Fungal infection in neural tissue of patients with amyotrophic lateral sclerosis

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease and the main cause of motor neuron pathology. The etiology of the disease remains unknown, and no effective therapy exists to halt the disease or improve the quality of life. Here, we provide compelling evidence for the existence of fungal infection in ALS. Immunohistochemistry analysis using a battery of antifungal antibodies revealed fungal structures such as yeast and hyphae in the motor cortex, the medulla and the spinal cord, in eleven patients with ALS. Some fungal structures were localized intracellularly and even intranuclearly, indicating that this infection is not the result of post-mortem colonization. By contrast, this burden of fungal infection cannot be observed in several CNS areas of control subjects. PCR analysis and next generation sequencing of DNA extracted from frozen neural tissue identified a variety of fungal genera including Candida, Malassezia, Fusarium, Botrytis, Trichoderma and Cryptococcus. Overall, our present observations provide strong evidence for mixed fungal infections in ALS patients. The exact mixed infection varies from patient to patient consistent with the different evolution and severity of symptoms in each ALS patient. These novel findings provide a logical explanation for the neuropathological observations of this disease, such as neuroinflammation and elevated chitinase levels, and could help to implement appropriate therapies.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder characterized by progressive muscular dystrophy and weakness caused by the degeneration of motor neurons (Saberi et al., 2015; Huynh et al., 2016). ALS is the most frequent motor neuron disease and commonly leads to death from respiratory failure within a few years of diagnosis. ALS typically exhibits a focal clinical presentation with a progressive dysfunction of both cortical (upper) and spinal (lower) motor neurons across multiple body regions (Ravits and La Spada, 2009; Turner et al., 2013). ALS is considered a heterogeneous disease, with many variants and overlapping symptoms; accordingly, modifications in neurons of the frontal cortex, temporal cortex, hippocampus, cerebellum and striatum have been identified (Saberi et al., 2015). Additionally, cognitive and behavioral impairment are recognized in 30% of ALS cases and 14% also have the related disorder frontotemporal dementia (FTD) (Turner et al., 2013). As with most neurodegenerative diseases, the etiology of ALS remains unidentified despite intensive research. The great majority of ALS cases are classified as sporadic (about 95%), whereas only a few are familial (Iguchi et al., 2013). A number of mutations in multiple genes have been described in familial ALS. The first described mutation was found in the Cu/Zn-superoxide dismutase (SOD1) gene. Further studies have uncovered many others and the list of potential genes related to ALS and its variants is steadily growing (Leblond et al., 2014; Renton et al., 2014; Tan et al., 2017). Among these mutations, a large hexanucleotide repeat expansion in the non-coding region of the C9orf72 gene is thought to account for a number of familial ALS cases (Renton et al., 2014). The causes of sporadic ALS are far less well understood.

In many neurodegenerative diseases, including ALS, several proteins have been identified to form cytoplasmic aggregates in affected brain regions in the majority of patients. In ALS this is exemplified by the presence of ubiquitinated inclusions, some of them containing trans-active response (TAR)-DNA binding protein 43 (TDP-43), and others containing the protein fused in sarcoma (FUS) (Saberi et al., 2015; Huynh et al., 2016). Both TDP-43 and FUS are widely expressed in most cell types and exhibit a predominant nuclear localization, shuttling between the nucleus and cytoplasm (Wang et al., 2004; Lagier-Tourenne et al., 2010). Both proteins are implicated in several steps of nucleic acid metabolism and can directly interact with RNA.
Under abnormal conditions, these proteins accumulate in the cytoplasm as components of stress granules (Fan and Leung, 2016). Together with TDP-43 and FUS, several additional proteins are under investigation as potential participants in ALS pathogenesis, including hnRNP A1, hnRNP A2/B1, hnRNP A3, TAF15 and EWSR1 (Neumann et al., 2011; Ugras and Shorter, 2012; Thomsen et al., 2013; Le Ber et al., 2014). The presence of these specific cytoplasmic aggregates constitutes the basis for classification of different subtypes of both ALS and FTD (Mackenzie et al., 2010; Lashley et al., 2015; Caroppo et al., 2016). Accordingly, the central concept of ALS pathology is that normal or mutated nuclear proteins accumulate in the cytoplasm and, after an undefined stress, aggregate in stress granules that become pathological for the correct functioning of motor neurons (Iguchi et al., 2013; Saberi et al., 2015; Huynh et al., 2016). This accumulation is likely a consequence of the impairment of protein transport between the nucleus and cytoplasm (Jovicic et al., 2016; Prpar Mihevc et al., 2017). It has been proposed that the hexanucleotide repeat expansions in C9orf72 and aging converge to block nuclear-cytoplasmic transport (Jovicic et al., 2016). Aggregation of TDP-43 in cytoplasmic granules has also been observed in the mesiotemporal lobe structure in 30% of normal individuals older than 65 years without mental deterioration (Geser et al., 2010a). An open question in the field is why mutations in different genes of familial ALS manifest after several decades of the patient's lifetime, and also why these inclusions are specifically pathological for motor neurons and are not observed in other cell types. Additionally, it is unknown why normal TDP-43 accumulates in stress granules in non-Caucasian ALS patients that do not harbor the hexanucleotide repeats in C9orf72 (He et al., 2015; Mukherjee et al., 2015).

The classification of ALS and related diseases is not an easy task since there is clinical overlap between these pathologies and cytoplasmic aggregates may be common to some of them (Mackenzie et al., 2010; Mackenzie et al., 2011). In this sense, similarities exist between ALS and Alzheimer's disease (AD) with regards to the presence of hyperphosphorylated tau protein in some neural cells and the formation of amyloid peptide (Yang and Strong, 2012; Muresan et al., 2014). In addition, cytoplasmic inclusions containing TDP-43 are also observed in AD, Lewy body diseases and Guamanian-parkinsonism dementia complex (Saberi et al., 2015). We have recently reported that mixed microbial infection may play a role in the development of AD (Alonso et al., 2014; Alonso et al., 2015; Pisa et al., 2015a; Pisa et al., 2015b; Carrasco et al., 2017). Accordingly, fungal proteins and DNA were detected in brain samples from AD patients, and direct visualization of intracellular fungal infection was demonstrated with specific antifungal antibodies. Interestingly, chitin-like polysaccharides have been detected in AD brains and chitinase is elevated in cerebrospinal fluid (CSF) from both AD and ALS patients (Castellani et al., 2005; Sotgiu et al., 2008; Watabe-Rudolph et al., 2012; Varghese et al., 2013; Pagliardini et al., 2015). More recently, using specific anti-chitin antibodies, we provided strong evidence for fungal structures in the brain of AD patients (Pisa et al., 2016b). These findings have led us to postulate that AD and ALS may share a similar microbial etiology, although the exact microbial species, the route of infection and the specific CNS areas affected, may differ (Alonso et al., 2015; Carrasco et al., 2017). Our previous work demonstrated that fungal proteins and DNA exist in ALS brain tissue (Alonso et al., 2015). Notably, *corpora amylacea* (CA) from both AD and ALS brain tissue was found to contain fungal proteins (Pisa et al., 2016a). In the present study, we provide compelling evidence for the existence of fungal infection in different regions of the central nervous system (CNS) of ALS patients. These novel findings could be important to implement appropriate therapies for these patients.

### 2. Materials and methods

#### 2.1. Description of ALS patients

Samples were evaluated from patients diagnosed with ALS and from control subjects. The age and gender of the subjects are listed in Supplementary Table I. Patient ALS1 was diagnosed with both ALS and FTD, whereas patient ALS8 was diagnosed with ALS and hippocampal sclerosis. Samples of tissue sections and frozen CNS tissue were supplied by a brain bank (Banco de Tejidos CIEN, Madrid) and were analyzed anonymously. The transfer of samples was carried out according to national regulations concerning research on human biological samples. The Ethics Committee of the Universidad Autónoma de Madrid approved the study and written informed consent was obtained in all cases. All samples were processed according to a common postmortem protocol followed by Banco de Tejidos CIEN. Briefly, rapid neuropathological autopsy was performed upon call by the donor's proxies (mean postmortem interval, 4.5 h). Immediately after extraction, the CNS was processed as described (Alonso et al., 2017). Neuropathological diagnosis and staging of all disease entities was performed according to consensus criteria. Samples from the frozen tissue were obtained with sterile instruments in a laminar flow hood, taking all measures to avoid contamination.

#### 2.2. Immunohistochemistry analysis

The antifungal antibodies employed in this study have been described in previous works (Pisa et al., 2015b; Pisa et al., 2016a). Antichitin antibodies were a generous gift from Dr. M.N. Horst (Mercer University, GA, USA). CNS tissue was embedded in paraffin following standard techniques and cut into 5 μm sections using a microtome (Microm HM355; Microm, Walldorf, Germany). A previously-described protocol was followed for immunohistochemical analysis (Pisa et al., 2016b). Most of the images were collected on a Zeiss LSM710 multiphoton confocal laser scanning microscope equipped with the upright microscope stand AxioImager.M2 (Zeiss), running ZEN 2010 software. Stacks of three-dimensional images were collected with the high-speed, high-resolution A1R+ confocal microscope (Nikon) combined with an inverted microscope, running NIS Elements 4.40 software. Wide-field analysis was collected on the LSM710 microscope coupled to an inverted microscope (Axio Observer, Zeiss), running Zeiss Zen2010B sp1 software. Images were deconvoluted using Huygens software (4.2.2 p0) and visualized with ImageJ (NIH).

#### 2.3. DNA extraction from frozen CNS tissue and nested PCR

DNA was extracted from frozen samples of different CNS regions using the QIAmp Genomic DNA Isolation Kit (Qiagen) as previously described (Alonso et al., 2017). Samples were analyzed by nested PCR using several primer pairs as reported (Alonso et al., 2017).

#### 2.4. Next-generation sequencing

NGS was performed as recently described (Alonso et al., 2017). Briefly, the region between the internal 1 primers was amplified with specific primers joined to linker sequences in a first round of PCR (specific product of ~300 nt). A second PCR was performed on this product using fusion primers containing Illumina and linker sequences. PCR products were sequenced on a MiSeq sequencing platform (Illumina). PCR and sequencing were performed by the Genomics Unit at the Scientific Park of Madrid. Quality analyses were performed over reads using FastQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). All sequences have been submitted to European Genome-phenome Archive with the accession number EGAS00001002473 and LT854655-LT854702.
2.5. Computational analysis

Once sequence set-up was established, we performed a metagenomic-type analysis that consisted of several steps (http://nbviewer. ipython.org/github/biocore/qiime/blob/1.9.1/examples/ipython/ Fungal-ITS-analysis.ipython). As a reference, we used the most recent version of the Qiime Fungal ITS data-base (ftp://ftp.microbio.me/ qiime/tutorial_files/its_12_11_otus.tgz). The sequences of all samples were grouped to define the Operational Taxonomic Units (OTUs) using the pick_open_reference_otus.py workflow (http://qiime.org/) with a percentage identity of 97%. We obtained a total number of 3603 OTUs from the total analysis of the 11 samples. Bray-Curtis distance matrix and the weight score of each principal component were calculated using the QIIME script core_diversity_analyses.py.

3. Results

3.1. Fungal cells in the CNS of ALS patients

Different areas of the CNS can be affected in ALS, including the motor cortex (MC) and the spinal cord (SC). To assess fungal infection in CNS regions, tissue sections from the MC, SC and the medulla (MD) from one patient with ALS (ALS1) were immunostained with antibodies against Candida albicans or Phoma betae, and with a second antibody against human α-tubulin to identify microtubule structures. As shown in Fig. 1, several structures resembling yeast cells and hyphae were immunopositive for the anti-fungal antibodies in the three areas examined. Accordingly, yeast-like cells were detected in MC and SC sections with an anti-C. albicans antibody (green). DAPI staining of the yeast nucleus (blue) was also evident in the MC sections. Further, hyphae were detected in MD sections using an anti-C. albicans antibody (Fig. 1). Several small yeast cells were also detected in MD sections using an antibody against P. betae, which also revealed hyphal structures together with punctate material in MC sections. It should be remembered that these polyclonal antibodies can cross-react with other fungal species (Pisa et al., 2015a; Pisa et al., 2015b). These findings clearly indicate the presence of mycotic structures in the three regions of the CNS from patient ALS1. To validate these findings, we used additional rabbit polyclonal antibodies raised against C. glabrata, Syncephalastrum racemosum, Penicillium notatum and fungal β-tubulin and chitin (Supplementary Fig. 1). CNS sections were also incubated with a second antibody against human neurofilament to mark neuronal fibers. The presence of fungal structures was evident with all antifungal antibodies examined. Of particular interest was the finding that the antichitin antibody immunoreacted with yeast-like cells in all three areas of the CNS analyzed. These observations support the notion that the immunostained structures are fungal in origin since they contain chitin, and are consistent with the idea that mycoses occur in the CNS of the ALS patient examined.

To extend this analysis, we assessed the three CNS areas (MC, MD and SC) in ten additional ALS patients (ALS2–ALS11). Remarkably, in all patients examined, fungal cells were detected in all three areas using anti-C. albicans (Fig. 2), anti-P. betae (Supplementary Fig. 2) and antichitin (Supplementary Fig. 3) antibodies. Accordingly, yeast-like cells could be detected in patient ALS2, ALS3, ALS8, ALS9, ALS10 and ALS11, whereas hyphal structures were evident in patient ALS3, ALS4, ALS5, ALS6, ALS7 and ALS11. Curiously, there was a wide variation in the size of the yeast cells in the sections examined: from 1–2 μm in diameter (MD ALS3, MC ALS4, MC ALS8 and MD ALS9), to 5 μm (MD ALS2, MC ALS3, MD ALS8, SC ALS9, MD ALS10 and MD ALS11). These findings may reflect yeast-like cells of different fungal species. Most of the fungal structures were clearly located extracellularly, whereas in some occasions they appeared to be intracellular and some yeast-like cells were intranuclear (MD ALS3). On other occasions, immunopositive structures had no discernable fungal morphology. To reinforce these observations, we used a range of antifungal antibodies to stain sections from different ALS patients (Supplementary Fig. 4). A variety of fungal structures were detected by their immunoreactivity against the panel of antibodies: immunoreactivity against C. albicans in ALS1, ALS2, ALS5, ALS7, ALS8, ALS9 and ALS10 (see panels A, B, C, E, F, G, K, L, M, R, S, T, U, V, W and Z); immunoreactivity against C. glabrata in ALS1 and ALS7 (see panels D, N and Q); immunoreactivity against β-tubulin in ALS7 (see panels H, I, J, O, P and Y); and immunoreactivity against P. notatum in ALS1 (see panel X). Notably, in some instances immunoreactive cells were found in close contact with the neural nucleus (see panels A, B, C, D, E, N, O, Q, V, W and Z), whereas on other occasions fungal material of undefined morphology was observed around the nucleus (see panels H, J, L, M, O, R and U). These observations resemble those previously reported by our laboratory using brain samples from AD patients, although in the latter the abundance of these mycoses was higher (Pisa et al., 2015a; Pisa et al., 2015b; Pisa et al., 2016a; Pisa et al., 2016b).

To demonstrate that some neural cells contained fungi inside the nucleus we used an orthogonal projection approach. Yeast-like cells could be detected intranuclearly (Fig. 3, panels A, B and C), and in three-dimensional images (panel G). In other cases, the fungal structures were detected closely adhered to the nucleus (Fig. 3, panels D, E, F and H). It is known that yeast can enter human cells when they are metabolically active, which is consistent with the idea that mycosis occurred when patients were still alive and is not the result of contamination.

Further support for the notion that fungal infection exists when the disease is evolving comes from the finding that CA contain fungal proteins (Pisa et al., 2016b). Thus, immunostaining of CA from the SC of patient ALS5 with an anti-C. albicans antibody (shown in green) demonstrated that some of these bodies are clearly immunopositive, whereas other CA structures contain less fungal proteins (Fig. 4). A general idea of the numerous CA bodies detected in patient ALS5 is depicted in the wide-field image shown in Fig. 4, which also shows that many of them are immunopositive for C. albicans. Higher magnification images of two fields of this wide-field image are shown to more clearly illustrate the numerous CA present in this SC section. Of note, some CA contain fungal proteins (green and yellow staining), while other CA do not immunoreact with the anti-C. albicans antibody (red staining). Moreover, some CA are immunolabeled with both antibodies (shown in yellow), indicating that they contain human α-tubulin and fungal proteins. Ostensibly, these immunopositive CA have accumulated fungal proteins during the evolution of the disease. As a control, we have analyzed SC sections from ALS5 immunostained with the anti-C. albicans antibody or without this primary antibody and only incubated with the secondary antibody. Supplementary Fig. 5 shows that indeed only when the anti-C. albicans antibody is present, some CA are stained (green), whereas no fluorescence is detected when tissue sections are incubated only with the secondary antibody.

3.2. Analysis of fungal structures in control subjects

In previous reports from our laboratory, we have tested for fungal structures in brain sections from several control subjects (Pisa et al., 2016a; Pisa et al., 2016b). We noted that occasionally a few yeast-like cells and hyphae could be observed in some control sections, albeit at a much lower frequency than that detected in AD patients. Nevertheless, we extended our current analysis to four control subjects (C1–C4). We examined four CNS areas in each of the controls: frontal cortex (FC), entorhinal cortex/hippocampus (ERH), medulla (MD) and spinal cord (SC). The results of immunostaining with anti-C. albicans, anti-P. betae and anti-chitin antibodies are shown in Supplementary Figs. 6–8, respectively. In some sections, we could detect a single fungal structure, for example with the anti-C. albicans antibody (see Supplementary Fig. 6: panels FC C1, ERH C3 and FC C4); with the anti-P. betae antibody, (see Supplementary Fig. 7: panels FC C3, SC C3 and FC C4); and with the anti-chitin antibody (see Supplementary Fig. 8: panels FC C1,
SC C3, FC C4 and SC C4). However, even on the rare occasions when these structures were detected, their burden was very low. To illustrate this point in more detail, Fig. 5 shows a wide-field image comparing the SC area of patient ALS7 with that of control C4. Whereas fungal infection was clearly detected in the ALS patient, no immune positive structures were evident in the control section. Thus, consistent with our previous studies (Pisa et al., 2016a, Pisa et al., 2016b), the difference in the fungal burden between control subjects and ALS patients can be clearly distinguished by immunohistochemistry.

3.3. Identification of fungal species in the different CNS regions of ALS patients

Although our results clearly demonstrate that various fungal cells
can be detected in the CNS of ALS patients, the specific species present cannot be determined based solely on morphology. Thus we used DNA sequencing for species identification. To this end, frozen tissue from the three CNS areas described earlier was obtained from the eleven ALS patients. After DNA extraction, we used a nested PCR technique to amplify specific fungal regions for subsequent DNA sequencing. The genomic regions chosen were the intergenic sequences located between the ribosomal RNA genes. The primer sequences and the technique carried out to analyze fungal species in complex DNA samples that mostly contain human DNA have been described previously (Alonso et al., 2015, Alonso et al., 2017). The two intergenic regions, ITS-1 and ITS-2, were amplified using universal oligonucleotide primers in

Fig. 2. Immunohistochemistry analysis of several CNS regions from different ALS patients.

Three CNS regions (MC, MD and SC) from ten ALS patients (ALS2–ALS11) were processed for immunohistochemistry as described in Fig. 1. Samples were immunostained with a rabbit polyclonal anti-C. albicans antibody (1:100 dilution) (green) and a mouse monoclonal antibody against human α-tubulin (1:50 dilution) (red). Nuclei were stained with DAPI (blue). Scale bar: 5 μm.
Fig. 3. Orthogonal projections and three-dimensional images.
Orthogonal projections (panels A, B and C) and different stacks of a 3D image (D, E, F, G and H) from different ALS patients. Samples were immunostained with a rabbit polyclonal anti-C. albicans antibody (1:100 dilution) (green). Nuclei were stained with DAPI (blue). Scale bar: 5 μm.
individual nested PCR assays (see scheme in Fig. 6A). All amplified fragments were extracted from agarose gels and sequenced. To illustrate the results obtained, the nested PCR of ITS-1 of ALS patients ALS3–ALS8 is shown in Fig. 6 Panel B, and the PCR of the human β-globin gene is shown in Fig. 6 Panel C. The results obtained after sequencing of all products (from ITS-1 and ITS-2) are summarized in Supplementary Table II. Notably, the vast majority of DNA samples rendered at least one amplified fragment. Importantly, control PCR assays and the controls for DNA extraction were always negative for DNA amplification, indicating that no contamination occurred during the extraction protocol and the PCR assay. The fungal species identified by DNA sequencing belonged to several genera; the most prevalent of which were Malassezia, Candida, Cryptococcus, Penicillium, Cladosporium and Davidiella. Some species such as C. famata (also known as Debaryomyces hansenii), Cladosporium sp., Malassezia globosa, Penicillium sp., Rhodotorula mucilaginosa, Trichoderma sp., and uncultured sporidiobolus were also detected by next generation sequencing (NGS, see below), although the percentage was < 1% and they were therefore not listed in the results obtained by NGS. It should be borne in mind that although the primers utilized here are universal, the range of species amplified by each set of primers is different.

The most powerful technique currently available to identify fungal DNA in clinical samples is NGS. We performed a PCR assay of the ITS-1 region using universal primers. The amplicons obtained were sequenced with the Illumina platform employing the primers bound to their corresponding linkers. We analyzed the DNA samples obtained as indicated previously from the three CNS regions of the eleven ALS patients. The number of sequences obtained in each sample ranged from 513,554 to 926,101, providing a good confidence level for the results obtained. A variety of fungal species were found with this technique. The fungal species found in all samples with a percentage > 1% are listed in Supplementary Table III. Computational analyses of the sequences using Qiime and Blast classified the data from all CNS samples into phylum, class, order, family, and genus. The fungal families and genera from all patients for each area are shown in Fig. 7. Strikingly, in some patients the most prevalent species was found in three or at least two of the areas examined, suggesting that this was the dominant fungal infection in that patient. The six most prevalent genera in all the samples examined, in decreasing order, were Candida, Malassezia, Fusarium, Botrytis, Trichoderma and Cryptococcus. Some of these genera were also found in AD patients examined in previous studies from our group, although the particular fungal species occasionally differed (Alonso et al., 2017). Curiously, however, Trichoderma was not found in AD patients, whereas Alternaria was detected in only one sample (SC1) of patient ALS5. Of particular interest was the finding that the fungal species detected varied between ALS patients. Accordingly, in some patients a single fungal species appeared in a high proportion, as shown for Phaeosphaeria sp. from the MC region of ALS9, representing 92.5%, and Trichoderma sp. from SC1 of ALS10, that corresponded to 89%. Overall, our results suggest that there is a wide variety of fungal species in each patient and these species vary from to patient to patient. It is possible that the variation in fungal species detected may constitute the basis to explain the differences in the evolution and severity of the disease in each ALS patient.

4. Discussion

Much effort has been made in the past years to understand the pathology of ALS and related diseases at the molecular level. As a result, a number of mutations have been reported in several genes in familial...
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ALS has been put forward by our group based on the findings by providing compelling evidence for the presence of fungal infection in different CNS regions of ALS patients. Direct visualization of yeast-like cells and hyphae was achieved in all ALS patients analyzed using a range of antifungal antibodies. Moreover, NGS identified several fungal species present in these patients.

Our hypothesis that the etiology of ALS is microbial in origin can explain most if not all the findings reported for the neuropathology of the disease. First, the sudden appearance of the disease after several decades of life and its focal presentation followed by its spread to other neighbouring CNS areas can be explained if ALS is a microbial disease. Second, the induction of stress granules containing different proteins can also be reconciled by the fact that some of the fungal cells detected are intracellular (endomycosomes) (Alonso et al., 2015; Pisa et al., 2015b). Clearly, fungal infection could trigger a stress response and block the shuttling of proteins between the nucleus and cytoplasm, leading ultimately to neuron injury and death. Third, the existence of microbial infection is consistent with the reports of neuroinflammation in ALS patients, with a clear infiltration of T lymphocytes (Henkel et al., 2004; Hooten et al., 2015; Ransohoff, 2016). Indeed, the decrease in Th2 lymphocytes correlates with the rapid progression of the disease (Henkel et al., 2013). Therefore, the presence of mycoses would lead to the infiltration of immune cells into the infected tissue. Fourth, high levels of chitinase, the enzyme that cleaves the fungal polysaccharide chitin, are present in the CSF of ALS patients (Varghese et al., 2013; Pagliardini et al., 2015). The most plausible explanation for this finding is that fungal chitin in the CNS of ALS patients induces the synthesis of chitinase. Finally, the genetic predisposition for ALS can also be explained because the genetic background of a given individual also affects the susceptibility to fungal infection (Carvalho et al., 2010; Glocker and Grimbacher, 2010; Ok et al., 2011; Maskarinec et al., 2016). In this regard, the first mutated protein discovered in familial ALS was SOD1, which forms part of the innate immune response and is crucial for the defense against fungi (Moise and Strong, 2006; Lionakis, 2014). Therefore, to our knowledge, none of the known pathological events of ALS discard the possibility that this spectrum of diseases is caused by microbial infection. Moreover, our hypothesis can accommodate the pathological similarities between ALS and other neurodegenerative diseases. The fact that there are large differences between ALS patients regarding survival after diagnosis and the different CNS regions affected in each patient, is also consistent with the idea that mixed microbial infections are responsible for the disease. Conversely, we would submit that none of the other hypotheses put forward to account for the pathology of ALS is sufficiently strong to provide a logical explanation for all the described observations. In particular, the induction of chitinase and the neuroinflammation with infiltrates of immune cells cannot be explained by a defect in protein transport between the nucleus and cytoplasm.

Aside from the above arguments, the most compelling evidence that ALS patients develop fungal infection is provided by our current observations of immunopositive fungal structures in the CNS using specific antifungal antibodies. These antibodies recognize yeast-like and hyphal structures, which can also be detected with anti-chitin antibodies. Infection by fungi is often clearly observed inside the nucleus, pointing to the idea that the cell was infected while it was alive since fungal cells do not enter human cells that have died and are metabolically inactive (Neglia et al., 2006; Pacheco et al., 2007; Gilbert et al., 2014; Kasper et al., 2015; Munoz-Duarte et al., 2016). Consistent with this idea is the finding that CA from ALS brain tissue contains fungal proteins (Pisa et al., 2016a). It is known that these bodies are formed over extended periods of time and therefore fungal proteins presumably amassed during their establishment, and is not a consequence of contamination during sample collection. Our proposal that both AD and ALS can be caused by mixed microbial infections is supported by a number of experimental findings, including the direct visualization of fungal cells in the CNS of both AD and ALS patients (Alonso et al., 2015; Pisa et al., 2015a; Pisa et al., 2015b). It is clear that different microbial infections could account for the different pathologies. If some neurodegenerative diseases are triggered by mixed microbial infections, the variety of pathologies can be the result of: 1) the different microbial...
species infecting the patient; 2) the route of entry and spread of the infection; 3) the patient’s genetic background; and 4) the patient’s lifestyle, including diet and the immune status. The challenge in the detection of these infections in patients with neurodegenerative diseases can be met by using specific antibodies and PCR or NGS techniques. If some of these diseases are indeed caused by mixed microbial infections, many antimicrobial compounds are available for use in humans and there is no need to wait for other therapies. Along this line, the only approved compound to treat ALS, riluzole, exhibits partial antifungal activity (LaFleur et al., 2011). Thus, the action of riluzole on ALS may be due to its limited activity against fungi. If ALS is provoked by a mycosis, a much better choice of treatment would be the more potent antifungal compounds, such as the echinocandins or new generation azoles. Aside from riluzole, it should be stressed that at present no therapy is available to halt or even reverse the course of this devastating illness. Therefore, future clinical trials with already approved antimicrobial compounds could offer clinical benefits to stop or even reverse this disease.

5. Conclusions

Direct visualization of fungi in neural tissue from ALS patients can be achieved by immunohistochemistry using a battery of antifungal antibodies. Thus, yeast-like and hyphae are detected in different CNS regions, including the motor cortex (MC) and the spinal cord (SC). Some rounded fungal cells are observed inside the nucleus of neurons indicating that they do not represent a post-mortem colonization. Moreover, this fungal infection is not observed in control subjects. Identification of the fungal species in the different CNS regions by nested PCR and by NGS reveals a variety of species in each patient. These fungi vary from patient to patient possibly reflecting the evolution and severity of symptoms in each ALS patient. These findings open a new field of research in ALS and may provide an adequate therapy to combat this disease.

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Author information

R.A. and D.P. contributed equally to this work.

Contributions

D.P. and A.M.F. performed immunohistochemistry analyses. R.A.

Fig. 6. Nested PCR analysis of DNA extracted from ALS patients. PCR analysis was carried out as described in Materials and methods. Panel A: schematic representation of fungal rRNA genes (18S, 5.8S and 28S rRNA) and the ITS-1 and ITS-2 regions. Location of the primers employed for the different nested PCRs: external 1 and external 2 employed in the first PCR; ITS-1 and ITS-2 employed in the second PCR to amplify ITS-1; ITS-3 and ITS-4 employed in the second PCR to amplify ITS-2. Panel B: agarose gel electrophoresis of the DNA fragments amplified by nested PCR. PCR analysis of three regions (MC, MD and SC) from patients ALS3–ALS8 using primers ITS-1 and ITS-2 to amplify the ITS-1 region. Panel C: PCR analysis of DNA extracted from the samples tested in panel B using human β-globin oligonucleotide primers. Control PCR: PCR without DNA. CE: control of DNA extraction without DNA. MC: motor cortex; MD: medulla; SC: spinal cord.
Fig. 7. Distribution of fungal families and genera obtained by NGS of DNA from eleven ALS patients.
Computational analyses of the sequences obtained on the Illumina platform using Qiime classified the data into fungal families and genera. Left panels show the results of fungal families obtained from three CNS regions (MC, MD and SC). Right panels show the results of fungal genera obtained from three CNS regions (MC, MD and SC). Asc: Ascomycota; Bas: Basidiomycota; Chy: Chytridiomycota.
carried out the PCR and NGS analysis. A.R. selected the ALS patients and control subjects and prepared the paraffin sections and frozen samples. L.C. designed the study and wrote the paper. All authors discussed the results and commented on the manuscript.

Competing interests
The authors declare no competing financial interests.

Appendix A. Supplementary data
Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nbl.2017.09.001.

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