and 12 weeks (40.5 ± 1.3%; $p = 0.001$). This gradient was less pronounced at the later stages of the disease, probably due to a more severe and spread degeneration in all sites (Fig. 5).

Discussion

Sensory axons were evaluated in the footpad of control mouse and of SOD1^{G93A} mouse at different

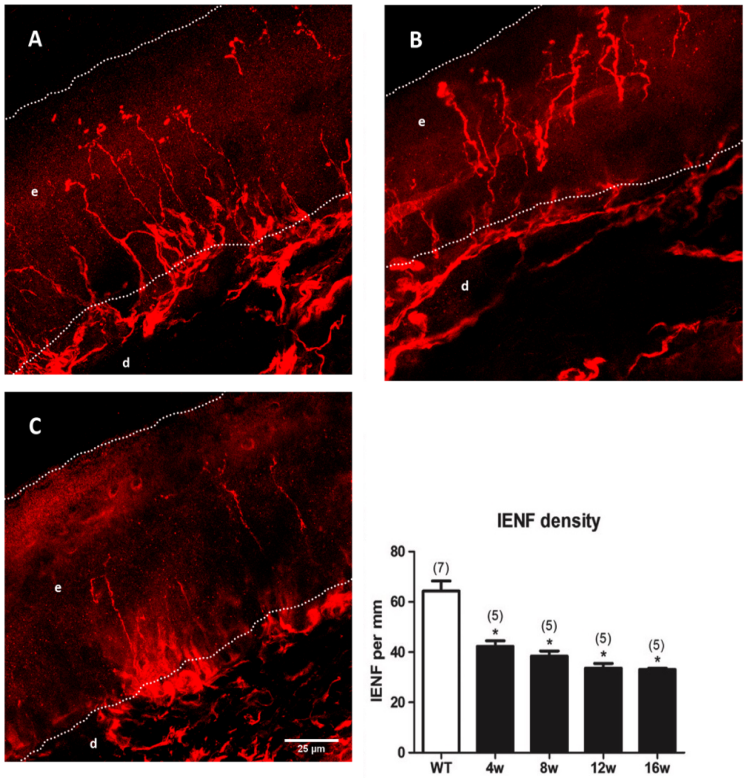

Figure 2. Intraepidermal nerve fiber (IENF) density from wild-type (A), SOD1^{G93A} mice of 8 (B) and 16 (C) weeks. Dotted lines mark limits of epidermis (e) and dermis (d). Histogram of IENF density showing an early decline of PGP9.5 fibers in the SOD1^{G93A} mouse. Data are mean ± SEM. Number in parentheses indicate number of animals examined (*$p < 0.001$ vs. wt).

ages and in different layers of the skin. We have found an overall impairment of sensory axons, including intraepidermal unmyelinated fibers, Meissner's corpuscles, SENP, and dermal fibers in the SOD1^{G93A} mice. The degree of axonal loss varied with the depth of the location in the skin studied and along time of evolution, with a distal to proximal pattern. At 4 and 8 weeks, dermal innervation was spared, while epidermis and SENP showed a marked axonal loss. Only at late stages of the disease, the innervation in the dermis showed significant impairment. These findings indicate predominantly distal axonal alterations in the early and pre-symptomatic stages of ALS, as demonstrated by the decrease of IENF and Meissner's corpuscles density. A distal axonopathy has been described as responsible for the degenerative process in motor nerve fibers in both mice and men, also evidenced in pre-symptomatic stages *(Fischer et al., 2004; Moloney et al., 2014)*. The predominantly distal sensory axonopathy would most likely be caused by the same degenerative process as in the motor fibers. Different mechanisms have been suggested for the "dying-back" pattern leading to progressive loss of motor neurons in ALS *(Dadon-Nachum et al., 2011)*, including alterations in mitochondrial function, abnormal aggregation of proteins, accumulation of neurofilaments in the cell body and subsequent alteration in the distal cytoskeleton, leading to axonal transport impairment *(Mancuso and Navarro, 2015)*.

As a similar loss of skin innervation has been described in ALS human patients *(Weis et al., 2011; Dalla Bella et al., 2016)*, our findings reinforce the concept that ALS extends beyond the corticospinal and neuromuscular motor systems into other areas. Evidence of sensory system involvement has been also reported previously in mutant SOD1 rodents, showing damage to dorsal root ganglia and dorsal roots associated with degeneration of sensory axons

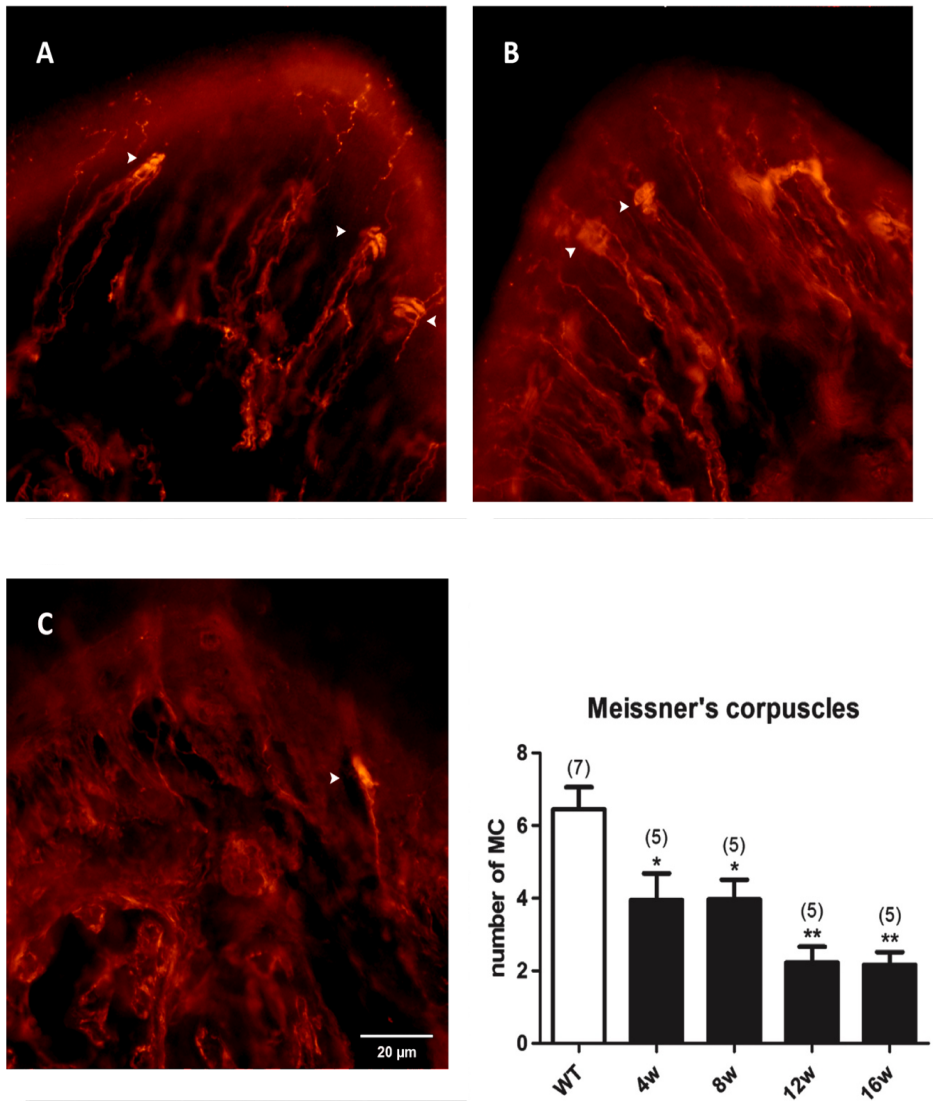

Figure 3. Meissner's corpuscles (MC) (arrows) from wild-type (A), SOD1^{G93A} mice of 8 (B) and 16 (C) weeks. Histogram of MC density. Data are mean ± SEM. Number in parentheses indicate number of animals examined (*$p < 0.02$ vs. wt; **$p < 0.001$ vs. wt).

(Fischer et al., 2005; Guo et al., 2009), and slight slowing of sensory nerve conduction velocity *(Mancuso et al., 2011)*. It was recently reported the accumulation of misfolded SOD1 in proprioceptive neurons together with sensory fiber degeneration in the transgenic animals *(Sábado et al., 2014)*. An intraepidermal axonal loss has been shown in the footpad of a chimeric SOD1^{G93A}-UCHL1-eGFP mice *(Genç et al., 2015)*, but in contrast to our findings, the degeneration occurred only at the end stage of the disease. However, the present results are the first evidence of a wide impairment of cutaneous sensory innervation in the

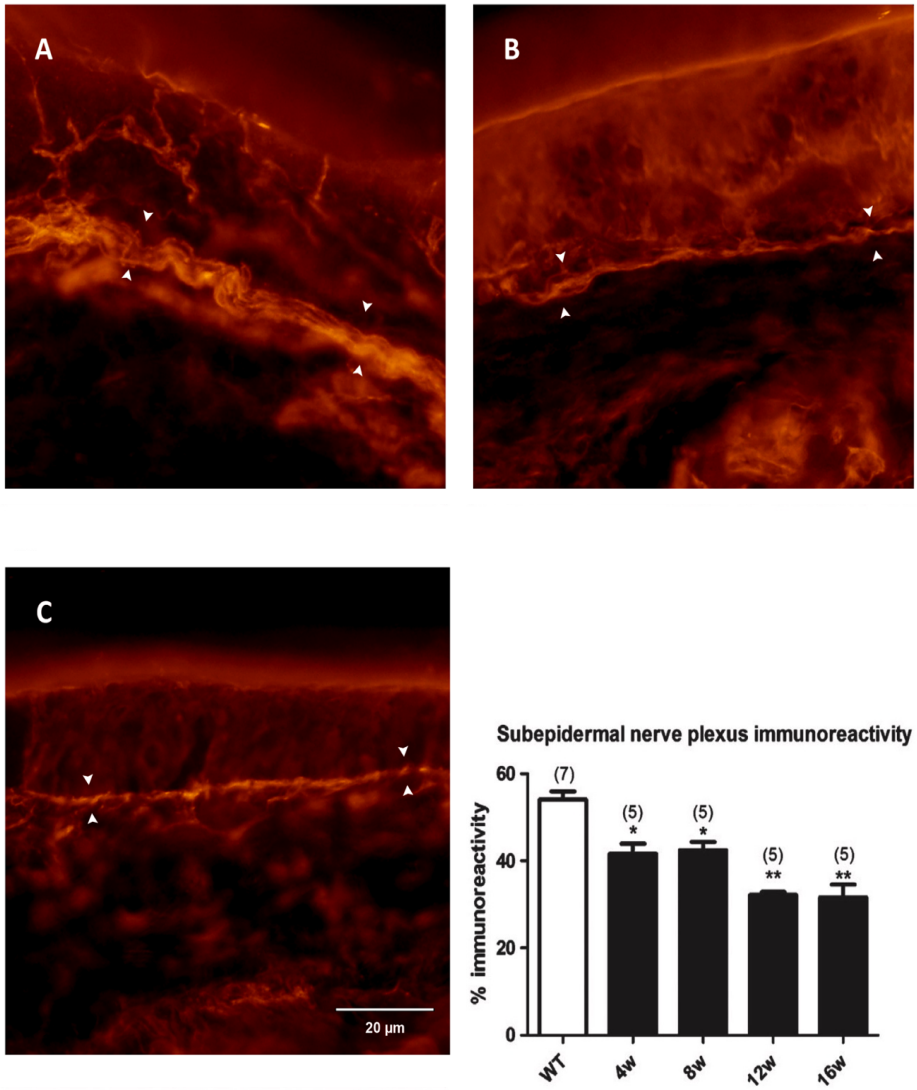

Figure 4. Subepidermal nerve plexus (arrows) from wild-type (A), SOD1^{G93A} mice of 8 (B) and 16 (C) weeks. Histogram of subepidermal nerve plexus density. Data are mean ± SEM. Number in parentheses indicates number of animals examined (*$p = 0.001$ vs. wt; **$p < 0.001$ vs. wt; and $p < 0.05$ vs. 4w and 8w).

SOD1^{G93A} mouse, not only limited to epidermal, but also to dermal sensory axons.

Regarding the early decrease of sensory skin innervation, previous works have found histological damage in dorsal root and dorsal funiculus *(Guo et al., 2009)*, and degeneration of proprioceptive sensory nerve endings in the SOD1 transgenic mouse model *(Vaughan et al., 2015)*, even before the symptomatic phase of the disease, independently of α-motor axon loss, suggesting also a distal sensory axonopathy. In ALS patients,

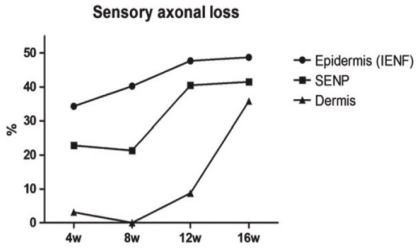

Figure 5. Plot of sensory axonal loss at epidermis, subepidermal nerve plexus and dermis, normalized as % of wild-type mice values. A gradient of distal to proximal axonal loss is shown at all ages except in the late stage of the disease.

the combination of electrophysiological and imaging techniques has also shown subclinical sensory impairments at early stages of the disease *(Iglesias et al., 2015)*.

Our results also point out that cutaneous sensory axonal loss progresses over time, suggesting that it correlates with the progression of the disease. Previous pathological studies on sural nerve biopsies of ALS patients also suggested that the degree of sensory alterations might correlate with the severity of the disease *(Heads et al., 1991)*. Recently, an *in vivo* analysis of corneal axons in ALS patients found a relationship between small fiber sensory neuropathy and loss of bulbar functions *(Ferrari et al., 2014)*. Reduction of IENFs in skin biopsies of the distal leg has been used as diagnostic tool for small fiber neuropathy *(Kennedy et al., 2005)*. In ALS patients, this has been proved useful for the determination of reduced intraepidermal and subepidermal innervation *(Weis et al., 2011)*. Although predominant involvement was reported in spinal-onset ALS patients *(Truini et al., 2015)*, a larger study showed IENF loss in 75% of ALS patients without correlation with onset, phenotype, and severity of the disease *(Dalla Bella et al., 2016)*. Therefore, our data indicates that the SOD mouse offers an interesting model for the study of potential mechanisms involved in motor and sensory distal axonal degeneration.

Acknowledgements

This work was supported by funds from CIBERNED and TERCEL, Instituto de Salud Carlos III of Spain, and grant TV3201428-10 of Fundació La Marato-TV3. We thank Marta Morell and Jessica Jaramillo for their technical help.

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RESULTS

CHAPTER 2
Characterization of somatosensory neurons involvement in the SOD1^{G93A} mouse model

Characterization of somatosensory neurons involvement in the SOD1^{G93A} mouse model

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Abstract:

Introduction. SOD1^{G93A} mice show loss of cutaneous small fiber as in ALS patients. Our objective is to characterize the involvement of different somatosensory neuron populations, as well as its temporal progression. We aim to analyze at the same time points the neuronal bodies located in the dorsal root ganglia (DRG) and the distal part of their axons in the skin, in order to discern the pattern of degeneration.

Methods. We performed immunohistochemical analysis of peptidergic (CGRP), non-peptidergic (IB4) fibers in epidermis, as well as sympathetic sudomotor fibers (VIP) in the footpads of SOD1^{G93A} mice and wild type littermates at 4, 8, 12 and 16 weeks of age. We also immunolabeled and quantified neuronal bodies of IB4, CGRP and parvalbumin (PV) positive sensory neurons in lumbar DRG at the same time points.

Results. We detected a loss of intraepidermal nerve fiber density in the SOD1^{G93A} mice of both peptidergic and non-peptidergic axons, although the latter showed earlier and more marked involvement. Sweat gland innervation was also decreased in the SOD1^{G93A} mouse at 12 weeks. Nonetheless, the number of DRG neurons from the different sensory populations studied remained unchanged during the time lapse evaluated.

Conclusions. Cutaneous sensory axons are affected in the SOD1^{G93A} mouse, with non-peptidergic being slightly more vulnerable than peptidergic axons. Loss of the distal portion of sensory axons with preservation of the corresponding bodies suggest a distal axonopathy as the cause.

Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder that mainly involves the motor neurons in the spinal cord and the cerebral cortex. Nevertheless, in the last years, cumulative data have shown that there are other neural structures besides motor neurons that are also affected. Aside cognitive impairments in up to 50% of patients, other non-motor manifestations have been reported, including extrapyramidal, autonomic, and even sensory abnormalities (McCombe et al., 2017; Tao et al., 2018). Particularly, a small fiber sensory neuropathy has been observed in several studies in ALS patients (Weis et al., 2011; Truini et al., 2015; Dalla Bella et al., 2016; Nolano et al., 2017) and in animal models (Genç et al., 2015; Sassone et al., 2016). We have previously reported that in the SOD1^{G93A} mouse, the most common animal model for the disease, there is a significant axonal loss of sensory axons in the skin of the footpads, even at the presymptomatic stage of the disease (Rubio et al., 2016), and showing a gradient of involvement from the epidermis (most affected) to the deepest dermis (less affected). However, although peripheral sensory involvement has been confirmed in both humans and animal models, characterization of the neuronal populations most involved, the evolution overtime and the degenerative mechanism are still lacking.

Cutaneous nerve endings are the terminal portions of axons that raise from the body of the pseudo-unipolar neurons located in the dorsal root ganglia (DRG). These neurons and their sensory fibers are classified in subpopulations according to anatomy, physiology, and neurochemistry considerations and present different molecular marker expressions, distinctive receptor characteristics and functions that could have different vulnerability to the neurodegenerative process in ALS. Based on the size and the molecular characteristics, DRG neurons can be divided into three groups: large, myelinated neurons (Aβ mechanoreceptors fibers and Aaβ proprioceptors, the latter expressing parvalbumin; PV), small myelinated (Aδ fibers), and unmyelinated (C fibers) that may be peptidergic neurons (expressing substance P and calcitonin gene-related peptide CGRP) and non-peptidergic (labeled with isolectin B4, IB4) neurons (Usoskin D et al., 2014; Le Pichon et al., 2014; Vukojevic K et al., 2016).

Our objective was to better define the peripheral sensory involvement in the SOD1^{G93A} mice from the epidermal terminations to the soma in the DRG,

distinguishing between the different populations of sensory neurons affected. To achieve this aim we have labeled and quantified the presence of peptidergic and non-peptidergic intraepidermal nerve fiber (IENF), as well as the sweat gland nerve fiber (SGNF) density as a comparative autonomic innervation, at different stages of the disease, and compared with their wild type (WT) littermates. In parallel we have studied the proportion of neuronal somas of the different sensory populations in the DRG.

Methods

Transgenic SOD1^{G93A} mice

Transgenic female mice with the G93A human SOD1 mutation [B6SJL-Tg(SOD1-G93A)1Gur] were obtained from Jackson Laboratories, and maintained at the Animal Service of the Universitat Autònoma de Barcelona. The offspring was identified by PCR amplification of DNA extracted from the tail. Non-transgenic littermates were used as controls. The experimental procedure was approved by the Ethics Committee of the Universitat Autònoma de Barcelona and followed the guidelines of the European Commission on Animal Care.

Footpad sample collection and processing

Animals were euthanized under anesthesia with pentobarbital (50 mg/kg i.p.) and transcardially perfused with 4% paraformaldehyde in PBS. The plantar pads of the hindfoot of SOD1^{G93A} mice and WT mice were carefully extracted at 4, 8, 12 and 16 weeks of age (4 mice per age and group). Footpads were then kept frozen in a cryoprotective solution (PBS with 30% sucrose and sodium azide). 60 µm-thick sections were serially cut with a cryotome (Leica) and collected by free-floating in PBS medium.

For immunohistochemistry, sections were blocked with PBS-Triton 0.3%-normal donkey serum 1.5% and normal goat serum 10% with 1% bovine serum albumin (BSA) and primary incubated in lectin (unconjugated Griffonia Simplicifolia Lectin I) at 4ºC overnight, and subsequently incubated overnight at 4º C with anti-protein gene product 9.5 (PGP 9.5, 1:800, Ultraclone), anti-CGRP (1:200, PC205L Millipore), or anti-lectin (1:500, anti-Griffonia Simplicifolia Lectin I, unconjugated, L-1104 Vector) as primary antibodies. Additionally, samples from both SOD1^{G93A} and WT at 4 and 12 weeks were incubated with anti-VIP (1:500, 20077 Immunostar) as

a selective marker of the sympathetic innervation of the sweat glands. After washes, sections were incubated overnight at 4ºC with Cy3 (1:200, Jackson Immunoresearch), Alexa 488 (1:200, Thermofischer), and donkey anti-rabbit Cy5 (1:200, Swant) secondary antibodies. After immunohistochemical processing, sections were adhered to gelatinized slides and mounted with Fluoromount G (Southern Biotech). To assess antibody specificity, control samples were processed in parallel as described but without primary antisera.

Intraepidermal nerve fiber quantification

Footpad samples were viewed under an Olympus BX-51 microscope equipped for epifluorescence using appropriate filters. For the quantitative analyses of IENF, individual fibers were counted as they pass through crossed the basement membrane. Branching occurring within the epidermis was not considered. IENF were counted at the lateral flat side of the footpad and expressed as number of fibers per 1 mm length of epidermis. The average density of IENF per animal was then derived. Confocal images were taken with a Leica microscope for IENF counting. IENFs were also counted for CGRP and IB4 intraepidermal positive fibers separately.

Sweat gland nerve fiber density

Digital images were taken of the sweat glands of the footpad of each animal, and nerve fibers in the gland were selected using computer assisted image analysis. The SGNF density was estimated as the percent area of nerve fibers within the area of interest (Vilches et al., 2002; Gibbons et al., 2010). The area occupied by single glands (area of interest) was manually delineated, and the VIP-labeled nerve fibers within were quantified subtracting background based on the pixels above threshold, using ImageJ software. A mean of 19 glands per animal were analyzed, and for each animal (WT and SOD1^{G93A}, at 4 and 12 weeks of age) we calculated the median percent area of SGNF.

DRG sample processing and immunohistochemical procedure

L4 and L5 DRG were extracted from wild type and SOD1^{G93A} mice at 4, 8, 12 and 16 weeks. DRG were maintained at 4ºC in a cryoprotective solution and then were embedded in OCT (optimal cutting temperature compound) and serially cut with a cryotome in 20-µm thick sections, collecting the slices in gelatinized slides.

For immunofluorescence, slides were blocked as above and incubated in lectin (unconjugated Griffonia Simplicifolia Lectin I) at 4ºC overnight, and subsequently incubated overnight at 4º C with anti-PGP9.5, anti-CGRP, anti-parvalbumin (1:1000, PV-28 Swant) and anti-lectin. After washes, sections were incubated overnight at 4ºC with Alexa Fluor 488, donkey anti-rabbit Cy5, donkey anti-mouse Alexa Fluor 594 as secondary antibodies. DAPI staining was also used to ensure correct recognition of tissue structures. Slides were mounted in Fluoromount G (Sourthern Biotech). To assess antibody specificity, control samples were processed in parallel as described but without primary antibodies.

DRG sensory neurons quantification

Photographs of the entire area of the DRG were taken using a confocal microscope. Every second serial section from the DRG was analyzed (with a distance of 20 μm between sections), and a total of 6 sections was analyzed for each DRG (comprising a total thickness of 320 μm, as representative area of the ganglia). The number of immune-positive DRG neurons was determined counting neurons that contained a nucleus and showed a strong signal intensity in the cytoplasm. Area of these DRG sections was also calculated and the density of neurons per mm^2 was derived. The total number of neurons, as well as the number of CGRP+, PV+, IB4+ and double IB4+/CGRP+ neurons were counted. Percentage of each neuronal class was then derived.

Data analysis

Data are expressed as mean ± SEM. Means were compared by t-test and ANOVA applying Tukey's post hoc test when necessary (SPSS statistics 25 software). The level of significance was set at $p < 0.05$.

Results

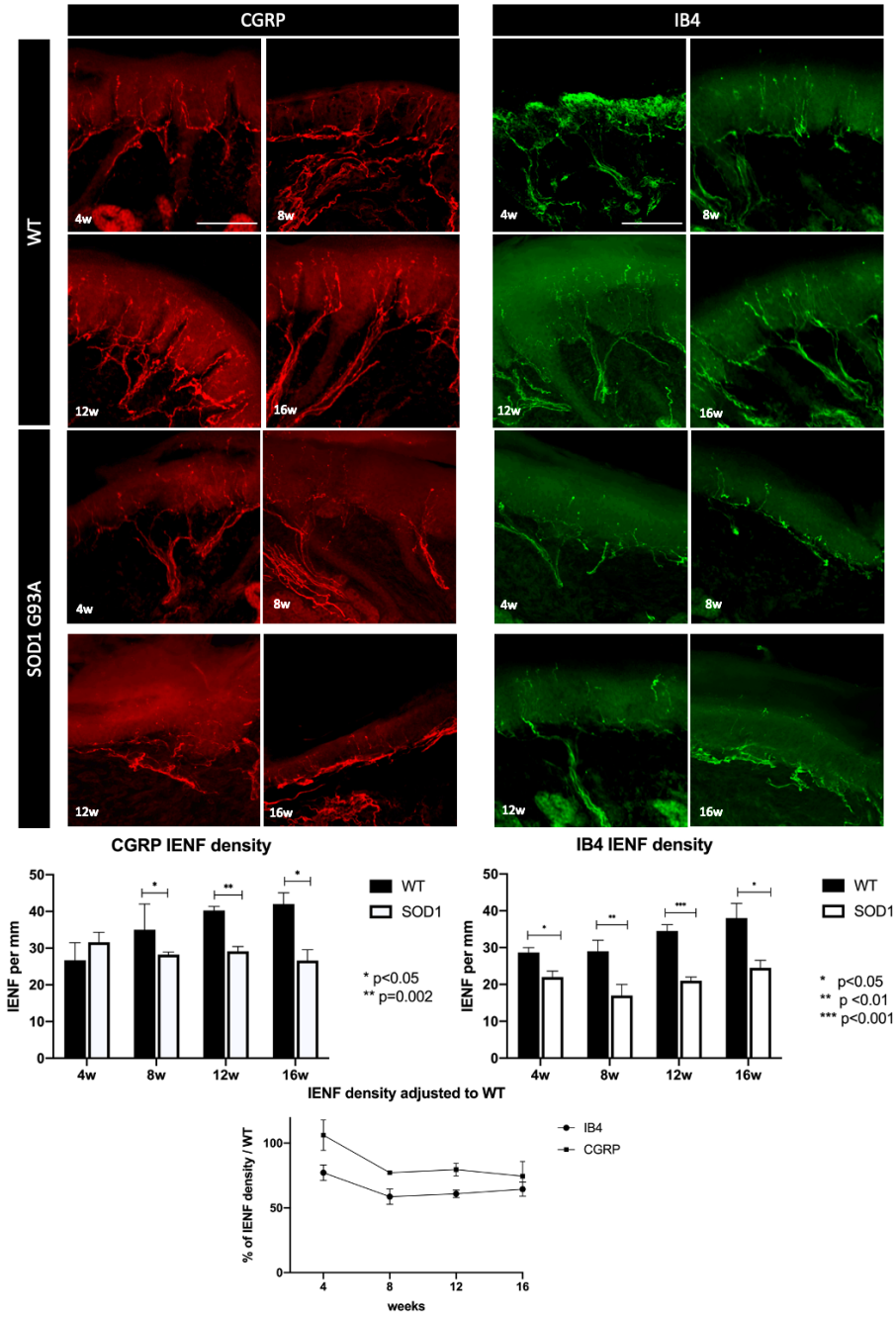
Intraepidermal nerve fiber density

We quantified IENF density of two populations of sensory neurons: nociceptive peptidergic (CGRP+) and non-peptidergic (IB4+). In the WT mice, both peptidergic and non-peptidergic fibers density showed an increase with age, although without significant differences between weeks of age. In the WT mice, the mean number of CGRP+ and IB4+ axons at 4 weeks of age were 27±5 and 29±1 per mm of epidermal

length respectively and increased progressively to 42±3 and 38±4 respectively at 16 weeks (Figure 1). The number of peptidergic intraepidermal fibers per length of epidermis was slightly higher than that of non-peptidergic fibers.

In the SOD1 transgenic mice both populations of IENF remained stable overtime, and again the peptidergic fibers were marginally higher than the non-peptidergic ones. We found a lower density of intraepidermal IB4+ fibers (22±2 fibers per mm, p=0.034) in the 4 weeks SOD1^{G93A} mice as compared with corresponding WT mice, but not in the CGRP+ population (31.6±2.7 fibers per mm, p=0.396). At 8, 12 and 16 weeks of age, the SOD1^{G93A} mice presented significantly lower density of both subpopulations of IENF compared to the WT mice ($p<0.05$) (Figure 1). Comparing the loss of IB4 and CGRP epidermal axons, we found that at 4 weeks non-peptidergic loss was more marked, and as the disease progressed, the decrease of IENF density between the two neuronal populations became similar.

Figure 1 (next page). IENF loss of both sensory epidermal populations in SOD1^{G93A} mice. ***Top***, representative microphotographs of epidermal innervation of CGRP+ and IB4+ fibers of WT and SOD1 mice, at 4, 8, 12 and 16 weeks. Scale bar: 100 μm. ***Bottom***, bar charts representing peptidergic (CGRP+) and non-peptidergic (IB4+) IENF density. CGRP+ fibers were significantly lower in SOD1^{G93A} compared to WT mice at 8, 12 and 16 weeks of age, whereas IB4+ fibers were reduced already at 4 weeks of age. Comparison of percentage of IENF density loss adjusted to the respective WT values show a more marked loss of IB4+ fibers in the presymptomatic stage, but as disease progresses, both CGRP+ and IB4+ fibers are similarly affected. Data are expressed as mean±SEM. IENF: intraepidermal nerve fiber.


CGRP
IB4
WT
SOD1 G93A
4w
8w
12w
16w
CGRP IENF density
IB4 IENF density
IENF per mm
WT
SOD1
* p<0.05
** p=0.002
* p<0.05
** p <0.01
*** p<0.001
IENF density adjusted to WT
% of IENF density / WT
IB4
CGRP
weeks

Sweat gland innervation

At 4 weeks (presymptomatic stage), sweat gland innervation was similar between the SOD1^{G93A} and the WT mice. However, we detected a decrease of SGNFD at 12 weeks with a 29 % loss of innervation ($p<0.05$) in the SOD1^{G93A} mice. We also analyzed sweat gland size and did not find differences between the two groups mice neither in the presymptomatic nor in the symptomatic stage (Figure 2).

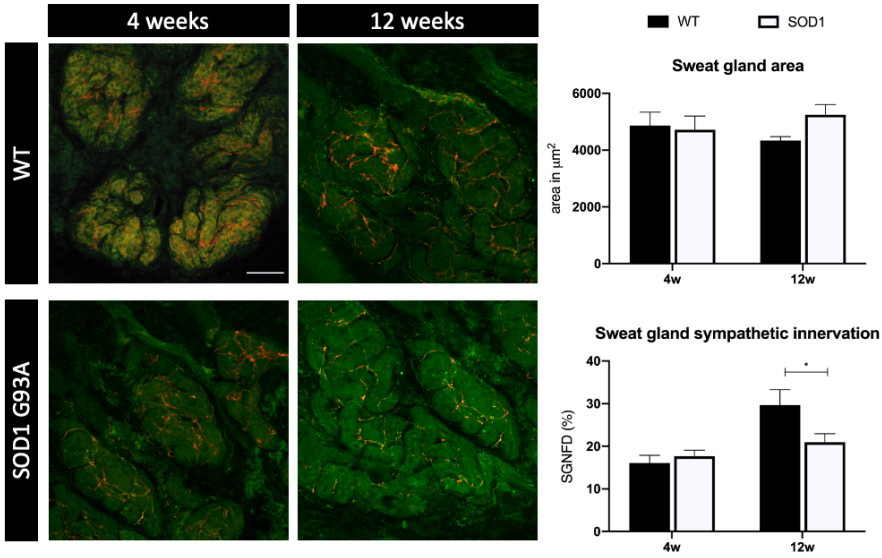


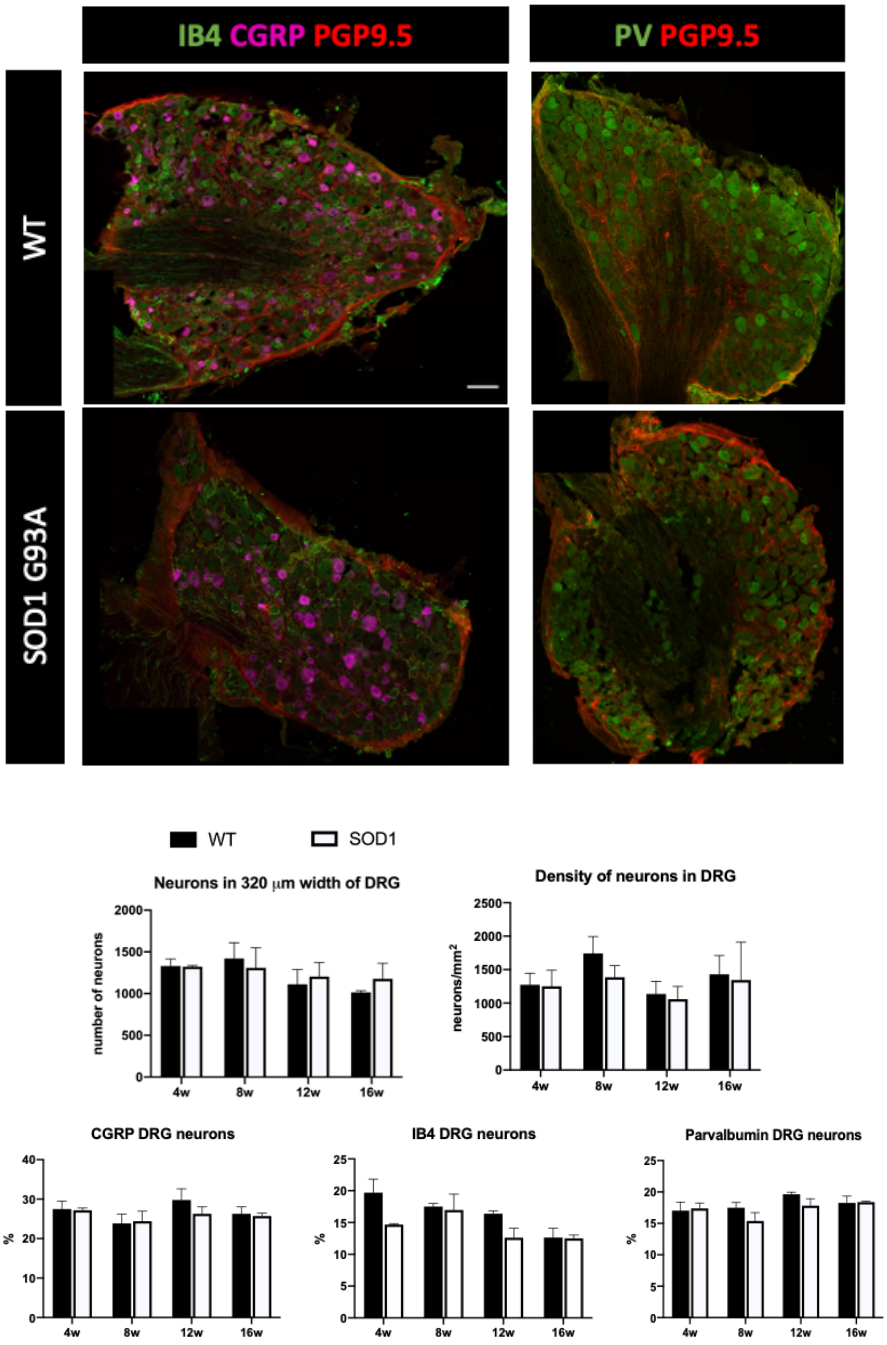
Figure 2. Sweat gland nerve fiber innervation decreases in the SOD1^{G93A} mice. ***Left***, representative microphotographs of sweat gland innervation (VIP) in SOD1 and WT mice at 4 and 12 weeks. ***Right***, the SGNFD was similar in SOD1^{G93A} and WT mice at 4 weeks of age, but at 12 weeks, it was significantly lower in the SOD1^{G93A} mice. Scale bar: 50 µm. Data are mean±SEM. *p<0.05. SGNFD: sweat gland nerve fiber density. Sweat gland area remained stable overtime in both SOD1^{G93A} and WT mice.

Dorsal root ganglion sensory populations

Firstly, to ensure that the percentage comparisons of the different sensory populations were not affected by neuronal loss, we quantified the total number of neurons in 6 sections covering a 320μm thickness of each DRG, a representative volume of the DRG, and the density of total neurons per mm^2 in the sections studied, of each mouse at each stage. We did not detect significant differences between groups at any age, neither in the total number of neurons nor in the density of neurons (Figure 3). Then, we calculated the percentage of IB4+, CGRP+ and PV+ positive neurons of the total DRG neurons of SOD1^{G93A} mice and WT littermates.

In the WT mice, CGRP+ neurons represented 26.9% (IQR 24.2-30.1), IB4+ 16.8% (IQR 15.2-17.9), and PV+ 17.8% (IQR 16.2-19.6) of total DRG neurons. A small fraction of neurons (1.26%, IQR 0.89-2.2) presented doble labeling for CGRP and IB4. In the SOD1^{G93A} mice the results were similar: CGRP+ 26.4% (IQR 24.8-27.7), IB4+ 14,3% (IQR 12.2-15.2), PV 18.0% (IQR 15.6-18.5), and CGRP+/IB4+ 0.86% (0.6-1.05). Therefore, the number of DRG neurons and the estimated proportions of CGRP+, IB4+ and PV+ did not change overtime and did not show differences between the WT and the SOD1^{G93A} mice.

Figure 3 (next page). Neuronal bodies are preserved in the DRG of the SOD1^{G93A} mice. ***Upper panel***, representative images of DRG of WT and SOD1^{G93A} mice showing CGRP, IB4 and PV sensory populations. Scale bar: 100 μm. ***Medium figures***, total number of neurons in 320 μm width of the DRG (counted in 6 sections of the DRG), and density of neurons per mm^2 in the DRG. There were no differences in the overall number nor in the density of DRG neurons. ***Lower figures,*** percentage of different DRG sensory populations of SOD1^{G93A} mice compared to the WT. There were no differences between groups and overtime in the proportion of CGRP+, IB4+ and PV+ neurons. Data are expressed as mean±SEM.


IB4 CGRP PGP9.5
PV PGP9.5
WT
SOD1 G93A
WT
SOD1
Neurons in 320 μm width of DRG
number of neurons
2000
1500
1000
500
0
4w
8w
12w
16w
Density of neurons in DRG
neurons/mm²
2500
2000
1500
1000
500
0
4w
8w
12w
16w
CGRP DRG neurons
%
40
30
20
10
0
4w
8w
12w
16w
IB4 DRG neurons
%
25
20
15
10
5
0
4w
8w
12w
16w
Parvalbumin DRG neurons
%
25
20
15
10
5
0
4w
8w
12w
16w

Discussion

In this study we found an early loss of IENF of both peptidergic and non-peptidergic neurons in the SOD1^{G93A} mice compared with their WT littermates. Although at advanced stages of the disease the loss of both populations was similar, initially IB4+ fibers were the most affected. Axons from both sensory populations share some similarities but have some differences. All form free endings in epidermis and dermis. CGRP+ sensory neurons are small myelinated (Aδ type) and unmyelinated (C type), use glutamate as main neurotransmitter and CGRP and some substance P (SP) as neuromodulators, and are regulated during development by nerve growth factor (NGF) (van Rossum, et al., 1997; Iyengar et al., 2017). Somatic CGRP sensory fibers act as polymodal nociceptors, responding to high-threshold mechanical, thermal, and chemical stimuli (McCarthy and Lawson 1990, 1997; Lawson et al., 1996). Unmyelinated CGRP+ fibers project predominantly to laminae I and IIo (outer layer) in the ventral horn of the spinal cord, whereas small myelinated CGRP axons innervate laminae I and III/IV. IB4+ axons correspond to unmyelinated C nociceptors (Stucky et al., 1999). These neurons are regulated by glial cell line derived neurotrophic factor (GDNF), although they are dependent on NGF until early postnatal stage when they switch dependence to GDNF (Molliver et al., 1997). IB4+ central projections synapse into the IIi (inner layer) Rexed lamina (Silverman and Kruger 1990; Alvarez et al., 1991).

Different factors may contribute to the different vulnerability between peptidergic and non-peptidergic axons in the early stage of the disease. It is worth mentioning that such differences are also seen after peripheral nerve damage, where IB4+ projections in the dorsal horn tend to regress, while peptidergic projections are less affected (White et al., 1990; Bennett et al.,1998; Bailey and Ribeiro-da-Silva, 2006; Casals-Díaz et al., 2007). IB4 neurons lack α7-integrin, a laminin receptor essential for optimal axonal regeneration in the peripheral nervous system, while it is expressed in CGRP and NFH (neurofilament heavy-chain) neurons (Gardiner et al., 2005). Also, cultured DRG IB4 neurons, although they respond to GDNF, exhibit a lower neurite outgrowth compared to other sensory neuronal populations (Leclere et al., 2007). In line with this, although after a peripheral nerve lesion NGF and GDNF are both upregulated (Dethleffsen et al., 2002; Funakoshi et al., 1993; Hoke A et al., 2000, Hoke A et al., 2002), the latter shows lower levels in the DRG (Leclere

et al., 2007; Cobianchi et al., 2013). In the SOD1^{G93A} mice, GDNF mRNA expression in the hindlimb muscles is indetectable at the presymptomatic stage, increases by the onset of motor symptoms and continues with a marked decrease overtime, while in ALS patients GDNF mRNA expression tends to reduce in advanced muscle pathology, and in serum from ALS patients, the levels of GDNF are markedly lower than in healthy subjects (Yamamoto et al., 1999; Stanga et al., 2018). In the skin of adult animals, GDNF and NGF are also both expressed, but in the case of GDNF at low levels, and changes of its expression after tissue damage or inflammation are unclear (Botchkareva et al., 2000). In contrast, NGF is clearly increased after skin injury (Ueda et al., 2002; Constantinou et al., 1994), causing collateral axonal sprouting and contributing to nociceptor sensitization and hyperalgesia (Diamond et al., 1992; Shu and Mendell, 1999; Reynolds and Fitzgerald, 1995). Therefore, it seems that although at advanced stages both sensory fiber populations are similarly affected, intrinsic difficulties of IB4 neurons for axonal regeneration and sprouting may explain their earlier susceptibility.

In addition to the epidermal fiber loss, our findings showed a decrease in the sweat gland innervation in the SOD1^{G93A} mice. This observation is in correspondence with reports in ALS human patients showing involvement of unmyelinated postganglionic sympathetic fibers and sweating deficiencies (Ren et al., 2018; Beck et al., 2002; Beck et al., 2005; Santos-Bento M et al., 2001). Axons innervating the sweat glands show degeneration, TDP-43 accumulation, increased intracytoplasmic granules and synaptic vesicles accumulation, in parallel to decrease of pilomotor nerve fiber density (Ren et al., 2018; Nolano et al., 2017; Provinciali et al., 1994).

Whereas the skin innervation showed a reduced number of IENF (Rubio et al., 2016), affecting both peptidergic and non-peptidergic fibers (present results) and also reduced innervation of Meissner corpuscles in SOD1^{G93A} mice, DRG analyses showed that different sensory populations are preserved, with only a marginal decrease of doble positive neurons (CGRP+ and IB4+) at 12 weeks. Regarding proprioceptors, degeneration of sensory Ia/II fibers of the muscle spindles has been observed in SOD1^{G93A} and TDP43A315T mutated mice at presymptomatic stage (Vaughan et al., 2015), despite there is no loss of proprioceptive neurons in the DRG. Therefore, the loss of distal axons with preservation of the neuronal soma suggests that distal axonopathy is the main physiopathological mechanism involved in the sensory involvement. A motor distal axonopathy has already been proposed

based on the findings of early loss of neuromuscular junctions in both animal and human ALS, before the onset of clinical symptoms, and even before motor neuron loss in the anterior horn of the spinal cord (Fischer et al., 2004; Moloney et al., 2014; Sharma et al., 2016; Xia et al., 2012; Fischer et al.,2012; Jokic et., al 2006; Teng FY et al., 2008).

Despite there is no evidence of significant neuronal loss in the DRG of ALS mice, it has been reported the occurrence of pathological changes such as cytoplasmic fragmentation, microvacuolization, macrophage and microglia recruitment (Sábado et al., 2014) and swollen mitochondria (Guo et al., 2009). Also, SOD1 protein accumulation has been observed in PV+ neurons, but not in CGRP, IB4 and SP neurons of the SOD1^{G93A} mouse. However, it has been described a loss of 53% of dorsal root axons (Fischer et al., 2005) and signs of Wallerian degeneration (Guo et al., 2009), that could be due to particular vulnerability of the sensory axons. Other studies have confirmed sympathetic preganglionic neuronal loss in the intermediate lateral column (IML) of the spinal cord (Konno et al., 1986; Kennedy et al., 1985) but characteristically with preservation of sympathetic ganglion cells (Itoh et al., 1992).

There is an increased amount of evidence regarding the role of axon homeostasis in the physiopathology of ALS: from mitochondrial dysfunction, increase in the oxidative stress radicals and deficits in axonal transport. Several genes involved in motor neuron disease in general, and in ALS in particular, are tightly involved in axonal transport (Brown et al., 2017; Castellanos-Montiel et al., 2020). Indeed, in ALS mouse models, deficit in both anterograde and retrograde transport are reported as early events (Williamson and Cleveland 1999; Bilsland et al., 2010). An increased intrinsic susceptibility to oxidative stress of the most distal portion of axons with a greater degree of oxidative stress in its surroundings was also described (Muller et al., 2006; Muller et al., 2007). Moreover, TDP-43 protein, a hallmark of both sporadic and familial forms of ALS, may affect the function of transport along the axon (Arnold et al., 2013), and can contribute to protein aggregation through axonal translational disturbances (Nagano et al., 2020).

In summary, we have found that sensory involvement in the SOD1^{G93A} mouse is located in the distal part of the axon, affecting all types of small nerve fibers, and also sympathetic sudomotor fibers. There are slight differences in the rate of IENF

reduction that could translate intrinsic susceptibility to injury. Furthermore, analysis of peripheral axonal endings and their neuronal bodies in the DRG, confirm that while there is a significant loss of the distal part of the axons, their somas are preserved, suggesting a distal sensory axonopathy as underlying process, similarly to what was confirmed in the motor counterpart of the disease.

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RESULTS

CHAPTER 3
TDP-43 cytoplasmic translocation in intraepidermal axons and in skin fibroblasts of ALS patients

TDP-43 cytoplasmic translocation in intraepidermal axons and in skin fibroblasts of ALS patients

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Abstract

Objective. Diagnosis of ALS is based on clinical symptoms that appear when motoneuron degeneration is significant and difficult to revert. Therefore, new approaches for early diagnosis are needed. We aimed to assess if alterations in appearance and cellular localization of cutaneous TDP-43 in cutaneous axons and in non-neural structures may represent a biomarker for ALS.

Methods. Skin biopsies from 64 subjects were analyzed: 44 ALS patients, 10 healthy controls (HC) and 10 neurological controls (NC) (5 patients with Parkinson's disease and 5 with multiple sclerosis). TDP-43 immunoreactivity and co-localization with epidermal fibers (PGP 9.5) was studied, as well as the presence of TDP-43 in epidermis and dermis. The percentage of cells with TDP-43 cytoplasmic localization in defined areas of epidermis and dermis (papillary and reticular) was quantified. ROC analyses were also performed. A subset of ALS patients was again biopsied 12 months later for comparison over time.

Results. We did not find co-localization of TDP-43 and intraepidermal fibers. We detected higher amount of TDP-43 in epidermis ($p<0.001$) and in both layers of dermis ($p<0.001$), as well as higher percentage of TDP-43 cytoplasmic positive cells ($p<0.001$) in the ALS group compared to HC and NC groups. Dermal cells containing TDP-43 were fibroblasts as identified by co-labeling against vimentin. ROC analyses (AUC 0.867, $p<0.001$; CI 95% 0.800-0.935) showed that detection of 24.1% cells with cytoplasmic TDP-43 positivity in the dermis had 85% sensitivity and 80% specificity for detecting ALS. We did not find significant correlation with clinical features.

Conclusions. We have not identified the presence of TDP-43 in sensory epidermal fibers, but we have found significantly increased TDP-43 levels in epidermis and in the cytoplasm of dermal cells of ALS patients. Our findings provide support to the use of TDP-43 in skin biopsies as a potential biomarker.

Key words: amyotrophic lateral sclerosis, dermis, skin biopsy, TDP-43, biomarker

Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder affecting motor neurons from cortex, brainstem, and spinal cord. While most are sporadic cases, 10-15% are familial forms (1,2). Studies derived from the genetic forms have expanded the spectrum of the disease to extra-motor manifestations like cognitive impairment, extrapyramidal or neuropsychiatric symptoms (3-6). Diagnosis is based on clinical symptoms and, often is established relatively late; therefore, there is a need to identify biomarkers for the early diagnosis of ALS that could allow to monitor disease progression and to start early neuroprotective treatment to prevent the motoneuron degeneration. The 43-kDa TAR DNA-binding protein (TDP-43) is a ubiquitous DNA binding protein with multiple functions encoded by the *TARDBP* gene, and its mutations have been associated with autosomal dominant ALS and frontotemporal dementia (FTD) (7,8). However, the more than 50 missense mutations identified in *TARDBP* only account for 1-2% of total ALS cases (9). Nevertheless, the importance of TDP-43 in ALS relies on the fact that it is a major component of the ubiquitinated insoluble cytoplasmic inclusions, concomitant with a loss of nuclear TDP-43 in upper and lower motor neurons and in other regions of the central nervous system in most patients (both sporadic and familial, with or without *TARDBP* mutations) (10). These inclusions are widespread, regardless the location of symptoms onset, and considered a pathological hallmark of ALS-FTD spectrum (11,12). In healthy motoneurons TDP-43 protein is located in the nucleus, suggesting that the increased TDP-43 cytoplasmic translocation may be a potential pathological biomarker. However, monitoring this alteration using biopsies from the central nervous system is unpractical. Nevertheless, alterations in the skin and other tissues may precede or appear concomitantly with neurological symptoms in some neurodegenerative diseases (13). Loss of intraepidermal nerve fibers has been described in ALS patients (14-16) and in animal models (17-19). However, it is not known whether, in addition to sensory axonal loss, other pathological changes are also present, as in motor neurons, such as the cytoplasmic presence of TDP-43.

Also, despite the variability among the studies performed on either skin biopsies, cultured fibroblasts or engineered skin tissue, there is a common finding of TDP-43 cytoplasmic accumulation in the skin of sporadic and familial ALS patients (20-27). Nonetheless, there is not a well-defined relationship with disease progression and

other clinical features. Moreover, nuclear TDP-43 expression is found in skin cells, fibroblasts, keratinocytes, Langerhans cells and melanocytes, of healthy individuals, but importantly, much lesser amounts are found in the cytoplasm (28,29). Therefore, although cutaneous TDP-43 seems a promising candidate as a minimally invasive biomarker of the disease, its role is still undefined (30). Quantitative detailed studies are needed to define the role of skin TDP-43 and its relationship with clinical features. The objective of this study was to further investigate the cytoplasmic localization of TDP-43 in the skin of ALS patients, in sensory fibers and in non-neuronal structures, quantifying its accumulation to identify more accessible histopathological hallmarks of ALS.

Methods

Participants and samples

Forty-four subjects with definite ALS and 10 healthy controls (HC) were prospectively recruited by the ALS units from Hospital del Mar and Hospital de Bellvitge, Barcelona. A third group of 10 neurological controls (NC) were also selected. ALS patients fulfilled the diagnostic criteria (revised El Escorial criteria) (31) and were selected by experienced neurologists specialized in motor neuron diseases. ALS patients were 22 females and 22 males, with a median age of 66 years (IQR 58-73). Healthy subjects did not have any signs, symptoms, or history of neurological diseases; 6 were female, with a median age of 59 years (IQR 43-62). The NC group consisted of 5 patients with Parkinson disease and 5 with relapsing-remitting multiple sclerosis, 4 of them were female, and had a median age of 59 years (IQR 54-66). There were no statistically significant differences in sociodemographic variables between the groups. Clinical data as site of onset, time from onset of the disease to the biopsy and ALSFR-R slope were obtained for the ALS group. Of the ALS patients, 18 (40.9%) had spinal onset and 26 bulbar onset (59.1%), and the median ALSFR-R slope at the time of the skin biopsy was 1.29 (IQR 0.63-2.18). Median time from onset of ALS symptoms to biopsy was 11.5 months (IQR 8.52-19.13). All ALS patients were tested for the hexanucleotide expansion in the *C9ORF72* gene, and in all but one, no pathological expansion was detected. The study was approved by the local ethics committee of both participating hospitals. Written informed consent for skin biopsy was obtained from all subjects.

Any method detail and data not published within the article will be shared anonymized by reasonable request from any qualified investigator.

Skin biopsy and processing

Three-millimeter skin punch biopsies were obtained from the distal leg after local anesthesia with mepivacaine. Skin biopsies were fixed in paraformaldehyde solution at 4% and maintained at 4ºC in PB (phosphate buffer) with sucrose. Then, biopsies were cut by cryotome into 60-µm thick sections collecting the slices in PBS.

For immunofluorescence, sections were blocked with endogenous Biotin-Blocking Kit (Invitrogen) and TBS-Triton 0.3%-normal donkey serum 10%, and subsequently incubated overnight at 4ºC with anti-TDP-43 antibody (1:200, cat#12892-1-AP, Proteintech), anti-vimentin (1:200, Sigma) and rabbit anti-protein gene product 9.5 (PGP9.5, 1:50, Cedarlane) as primary antibodies. After washes, sections were incubated overnight at 4ºC with horse anti-rabbit biotinylated antibody (1:200, Vector Laboratories), conjugated streptavidin Alexa Fluor 488 (1:200, ThermoFischer) or streptavidin Alexa Fluor 594 (1:200, ThermoFischer) and donkey anti-rabbit cyanine 3 (1:200, Jackson Immunoresearch) as secondary antibodies. Slices were then transferred to gelatinized slides and mounted in Fluoromount G (Sourthern Biotech). To assess antibody specificity, control samples were processed in parallel as described before but without primary antibodies. DAPI staining was used to ensure correct recognition of tissue structures. The anti-TDP43 antibody used in this study recognizes the C-terminal cleavage product (20-30 KDa) and the native and phosphorylated forms of TDP-43. Antibodies against the non-phosphorylated form have been widely used to determine the TDP-43 translocation to the cytoplasm without the risk of missing different post-translational modifications and non-phosphorylated cytosolic distribution of TDP-43 (12, 22, 32). In the studies published to date on skin of ALS patients, only two have used antibodies against p-TDP-43, finding no labeling in one of them (33) and labeling only 1/3 of the cases in the other (34). The rest of the studies have successfully used antibodies against the non-phosphorylated form (20-27).

Intraepidermal innervation and TDP-43 nerve colocalization

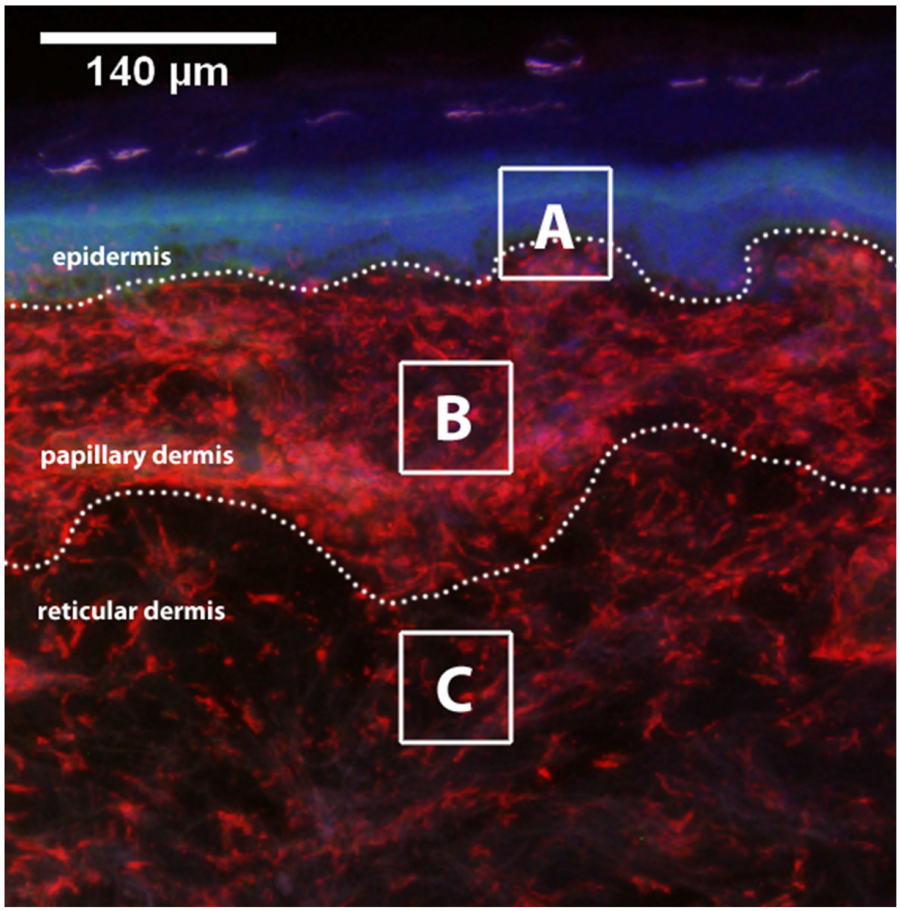
To study the possible colocalization of TDP-43 in the cutaneous innervation, we performed immunohistochemical co-labeling for PGP9.5 and TDP-43. Confocal images were taken with a confocal microscope for counting intraepidermal nerve fibers (IENF) and for TDP-43 colocalization assessment. IENF were measured in a subset of ALS and HC subjects. Individual fibers were counted as they pass through the basement membrane, whereas branching occurring within the epidermis did not increase the number of the IENFs counted. IENF density was expressed as the number of fibers per 1 mm length of the epidermis. Average density of IENFs in two images per sample was then derived.

Confocal imaging and measurements

Immunolabeled sections were first viewed under an Olympus BX-51 microscope equipped for epifluorescence using appropriate filters. Areas of interest were analyzed with a scanning confocal microscope (Figure 1).

Epidermal TDP-43 amount was measured in microphotographs taken from two representative areas of epidermis (101.61x101.61 μm^2 each) of each case, using ImageJ software. The mean of the two measures of the percentage of area with TDP-43 labeling was calculated after defining the threshold background correction.

The two layers of the dermis (papillary and reticular dermis) were evaluated separately. This distinction was made given that papillary and reticular cells have different pattern of protein synthesis and expression (32,35). Differentiation of both layers was based on visual recognition of different density of connective tissue, and the superficial vascular plexus at the boundaries between both layers. Images of two representative areas (101.61x101.61 μm^2 each) of papillary and reticular dermis were taken and analyzed. The levels of cytoplasmic TDP-43 were quantified as: 1) percentage of cytoplasmic TDP-43 immunoreactivity, and 2) percentage of cells with positive TDP-43 into the cytoplasm in each defined area. For calculating the percentage of cytoplasmic TDP43 immunoreactivity, first a threshold was defined for background correction, then the percentage of pixels in the area above threshold of TDP-43 labeling was measured using ImageJ software. Additionally, the mean percentage of cells with TDP-43 positivity within their cytoplasm was calculated in the analyzed confocal images. Results are expressed as the mean of the two measures per subject and layer.


140 µm
A
epidermis
B
papillary dermis
reticular dermis
C

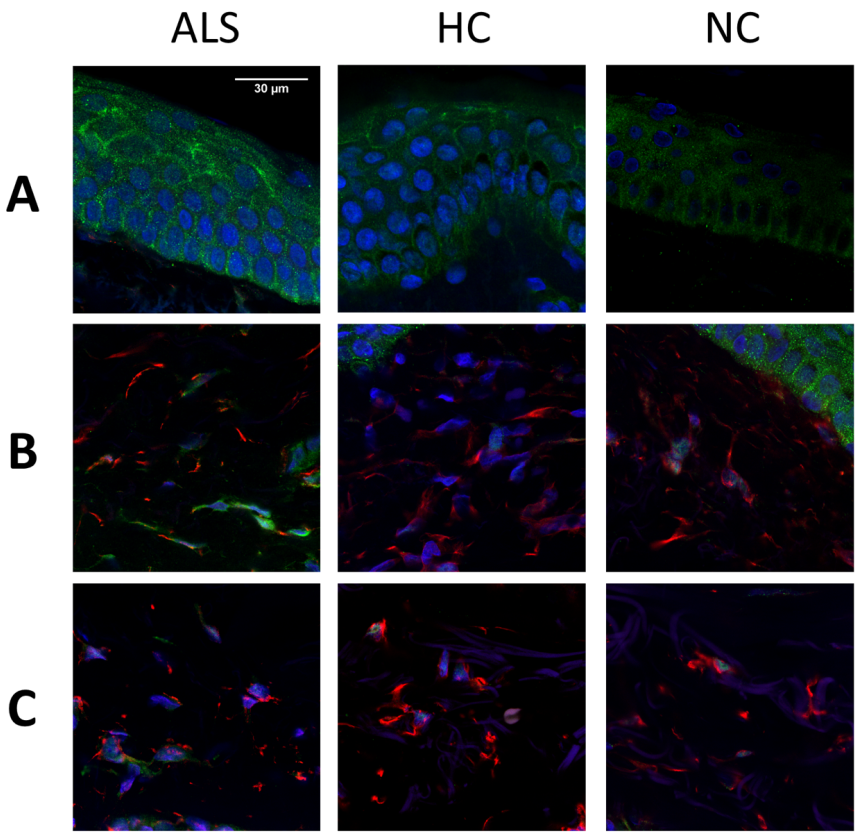


Figure 1. TDP-43 quantification in keratinocytes and dermal cells. Cytoplasmic TDP-43 immunoreactivity was analyzed in at least two areas of epidermis, papillary and reticular dermis (dotted rectangular area) per case (***first panel from previous page***). In the ***second panel*** representative confocal microscopy images of epidermis, papillary dermis and reticular dermis from ALS, HC and NC subjects are shown. TDP-43 was found profusely in epidermis in all cases although with higher density in the ALS group ($p<0.001$) compared to both HC and NC groups. In the dermis of HC and NC subjects TDP-43 was mostly seen in the nucleus, whereas cytoplasmic aggregations were more present in ALS cases in papillary and reticular dermis ($p<0.001$). ALS: amyotrophic lateral sclerosis, HC: healthy controls; NC: neurological controls.

Data analysis

Data are expressed as mean ± SEM. Means were compared by ANOVA applying Tukey's post hoc test when necessary (SPSS statistics 19 software). The level of significance was set at $p < 0.05$. Pearson's correlation coefficient was used to assess possible linear association between two continuous quantitative variables. In order to get an estimate of sensitivity and specificity of TDP-43 quantification regarding ALS diagnosis, ROC curves and area under the curve (AUC) were calculated and optimal cut-off values were selected using Youden's index.

Results

TDP-43 and epidermal nerve fibers

We analyzed the IENF density of 18 ALS patients and 6 controls. The ALS group presented lower values of IENF (7.78±0.80; p=0.010) compared with HC (12.75±2.20) and NC (10.89±1.77). However, no colocalization of TDP-43 was found in PGP9.5 labeled fibers either in ALS patients HC or NC (Figure 2).

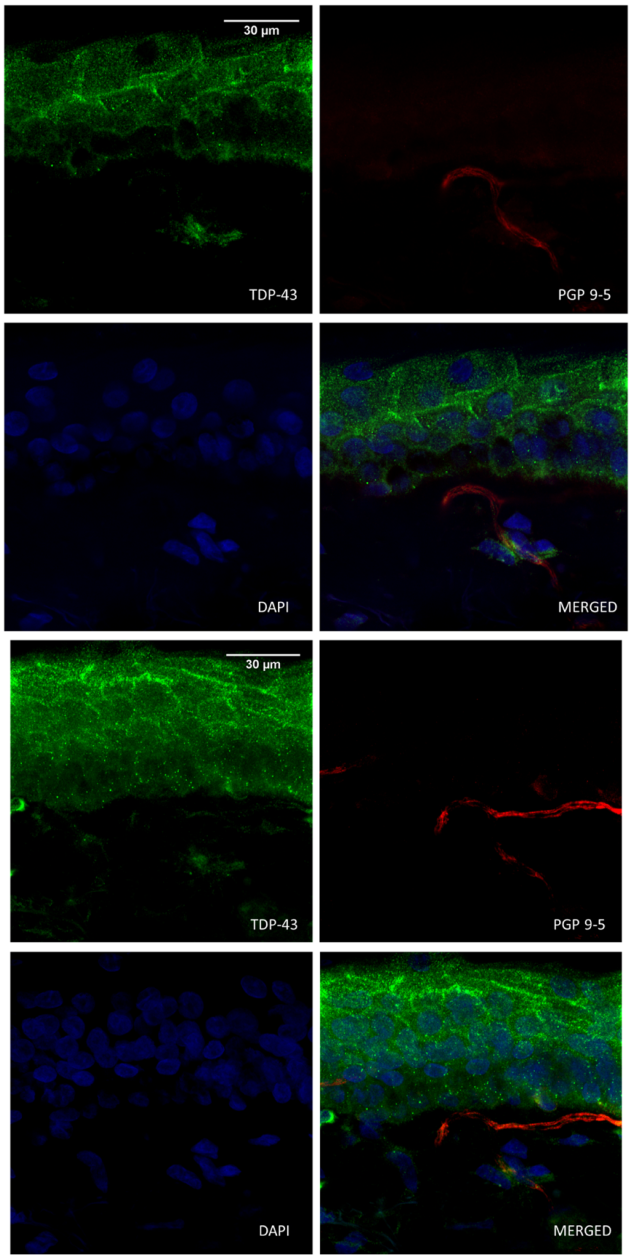


Figure 2. TDP-43 and epidermal nerve fibers do not colocalize. Representative images of ALS skin sections immunohistochemically labeled for PGP 9.5 (in red) to identify the innervation and for TDP-43 (in green). In the images, subepidermal nerve plexus and intraepidermal nerve fibers are shown with no colocalization of TDP-43 immunoreactivity.

TDP-43 cytoplasmic localization in ALS patients skin biopsies

To determine cytoplasmic TDP-43 accumulation, a signature of ALS pathology, skin biopsies were labeled with polyclonal antibody against TDP-43. The results of TDP-43 immunoreactivity levels and proportion of cells with cytoplasmic TDP-43 positivity in epidermis, papillary and reticular dermis are summarized in Table 1.

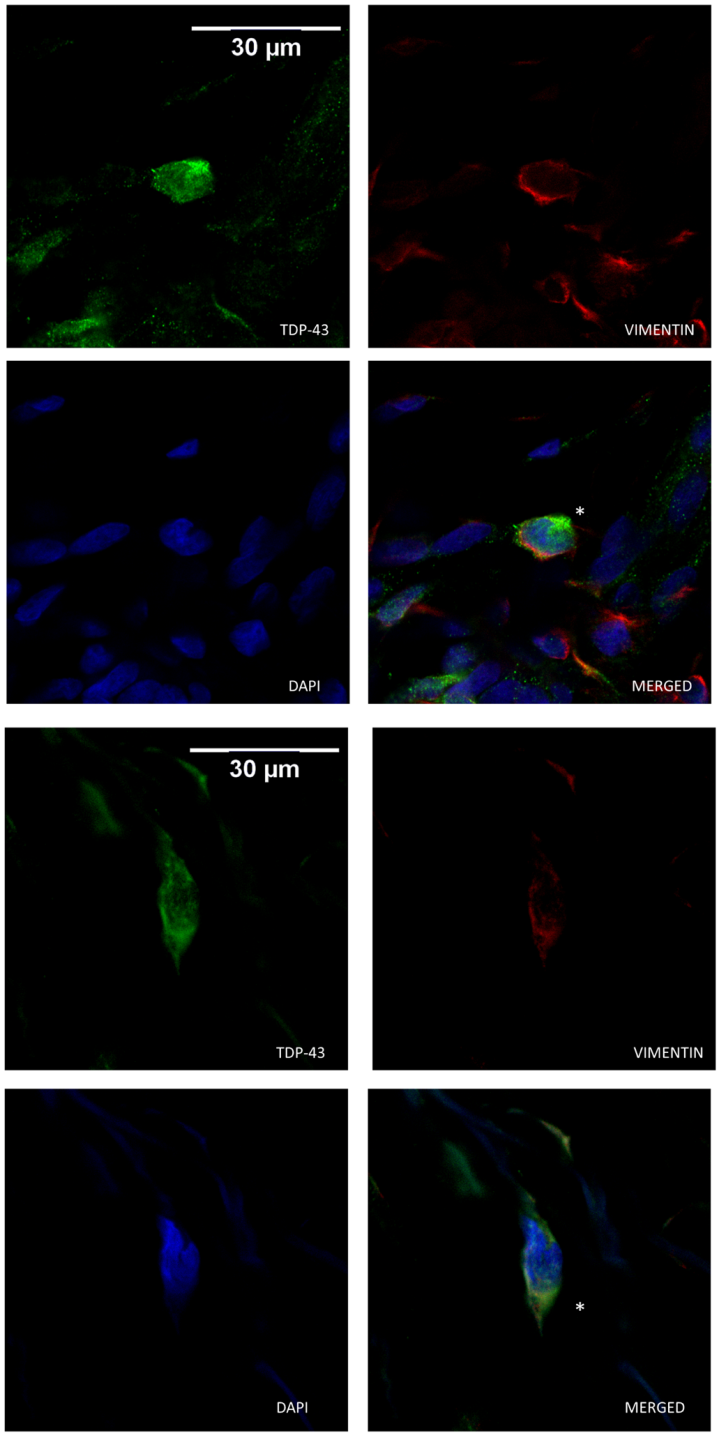
Table 1. Results of TDP-43 immunoreactivity and cells with cytoplasmic TDP-43 positive cells. Values are shown as mean and SEM. ALS: amyotrophic lateral sclerosis, HC: healthy controls; NC: neurological controls (5 multiple sclerosis and 5 Parkinson's disease), IR: immunoreactivity.

	ALS (N=44)	HC (N=10)	NC (N=10)	p value
Epidermis				
% TDP-43 IR	12.94 ± 1.05	7.19 ± 0.97	4.37 ± 1.25	<0.001
Papillary Dermis				
% TDP-43 IR	1.33 ± 0.17	0.13 ± 0.05	0.31 ± 0.07	<0.001
% Cytoplasmic TDP-43^{+} cells	50.88 ± 3.91	19.11 ± 5.2	16.58 ± 4.85	<0.001
Reticular Dermis				
% TDP-43 IR	0.82 ± 0.09	0.18 ± 0.07	0.10 ± 0.03	<0.001
% Cytoplasmic TDP-43^{+} cells	50.36 ± 3.64	21.73 ± 6.40	11.75 ± 5.88	<0.001

In the epidermis, TDP-43 was distributed in stratum basale and stratum spinosum. Absence of TDP-43 in outer layers of the skin may be explained by the process of terminal differentiation of keratinocytes in granulosum and corneum strata. We detected a higher expression of TDP-43 in epidermal cells ($p<0.001$) in ALS group (12.9±1.0%) compared to HC (7.2±1.0%) and NC (4.4±1.2%).

Cytoplasmic TDP-43 was detected in dermal cells of ALS patients and controls. However, a higher number of positive cells for cytosolic labeling of TDP-43 were found in ALS patients than in healthy and NC. Those dermal cells were identified as fibroblasts as they were positively marked with vimentin (Figure 3). Unlike what was seen in the epidermis, fibroblasts expressing TDP-43 were not widely distributed, and represented only a small percentage of dermal cells. The immunoreactivity against TDP-43 as well as the percentage of fibroblast cells with cytoplasmic label for TDP-43 were significantly increased in the ALS group compared with the HC and NC groups in both the papillary and reticular dermis (Table 1).

Figure 3 (next page). Dermal cells showing cytoplasmic TDP-43 are fibroblasts. Cytoplasmic TDP-43 positive cells were identified as fibroblasts based on vimentin immunoreactivity. Representative confocal microscopy images of ALS dermal fibroblasts co-immunolabeled for TDP-43 (in green), vimentin (in red) and DAPI nuclear staining (in blue).


30 µm
TDP-43
VIMENTIN
DAPI
*
MERGED
30 µm
TDP-43
VIMENTIN
DAPI
*
MERGED

We performed a ROC analysis based on the percentage of TDP-43 cytoplasmic positive cells of the overall dermis as well as on the percentage of immunoreactivity to achieve a theoretical cut-off point as a proof of concept of its value as a marker of the disease. ROC curves and AUC (0.867, p<0.001; CI 95% 0.800-0.935) were calculated for TDP-43 positive dermal cells, including measures from superficial/deep dermis, and cut-off values were selected according to optimal Youden's index. Detection of 24.14% of cells with cytoplasmic TDP-43 positivity in the dermis had 85.2% sensitivity and 80.0% specificity, with a positive predictive value of 90.4% and negative predictive value of 71.1% (Figure 4). Considering the proportion of TDP-43 immunoreactivity in the dermis, ROC analyses showed an AUC of 0.911 (p<0.001, CI 95% 0.862-0.960) and cut-off values of 0.26%, with 88.6% sensitivity, 82.5% specificity, 91.8% of positive predictive value and 76.7% of negative predictive value for detecting ALS.

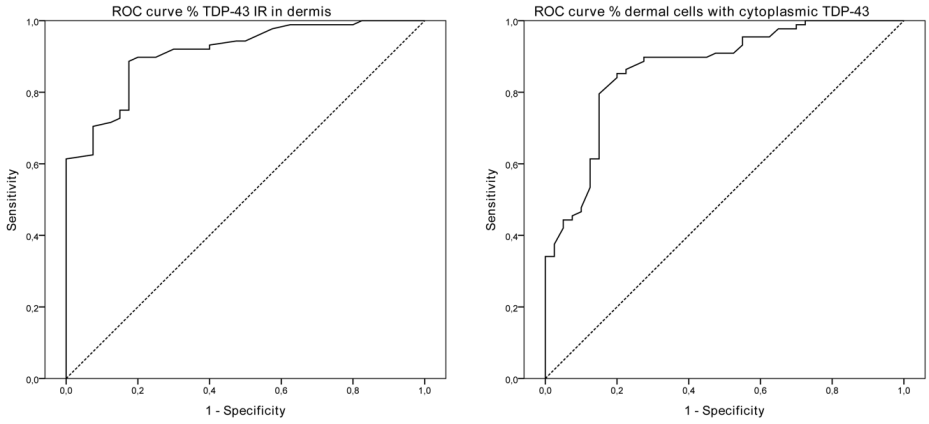


Figure 4. ROC curves of the percentage of TDP-43 immunoreactivity in both layers of the dermis (AUC 0.911, CI 95% 0.862-0.960, p<0.001) and of the percentage of dermal cells with cytoplasmic TDP-43 labeling (AUC 0.867, CI 95% 0.800-0.935, p<0.001) in ALS patients. ROC: receiver operation characteristic; AUC: area under the ROC curve.

There were no differences in TDP-43 levels (immunoreactivity and percentage of cells with cytoplasmic TDP-43 positivity) in epidermis and papillary dermis according to gender and site of onset in ALS patients. Only in the reticular dermis we found a significant lower TDP-43 immunoreactivity in bulbar onset patients, but no

differences were found considering percentage of cells with cytoplasmic TDP-43 localization. No significant correlations were found between TDP-43 expression measurements and age of onset of the disease, time from symptoms onset to biopsy and ALSFR-R slope (Table 2).

Table 2. TDP-43 quantification and clinical features of ALS patients (N=44). IR: immunoreactivity. R: Pearson correlation coefficient.

	Epidermis	Superficial Dermis		Deep dermis	
	% TDP-43 IR	% TDP-43 IR	% Cytoplasmic TDP-43$^+$ cells	% TDP-43 IR	% Cytoplasmic TDP-43$^+$cells
Gender					
male	14.66±1.33	1.24±0.19	47.37±5.60	0.85±0.12	50.96±5.34
female	11.21±1.57	1.43±0.29	54.39±5.49	0.81±0.13	47.95±4.50
	p=0.102	p=0.601	p=0.376	p=0.832	p=0.669
Site of onset					
spinal	14.31±1.21	1.33±0.20	46.57±5.30	0.98±0.12	49.02±4.40
bulbar	10.52±1.86	1.34±0.34	58.43±5.12	0.55±0.08	50.21±5.76
	p=0.083	p=0.991	p=0.147	p=0.017	p=0.871
Age	R=-0.050	R=-0.180	R=-0.199	R=0.043	R=-0.114
	p=0.747	p=0.243	p=0.194	p=0.780	P=0.460
Time from	R=0.071	R=0.032	R=-0.115	R=0.053	R=-0.119
onset to biopsy	p=0.646	p=0.836	p=0.457	p=0.731	p=0.440
ALSFR-R slope	R=-0.169	R=-0.205	R=-0.046	R=-0.086	R=0.057
	p=0.277	p=0.186	p=0.772	p=0.585	p=0.717

TDP-43 changes over time

A small subset of ALS patients (8 in total; 3 bulbar and 5 spinal onset) underwent a second skin biopsy 12 months later. There were no significant differences in levels of TDP-43 immunoreactivity in epidermis (17.29 ±5.33 vs 18.58±3.68; p=0.831), superficial dermis (1.17±0.34 vs 8.58±6.24; p=0.283) and deep dermis (3.85 ±3.17 vs 16.29±11.32 p=0.336) between the initial biopsy and 12 months later. The increase in levels of TDP-43 in the dermis at the second biopsy did not achieve statistical significance due to the low number of samples analyzed. However, the percentage of cells with cytoplasmic TDP-43 translocation in the dermis increased in the second biopsy, with differences being significant for the papillary dermis (34.82±7.79 vs 62.13±3.82; p=0.034), but not for the reticular dermis (49.73±10.01 vs 62.38±2.32; p=0.241).

Discussion

As expected, based on previous observations (14), we have found a lower IENF density in the ALS group, but we did not find any colocalization of TDP-43 in dermal or intraepidermal nerve fibers. To date only one study have shown deposition of TDP-43 in autonomic dermal fibers, but only in one third of the subjects studied (34). Also, only in one patient (of a total of 18) presented TDP-43 deposits in the subepidermal plexus and around hair follicles. It is unclear if the lack of such deposits is due to intrinsic features of the pathology in the sensory axons, whether it is related to the large distance between the nucleus of the cell and the distal portion of their axons, or whether the lack of detection is due to methodological and conformational aspects of the protein (36).

On the other hand, the results of this study show that cytosolic localization of TDP-43 is increased in the skin cells of ALS patients and can be detected by immunofluorescence of skin biopsies. TDP-43 presence in the cytoplasm of dermal fibroblasts is particularly relevant and represents a potentially useful biomarker of ALS.

TDP-43 cytoplasmic deposition and its clearance from the nucleus have been observed beyond motoneurons in other areas of the nervous system, and in extra-neural tissues (25, 37-40) . However, few studies have explored the presence of

TPD-43 protein in skin of ALS patients, and quantitative information from the dermis of skin biopsies was missing. Suzuki et al (20) analyzed skin biopsies from 15 ALS patients and 15 neurological controls, and found higher amount of TDP-43 in ALS group although they did not report on the nuclear/cytoplasmic localization of the protein. They also observed TDP-43 immunoreactivity in both neurological controls and ALS in blood vessels and glands, but lower in the case of controls (38). In a tissue engineered model derived from fibroblasts of 12 ALS patients (6 sporadic and 6 familial *C9ORF72* cases), it was found an increased accumulation of cytoplasmic TDP-43 in ALS patients (30%) compared to HC (4%) in epidermis, dermo-epidermal junction, and dermis (20). Wang et al (21) observed in patients harboring *TARDBP* A315T mutation that cytoplasmic TDP-43 presence occurs mainly in epidermis but also in dermis, although no quantification was performed. Another study with skin biopsies from 22 ALS patients and 26 neurological controls (neuropathies) (25) analyzed TDP-43 mRNA expression, epidermal TDP-43 immunoblot and percentage of TDP-43 positive cells in epidermis. They did not find differences in the immunohistochemical analysis, although differences in immunoblot TDP-43 expression with a reduction of mRNA in ALS epidermis suggested a dysregulation of protein expression.

Studies on cultured fibroblasts have shown abnormal TDP-43 cytoplasmic aggregations in ALS (sporadic and familial cases of *SOD1, TARDBP, FUS* and *C9ORF72* mutations) (23), even with different patterns regarding nuclear/cytoplasmic deposition ratio between patients harboring different mutations. Other studies have focused on the cytoplasmic aggregation in cultured fibroblasts under the presence of stress in both sporadic and familial cases (*UBQLN2, UBQLN1, TARDBP*) (24,26,27). Only one study to date based on cultured fibroblasts from sporadic ALS patients did not detect any TDP-43 accumulation (33). Authors speculated that methodological aspects could explain differences from other similar studies.

TDP-43 accumulation is not homogenous throughout the motor nervous system of ALS patients (12). Full length protein can be found in both cortical and spinal motor neurons, but C-terminal fragments have only been observed in brain neurons (41,42). These differences in affected neurons raise the question if TDP-43 proteinopathy may be heterogeneous also in non-neuronal cells (43).

We did not find correlation between TDP-43 deposition and clinical data. There are only few previous reports about clinical correlation, and only disease duration was analyzed with contradictory results. In the subgroup of patients with a second biopsy 12 months later, there was a high variability of TDP-43 immunoreactivity between first and second biopsies. Although changes were not significant, a higher number of dermal cells with cytoplasmic TDP-43 deposition were detected in the superficial dermis. However, the conclusions from this subgroup should be taken with caution due to the small number of patients, and the limited time interval between biopsies in a fast progression disease such as ALS.

Albeit having some distinctive histopathological features, ALS lacks an accessible biomarker. Diagnosis is based on clinical criteria and supported by neurophysiological studies (31, 45, 46). Although these criteria have been revised to achieve an early diagnosis with a proper sensitivity and specificity, the time from symptoms onset to diagnosis ranges from 8 to 16 months, and even a not negligible proportion of patients die without achieving a sufficient diagnostic certainty (47,48). Even in the absence of a curative treatment, benefit of a biomarker should not be overlooked, whether it is for the possibility of an earlier diagnosis in suspected cases, presymptomatic detection, and an optimal selection of candidates for clinical trials. For this reason, determination of TDP-43 in corporal fluids or tissue as a potential biomarker has been pursued (49). Different approaches have been made for the detection and quantification of TDP-43 in blood (50-52) and cerebrospinal fluid (CSF) in ALS and FTD (53-56). However, the ubiquitous nature of the TDP-43 protein makes its detection and interpretation more difficult compared to the detection of amyloid or tau (purely neuronal proteins) in Alzheimer's disease (57).

TDP-43 cytoplasmic localization is absent in intraepidermal axons but present in epidermal keratinocytes and in dermal fibroblasts of ALS and HC; and quantitative analyses show significantly larger amounts in ALS patients. Quantitative values of dermal cells with TDP-43 appear to offer a sensitive and minimally invasive biomarker. Further experiments will be needed to explore specifically the levels of post-translational modifications of TDP-43 in these samples, such as phosphorylation, ubiquitylation and truncated forms. Despite the number of skin samples from ALS patients represents the larger series reported to date, a higher amount is needed to achieve practical cut-off values of TDP-43 deposition for a diagnosis purpose. Nevertheless, even considering the limitations, this work

represents the proof of concept of cutaneous TDP-43 as a potential biomarker for ALS.

Funding. This work was supported by project RTI2018-096386-B-I00 from Ministerio de Ciencia, Innovación y Universidades and Agencia Española de Investigación of Spain, CIBERNED (CB06/05/1105) and TERCEL (RD16/0011/0014) funds from the Instituto de Salud Carlos III of Spain, co-funded by European Union (ERDF/ESF, "Investing in your future").

Ethical approval. All procedures were approved by the respective Ethics Committee and Review board of the participating centers.

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GENERAL DISCUSSION

The main objective of this thesis was to better characterize the involvement of the somatosensory system in ALS. Historically, ALS has been considered an exclusively motor disease with several manifestations believed to be mandatory absent such as cognitive impairment, bladder dysfunction and sensory involvement. However, to date, many of these statements have proven to be wrong, widening the concept of the disease. Sensory impairment has been confirmed in previous works involving both central and peripheral nervous system, but description of its evolution overtime and physiopathological mechanisms have been lacking. We have focused on peripheral sensory involvement, to parallel the results with the well-studied peripheral motor impairment.

Given that cutaneous free endings are the terminal part of peripheral sensory afferences, and skin fiber loss has been described in ALS patients, we investigated if those changes were also present in the $SOD1^{G93A}$ mouse, the most used animal model for ALS. By using this animal model, it was possible to make deeper studies that are not feasible in human cases and bring light into the sensory alterations associated to ALS. For this purpose, we have analyzed IENF density, subepidermal plexus and dermal innervation as well as Meissner's corpuscles, the latter as an example of cutaneous large axons. We have performed immunohistochemical analyses of the footpads of the hindlimbs of $SOD1^{G93A}$ mice, along the life of the animals, and compared them with their wildtype littermates. In this first part we have found a decrease in IENF density, even at early, presymptomatic stages. Further analyses of the changes in epidermal, subepidermal plexus and dermal fiber density showed a gradient of loss from the most superficial layer of the skin to the deepest, that increased overtime. This suggested that loss of sensory innervation in the skin of the $SOD1^{G93A}$ mice follows a distal axonopathy pattern. Our study was the first to demonstrate the loss of terminal skin innervation in this model.

In the second part we aimed to further characterize the sensory involvement in the animal model of the disease, in terms of different sensory populations that could be affected, as well as its anatomical and temporal evolution. We performed immunohistochemical studies of footpads and DRG of SOD1^{G93A} mice at different stages of the disease (4, 8, 12 and 16 weeks) with their corresponding wildtype mice as controls. Sensory cutaneous populations analyzed were those expressing IB4 (non-peptidergic C fibers), CGRP (peptidergic C fibers), and VIP (cholinergic sudomotor C fibers) innervating eccrine sweat glands. Analysis of the dorsal root ganglia (DRG) allowed studying the soma of the IB4 and CGRP positive neurons, and also PV expressing neurons, corresponding to proprioceptors. Our results showed that in skin, although initially CGRP+ fibers were more preserved than IB4+ ones, all sensory populations were similarly affected with time. VIP fibers were also decreased in the SOD1^{G93A} mice at a symptomatic stage. Interestingly during the disease, none of the neuron populations in DRG were lost. These findings supported, as mentioned in the first part, the existence of a distal axonal neuropathy.

In the third part of our work, we studied the sensory cutaneous innervation in ALS patients, and aimed to find whether there were other pathological findings pathognomonic of ALS in addition to axonal loss. Given that in the previous studies we had not seen differences in the sensory populations affected, we focused on TDP-43 proteinopathy. TDP-43 deposits are considered a pathological hallmark of the ALS-FTLD spectrum. TDP-43 cytoplasmic translocation is present in sporadic and familial forms of ALS, except for those secondary to *SOD1* and *FUS* mutations (Neumann et al., 2006; Kwong et al., 2007). As expected, the ALS group presented a lower IENF density than the control subjects, but we did not find co-labeling of TDP-43 in the cutaneous sensory fibers. This observation may suggest that, at least, TDP-43 proteinopathy does not play a role in sensory distal axonopathy in humans. One interesting finding from that work is that TDP-43 was found expressed in the cytoplasm of dermal fibroblasts in a high proportion of cells in ALS patients. We quantified cytoplasmic immunoreactivity and the number of fibroblasts with cytoplasmic TDP-43 deposits and found them significantly increased in the ALS group compared to both healthy and neurological control groups. Moreover, ROC and AUC analyses showed that detection of 24.1% of dermal cells with TDP-43 cytoplasmic translocation represents 85% sensitivity and 80% specificity for ALS.

In humans, a few studies have previously explored the involvement of small sensory fibers in ALS and did not find correlations with clinical and epidemiological data (Dalla Bella et al., 2016). We have identified that loss of cutaneous small sensory fibers increased overtime, and that is already present at presymptomatic stage of the SOD1^{G93A} mouse. Since the diagnosis of ALS is made when disease is already well-developed and advanced, it seems unreliable to correlate its findings with chronological variables such as survival, rate of progression and disease duration.

Moreover, it is important to stress that not only small somatic sensory axons are affected in the SOD1^{G93A} mouse, but also autonomic axons and myelinated axons forming Meissner corpuscles. The latter are of special interest since they are the terminals of Aβ axons, larger than the intraepidermal ones, although it has been described that are intertwined with both peptidergic and non-peptidergic C-fiber endings with nociceptive functions, in addition with the mechanoreceptive properties (Paré et al., 2001). Other authors have described disturbances in autonomic axons in the skin of ALS patients such as swellings, intracytoplasmic granules and accumulation of synaptic vesicles, and even axonal loss and TDP-43 deposits in eccrine sweat glands, as well as denervation of arrector pili muscles (Ren et al., 2018; Nolano et al., 2016; Provinciali et al., 1994).

Sensory neurons are more diverse in function, morphology, and molecular expression than motor neurons. For this reason, the study of selective vulnerability between different sensory neuronal populations may be of particular interest. Although all sensory distal axons showed lastly and overall impairment, non-peptidergic were found to be the most vulnerable. Even though it could be argued whether this is due to a special resilience of the peptidergic axons and/or fragility of the non-peptidergic axons, there is plenty of evidence pointing to the latter. IB4+ DRG sensory neurons present an altered neurite outgrowth (Leclere et al., 2007) even in the presence of GDNF, its neurotrophic factor in post-embryonic life (Molliver et al., 1997), that has been linked to lack of α7-integrin and GAP43 (Gardiner et al., 2005, Leclere et al., 2007). Also, IB4 neurons are more vulnerable to axonal lesions, and their axons in the dorsal horn degenerate while CGRP ones are significantly less affected (White et al., 1990; Bennett et al., 1998; Bailey and Ribeiro-da-Silva, 2006).

Despite the distal involvement of sensory axons in the skin, we did not find loss of sensory neuronal somas in the DRG. This resistance of the sensory neuron body is in line with the classical conception of the sparing of the main sensory pathways in ALS. Data from necropsies revealed that DRG neuronal loss is scarce and most likely occur in larger axons of the dorsal root (Kawamura et al., 1981). Marked DRG neuronal loss has been reported in genetic forms, from both human and animal models, also including pronounced sensory symptoms, predominantly in the form of gait ataxia (Camdessanché et al., 2011; Nardone et al., 2016; Awano et al., 2009). In this regard DRG sensory neurons of the $SOD1^{G93A}$ mice show structural alterations, seen under electron microscopy, such as swollen mitochondria, cytoplasmic fragmentation and microvacuolization (Sábado et al., 2014; Guo et al., 2009). Therefore, it may be suggested that sensory impairment in ALS is not a qualitative feature, with the dichotomy of being affected or unaffected, but a spectrum of a quantitative gradient of involvement.

In this study, it has been possible to simultaneously investigate distal peripheral sensory axons and the neuronal somas of certain sensory populations in DRG. The pattern of distal axonal loss with preservation of neuronal somas correspond to a typical distal axonopathy. We have not studied neuronal bodies of sympathetic neurons, nor the peripheral component of PV positive proprioceptors. However, other studies have confirmed preservation of sympathetic ganglion cells (Itoh et al., 1992), whereas we found decreased sympathetic sudomotor innervation in the skin. Similarly, the distal portion proprioceptive sensory fibers in the muscle spindles are degenerated in $SOD1^{G93A}$ and $TDP43^{A315T}$ mutated mice from a presymptomatic stage (Vaughan et al., 2015), and in the Drosophila $SOD1^{G85R}$ model (Held et al., 2019), even before motor neuron degeneration is established, and we did not find loss of proprioceptive neurons in the DRG.

A distal axonopathy feature has been described in the motor counterpart of ALS, where in animal models and in humans, early loss of neuromuscular synapses occurs even before α-motor neurons in the anterior horn of the spinal cord are loss (Fischer et al., 2004; Moloney et al., 2014; Sharma et al., 2016; Xia et al., 2012; Fischer et al., 2012; Jokic et al., 2006; Teng et al., 2008; Mancuso et al., 2011). As an example, in the $SOD1^{G93A}$ mice denervation of the tibialis anterior muscle occurs 14 to 30 days prior to motor neuron degeneration (Vinsant et al., 2013). Also, in the

FUS mice and in the *FUS* Drosophila models, there are early synaptic changes at the neuromuscular junction that lead to progressive denervation (Sharma et al., 2016, Xia et al., 2012). Another finding that support the role of axons early in the disease is the presence of morphologic abnormalities in distal motor axons, varicosities along the intramuscular nerve fibers, terminal swellings, and even the presence of sprouting in the absence of significant denervation in the SOD1 *knockout* mice (Fischer et al., 2012).

Although it has been described similar loss of small fibers in the skin of distal leg and thigh in ALS patients (Nolano et al., 2018), it does not argue against the distal axonopathy model. Length-dependent axonal disturbances translate into a gradient of manifestations from distal (more profound) to proximal (less affected) and are related to distal axonopathies, but that does not imply that the inverse is right. Distal axonopathy is a pattern of neuronal degeneration, where different mechanisms can be involved. In line with this, SOD1 *knockout* mice showed that motor axons with similar length degenerate differently (Fischer et al., 2012). Tibialis anterior muscle became significantly denervated (30%) by four months, while in soleus muscle no significant denervation was seen until 12 months of age. On that case, axon length was not a determinant factor, while type of muscles/synapses was more relevant, being the fast twitch muscles of the hind limbs the ones that exhibited greater atrophy over time than slow twitch muscles (Vinsant et al., 2013).

The role of early denervation of the neuromuscular junction has raised the possibility of the non-autonomous cell death as a key factor in the neurodegeneration process in ALS. As an example, muscle expression of Nogo-A is correlated negatively with disease duration, and positively with disease progression rate and proportion of denervated neuromuscular junctions (Bruneteau et al., 2015; Jokic et al., 2006; Teng et al., 2008). Another link has postulated that the expression of semaphorin 3A (Sema3A), an axonal chemorepellent molecule, in the terminal Schwann cells associated to the neuromuscular junction of the most vulnerable fast-fatigable motor units would explain initial denervation and later motoneuron death (De Winter et al., 2006).

Beyond the possible role of skeletal muscle and terminal Schwann cells in the denervation process, different mechanisms have been described to contribute in ALS motor distal axonopathy. Deficits in axonal transport are reported to contribute

to many neurodegenerative diseases. Axonal function heavily depends on a healthy cytoskeleton composed of microfilaments and intermediate filaments (predominantly for structural support) and microtubules. Aggregates or abnormalities in neurofilaments have been directly linked to the ALS phenotype and pharmacological stabilization of microtubules decreases motor neuron death and improves life expectancy in ALS mice (Fanara et al., 2007). In ALS mouse models, deficits in both retrograde and anterograde transport have been reported, even independently of denervation (Bilsland et al. 2010; Marinkovic et al. 2012; Vinsant et al. 2013). In the $SOD1^{G93A}$ mice, there is a significant impairment in retrograde transport at a presymptomatic stage, that worsens overtime (Williamson and Cleveland 1999). Also, *in vivo* analysis showed an altered transport of mitochondria in the presymptomatic SOD1 mouse (Bilslband et al., 2010). However, no early differences in the rate of retrograde transport in either tibialis anterior or soleus innervating axons in SOD1 mice has been found.

Some genetic forms of ALS have some links with axonal function. Genes related to monogenic forms of the disease can be classified into four groups depending on the cellular pathways that are involved: protein homeostasis, RNA homeostasis and trafficking, mitochondrial function, and cytoskeletal dynamics (Mathis et al., 2019; Suzuki et al., 2020; Morfini et al., 2009; Sau et al., 2011; Brown et al., 2017; Castellanos-Montiel et al., 2020). Those genes related to ALS (and other motor neuron disorders) and involved in cytoskeletal dynamics are summarized in **Table 4**.

Table 4 (next page). ALS and other motor neuron diseases related genes involved in cytoskeletal dynamics and its physiological functions. SBMA: spinal-bulbar muscle atrophy. SPG30: hereditary spastic paraplegia type 30.

Genes involved in cytoskeletal dynamics	Protein and function
***SOD1*. 21q22.11 Dominant/Recessive**	Superoxide dismutase 1. Role in mitochondria transport, microtubule stability and modulation of motor proteins via p38 MAP kinase
***ALS2*. 2q33.1 Recessive**	Alsin. Implicated in the regulation of endosomal dynamics. Promotes neurite outgrowth.
***SPG11*. 15q21.1 Recessive**	Spatacsin. Tubulin acetylation, anterograde vesicle transport, axonal stabilization.
***VAPB*. 20q13.32 Dominant**	Vesicle-associated membrane protein-associated protein B. Mitochondria and vesicle transport
***TARDBP. 1p36.22* Dominant**	TAR DNA-binding protein 43. Transport of mitochondria and mRNP granules. Microtubule stability and acetylation.
***CHMP2B. 3p11.2* Dominant**	Charged multivesicular body protein 2B. Endocytic trafficking and signaling of endosomes.
***DCTN1*. 2p13.1 Dominant**	Dynactin Subunit 1. Microtubules anterograde and retrograde transport.
***ANXA11 10q22.3* Dominant**	Annexin Xl. Tethering between lysosomes and RNA granules in axon
***C9ORF72. 9p21.2* Dominant**	C9orf72. Mitochondrial transport
***PFN1*. 17p13 Dominant**	Profilin 1. Polymerization of actin filaments.
***FUS*. 16p11.2 Dominant**	FUS. Microtubule acetylation, mitochondrial transport, axon branching.
***KIF5A*. 12q13 Dominant**	Kinesin family member 5A. Mediates anterograde transport in microtubules.
***NF-L*. 8p21.2 Biomarker**	Light-Neurofilament. Main component of motor neuron intermediate filaments.
***NF-H.* 22q12.2 Dominant/Recessive. Biomarker**	Heavy-Neurofilament. Main component of motor neuron intermediate filaments.
***PRPH.* 12q13.12. Dominant/ Recessive**	Peripherin. Main component of motor neuron intermediate filaments.
***SPAST*. 2p22.3. Dominant**	Spastin. Microtubule's disassembly
***TUBA4A.* 2q35. Dominant**	Tubulin Alpha 4a. Component of microtubules, providing stability to the microtubule network.
***PFN1*. 17p13.2. Dominant**	Profilin 1. Mediator of actin dynamics.
***KIF1A*. 2q37.3 Dominant/Recessive (SPG30)**	Kinesin Family Member 1A. Kinesin-3 mediated transport.
***AR*. Xq12 X-linked (SBMA)**	Androgen receptor. Retrograde and anterograde transport.

In addition to deficits in axonal transport, other mechanisms can explain pathological findings such as axonal swellings found in motor and sensory axons. In physiological conditions, axonal local translation contribute to axonal efficiency and compartmentalization (Spaulding et al., 2017). Whereas terminal swellings seen in ALS are also seen in other models of motor neuropathy and Wallerian degeneration, they are usually related to protein aggregation, related with post translational alterations and not only due to transport deficits. On this regard, it has been recently revealed that TDP-43 controls axonal transport of ribosomal protein mRNAs and local translation by ribosomes, essential to maintain morphological integrity of axons (Nagano et al., 2020).

In addition, it is hypothesized that distal portion of axons could have an intrinsic susceptibility to oxidative stress and/or that microenvironment conditions surrounding distal axons could present increased oxidative stress (Fischer et al., 2007; Muller et al., 2006; Muller et al., 2007). Interestingly, the SOD1 *knockout* mouse, a model of chronic oxidative stress, was originally thought to lack a motor phenotype until it was discovered that these mice depict a model of distal axonopathy, with the pathology restricted to the distal axon, and a very early involvement of the fast-twitch muscles (Fischer et al., 2012). In this animal, characteristically epidermal sensory fibers are preserved, in contrast with the $SOD1^{G93A}$ mice (Rubio et al., 2016). This may suggest that sensory axons could be more resilient or that they are less exposed to oxidative stress. Gain of function *SOD1* mutation could contribute to sensory axonopathy (Bunton-Stasyshyn et al., 2015; Ilieva et al., 2009; Saccon et al., Sau et al., 2007; Ekhtiari Bidhendi et al., 2018; Miller et al., 2020). Toxic effects in the distal portion of axons of mutated *SOD1* have been suggested based on the improvement and increased survival in the crossbred mice between $SOD1^{G93A}$ and the Loa mice (an animal model with mutated cytoplasmic dynein that results in deficits of the retrograde transport) (Hafezparast et al., 2003; Kieran et al., 2005), although similar results were not found in $SOD1^{G85R}$ and $SOD1^{G73R}$ transgenics (Ilieva et al., 2008).

Moreover, diverse well-known motor and sensory-motor distal axonopathies share genetic links with certain forms of ALS, which are summarized in the following table (**Table 5**).

Table 5. Motor and Sensory-Motor distal axonopathies and ALS allelic disorders. AOA2: ataxia-oculomotor apraxia type 2. CMT: Charcot-Marie-Tooth neuropathy. PLS: primary lateral sclerosis. SMAJI: spinal muscular atrophy infantile James Type. SPG: hereditary spastic paraplegia

Distal axonopathy disease	Gene. Chromosome	Comments
Infantile ascending hereditary spastic paraplegia (IAHSP)	*Alsin2* (*ALS2*) 2q33.1 Recessive	Vesicle transport Allelic with ALS2, juvenile PLS
SPG4	*SPAST (SPG4)* 2p22.3 Dominant	Microtubule's dynamics
SPG6	*NIPA1*. 15q11.2 Dominant	
SPG10	*KIF5A* (kinesin heavy chain 5 A). 12q13.3 Dominant	Mediates transport of RNA and RNA-binding protein granules, VAPB mitochondria Allelic also with ALS and CMT2
SPG19	*SPG19*, 9q33-q34 Dominant	
SPG11, Spastic paraplegia with thin corpus callosum (ARHSP-TCC)	*Spatacsin*, 15q21.1 Recessive	Located in axons and dendrites. Role in axon maintenance controlling cargo trafficking. Present in cytoskeleton and synaptic vesicles.
SPG18	*ERLIN2* (endoplasmic reticulum lipid raft-associated protein 2) 8p11.23 Recessive or Dominant	Endoplasmic reticulum-associated degradation pathway
SPG78 – Spastic Ataxia	*ATP13A2* (*PARK9*) 1p36.13 Recessive	Located in lysosomes and endoplasmic reticulum. Transporter in endolysosome system. Interaction with heavy metals.
5,10-Methylenetetrahydrofolate Reductase Deficiency	*MTHFR* 1p36.22 Dominant	Spastic paraparesis and increased sporadic risk of ALS in females. MTHFR protein; methyl donor for homocysteine re-methylation to methionine

Distal hereditary motor Neuropathy, HMN1	7q34-36 Dominant	
Distal hereditary motor neuropathy with upper motor neuron signs	*Senataxin* (*SETX*) 9q34.13 Dominant	Allelic: ALS, AOA2 Similar locus to SPG19
CMT2D	Glycyl tRNA Synthetase (*GARS*) 7pq14.3 Dominant	Allelic: Distal Motor neuropathy, CMT2K, SMAJI, Bulbar ALS GARS protein; ubiquitous, located in mitochondria and cytoplasm. Involved in tRNA changes.
CMT2F	*HSPB1*; 7q11.23; Dominant or Recessive	Allelic to ALS and distal motor neuropathy 2B Regulation and maintenance of cytoskeleton and microtubule regulator (transport). Interacts with intermediate filament proteins.
CMT4J (axonal + demyelinating)	*FIG4 (SAC3*) 6q21 Recessive	FIG4 protein. Located in vacuolar membranes. Role in endolysomal trafficking and autophagy.

In conclusion, there is a global involvement of somatic small and large sensory axons, and also of autonomic axons in the $SOD1^{G93A}$ ALS mice, that progresses with time. Parallel study of their neuronal bodies shows a pattern of distal sensory axonopathy, with a degeneration of the most distal portion of the axon, while the soma is preserved. Similar pattern of degeneration has been accepted in the neuromuscular system in ALS, reflecting similarities in terms of disease pathophysiology between such diverse neurons.

CONCLUSIONS

Chapter 1. Involvement of sensory innervation in the skin of SOD1^{G93A} ALS mice.

- SOD1^{G93A} mice present reduced IENF density, already at an early stage.
- There is loss of Meissner's corpuscles in the dermal papillae, and loss of axons in the subepidermal nerve plexus and in the dermis.
- There is a gradient of distal to proximal axonal loss through the skin, at all ages, except in the late stage.

Chapter 2. Characterization of somatosensory neurons involvement in the SOD1^{G93A} mouse model

- SOD1^{G93A} mice present a decreased IENF density of both peptidergic and non-peptidergic axons, already at the presymptomatic stage.
- Intraepidermal non-peptidergic axons loss appears earlier and to a larger amount than that of the peptidergic ones.
- SOD1^{G93A} also show a reduction in sympathetic innervation of sweat glands.
- There is no loss of any subtype of sensory neurons in the DRG throughout the course of the disease in the SOD1^{G93A} mouse.
- Loss of most distal part of the sensory axons with preservation of the neuronal somas points to a distal axonopathy as the underlying process involved in peripheral sensory involvement in the SOD1^{G93A} mice.

Chapter 3. TDP-43 cytoplasmic translocation in intraepidermal axons and skin fibroblasts of ALS patients

- In our ALS patients, we corroborated a lower IENF density in the skin compared with healthy controls and patients with other neurological diseases.
- There is no localization of TDP-43 in the epidermal sensory fibers.
- Cytoplasmic TDP-43 is found aggregated in keratinocytes and fibroblasts of the skin of ALS patients.
- ALS patients present higher cytoplasmic TDP-43 expression in dermal fibroblasts than the controls.
- The amount of cytoplasmic TDP-43 in skin cells does not correlate with clinical features of ALS patients.

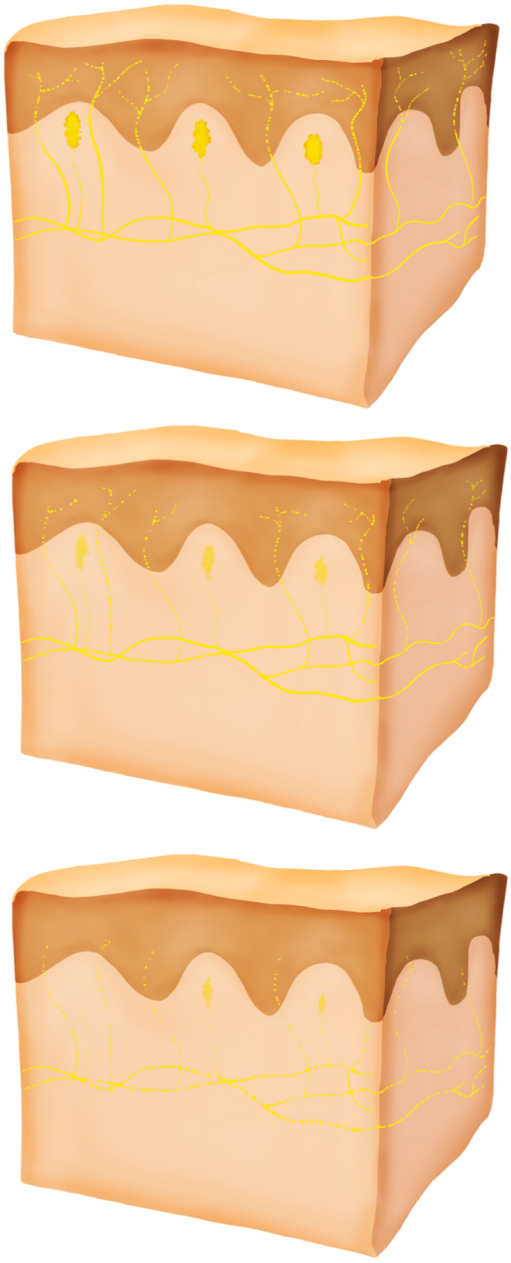

Figure 1. Chapter 1. Involvement of sensory innervation in the skin of SOD1^{G93A} ALS mice. Progressive loss of IENF density and Meissner's corpuscles, with a gradient from distal to proximal through the skin.

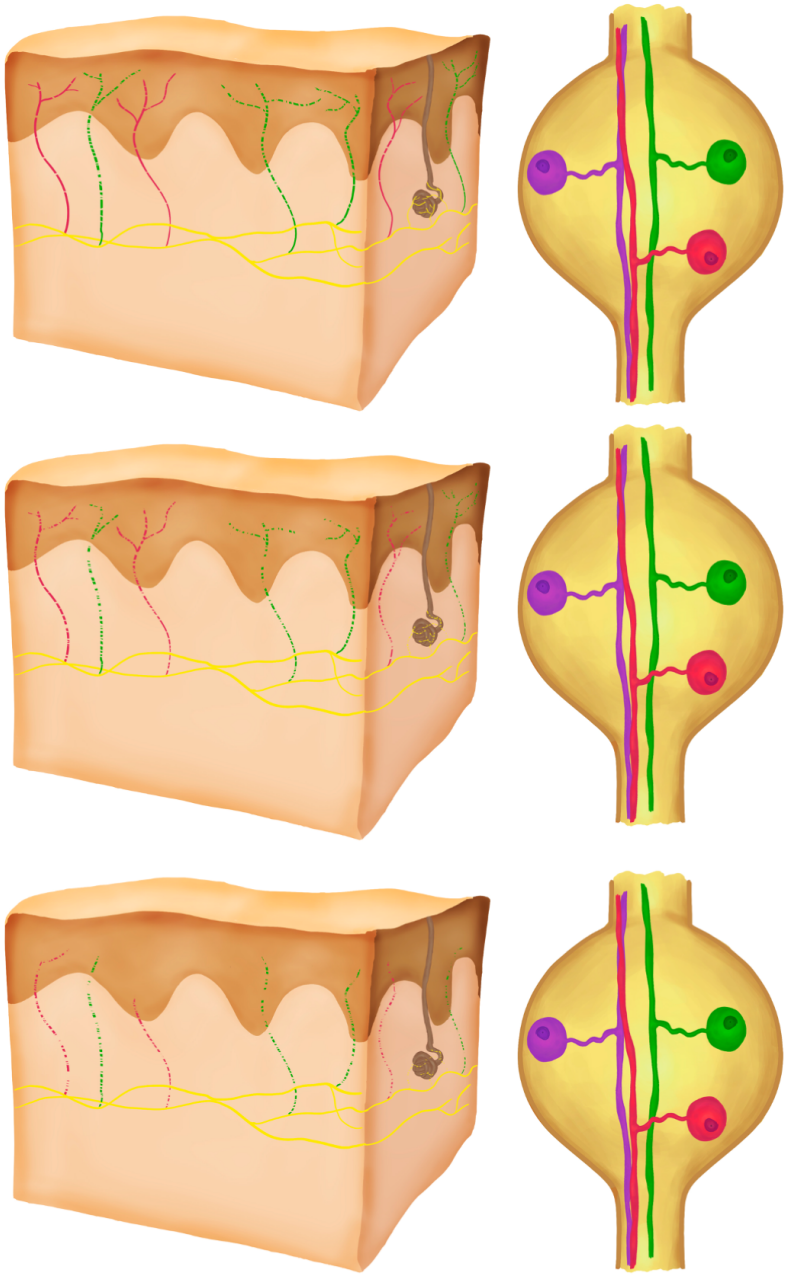

Figure 2. Chapter 2. Characterization of somatosensory neurons involvement in the SOD1^{G93A} mouse model. Loss of peptidergic (red) and non-peptidergic axons (green) in the skin, but with earlier and larger loss in the latter. Somas of all sensory populations in the DRG are preserved. There is also a reduction of sympathetic innervation in the skin.

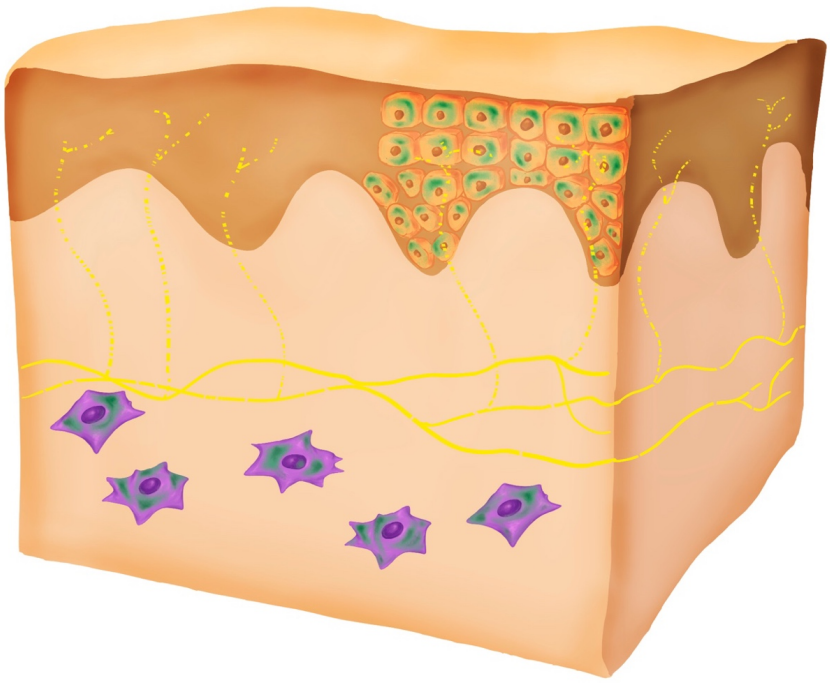

Figure 3. Chapter 3. TDP-43 cytoplasmic translocation in intraepidermal axons and skin fibroblasts of ALS patients. ALS patients presented lower IENF density. No TDP-43 localization was found in sensory fibers. Cytoplasmic TDP-43 (green) was found in keratinocytes and in dermis fibroblasts.

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ACKNOWLEDGMENTS

“A thesis is never late, nor is it early, it arrives precisely when it means to.”

Tot i que a l’autoria d’aquesta tesi només consti un sol nom, per la seva realització ha sigut indispensable l’ajut i el recolzament de moltes altres persones a les quals els hi vull agrair. Segurament també em deixaré moltes altres que espero em sàpiguen perdonar per no fer-los menció.

Primer de tot agrair a Xavier Navarro per obrir-me les portes del seu laboratori i oferir-me aquesta oportunitat. La possibilitat de retrobar-me amb els aspectes més fonamentals de la fisiologia en general i la neurologia en particular ha suposat un punt d’inflexió en la meva visió com a neuròleg clínic.

A la Mireia Herrando, per la seva infinita paciència i per ser la millor guia que un profà en les tècniques de laboratori pot tenir.

També a tota la gent del laboratori que sempre que me’ls he trobat entre micros, criostats i poyatas m’han acollit, ofert un somriure i donat un cop de mà (Nuria, Olaia, Aina, Guillem, Quim...).

Als malalts i les seves famílies, no solament per fer possible aquest treball, sinó per tot lo que aprenc d’ells en el dia a dia i per ser una viva demostració d’inspiració, superació i coratge.

A Bernat Bertran, que ha sigut peça fonamental per a poder finalitzar els treballs de la tesi en el moment idoni, però sobretot per fer-me sentir afortunat de tenir-lo de company.

A Elvira Munteis per moltes coses. Per citar un parell; per la seva ajuda sempre que la he necessitat i per ser la meva eterna referència clínica. Quant he après de tu i lo que em queda!

A Jaume Roquer per la seva confiança depositada tots aquests anys.

Tots aquests anys de feina no haguessin sigut lo mateix sense el suport, la simpatia, l'humor i l'estima de la Greta i l'Aida. Moltes gràcies per ser-hi sempre.

Agrair a tots el companys del despatx de la planta 20 (Ángel, Elisa, Irene) pel seu suport, per la seva paciència durant les reunions i classes telemàtiques, i per tenir la màquina de cafè sempre a punt.

Als companys de la Unitat d'ELA de l'hospital del Mar; Juana Martínez, Ana Balañá, Anna Guillen, Montse Villatoro. Treballar en equip mai havia sigut ni tan fàcil ni tan gratificant. Sempre a més!

A la resta de companys del servei de Neurologia de l'Hospital del Mar; adjunts, residents i ex-residents; Ana, Victor, Carla, Laura...

Al meu pare, perquè el seu cos no estarà present, però el seu record, la seva estima, exigència, manera de fer les coses, i el seu enginy sempre m'acompanyen.

A la meva mare i al meu germà pel suport incondicional.

I sobretot a Maria i a les meves filles, Sígrid i Enid per aportar la seva màgia a la meva realitat i per ser les superheroïnes que necessito, no les que mereixo.