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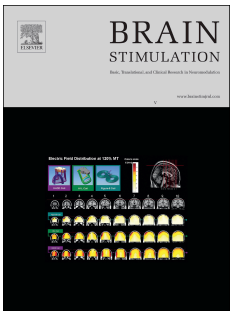

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Modulation of human corticospinal excitability by paired associative stimulation in patients with amyotrophic lateral sclerosis and effects of Riluzole

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Abstract:

BACKGROUND: Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that causes an impairment in both the upper and lower motor neurons. The recent description of numerous non-motor signs points to an involvement of the neocortex networks that is more complex than was previously believed. Paired associative stimulation (PAS), a combination of transcranial magnetic stimulation (TMS) and peripheral nerve stimulation, can enhance motor output in the contralateral hand through an NMDA-mediated sensorimotor mechanism.

OBJECTIVE: To describe the effects of PAS on ALS patients before and after Riluzole intake compared with healthy subjects.

METHODS: PAS was used to detect differences between 24 newly-diagnosed ALS patients and 25 age-matched healthy controls. MEP amplitude from the abductor pollicis brevis was considered before PAS, immediately after (T0) and after 10 (T10), 20 (T20), 30 (T30) and 60 (T60) minutes. Statistical significance was calculated using RM-ANOVA.

RESULTS: In healthy controls, PAS significantly increased MEP amplitude at T10, T20 and T30 ($p<0.05$). In ALS patients, a significant increase in MEP amplitude was also observed after 60 minutes ($p<0.05$), thus demonstrating NMDA-mediated enhanced facilitatory plasticity. After two weeks of riluzole intake, no MEP amplitude increase was evident after PAS at any time point. In three monomelic-onset ALS patients, sensorimotor facilitation was evident only in the hemisphere corresponding to the affected side and appeared in the opposite hemisphere when the patients manifested contralateral symptoms.

CONCLUSIONS: PAS may be considered a useful tool when investigating NMDA-mediated neocortical networks in ALS patients and the modulation of such networks after anti-glutamatergic drug intake.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is considered a rare disease, with a reported worldwide incidence of between 1.5 and 2.7 per 100,000 people per year and a prevalence of 1 per 20,000 [1,2]. It is an adult onset neurodegenerative disorder that causes a premature loss of motor neurons in the cerebral cortex, brainstem and spinal cord. While the aetiology of ALS is unknown, current evidence suggests that multiple interacting factors contribute to motor neuron injury in ALS. One of the hypothesized pathogenetic mechanisms is glutamate-driven excitotoxicity in the motor cortex [3]. A considerable amount of significance has recently been assigned to non-motor aspects of motor neuron disease, such as cognitive impairment [4], gastrointestinal dysfunction [5], small fiber neuropathy [6] and laryngeal sensitivity [7]. The aim of this trial was to investigate pathogenetic mechanisms in ALS that involve not only motor function but also the cortical networks, which are responsible for some of the non-motor signs in this disease.

Until recently, the only approved therapy for ALS was Riluzole, an antiepileptic drug with numerous pharmacodynamic mechanisms, such as inhibition of persistent Na^+ current, potentiation of calcium-dependent K^+ current, inhibition of neurotransmitter release, inhibition of fast Na^+ current, inhibition of voltage-gated Ca^{++} current and inhibition of voltage-gated K^+ current [8]. The activation of Na^+ current enhances NMDA receptor function [9]. Riluzole also acts on muscle Na^+ channels in myotubes [10].

There has recently been a surge in interest in electrophysiological techniques that promote and investigate short-term changes in human cerebral cortex excitability; within this context, paired associative stimulation (PAS) has drawn attention both as a therapeutic intervention [11,12] and as an experimental method to investigate Hebbian principles of synaptic plasticity. In the prototypical form of PAS [13], a single electrical stimulus is directed to a peripheral nerve before a magnetic stimulus is delivered to the contralateral primary motor cortex (M1). The inter-stimulus interval is adjusted to ensure that inputs to M1 initiated by the afferent volley arising from the nerve

stimulation occur simultaneously with the magnetic stimulation. Repeated pairing of the two sources of stimulation (i.e. association) over an extended period of time increases the excitability of corticospinal projections from M1. A reduction in corticospinal excitability has been reported when the inter-stimulus interval is adjusted so as to allow a corollary of the afferent volley to reach M1 after the magnetic stimulus [14].

This neuroplastic adaptation revealed by PAS appears to exhibit several of the criteria designated for long-term potentiation (LTP) and long-term depression (LTD): its effects evolve quickly, are reversible and persist beyond the period of stimulation [15,16]. Pharmacological agents that interact with NMDA-receptor activity interfere with the outcomes of PAS, thereby supporting the hypothesis that LTP-like changes are implicated [16]. In view of these properties, and the widespread belief that the alterations in excitability induced by PAS are restricted to cortical representations of the muscles innervated by the peripheral nerve that has been stimulated electrically, it has been suggested that PAS-induced adaptation represents a form of associative LTP (and LTD) that is synapse-specific [17] and behaves in accordance with Hebbian principles [13,18,19].

Following the first description of this technique by Stefan and colleagues in 2000, there have been a wide range of derivative investigations concerning, among other features, the most effective inter-stimulus intervals (ISIs) [20,21], the muscles in which the effects can be elicited [13,22,23] and variations in the extent to which they can be induced in various clinical populations [12]. As this corpus of work has accumulated, large inter-individual differences in response to PAS have been observed [24]. This has led to investigations on potential mediating factors such as age [25], cortical anatomy [26] and the role of specific genetic polymorphisms [27].

In this study, we used the PAS paradigm to test whether plasticity within the sensorimotor cortex is abnormal in ALS patients and to investigate the effects of Riluzole on the outcome of this stimulation. PAS was also used in three patients with monomelic onset to determine whether there

are any differences in cortical excitability between the two hemispheres. Two of these three patients were re-tested shortly after contralateral lower motor neuron signs had appeared.

MATERIALS AND METHODS

We recruited 24 newly-diagnosed patients affected by probable and definite ALS [28]. The patients' demographic characteristics are shown in Table 1. None of the patients were taking any specific therapy when they were recruited; any psychotropic drugs were suspended at least 2 weeks before the trial started. Twenty-five, healthy, age-matched controls were also tested.

Every ALS patient was tested before and after taking 100 mg/die of Riluzole for two weeks. When PAS was performed while the patients were taking Riluzole, the drug was administered 12 hours before PAS so as to be able to investigate the chronic effects, as opposed to the acute effects, of Riluzole. In every subject, we measured the resting motor threshold (RMT), the sensory threshold over the median nerve of the non-dominant hand (assessed by means of the Edinburgh Handedness Inventory scale) at 500 μs of duration, the maximal motor evoked potential (MEP) and the mean MEP amplitude, which was based on 10 stimuli at 0.1 Hz at 120% of the RMT, delivered before PAS stimulation, immediately after (T0) and after 10 (T10), 20 (T20), 30 (T30) and 60 (T60) minutes. The RMT was defined as the lowest intensity able to evoke an MEP of more than 50 μV in at least 5 out of 10 consecutive trials in the abductor pollicis brevis (APB) of the non-dominant hand. Transcranial magnetic stimulation (TMS) was delivered through a high-frequency magnetic stimulator (Magstim Rapid – The Magstim Company Ltd, Whitland, South West Wales, UK) connected to a figure-of-eight coil. We also measured the N20 wave of the somatosensory evoked potential (SEP) in each patient. PAS stimulation consisted of 200 paired stimuli at 0.3 Hz. Magnetic stimuli were delivered over the hotspot for the APB of the non-dominant hand at 100% of the RMT. The coil was held tangentially to the scalp with the handle pointing back and away from the midline at 45°. Electrical stimulation was delivered over the median nerve of the non-dominant hand at 500 μs of duration, at an intensity equivalent to 300% of the sensory threshold. The time between the electrical and magnetic stimuli (inter-stimulus interval - ISI) was calculated by adding 6 ms to the

N20 component of each patient. Previous studies have demonstrated that the ISI plays a key role in determining MEP amplitude changes after PAS [29], thereby highlighting the importance of personalizing this test.

A PAS session was performed the following day in the 8 healthy controls using the same parameters (mean MEP amplitude evoked by 10 stimuli before PAS stimulation, immediately after (T0) and after 10 (T10), 20 (T20), 30 (T30) and 60 (T60) minutes), adopting a testing stimulus intensity of 110% instead of 120% of the RMT in order to ensure that the number of motor neurons activated did not affect the PAS.

In three patients with monomelic ALS onset who presented first and second motor neuron involvement in the same upper limb, PAS was performed by stimulating the right and left cortex on two consecutive days while they did not take Riluzole. The diagnosis of probable or definite ALS was subsequently confirmed by disease progression. Two of these three patients did not take Riluzole and were tested again when signs of ALS appeared in the opposite arm.

STATISTICAL ANALYSIS

The mean RMT was calculated in the patient group before and after they took Riluzole as well as in the control group. Mann-Whitney for independent samples was used to compare the RMT amplitude in patients not taking Riluzole and in the control group. ANOVA for repeated measures (RM-ANOVA) was used to detect any effect of PAS on the absolute MEP amplitude immediately after PAS (T0) and 10 (T10), 20 (T20), 30 (T30) and 60 (T60) minutes later; a within-subject and between-subject analysis was performed to detect any variation in the mean MEP amplitude between pre-stimulation and post-stimulation values and between patients and controls. Corrections for the disease duration, cMAP amplitude from the median nerve of the non-dominant hand, ALSFRS-R score and product between ALSFRS-R score and disease duration were applied to the results when they significantly affected them. Statistical significance was set at $p<0.05$. All the data analyses were performed by means of IBM SPSS Statistics 22.

RESULTS

The mean RMT in the patients before and after Riluzole intake as well as in the control group are shown in Table 2. The Mann-Whitney test revealed a markedly higher RMT in ALS patients than in age-matched controls ($p<0.01$), though no significant difference emerged between the pre- and post-Riluzole sessions ($p>0.05$). The MEP amplitude at 120% of RMT was higher in healthy controls than in ALS patients regardless of whether they took Riluzole, though the difference was not statistically significant. A one-way RM ANOVA was conducted to compare the effect of PAS on MEP amplitude at the different time points. Our data violated the assumption of sphericity for both controls and ALS patients regardless of whether the latter took Riluzole. The results of the RM ANOVA with a Huynh-Feldt correction showed that the mean MEP amplitude differed significantly at the different time points in the control group ($F(3.173, 2.376) = 2.820$, $p < 0.05$); no significant difference in MEP amplitude between the time points emerged in ALS patients regardless of whether they took Riluzole, although a trend to significance was detected in patients who did take Riluzole ($p=0.064$). The within-subject analysis performed in the control group demonstrated a significant post-stimulation increase in the mean MEP amplitude at T10 ($p=0.027$), T20 ($p=0.040$) and T30 ($p=0.024$) compared with the pre-stimulation mean MEP amplitude. The within-subject analysis in the ALS patients who did not take Riluzole revealed a significant increase in the mean MEP amplitude at T20 ($p=0.036$), T30 ($p=0.017$) and T60 ($p=0.031$) compared with the pre-stimulation mean MEP amplitude. The within-subject analysis in ALS patients taking Riluzole demonstrated a significant decrease in mean MEP amplitude at T0 ($p=0.009$), T20 ($p=0.042$) and T30 ($p=0.039$) compared with the pre-stimulation mean MEP amplitude, with a trend to significance at T10 ($p=0.077$) and T60 ($p=0.056$) (fig.1). The between-subject analysis did not detect any significant difference between controls and ALS patients who did not take Riluzole; this finding is likely to be due to the small sample size.

We also performed a within-subject analysis on the effect of PAS on the ratio between the MEP amplitude at different time points and the baseline MEP amplitude in order to exclude any effect of the baseline MEP amplitude on the results. The within-subject analysis performed in the control

group revealed a significant post-stimulation increase in the mean MEP amplitude at T10 (p=0.001), T20 (p=0.022) and T30 (p=0.003); the within-subject analysis in ALS patients who did not take Riluzole also revealed a significant post-stimulation increase in the mean MEP amplitude at T20 (p=0.025), T30 (p=0.016) and T60 (p=0.013).

The same statistical evaluations were performed in healthy controls, who were tested at 110% of the RMT intensity. No substantial differences were detected between this session and that performed at 120% of the RMT intensity (when compared with pre-stimulation MEP values, values were statistically significant at T10, T20 and T30; respectively p=0.030, p=0.028, p=0.003). All the results of RM ANOVA were covariated for the disease duration, ALSFRS-R score, cMAP amplitude from the median nerve of non-dominant hand and product between disease duration and ALSFRS-R score, as a parameter of decline speed. None of these parameters significantly corrected the RM ANOVA results (p>0.05).

In the three patients with monomelic upper limb onset, PAS was performed both on the affected and unaffected sides. On the affected side, MEP behaved in the same way as in ALS patients who did not take Riluzole, with an increase in amplitude that persisted for 60 minutes post-stimulation. By contrast, the unaffected side yielded an increase in MEP amplitude that disappeared by T60, thus resembling the behavior observed in healthy subjects (Fig. 2). Two of these three patients did not take Riluzole and were tested again, shortly after a contralateral loss of strength and EMG signs of peripheral involvement had appeared. The originally unaffected arm yielded the same results as the affected side (Fig. 3).

DISCUSSION

ALS is a rare disease with a non-defined etiology. Numerous theories on its causes have been proposed, ranging from genetic mutations [30,31] to inflammatory damage [32], mitochondrial dysfunctions [33] and ubiquitin-proteasome system alterations [34]. Many of these hypotheses converge upon an increase in glutamate levels, which results in excitotoxic damage mediated by NMDA-channels: the opening of this receptor generates a Calcium inflow, which activates caspases

and nitroxide synthase-mediated apoptosis. Increased glutamate levels and decreased glutamate transporter levels have been observed in the CSF of ALS patients [35,36,37]; glutamate leads to an increase in intracellular calcium levels, which in turn causes neuronal death. PET studies have revealed enhanced expression of NMDA receptors in ALS mouse models [38]. Riluzole, the only drug that has been approved for the treatment of ALS in many countries, has an anti-glutamatergic effect that is mediated by Na^+ channel inhibition [5] and is considered to prevent excitotoxic damage [39,40]. Riluzole also exerts effects on a number of ion channel currents [8].

Human motor cortex excitability, as tested by means of a RMT assessment, is related to glutamatergic transmission via non-NMDA glutamatergic channels, such as AMPA and Kainate [41]. A reduced RMT has been observed in Alzheimer's disease [42]. Some authors have reported that the RMT is higher in ALS patients than in healthy subjects, which confirms previous data by suggesting that cortical excitability is reduced in ALS [43]. Other authors have pointed out that ALS patients may even be affected by a reduced RMT, though this generally occurs in the early stages as a result of corticomotor neuron hyperexcitability induced by glutamate, i.e. before upper motor neurons signs appear [44]. Riluzole intake did not have any effect on the RMT in the sample enrolled in this study. PAS has been shown to represent a form of associative LTP that is synapse-specific [17] and thus exploits a NMDA-mediated mechanism. In our study, PAS increased the mean MEP amplitude 10 minutes after stimulation in the control group, though this effect had worn off 60 minutes post-stimulation. Although no direct difference between ALS patients who did not take Riluzole and the control group emerged, a significant increase in mean MEP amplitude was observed after 20 minutes and persisted beyond 60 minutes in ALS patients regardless of their baseline MEP amplitude (Fig. 1a and 1b). When the same ALS patients underwent PAS two weeks after Riluzole intake, a paradoxical reduction in MEP amplitude was observed immediately after stimulation as well as 20 and 30 minutes post-stimulation, with the reduction approaching significance at the other time points. Similar results were observed when the MEP amplitude was tested in healthy subjects at a stimulation intensity corresponding to 110% of the RMT. This spin-

off analysis was performed to simulate the effects of PAS on a reduced number of motor neurons, as occurs in ALS patients. The fact that the results yielded by both the 110% and 120% sessions were comparable demonstrates that the effects of PAS are not dependent on the number of motor neurons activated and consequently explain the similar results obtained in healthy subjects and ALS patients.

All these data point to a reduction in AMPA and Kainate-mediated glutamatergic transmission, with greater NMDA-mediated facilitatory plasticity being observed in ALS patients than in the control group; indeed, the enhancing effect exerted by PAS on the MEP amplitude persisted 60 minutes post-stimulation (Fig. 1), whereas control group values at the same time point had returned to pre-stimulation conditions. Two weeks of Riluzole intake did not modify non-NMDA glutamatergic channel-mediated RMT, but did break down NMDA-mediated enhanced facilitatory plasticity in ALS patients, who even presented a paradoxical reduction in MEP amplitude following PAS. We may also speculate that Riluzole exerts an effect on the Na^+ channel, which would in turn impair NMDA receptor function [9]; this hypothesis is supported by findings in other studies, according to which some antiepileptic drugs that block the Na^+ channel reduce cortical excitability by acting on NMDA-related mechanisms [45,46].

The potential limitations of this study are the variability of the response to PAS and the lack of correlation between our results and Riluzole blood levels. Nevertheless, it should be borne in mind that the results were based on a cross-over trial in which the same patients were analyzed before and after Riluzole intake; moreover, the physicians ensured that Riluzole had been ingested regularly before every PAS session. Lastly, besides acting on the glutamatergic system, Riluzole has numerous other mechanisms of action that may modify the effects of PAS; nevertheless, the fact that PAS appears to induce NMDA-mediated LTP-like changes and that its effect is modified by other pharmacological agents that interact with NMDA-receptor activity [16] suggests that the most important of the effects exerted by Riluzole on PAS is due to the anti-glutamatergic mechanism of this drug. Finally, other authors demonstrated that sodium and calcium channel blockers without

considerable neurotransmitter properties (carbamazepine, lamotrigine, losigamone) elevated RMT but did not change intracortical excitability [47]. Riluzole did not elevated RMT, thus excluding a relevant effect on ion channels.

Other authors have described alterations in intracortical plasticity in ALS patients, with a decrease in short interval intracortical inhibition (SICI) and an increase in intracortical facilitation (ICF) being reported [48,49,50]. In our study, we observed an increase in plasticity involving the sensory and motor areas in ALS patients that disappeared after Riluzole intake, which thus confirms the protective role of this drug.

Facilitation of plasticity in a network that is highly complex, as is the sensorimotor network, may also explain some of the non-motor signs described in motor neuron diseases, such as behavioral and cognitive changes [51].

PAS was delivered over both hemispheres in three ALS patients with monomelic onset to investigate any differences between the affected and unaffected side. A diagnosis of either probable or definite ALS was subsequently confirmed in all three patients. Given the rarity of this condition and the small sample we studied, no statistical analysis could be performed; nevertheless, the trend in MEP amplitude after PAS in all three patients resembled that observed on the unaffected side in healthy subjects and on the affected side in ALS patients who did not take Riluzole (Fig. 2).

A dying-back phenomenon has been observed in many ALS mouse models, with an early involvement of the muscle and neuromuscular junction that subsequently spreads to the second and first motor neurons [52,53,54]. An MRI and histological study on G93A-SOD1 mice revealed an early reduction in muscle volume but visible alterations in M1 cortex only considerably later [55]. Other authors have instead hypothesized a dying-forward model, with a reduction in SICI and an increase in ICF preceding lower motor neuron impairment [56]. Moreover, Fogarty observed an increase in dendritic spine density in the pyramidal motor neurons of ALS patients, thereby explaining the increased excitatory neurotransmission [57]. In our small sample of monomelic onset patients, the unaffected side may be considered as a pre-symptomatic model of disease; indeed, the

loss of focal strength loss in all three patients began with peripheral features, including muscle atrophy and fasciculations. Upper motor neuron signs in the same arm only appeared later, thus pointing to a dying-back mechanism in such patients. PAS in these monomelic patients induced a behavior that was similar to that observed in other ALS patients on the affected side and in healthy controls on the unaffected side. When the initially unaffected side was tested again in two patients following the appearance of loss of strength, the findings were similar to those observed on the opposite side, thus suggesting that changes in sensorimotor network plasticity do not precede lower motor neuron signs.

We may speculate that the disease is transmitted between the upper and lower motor neurons in various ways that are dependent on the onset, though further investigations are needed to shed light on this hypothesis.

CONCLUSION

In this study, PAS showed that facilitatory plasticity of the sensorimotor network is greater in ALS patients than in healthy subjects; this effect disappears after Riluzole intake, thereby confirming glutamate toxicity in this pathology. In monomelic patients, the effects of PAS on the affected side were found to be the same as those observed in ALS patients, while those on the unaffected side were the same as those observed in healthy controls. Enhanced plasticity of the sensorimotor network cannot, therefore, be considered to precede lower motor neuron signs in these monomelic patients. To sum up, PAS should be taken in consideration as a tool to check the efficacy of new anti-glutamatergic drugs and to improve the diagnostic approach to motor neuron diseases.

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Tab. 1 Demographic characteristics of ALS patients.

Age (years)	68 (±8.6)
Gender (M/F)	16/8
ALSFRS-R score	39.9 (±4.8)
Disease duration (months)	17.2 (±13.7)
Median cMAP amplitude (mV)	8.1 (±3.7)

Tab. 2 MEP amplitude±SEM before and after PAS (T0, T10, T20, T30 and T60) in healthy controls and ALS patients without and with Riluzole, stimulated at 110 and 120% of RMT.

	Healthy subjects	ALS Patients	
		Without Riluzole	With Riluzole
RMT	59.96±2.53	70.14±2.69	68.50±2.90
MEP pre-stimulation	0.87±0.17	0.50±0.13	0.48±0.11
MEP T0	0.93±0.18	0.47±0.13	0.38±0.10*
MEP T10	1.14±0.21*	0.75±0.20	0.39±0.09
MEP T20	1.11±0.24 *	0.70±0.19*	0.38±0.11*
MEP T30	1.30±0.27 *	0.70±0.18*	0.37±0.09*
MEP T60	0.94±0.17	0.68±0.15*	0.41±0.10
MEP pre-stimulation 110%	0.32±0.14		
MEP T0 110%	0.37±0.15		
MEP T10 110%	0.98±0.30*		
MEP T20 110%	0.61±0.19*		
MEP T30 110%	0.65±0.14*		
MEP T60 110%	0.36±0.14		

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Fig. 1a and b: Absolute (a) and normalized (b) MEP amplitude before and after PAS (T0, T10, T20, T30 and T60) in healthy controls and ALS patients without and with Riluzole. * $p<0.05$, compared with pre-stimulation.

Fig. 2: Normalized MEP amplitude before and after PAS (T0, T10, T20, T30 and T60) in affected and unaffected side of three monomelic onset patients not taking Riluzole.

Fig. 3: Normalized MEP amplitude before and after PAS (T0, T10, T20, T30 and T60) in two monomelic onset patients: comparison between the same arm before and after developing lower motor neuron signs.

None of the authors disclose any financial and personal relationships with other people or organizations that might bias this study. This clinical trial was approved by the local ethics committee and respected the declaration of Helsinki.

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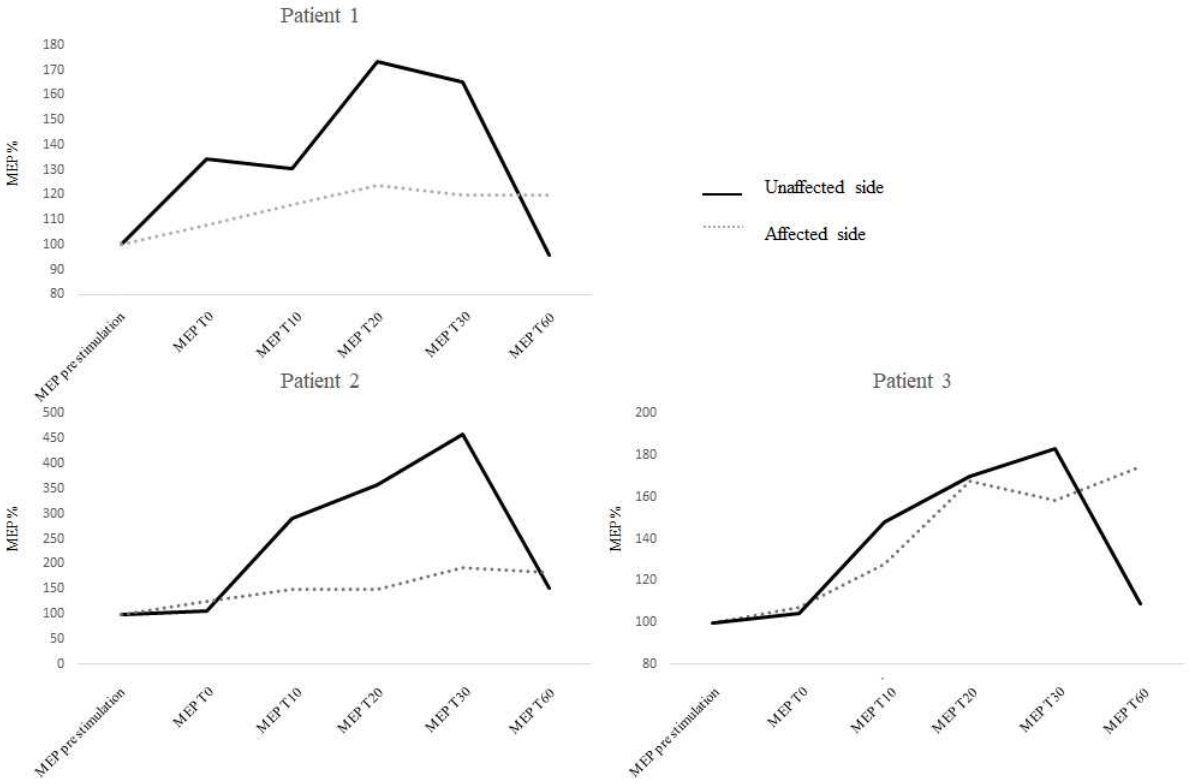

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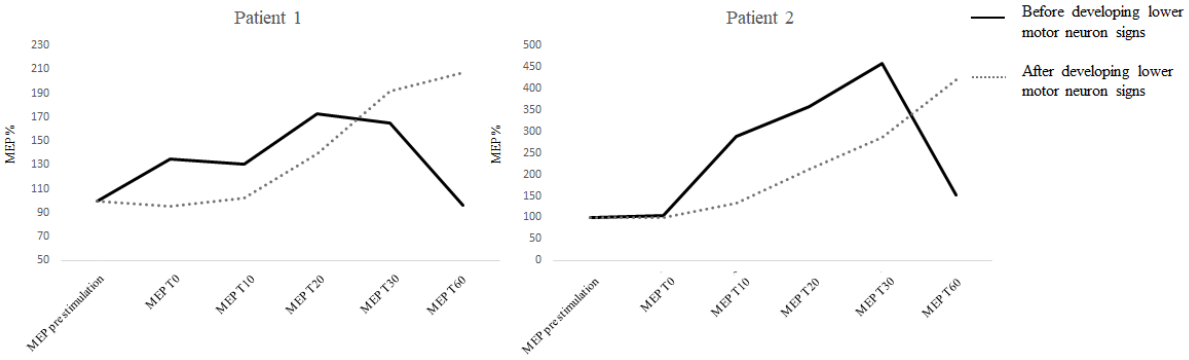

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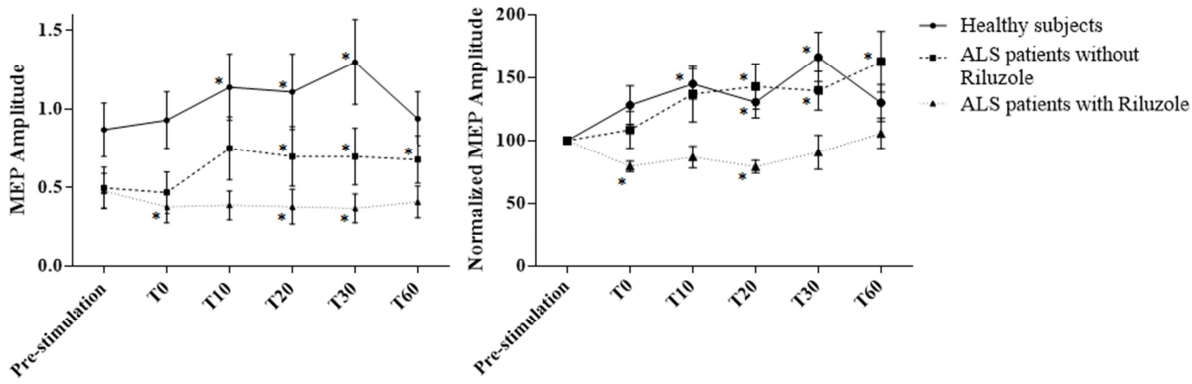

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- Integrative networks in ALS are evaluable by paired associative stimulation (PAS)
- The MEP amplitude increase after PAS lasts one hour in ALS, 30 minutes in controls
- Riluzole assumption nullifies MEP increase in ALS patients
- Integrative networks are facilitated in ALS patients by glutamatergic mechanisms

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