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Running title: Visually guided saccades in ALS

Abstract

Two clinical phenotypes characterize the onset of amyotrophic lateral sclerosis (ALS): the spinal variant, with symptoms beginning in the limbs, and the bulbar variant, affecting firstly speech and swallowing. The two variants show some distinct features in the histopathology, localization and prognosis, but to which extent they really differ clinically and pathologically remains to be clarified. Recent neuropathological and neuroimaging studies have indicated a broader spreading of the neurodegenerative process in ALS, extending beyond the motor areas, toward other cortical and subcortical regions, many of which are involved in visual processing and saccadic control. Indeed, a wide range of eye movement deficits have been reported in ALS, but they have never been used to distinguish the two ALS variants.

Since quantifying eye movements is a very sensitive and specific method for the study of brain networks, we compared different saccadic and visual search behaviours across spinal ALS patients (n=12), bulbar ALS patients (n=6) and healthy control subjects (n=13), along with cognitive and MRI parameters, with the aim to define more accurately the two patients subgroups and possibly clarify a different underlying neural impairment.

We found separate profiles of visually guided saccades between spinal (short saccades) and bulbar (slow saccades) ALS, which could result from the pathologic involvement of different pathways.

We suggest an early involvement of the parieto-collicular-cerebellar network in spinal ALS and the fronto-brainstem circuit in bulbar ALS. Overall, our data confirm the diagnostic value of the eye movements analysis in ALS and add new insight on the involved neural networks.

Keywords: motor neuron disease; eye movements; cognitive dysfunctions; executive functions; quantitative neuroimaging

Abbreviations: ALS: Amyotrophic Lateral Sclerosis; sALS: spinal ALS variant; bALS: bulbar ALS variant; ALS-FRS: Amyotrophic Lateral Sclerosis Functional Rate Scale; ALS-FTSD: motor neuron disease–FTD spectrum disorder; AS: antisaccades; BG: basal ganglia; DLPFC: dorsolateral prefrontal cortex; ECAS: Edinburgh Cognitive and Behavioral ALS Screen; FC: frontal cortex; FEF: frontal eye fields; FTD: fronto-temporal dementia; MGS: memory-guided saccades; PEF parietal eye fields; PPC posterior parietal cortex; SEF: supplementary eye fields; SC: superior colliculus; std: standard deviation; VSS: visual sequential search; VGS: visually guided saccades

Introduction

Amyotrophic lateral sclerosis (ALS) is a lethal neurodegenerative disease, characterized by the combined degeneration of first and second motoneuron[1]. Classically, ALS may have two clinical onset presentations: the most prevalent spinal variant (sALS), with symptoms beginning in the arms or legs, and the more severe bulbar variant (bALS), affecting firstly speech and swallowing. Although nearly 80% of sALS patients develop bulbar signs with disease progression[1], the two variants show differences in the underlying histopathology, anatomical localization and progression, suggesting the occurrence of specific, not yet elucidated, pathological mechanisms[2-5].

ALS symptoms spread beyond the pyramidal system, demonstrating structural and functional involvement of other motor, cognitive and behavioural networks[1,6-9]. In this respect, intra-cytoplasmic inclusions of phosphorylated 43-kDa TAR DNA-binding protein (pTDP-43) causing neuronal death, and closely associated with oligodendroglia degeneration and altered white matter connectivity[10-15], can expand from the motor neuron system to the frontotemporal and parietal cortices and deep grey matter[16]. Indeed, even non-demented ALS patients may show cognitive impairment related to frontal lobe dysfunctions[17-19], including deficit in executive functions, verbal fluency, language[7,20,21] and alterations of antisaccades[22,23]. To which extent this broader involvement differs in the two variants is not well clarified.

Oculomotor abnormalities are not traditionally considered a predominant sign in ALS, but a wide range of eye movement deficits has been described. However, no studies have investigated the saccadic profiles in ALS variants and used them to understand their underlying patho-mechanism.

Conversely, testing the saccadic behaviour with standardized protocols offers many advantages in the study of neurodegenerative diseases, including ALS. First, the neural circuits underlying saccadic system are among the best understood and second, new devices make the recording of saccades technically easy and provide robust, repeatable, and interpretable results.

Saccades are initiated by two main cortical areas: the frontal eye fields (FEF) in the lateral frontal cortex (FC), which mostly act in synergy with the basal ganglia to generate voluntary saccades, and the parietal eye fields (PEF) of the posterior parietal cortex (PPC), more specifically involved in visually reflexive saccades. Both pathways converge into the superior

colliculus (SC), where the command signal for a saccade is sent to the brainstem oculomotor network, to which signals from the cerebellum also converge.

The neural substrate contributing to these saccadic behaviours can be explored by testing specific saccadic paradigms. Reflexive visually guided saccades toward a peripheral stimulus (VGS) test the parieto-collicular network capacity to select and localize, spatially, a salient target and the ability of the SC to react to new stimuli by disengaging fixation (Gap and Overlapp paradigms). Antisaccades (AS), (saccades to the opposite direction than the stimulus) and memory guided saccades (MGS), (saccades directed to a remembered target position) test the voluntary fronto-BG-collicular circuit, visual working memory system and the inhibition of reflexive movements by the dorso-lateral prefrontal cortex (DLPFC)[24-26].

Thus, while measuring saccade dynamic and metric parameters precisely indicates the functioning of groups of neurons in the brainstem and cerebellum[26], the characterization of saccadic behaviour with specific tasks provides insights on those cortical-subcortical brain networks.

In this perspective, the current study aims to evaluate specific saccadic features that could help to clarify the underlying neural network in the two different types of ALS. To pursue this objective, we compared the eye movement profiles, as resulting from reflexive and voluntary saccades and visual sequencing, with clinical and cognitive features, and quantitative brain MRI, in 18 ALS patients and 13 control subjects, investigating possible differences between sALS and bALS groups.

Materials and Methods

Participants

Eighteen patients were recruited between 2017 and 2018 from the referral motor neuron diseases Centre of the University of Siena. Data were compared to that collected from thirteen healthy age-matched subjects. Diagnosis of ALS was formulated according to the revised El Escorial diagnostic criteria (EEDCr,1998) by two experienced neurologists, considering four classifications: possible, probable, probable laboratory supported and definite[27]. Disease stage was evaluated according to the King's College Staging System (score ranging from 1 to 4, with higher scores reflecting more spread disease)[28] and ALS-MITOS Staging System (score ranging from 0 to 5, with higher scores reflecting greater disability)[29]. Exclusion criteria were diagnosis

of primary lateral sclerosis, progressive motor atrophy and non-classic motor neuron diseases, inability to maintain the sitting position during the eye-tracking session and severe cognitive impairment at the time of the enrolment. Disease onset was recorded as spinal or bulbar. If the medical history showed the simultaneous presence of limb weakness and bulbar signs at the beginning of the disease, the patient was categorized into the bulbar group. Data was collected during the first diagnostic work-up, when patients were not yet under treatments potentially interacting with cortical or saccadic performances. Global disability was assessed by the Amyotrophic Lateral Sclerosis Functional Rate Scale, revised (ALS-FRS, range 0–48 with lower scores reflecting greater disability). Systematic genotyping evaluated potential mutations known to be associated to genetic forms. Two patients were diagnosed with familiar ALS, carrying a *C9ORF72* hexanucleotide repeat expansion. Their family history was remarkable for ALS spinal–onset and pure FTD. All subjects had normal ophthalmic examination. The control group included thirteen age- and sex-matched healthy subjects, not suffering from any genetic, cerebrovascular or acquired neurological disease or ocular disturbances. All subjects were free from treatments affecting ocular or neurological functions and had no past history of ocular or neurological diseases. The only treatments admitted were antiplatelet and statins 6/13 and anti-hypertensive drugs (5/13). After giving a signed informed consent all patients and controls underwent to the same protocol. The study was performed according with the criteria of the Declaration of Helsinki, and it was approved by the local Ethical Committee Azienda Ospedaliera Universitaria Senese, EVAlab protocol CEL no. 48/2018.

Experimental protocol

Cognitive assessment

The Edinburgh Cognitive and Behavioural ALS Screen (ECAS) is a twenty minutes examination that includes ALS-non-specific (memory and visual-motor skills) and ALS-specific (speech, fluency and executive functions) tasks[27,30,31]. Memory tasks consisted in immediate recall, delayed retention, and delayed recognition of a short story. Visual-motor skills were evaluated asking the subject to count cubes and dots, and to locate numbers. Speech evaluation included naming, language comprehension and spelling words. Verbal fluency was explored recalling free-words beginning with the letter S and restrained-four letters-words beginning with the letter C. Executive functions consisted in reverse digit span, alternation, inhibitory sentence completion and social cognition tasks. In the reverse digit span, subjects were presented with

sequences of numbers (digits) that they had to reproduce immediately after presentation in the reverse temporal order. The length of the sequence was progressively increased, and the span (a measure of short-term memory store capacity) was the longest sequence correctly reproduced. In the alternation task, the subject was required to alternate numbers and letters to complete a progressive sequence, *i.e.* 1-A, 2-B, 3-C. In the sentence completion task, the subject was asked to complete sentences logically (in the context) and then illogically (out of the context). In the social cognition task, the subject was shown six groups of images (four images in each group) and invited to refer which one they preferred. Then the patient was required to say toward which image a smiling face was looking to.

Additionally, the ECAS investigated behavioural changes and psychotic symptoms with two separate career interviews: by a checklist of ten behaviours across five domains and three questions for the presence of psychotic symptoms. An ALS-specific score (maximum 100 points), an ASL-non-specific score (maximum 36 points) and a total ECAS score (maximum 136 points) were calculated. The scores were corrected for age and education.

Neuroimaging procedure

T1-weighted 3D-MPRAGE sequences (TR = 1,880 ms, TE = 3.38 ms, TI = 1,100 ms, FA = 15, number of slices = 176, thickness = 1 mm, gap = 0 mm, and imaging matrix = 256 ⇥ 256). Global and regional brain parenchymal volumes were evaluated through a modified version of the SIENAX (Structural Image Evaluation using Normalization of Atrophy) software, part of FSL (Oxford FMRIB Software Library. https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/FSL)[32-34]. The skull-stripped brain image was affinely registered onto MNI152 standard-space image[35,36]. We obtained measures of global brain volume, WM volume, total and cortical grey matter (GM) volumes. Moreover, volumes of brain lobes (i.e., frontal, temporal, parietal, occipital and insular cortices) and cerebellum were obtained using manually edited standard-space masks. The ventricular cerebrospinal fluid volume was computed by subtracting the parenchymal volume from the global brain volume. All volume measures were normalized for the subject's head size.

Recordings of eye movements

Eye-movements were recorded using an ASL-504 eye-tracker device (Applied Science Laboratories, Bedford, MA, USA) sampling the image of the eye at 240 Hz. Data acquisition and visual stimulation were controlled by a PC (3 GHz Pentium) running a custom software dedicated to real-time data acquisition. An interactive procedure was used for eye calibration,

based on nine static points disposed in various positions on the screen, and the following validation of the recording. The visual stimulus was presented on a 310×510 mm LCD screen (frame rate 60 Hz) having a resolution of 1024×768 pixels and placed at 720 mm from the eyes of the subject. The subject's head was immobilized by a chinrest. All recordings were conducted in complete darkness. Stimuli were seen binocularly, but only one eye was recorded (randomly left or right).

The eye-tracking protocol lasted about twenty minutes and consisted of four tasks: visually guided saccades (VGS), antisaccades (AS), memory-guided saccades (MGS) and the visual sequential search (VSS) test, developed in EVALAB to study the top-down mechanisms of visual search[37].

In the VGS task (GAP paradigm), after 200 ms (GAP) a central fixation point was switched off, participants had to make a saccade as soon as an eccentric target appeared (±10 deg or ±18 deg, right or left; or ±8 deg, up or down; gap 200 ms, exposition 1500 ms).

In the AS task, after a central fixation point, participants had to make a mirror saccade with respect to the position of an eccentric horizontal target appearing for 2500 ms (±10 deg or ±18 deg, right or left).

In the MGS task, while the participants were in central fixation, another stimulus rapidly (200 ms) flashed eccentrically (±10 deg and ±18 deg, right and left). In this first phase (memorization phase) the participants had to suppress any reflexive saccade towards the flash. After the complete extinguishment of the central fixation target (go signal), the participants had then to make a voluntary saccade toward the memorized position of the flashed eccentric target. At last, an eccentric target turned-on in the same position of the flash and the participants were required to correct their position accordingly (memorization error).

Each saccadic task consisted of ten trials for each target position. The positions were randomized.

The VSS task investigated top-down gaze strategies adopted during the exploration of alphanumerical strings[38,39]. Subjects were required to connect by gaze a number with its respective letter following the alphanumeric sequence, *i.e.* 1—A—2—B—3—C—4—D—5—E. After a central fixation period of 1000 ms, numbers and letters appeared on the screen for 20000 ms. Each letter/number in red, (2.0 cd/m2) on a black background, subtended about 2-3 deg of visual angle and was arranged in a random position. The task was repeated four times with four

different randomized positions of the letters and numbers. Nevertheless, the overall geometry of the visual targets (spatial position of each target) was kept the same for all tests. Prior to perform the VSS task, the subjects were trained using the written pencil trail.

Signal processing and data analysis

X- and y-coordinates of the gaze and of the position of the stimulus were exported in .csv format to be later processed in Matlab (v2020b). The signal was de-blinked (pupil size equal to zero for more than 40 ms), interpolated and smoothed through a third-order Butterworth low-pass digital filter (−3 dB attenuation at 25 Hz cut-off frequency). Saccades and fixations were extracted from the signal through a velocity-based discrimination algorithm (threshold of 10 deg/s)[40]. Eye velocity was obtained with an eight-point central difference derivative algorithm having a bandwidth larger than 70 Hz at a digitization frequency of 240 Hz. For the identification of the relevant movements, a semi-automatic algorithm was employed. A visual check of the signal was performed by a trained neurologist to ensure the correct selection of the movements.

VGS tasks

For each saccadic movement, we computed amplitude, gain, duration, peak velocity and latency. Amplitude is the difference between the position of the eye at the start and at the end of the saccadic movement (degrees of visual angle). Gain is the ratio between the saccadic and the stimulus amplitudes. Peak velocity is the maximum speed achieved by the saccade (degrees of visual angle per millisecond). Duration is the difference between the onset time and the ending time of the saccade (milliseconds). Latency is the time delay between the appearance of the eccentric target and the onset of the saccadic movement. Correct movements with latencies less than 80 ms were flagged as anticipatory and excluded from the analysis.

AS and MGS tasks

For each AS task, we defined the latencies of correct movements and errors (*i.e.* time-lapse between the go-signal and the onset of the correct or erroneous saccadic movement).

For MGS task, we defined the memorization error as the amplitude of the correction from the position of the saccade toward the memorized position of the flash and the actual position of the target (last stage of the MGS task). Erroneous MGS were grasp saccades made in the direction of the flash before the go-signal.

For both the AS and MGS tasks performance rates were computed in terms of 1) percentage of correctly executed movement over the number of stimuli (%AS and %MGS); 2) percentage of

erroneous prosaccades over the number of stimuli (%ErrAS) and 3) percentage of reflexive saccades anticipating the go-signal in MGS (%ErrMGS); 4) percentage of corrective antisaccades over the number of erroneous movements (%CorrAS) and 5) the percentage of corrective saccades with respect to the memorization point (%CorrMGS).

For both AS and MGS tasks, correct movements with latencies less than 100 ms were flagged as anticipatory and excluded from the analysis.

VSS task

Numbers and letters were sampled as pre-defined squared regions of interest (ROIs) centered on letters and numbers with the width and height set to 3.5×3.5 deg[37]. The distribution of fixations and sequencing abilities were then evaluated. A generic fixation at time *t* was assigned to a ROI if the coordinates of its centroid were contained in that region. For each task, we evaluated a possible indicator of peripheral detection capacity during visual sequencing (*i.e.*, the distribution of fixations with respect to the ROIs, measured by means of the Euclidean distance in degrees for each fixation to the nearest ROI, DN); and an indicator of performance (*i.e.*, the distance of each fixation to the next target, DT). Moreover, for each subject, we measured the average duration of a fixation during the task (FIX_DURATION) and the average duration of the fixations landing on the target (FIX_DURATION_TARGET). To assess the sequencing abilities, a sequencing score (SEQ) was computed as the sum of all valid steps (n of correct connections between number and letter in the sequence) divided by the maximum score (maximum score = 10)[39,41].

Statistical analysis

All statistical tests were performed using the Matlab statistics toolbox. Results were considered significant for two-tailed p-values lower than 0.05. We considered the patient groups both separately (sALS and bALS) and as a whole (ALS-All). Data were first investigated for normality (Shapiro-Wilk test) and homoscedasticity (Fligner-Killeen test). Measures were then compared between pairs with Mann-Whitney-U-test and across groups with Kruskal-Wallis test or Welch tests on ranked data and Games-Howell Post-hoc multiple pair-wise comparison test.

First, differences in demographic, neuropsychological, clinical scores and brain volumes across groups were analysed. Then we compared means and variances of all saccadic parameters (duration, amplitude, peak velocity, mean velocity, latency, gain) among groups. For better definying saccade dynamics in each group, we assessed the relationship between the main

sequence of peak velocity versus amplitude and duration versus amplitude using exponential and linear model fitting[26]. The curve fits of patients were compared against those of the control group including 95 % confidence interval. In AS and MGS, the rates of correct or erroneous movements were compared among the groups through (Chi-square) χ^2 test followed by the post hoc Marascuilo procedure.

Spearman correlation coefficient (rho) was used to investigate the relationship between the saccadic and fixational metrics and motor disability scores, brain volumes, and cognitive profiles. When the comparison between the two groups of patients did not reach any significance, the correlation was performed after merging the two patient groups into one (ALS - All)

Data availability

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Results

Demographic, clinical, cognitive characteristics, and brain volumes

Demographic and clinical data, disease stage and level of diagnostic certainty, cognitive and volumetric MRI results are shown in Table 1.

ALS patients and the control group were matched for age: bALS 69 years (range 59-67); sALS 65 years (range 46-79); controls 64 years (range 52-73) and gender. ALSFRS-R and ECAS scores were adjusted for gender, age and education of the tested groups. bALS and sALS did not show significant differences in terms of disease duration nor in terms of ALSFRS-R and ECAS score. When compared to healthy controls, ALS patients demonstrated significant educational differences in years bALS: 8y (range 5-13y); sALS 8y (range 5-15y); All ALS 8 y (range 5-15y) vs Controls 14y (range 11-19y), respectively *p=0.0211, **p=0.0019, ***p=2.2966e-04. ALS patiens subgroups also showed significant reduction of ECAS scores with respect to controls (Table 1).

With respect to controls, bASL patients had smaller volumes in total peripheral grey bALS: 534.4 (503.7-549.0) vs Controls 627.3 (578.5-672.0) *p=0.0028; parietal lobe bALS 122.8 (104.8-131.9) vs Controls

140.1 (126.1-154.0) *p=0.0446, bALS temporal lobe 125.9 (113.3-138.4), vs Controls 152.2 (144.4-168.0) p=0.0051; and brain frontal lobe 186.0 (168.3-198) vs Controls 225.5 (206.9-238.0) *p=0.0039. No significant differences were found between bALS and sALS or between sALS and CNTRL.

Saccadic behaviour findings

Figure 1 shows examples of eye movements for each of the saccadic tasks proposed in this study for each tested group. In particular bALS patients show normal amplitude, slow VGS and great memorization error in MGS; sALS patients show hypometric two-three steps VGS and staircase AS.

Visually guided saccades (VGS) task

The boxplot of the distributions of the main saccadic parameters of the VGS task are reported in Figure 2-a. In detail, both bALS and sALS patients had greater latencies than controls. bALS had reduced speed and increased duration than controls and sALS for both the vertical and horizontal saccades. sALS had reduced horizontal and vertical amplitude than controls and bALS.

The horizontal and vertical main sequence relationships for each group of patients against the CNTRL group are shown in Figure 2-b and 2-c respectively. The saccade dynamic of bALS did not follow the main sequence of CNTRL for saccades within the range of 20 deg of amplitude.

For the bALS patients, vertical peak velocities inversely correlated with disease durations (rho = -0.88273, p = 0.044444), whereas horizontal and vertical amplitudes directly correlated with total grey matter volumes (rho = 0.94112, p = 0.016667).

For the sALS patients, horizontal durations and amplitudes directly correlated with parietal (rho = 0.92582, p = 0.033333) and total cerebellar volumes (rho = -0.94286, p = 0.016667), respectively; and inversely correlated with disease duration (rho = -0.59299, p = 0.042136).

Antisaccade (AS) task

The boxplot of the distributions of the latencies of the correctly executed antisaccades (AS), the erroneous prosaccades (ErrAS) and relative correction movements (CorrAS, intersaccadic latency) are shown in Figure 3-a. The latency of the correctly executed antisaccades and of the erroneously executed pro-saccades was not significantly different among the three study groups.

The rate of the correctly executed AS and of the errors and relative corrections in the AS task are reported in Table 2. ALS patients carried out more errors in the antisaccadic task with respect to

the CNTRL, with no significant differences between the patient's groups. No significant differences among groups were found in correcting erroneous prosaccades.

By pooling the AS data of all ALS patients, we found an inverse correlation between the latency of correctly executed AS and disease duration (rho = -0.7658, p = 0.00087362).

Memory-guided saccades (MGS) task

The boxplots of the distribution of the amplitude of the adjustments after a correctly executed MGS (memorization error magnitude) for the three groups are reported in Figure 3-c. At 10 deg the memorization error magnitude was increased in bALS with respect to both sALS and CNTRL. At 18 deg, the memorization error magnitude was higher in the sALS than in the CNTRL group. The memorization error was affected by the age in all study groups.

The rate of correctly executed MGS and of the errors and relative corrections are reported in Table 2. The rate of the correctly executed MGS was significantly lower in ALS patients, who also showed an increased error rate and reduced correction rate during the MGS task with respect to CNTRL. sALS showed higher percentages of correctly executed MGS than bALS. bASL performed with significantly higher error rate in MGS than sALS.

For sALS, the percentage of correctly executed MGS directly correlated with the parietal cortical GM volume (rho = 0.89865, p = 0.027778).

VSS Results

All groups were able to perform the pencil-based task before the VSS task. The distribution of fixations in the VSS task of controls and ALS-All is shown in Figure 4-a and Figure 4-b, respectively. ALS patients showed shorter and sparser fixations with respect to controls. Table 3 reports the main results of the metrics computed for the VSS task. The ability to perform visual sequencing (SEQ) was found significantly lower in bALS than in CNTRL. For all the other variables no significant differences were detected among groups.

For the ALS All patients, the distance to the next target (DN) inversely correlated with the ECAS scores (*i.e.* language rho = -0.52493, p = 0.030495; fluency: rho = -0.63645, p = 0.0060159, and ECAS total score: rho = -0.56319, p = 0.018571); the fixation duration inversely correlated with ALSFRS-R (rho = -0.58708, p = 0.013224) and the sequencing score (SEQ) correlated with the temporal lobe volume (rho = 0.74086, p = 0.014233).

Discussion

The main original result of this study, aimed to explore the involvement of different neural networks in spinal and bulbar ALS by means of saccadic behaviours, shows that bALS are associated with slow saccades, while sALS have hypometric and multistep saccades. Both parameters negatively correlate with disease duration, suggesting a link to the disease pathology. Moreover, according to the previous literature, we confirm an increased error rate and prolonged latency of volitional saccades in all patients vs controls. Several cross sectional and longitudinal studies have been focused on eye movements in ALS with two main objectives: to discover clinical markers of progression of disease and to find correlations between eye movements and cognitive deficits that may discriminate ALS from FTD and dementia[23,42-47]. These studies have confirmed that antisaccades and other volitional saccades are abnormal in ALS and correlate with abnormal structural and functional neuroimaging parameters, and with deficits in executive functions, verbal fluency and language[22,48,49]. Lacking convergence exists in the literature on VGS abnormalities[23].

VGS and Oculomotor Profile

ALS patients showed longer VGS latency than controls, that could reflect delayed visual processing, or target selection or motor programming in the parieto-collicular pathway[50,51].

When a reflexive movement is stimulated, the localization of the target in spatial coordinates is sent via parieto-collicular pathway to the saccade related neurons in the intermediated layer of the superior colliculus for fast gaze responses[52,53]. In ALS, neuropathological, structural and functional changes have been demonstrated in the parieto-occipital cortices, and their connections[4,11,12,16,54,55], possibly explaining the increased latency of VGS observed in our patients. Furthermore, a dysfunction of the SC, for an extension of the pathologic process to rostral midbrain, could be sufficient itself to explain the longer latency found in our patients, particularly because we use a gap paradigm, which tests the ability of the SC to disengage fixation, generating short latency saccades.

bALS patients, also showed reduced speed and increased duration of horizontal and vertical saccades. This dynamic change is well visible in (Figure 2-b), where the main sequence of bALS falls out the 95 % confidence limit of normal control. Slow saccades may be due to damage of the brainstem reticular formation, housing premotor burst neurons whose firing rate is strictly

correlated with saccade speed and whose projections are monosynaptically directed to the ocular motoneurons[56,57]. Finally, the loss of ocular motoneurons at the nuclear level is also plausible in bALS even if not yet demonstrated pathologically.

Lacking convergence exists in the literature on VGS abnormalities[23]. Eye movements are classically considered normal in ALS, since neurons in oculomotor nuclei (III, IV and VI) are more preserved compared with neurons of other cranial nerves (VII, XI and XII) and the lower motoneurons of the spinal cord[58-60]. Nevertheless, few reports have noticed slow reflexive saccades in bulbar onset and rapidly progressive forms[61,62] or in patients whose lives are prolonged by artificial ventilation[63]. Despite any relative resistance at the nuclear level[64], the discovery of pathological inclusions of Bunina bodies, spheroids and TDP-43 in the midbrain, pons and substantia nigra of ALS patients, would be compatible with the involvement of the brainstem ocular motor nuclei and reticular formation that houses the neural machinery for generating saccadic pulses[65,66]. According to the neuropathology, advanced neuroimaging studies also demonstrated structural, metabolic, neuroinflammatory and reactive changes in the brainstem of ALS that may occur early in bALS causing slow saccades[4,5]. A specific pattern of slow saccades mainly in the vertical plane was also found in a variant of ALS associated to progressive supranuclear palsy and extrapyramidal signs [45,67-69].

sALS patients showed normal velocity but hypometric, often multistep pattern of reflexive saccades, particularly for large target eccentricity (Figure 1). Hypometria of reflexive saccades could result from excessive SC inhibition[70], or, it may indicate a cerebellar deficit in controlling saccade duration[71,72] or may be related to an incorrect spatial localization due to target eccentricity. Both conditions would be supported here by the direct correlation between saccade amplitude and cerebellar and parietal lobe volumes[73,74]. Multistep VGS may also reflect an imbalance between the inhibition-facilitation of the brainstem saccade generator[75]. Finally, they could also just be the expression of a general facilitation in the execution of small saccades.

Furthermore, bALS saccade velocity and sALS amplitude showed an inverse correlation with the disease duration, as a further proof that these abnormalities rise from the dysfunction caused by the underlying pathological process.

Volitional saccades: AS and MGS characteristics.

The study of MGS and AS represents a good tool for monitoring executive functions, working memory and frontal activities in neurodegenerative diseases including ALS[44,55,76-78].

Our study confirms that all ALS patients have impaired AS and MGS, with a greater error rate than controls. According to previous reports[22,23,48], this behaviour was significantly more severe in bALS (Figure 3-b). However, most ALS patients self-corrected the direction error, revealing a still preserved motor program in both groups (Figure 3-b). Finally, bALS patients showed a higher magnitude of memorization error with respect to the sALS and healthy controls (Figure 3-d). Although not statistically evident, sALS showed a staircase pattern of voluntary saccades (Figure 1), confirming their fragmented gaze behaviour profile and suggesting an excess of inappropriate inhibition over SC from BG. In all patients, the rate of correctly executed MGS correlated to the frontal lobe volume.

The DLPFC, responsible for suppressing reflexive saccades, has been among the first functionally abnormal regions noted in ALS neuroimaging studies, particularly during tasks of executive function[79-83]. Furthermore, the frontal cortex has been recently shown to be less activated during antisaccade preparation in ALS patients[84].

Alternatively, the loss of suppression of reflexive behaviour could be also compatible with the widespread hyperreflexia, a diffuse facilitation of motor system which is a well-documented phenomenon in ALS[85-87].

We also found a correlation between latency of correctly executed AS and disease duration. Previous studies have confirmed that antisaccades and other volitional saccades are abnormal in ALS and correlate with abnormal structural and functional neuroimaging parameters, and with deficits in executive functions, verbal fluency and language[22,23, 48,49].

Visuo-sequential search abilities (VSS)

Although less impaired than language and fluency, visual search may be also abnormal in ALS[88,89].

VSS is an eye tracking task developed for studying top-down gaze strategies adopted during the exploration of alphanumerical strings which evaluates visual-spatial abilities, attentional switching, working memory and executive functions[37].

Here, ALS patients demonstrated a VSS strategy characterized by a greater number of sparse short fixations (Figure 4), with bALS patients having a significant reduced sequencing ability (Table 3). These results might indicate the need of resampling the element's position because of a deficit in spatial map, working memory or attention or difficulties in encoding the sequential string of letters and numbers. Overall, a successful VSS involves verbal fluency, working

memory and is mostly processed in the frontal networks[90,91] and optimized by the cerebellum[44]. This finding further confirms the early and prominent involvement of frontal areas in bALS and deserves further investigation, considering the relevance of language problems including verbal processing, naming, syntactic and single word comprehension occurring in ALS patients[92,93].

Conclusion

In conclusion, the abnormal saccadic profile observed in our ALS patients expresses a diffuse functional impairment of the brain, supporting the theory of a multi-system pathology that spreads from cortical to subcortical structures[6].

Furthermore, our results support the idea that testing volitional saccades is an effective method for monitoring frontal functions in ALS, but does not discriminate between subgroups. Conversely, we found separate profile of VGS between sALS (short multistep saccades) and bALS (slow saccades) ALS, which could result from the early pathologic involvement of different pathways, namely the cerebello-parieto-collicular network in sALS and the fronto-brainstem circuit in bALS. This finding is new and deserves to be further investigated for its diagnostic and prognostic implications. Ultimately, our data confirm the value of the eye movements analysis in the study of ALS and add new insight in the involved networks.

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Competing interests

The authors report no competing interests.

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Figure legends

Figure 1. Example of movements representative of the performance attained by the participants in the saccadic tasks. VGS: visually guided saccades; correct AS: correctly executed antisaccades; AS err/corr: erroneous pro-saccades and relative corrective saccades in the AS task; correct MGS: correctly executed memory-guided saccades; MGS err/corr: erroneous pro-saccades and relative corrective saccade in the MGS task; MGS error: erroneous pro-saccades without correction in the MGS task. bALS patients show normal amplitude, slow and long latency VGS and great memorization error in MGS; sALS patients show hypometric two-three steps VGS and staircase AS.

Figure 2. Visually guided saccades (VGS) parameters. (a) Boxplots of the distribution of the main saccadic parameters computed for the horizontal (10 and 18 degrees) and vertical (8 degrees) VGS task (CNTRL: control group; bALS: ALS-bulbar patients, sALS: ALS-spinal patients). When results of horizontal saccades at 10 deg and 18 deg are merged, horizontal latencies are reported (HOR). The statistical significances are reported ($p<0.05$, two-tailed). The boxplot reports: 25th and 75th interquartile ranges, box extremes; mean value, horizontal red line; median value, green cross; extreme data points, whiskers; outliers: red cross.

(b) Peak-velocity vs amplitude and duration vs amplitude main-sequence relationships for the horizontal VGS task. (CNTRL: solid black; 95% confidence interval of CNTRL: dashed black; (ALS-bulbar patients, blue; ALS-spinal patients: green). (c) peak-velocity vs amplitude and duration vs amplitude main-sequence relationships for the vertical VGS task. (CNTRL: solid black; 95% confidence interval of CNTRL: dashed black; ALS-bulbar patients, blue; ALS-spinal patients: green). (b-c) The fitting equations are reported on the graphs for each group (CNTRL: control group; bALS: ALS-bulbar patients, sALS: ALS-spinal patients). Peak-velocity versus Amplitude: $V_{peak}=c+V_{max}\times(1-e^{(A/c)})$; where V_{peak} = peak velocity; V_{max} = asymptotic peak velocity; A = amplitude, c = constant. Duration versus Amplitude: $(D=k+(b\times A)$; where D = duration, k = constant; b = slope of the fitted line; A = amplitude.

Figure 3. Antisaccade and Memory-Guided saccades parameters. (a) Boxplots of the distribution of the latencies of the correctly executed antisaccades (AS) for the control (CNTRL) and ALS groups (bALS and sALS). (b) Boxplots of the distribution of the amplitudes of the adjustment movement after a correctly executed memory-guided saccade (MGS) for the control (CNTRL) and ALS groups (bALS and sALS) at 10 and 18 degrees.

The boxplot reports: 25th and 75th interquartile ranges, box extremes; mean value, horizontal red line; median value, green cross; extreme data points, whiskers; outliers: red cross.

Figure 4. VSS parameters. (a) Distribution of fixations of CNTRL group. (b) Distribution of fixations of ALS-All patients. The colour map is reported and corresponds to the cumulative fixation duration (in milliseconds) over the image (averaged across subjects and trials). The plots qualitatively show a diverse visual strategy adopted by the analyzed groups when performing in the VSS task. ALS patients show sparser fixations with respect to controls.

Table 1 Summary of demographic and clinical cognitive and MRI characteristics of the subjects recruited in the study

	Bulbar ALS (n = 6)	Spinal ALS (n = 12)	ALS, All (n = 18)	Controls (n = 13)	*P*-value
Demographic and clinical measures					
Male/Female (N)	2/4	7/5	9/9	5/8	
Age (years)	69 (6.3), 59–77	65 (9.5), 46–79	67 (9), 46–79	64.4 (3.4), 52–73	
Education (years)	**8 (3.5), 5–13**	**8 (3.1), 5–15**	**8 (3.1), 5–15**	**14 (2.9), 11–19**	$*P = 0.0211$[a], $**P = 0.0019$[a], $***P = 2.2966 \times 10^{-4}$ ($U = -3.6839$)[b]
EEDCr (classN)	P: 1, PrL: 3, Pr: 1, D: 1	P: 3, PrL: 3, Pr: 4, D: 2	P: 4, PrL: 6, Pr: 5, D: 3	–	
ALS-FRS total score	38 (2.9), 33–41	37 (6.9), 23–46	38 (5.7), 23–46	–	
Genetics (N)	1	1	2	–	–
King (Score: Subjects)	1: 1, 2: 3, 3: 2, 4: 0	1: 5; 2: 1, 3: 5, 4: 1	1: 6; 2: 4, 3: 7, 4: 1	–	–
MITOS (Score: subjects) FVC reduced (N)	0: 5, 1: 1 3/6	0: 9, 1: 2, 2: 1 4/12	0: 14, 1: 3, 2: 1 7/18	–	–
Neuropsychological parameters					
Memory functions	15 (3.8), 9–20	**14 (3.8), 5–20**	**15 (3.7), 5–20**	**18.50 (7.10)**	$**P = 0.0312$, $***P = 0.0168$
Visual-spatial functions	**11 (1.6), 8–12**	**11 (1.0), 9–12**	**11 (1.2), 8–12**	**11.71 (0.34)**	$*P = 2.6870 \times 10^{-10}$, $**P = 4.6946 \times 10^{-13}$, $***P = 1.2615 \times 10^{-21}$
Language functions	**24 (5.3), 16–28**	**23 (3.7), 18–28**	**23 (4.2)**	**26.95 (2.01)**	$*P = 6.9406 \times 10^{-4}$, $**P = 1.2954 \times 10^{-12}$, $***P = 9.1026 \times 10^{-15}$
Verbal fluency	**15 (4.7), 8–22**	**14 (7.7), 0–24**	**14 (6.7), 0–24**	**21.00 (2.6)**	$*P = 9.1413 \times 10^{-10}$, $**P = 1.3161 \times 10^{-21}$, $***P = 9.1268 \times 10^{-30}$
Executive functions	**31.2 (7.0), 22–40**	**28 (9.2), 10–40**	**29 (8.5), 10–40**	**40.00 (4.32)**	$*P = 5.4827 \times 10^{-7}$, $**P = 1.7362 \times 10^{-22}$, $***P = 1.8355 \times 10^{-27}$
ECAS total score	**96 (17.4), 68–110**	**90 (17.8), 667–119**	**92 (17.4), 67–119**	**118.00 (4.62)**	$*P = 2.3967 \times 10^{-32}$, $**P = 9.7816 \times 10^{-101}$, $***P = 9.8810 \times 10^{-130}$
Brain Volumes					
Brain peripheral grey	**534.4 (20.4), 503.7–549.0**	577.0 (64.5), 456.0–644.5		**627.3 (29.2), 578.5–672.0**	$*P = 0.0028$[a]
Cerebellum	169.0 (20.2), 136.6–188.9	176.2(21.8), 143.2–195.8		179.9(21.4), 140.8–203.9	
Occipital Lobe	70.8 (6.1), 63.4–77.0	75.9 (10.7), 56.8–88.8		76.8 (9.3), 59.0–87.9	
Temporal Lobe	**125.9 (9.3), 113.3–138.4**	141.6 (14.9), 122.9–158.2		**152.2 (8.14), 144.4–168.0**	$*P = 0.0051$[a]
Insula	12.3 (0.9), 10.9–13.3	14.2 (2.5), 11.6–17.7		19.2 (6.4), 14.1–28.6	
Parietal Lobe	**122.8 (10.5), 104.8–131.9**	126.3 (13.8), 104.6–145.1		**140.1 (9.1), 126.1–154.0**	$*P = 0.0446$[a]
Frontal Lobe	**186.0 (12.8), 168.3–198.4**	199.9 (31.6), 147.6–232.5		**225.5 (9.8), 206.9–238.0**	$*P = 0.0039$[a]

Values are presented as mean (standard deviation), min–max. The groups significant differences are highlighted in bold (*bALS versus CNTRL, **sALS versus CNTRL, ***ALS(All) versus CNTRL). ALS: Amyotrophic Lateral Sclerosis group; sALS: spinal ALS variant group; bALS: bulbar ALS variant group; CNTRL: control group. - Not Applicable. EEDCr: El Escorial Diagnostic Criteria, revised (P=possible, Pr=Probable; PrL= Probable laboratory-supported, D=Definite). King: King's College Staging System. MITOS: Milano-Torino Staging System. FVC reduced: Forced Vital Capacity <80% of the prediction

[a]Games-Howell Post-hoc analysis after a significant Kruskal-Wallis test result ($p<0.05$, two-tailed).

[b]Mann-Whitney-U-test refers to comparison between ALS patients with spinal and bulbar onset and between ALS(All) and CNTRL ($p<0.05$, two-tailed).

Table 2 Mean and standard deviation of the performance rates computed for the AS and MGS tasks.

	bALS	sALS	CNTRL	*P*-value
%AS	**41 (35)**	**44 (28)**	**69 (24)**	***$P < 0.05$
%ErrAS	**59 (35)**	**56 (28)**	**31 (24)**	***$P = 1.141 \times 10^{-13}$
%CorrAS	98 (3)	99 (3)	98 (4)	-
%MGS	**42 (37)**	**56 (31)**	**80 (26)**	******$P = 2.4727 \times 10^{-14}$
%ErrMGS	**65 (33)**	**57 (32)**	**32 (25)**	***$P = 0.00031325$
%CorrMGS	**52 (32)**	**59 (29)**	**73 (29)**	******$P < 0.05$

ALS: Amyotrophic Lateral Sclerosis group; sALS: spinal ALS variant group; bALS: bulbar ALS variant group; CNTRL: control group; AS: anti-saccades task; MGS: memory-guided saccades task; %AS: percentage of AS correctly executed; %ErrAS: percentage of erroneous antisaccade movements; %CorrAS: percentage of corrections after an erroneous anti-saccade movement; %MGS: percentage of memory-guided saccades correctly executed; %ErrMGS: percentage of erroneously executed (reflexive) memory-guided saccades; %CorrMGS: percentage of corrections after an erroneous memory-guided saccade movement.
The groups significant differences are highlighted in bold (χ^2 test followed by the post hoc Marascuilo procedure, $p<0.05$, two-tailed, *bALS versus CNTRL, **sALS versus CNTRL, ***bALS versus sALS).

Table 3 Mean and standard deviation of the main metrics computed for the VSS task

	sALS	**bALS**	**CNTRL**	***P*-value**
SEQ	7.50 (2.14)	**7.33 (1.80)**	**9.54 (0.75)**	**P* = 0.0063
DN	4.69 (0.28)	4.86 (0.36)	4.78 (0.46)	-
DT	12.79 (2.97)	14.71 (2.23)	14.18 (4.08)	-
FIX_DURATION	270.66 (106.62)	327.51 (170.93)	517.52 (265.85)	
FIX_DURATION_TARGET	399.32 (165.66)	406.31 (201.43)	522.92 (174.85)	-

ALS: Amyotrophic Lateral Sclerosis group; sALS: spinal ALS variant group; bALS: bulbar ALS variant group; CNTRL: control group; VSS task: Visual Sequential Search; SEQ: sequencing score; DN: Euclidean distance for each fixation to the nearest region of interest (deg), DT: Euclidean distance for each fixation to next target (deg), FIX_DURATION: average duration of a fixation during the task (ms), FIX_DURATION TARGET: average duration of the fixations landing on the target (ms). The groups significant differences are highlighted in bold (Games-Howell Post-hoc analysis after a significant Kruskal-Wallis test result, p<0.05, two-tailed, *bALS versus CNTRL).

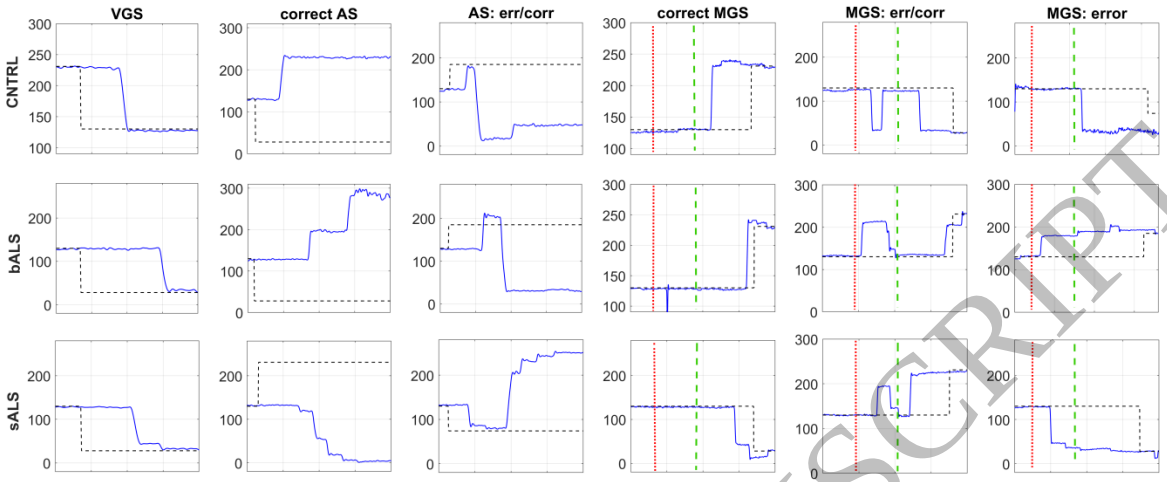

Figure 1
503x242 mm (6.7 x DPI)

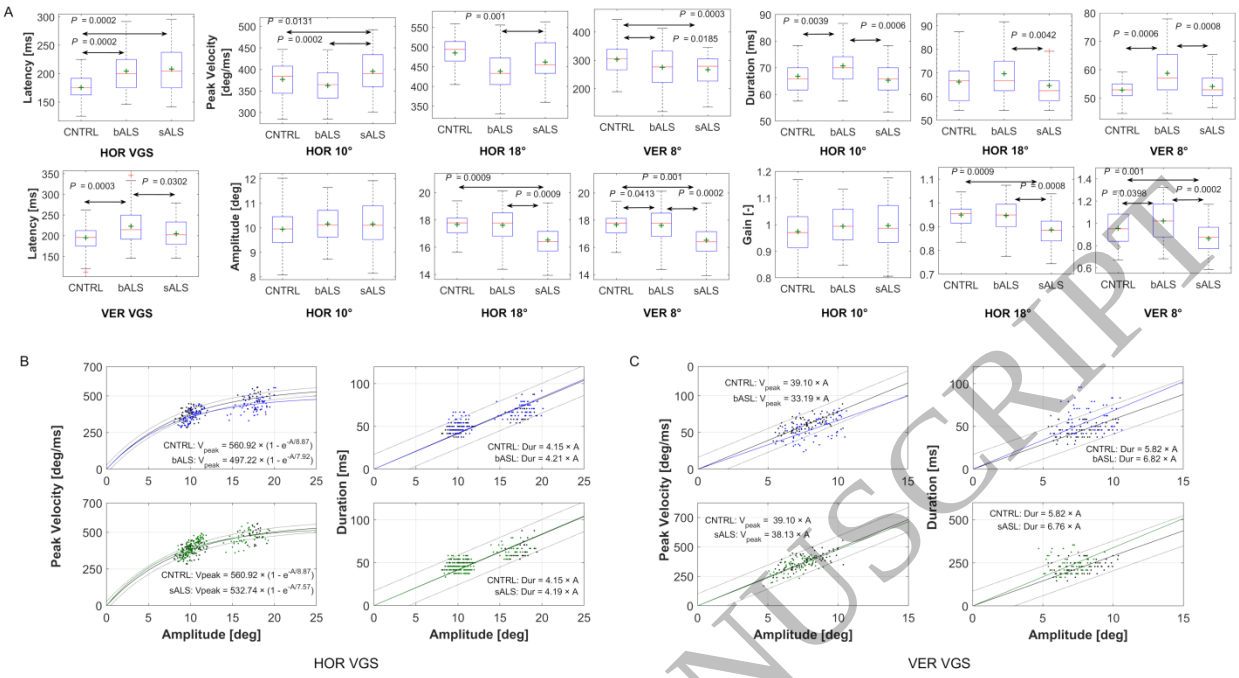

Figure 2
500x282 mm (6.7 x DPI)

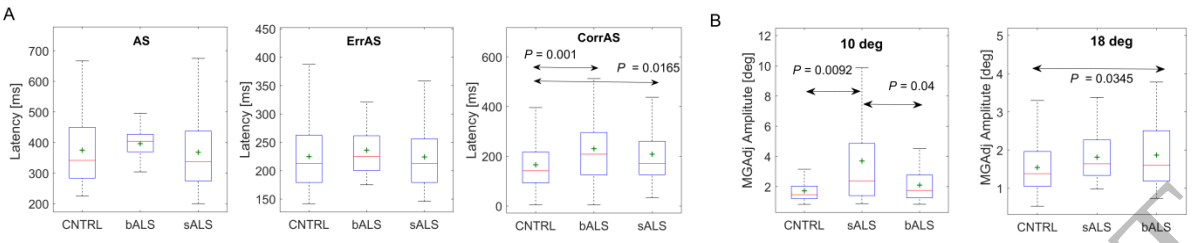

Figure 3
503x104 mm (6.7 x DPI)

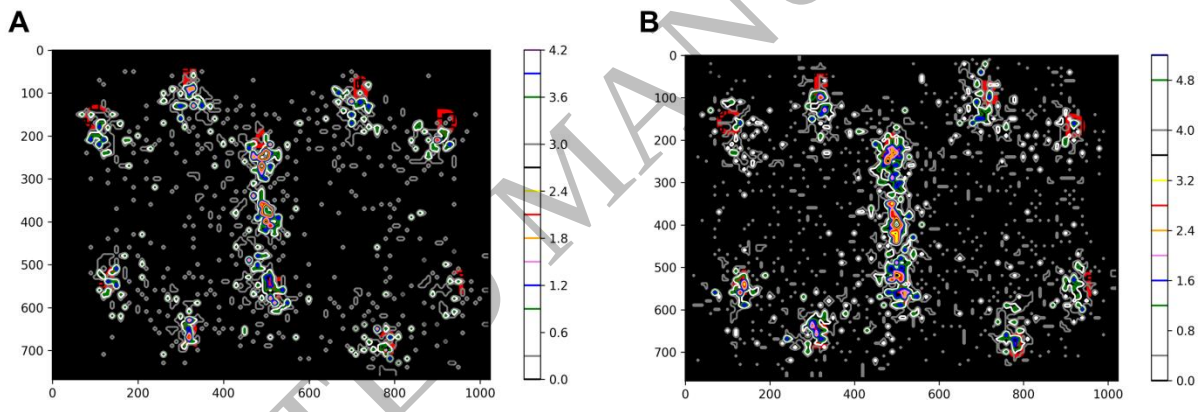

Figure 4
271x94 mm (6.7 x DPI)