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# Effect of amyotrophic lateral sclerosis serum on calcium channels related to spontaneous acetylcholine release

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**Abstract**

**Objectives**: The aim of this work was to further investigate the effect of sera from sporadic amyotrophic lateral sclerosis (ALS) patients on miniature end-plate potentials (MEPP) frequency, by the mouse passive transfer model, and to study whether the transferred serum induces any change in the sensitivity of the L-type voltage-dependent calcium channels (VDCC) to its specific blocker Nitrendipine.

**Methods**: A total of 35 CF1 mice were divided into 3 groups: (a) ALS group receiving sera from 15 patients that had been clinically and electromyographycally diagnosed as having sporadic ALS; (b) normal group receiving sera from 13 healthy volunteers and from 3 disease control patients, and (c) control group, which was kept untreated. Animals in groups (a) and (b) received daily intraperitoneal injection of 0.5–1 ml of serum for 3 days, and 24 h later the left hemidiaphragm was excised for electrophysiological recordings.

**Results**: Analysis of MEPPs frequency recorded from ALS group showed that 3 of them induced an increase in spontaneous neurotransmitter release while in 4 a decrease was observed, suggesting that sera alter spontaneous secretion as result of an increased or decreased $Ca^{2+}$ influx through the normally involved N-type or L-type VDCC, respectively. When the effect of Nitrendipine, an L-type VDCC blocker, was studied on ALS sera-injected mice, we found variable responses to the drug: only two mice showed control sensitivity to Nitrendipine, while in 7 its action was lower and surprisingly in 4 was greater than that without the drug.

**Conclusions**: These results suggest that ALS sera contain factor(s) that are able to modify spontaneous neurotransmitter release by altering calcium current through L-type and N-type VDCC, and even inducing changes in the sensitivity to the L-type VDCC blocker. 

*Keywords*: Amyotrophic lateral sclerosis; Spontaneous acetylcholine release; Calcium channels; Nitrendipine; Temperature

## 1. Introduction

Amyotrophic lateral sclerosis (ALS) is a degenerative incapacitating neuromuscular disease in which the upper and lower motor neurons are progressively destroyed. The sporadic form of the disease accounts for 90% of ALS. Although the pathogenesis of sporadic ALS remains undefined, several studies have implicated an autoimmune mechanism in the origin of the disease (Appel et al., 1991, 1993; Uchitel et al., 1988, 1992), where antibodies to voltage-dependent calcium channels (VDCC) in sera from ALS patients have been implicated in this hypothesis. There are evidences that immunoglobulin (Ig) from ALS patients increased frequency of miniature end-plate potentials (MEPPs) at the mice neuromuscular junction, both in vitro and after in vivo injection of Ig or serum from ALS patients (Uchitel et al., 1988, 1992; Appel et al.,1991; O'Shaughnessy et al., 1998; Mosier et al., 2000). As was shown in mammalian neuromuscular junctions, spontaneous acetylcholine release appears to be associated with opening of L-type and N-type VDCC, since Nifedipine and ω-Conotoxin-GVIA, specific channel blockers, decreased MEPP frequency by 49 and 35 %, respectively (Losavio and Muchnik, 1997). Thus, the above results would be related to an increased intracellular $Ca^{2+}$ due to a stimulatory effect of ALS Ig upon one or both of these VDCC. On the other hand, in other experiments, it was shown that ALS Ig decreased calcium currents (Delbono et al., 1991a,b; Appel et al., 1993), and that ALS patients presented autoantibodies to L-type VDCC, as demonstrated by enzyme linked immunosorbent assay (ELISA) and Western blots assays (Kimura et al., 1994; Smith et al., 1992). These results seem to be contradictory to the increased MEPP frequency observed in the previous experiments, since they should be associated to decreased or at least unmodified neurotransmitter release (see Vincent and Drachman, 1996).

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More consistent with the evidence of increased MEPP frequency, further findings demonstrated that ALS Ig induced death of a rat motor neuron/mouse neuroblastoma cell line, and these effects were prevented when the preparations were incubated with N-type VDCC specific blocker (Smith et al., 1994), which suggests a stimulatory effect of the Ig upon N-type VDCC. Although these findings were also referred to P-type VDCC (Llinás et al., 1993) and it was shown that antibodies from ALS patients increased the FTX-sensitive $Ca^{2+}$ current in a hybrid motoneuron cell line (Mosier et al., 1995), the participation of P/Q-type VDCC in the pathogenesis of the increased MEPP frequency appears improbable since this type of $Ca^{2+}$ channels are not related to spontaneous acetylcholine release, as was demonstrated by the failure of ω-Agatoxin TX, a specific blocker, in modifying MEPP frequency (Losavio and Muchnik, 1997).

The aim of the present work was to investigate the effect of sera from ALS patients on spontaneous acetylcholine release by the mouse passive transfer model and to study whether the transferred serum induces any change in the sensitivity of the L-type VDCC to its specific blocker Nitrendipine.

## 2. Methods

The sera used in this study were obtained from 15 patients (9 males, 6 females), in the age range from 35 to 77 years with a mean of 55.6 ± 4 years, who had been clinically and electromyographycally diagnosed as having sporadic ALS according to El Escorial criteria (1994). Control samples were collected from 13 healthy volunteers.

A total of 35 CF1 mice weighting 30 ± 5 g were divided into 3 groups: (a) ALS group receiving sera from patients suffering from ALS; (b) normal group receiving sera from healthy volunteers and from disease control patients (one patient with myasthenia gravis, one with brain glioma and one with stroke), and (c) control group, which was kept untreated. Animals in groups (a) and (b) received daily intraperitoneal injection of 0.5–1 ml of serum for 3 days, and 24 h later, they were anesthetized with sodium thiopental (50 mg/kg) and the left hemidiaphragm was excised for electrophysiological recordings.

Hemidiaphragm were transferred to a chamber filled with Ringer Krebs (mM): (NaCl 135, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 11, HEPES 5, pH 7.3–7.4), bubbled with $O_2$. The experiments were performed at room temperature (18 ± 2°C) and at 30 ± 2°C. Intracellular recordings were performed at the end-plate region of the muscle fiber with glass microelectrodes filled with 3 M KCl (resistance 5–10 MΩ). MEPP frequencies were recorded in not less than 10 fibers in each of the solutions tested. Muscle fibers with a resting membrane potential (RMP) less negative than −60 mV or MEPP with a rise time greater than 1 ms were rejected. MEPPs frequency recorded in 5 μM Nitrendipine (Sigma, USA) was done in darkness.

Statistical significance of differences between means was evaluated using two-tailed Student's *t* test corrected if necessary for multiple comparisons and by analysis of variance (ANOVA) analysis. In results, figures represent mean ± SE and *n* expresses number of muscles/number of fibers.

## 3. Results

### *3.1. Effect of the passive transfer of serum from ALS patients on MEPPs frequency*

As it was demonstrated in previous papers and in preparations incubated with Ig from other diseases (Losavio et al., 1989; Uchitel et al., 1988), MEPPs frequency was similar in mice injected with normal or disease control sera and in non-injected mice (group (b): 1.2 ± 0.1 (7/70), group (c): 1.3 ± 0.3 (13/130)). In mice passively transferred with ALS sera from 15 patients, keeping bath temperature at 18 ± 2°C, mean MEPPs frequency was not significantly different from the control group (group (c): 1.3 ± 0.3 (13/130), group (a): 1.2 ± 0.3 (15/150). However, as shown in Fig. 1, when each experiment was compared with the control group, some of the values were significantly different, as follows: 3 of them induced a significant increase in MEPPs frequency (1.6 ± 0.3; 1.7 ± 0.4; 1.6 ± 0.2), while other 4 showed a decrease in this parameter (0.7 ± 0.1; 0.7 ± 0.1; 0.7 ± 0.1; 0.8 ± 0.2). These results may be due to the presence of two populations of synapses, affected in a different manner by the ALS sera.

The values of RMPs and MEPPs amplitude in diaphragm fibers from groups (a), (b) and (c) were similar, regardless of their MEPPs frequency, except for the decreased MEPP amplitude obtained when injected sera from the myasthenic patient.

### *3.2. Effect of 5 μM Nitrendipine on MEPPs frequency recorded from mice transferred with sera from ALS patients*

In order to explain the different behaviors of ALS sera upon spontaneous acetylcholine release, the contribution of L-type VDCC, known to account for part of spontaneous secretion was studied. As mentioned above, Ig from ALS patients decreased calcium current through these VDCC and sera from some ALS patients had antibodies to L-type VDCC (Appel et al., 1993; Kimura et al., 1994; Smith et al., 1992). Thus, a possible speculation would be to find a decrease of the blocking effect of Nitrendipine, a specific L-type VDCC blocker, if these channels were previously blocked by ALS sera.

Fig. 2 shows the effect of 5 μM Nitrendipine on MEPPs frequency in control muscles and in those injected with normal and ALS sera. Animals injected with normal or disease control sera showed a sensitivity to Nitrendipine similar to the control group (percent of decrease in MEPPs frequency induced by Nitrendipine in control

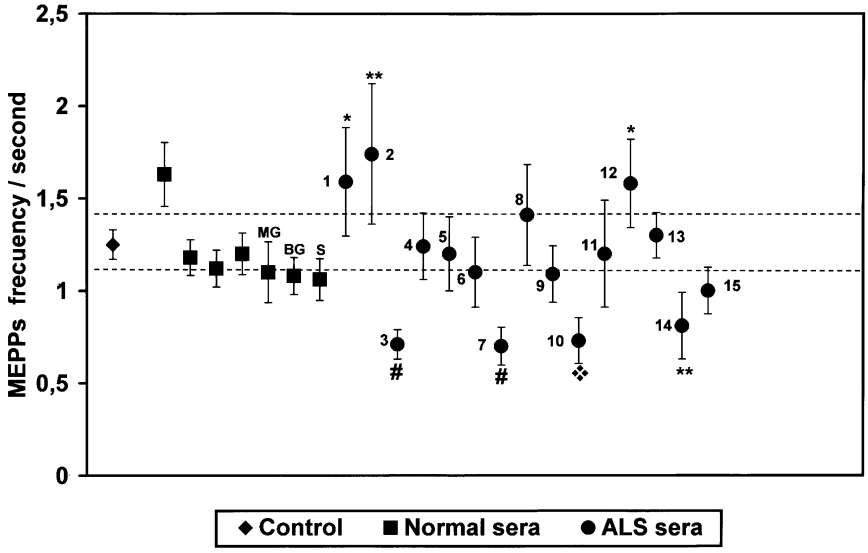


Fig. 1. MEPPs frequency recorded from diaphragms muscles from non-injected mice (group c: ◆) and from mice injected during 3 days with individual serum from healthy volunteers and patients with myasthenia gravis (MG), brain glioma (BG) and stroke (S) (group b: ■) and from ALS patients (group a: ●). Area between broken lines indicates mean ± 2 SEM of mean MEPPs frequency from control group. Each point in groups a and b represents the mean MEPP frequency ± SEM recorded in no less than 10 muscle fibers. Figures indicate individual ALS patients and symbols show significant differences compared to control group: $*P < 0.04$, $**P < 0.0075$, $\#P < 0.001$, ❖ $P < 0.002$. The analysis of the variance of group a compared with group c and b is not significantly different.

group: 41.0 ± 2.8 (9/95), normal group: 40.2 ± 0.9 (6/60)), while those injected with ALS sera showed a lower sensitivity to the L-type VDCC antagonist (8.8 ± 4.7 (13/130), $P < 0.001$). When each experiment was analyzed individually, we found that of 13 mice injected with ALS serum, only two caused a decrease in the rate of spontaneous acetylcholine release comparable to the control group (percentage of decrease in MEPPs frequency, 36.2 and 34.6); in 7 the sensitivity to the drug was lower, with a range of percentage of decrease in MEPPs frequency between 0 and 24.8 (mean 12.4 ± 3.4, $P < 0.0001$), and surprisingly in the remaining 4 experiments, acetylcholine secretion in presence of Nitrendipine was higher than that observed without the drug (percentage of increase in MEPPs

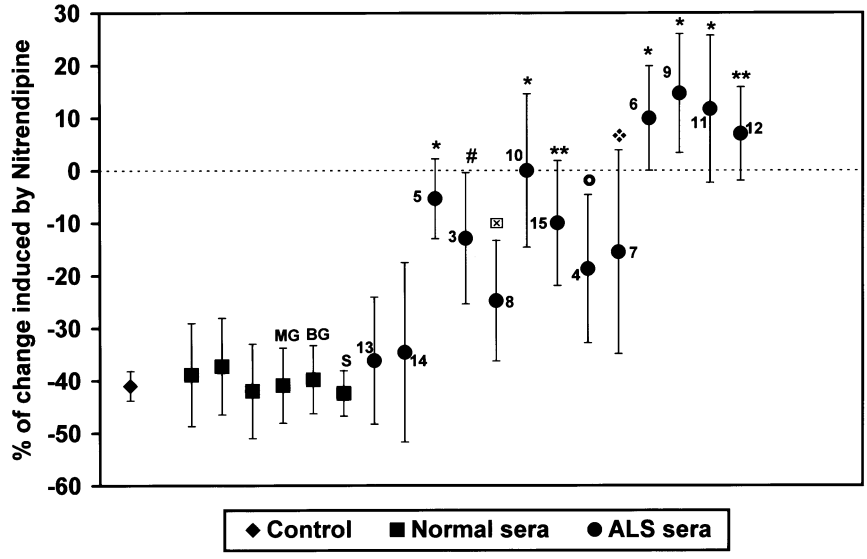


Fig. 2. Effect of 5 μM Nitrendipine upon MEPPs frequency in diaphragms from non-injected mice (◆), normal group (■) and ALS group (●). Each point (mean ± SEM) represents the percentage of change in MEPPs frequency induced by Nitrendipine compared with values obtained in the same preparation before addition of the VDCC antagonist.. Figures correspond to the same ALS patient depicted in Fig. 1 and symbols represent significant differences compared to control group: $*P < 0.0001$, $**P < 0.0003$, $\#P < 0.0007$, ❖ $P < 0.007$, ⊠ $P < 0.03$, ⭘ $P < 0.01$. The analysis of the variance of group a compared with group c and b is significantly different, with $P < 0.0001$.

frequency: range: 7.0–14.7, mean: 10.8 ± 1.9, $P < 0.0001$). Interestingly, in 3 of the 4 experiments in which ALS sera had shown a decrease in MEPPs frequency compared to the control group (see Fig. 1), a lower sensitivity to Nitrendipine was observed (percentage of decrease in MEPPs frequency in presence of Nitrendipine: 15.0, 12.9, and 0). Nitrendipine did not affect the RMP of the muscle fibers or the amplitude of the miniature potentials in all groups studied.

### *3.3. Effect of temperature change upon spontaneous transmitter release*

MEPPs frequency was demonstrated to be temperature-dependent (Elnqvist and Feldman, 1965; Hubbard et al., 1971). So, in a different group of experiments (shown in Fig. 3), spontaneous transmitter release was analyzed at 18 ± 2 and 30 ± 2°C. Mean MEPP frequency recorded from diaphragms of non-injected mice at 30°C was higher than that registered at 18°C (30°C: 3.5 ± 0.6 (4); 18°C: 1.5 ± 0.2 (4), $P < 0.02$). Similar results were obtained in mice injected with sera from ALS patients (30°C: 6.0 ± 1.3, $n = 4$; 18°C: 1.6 ± 0.1, $n = 4$, $P < 0.015$). Nevertheless, when control and ALS groups were compared, it can be observed that mice injected with ALS sera induced a higher increase in MEPPs frequency at 30°C than the control group at the same temperature ($P < 0.04$). Moreover, when mean MEPPs frequency recorded in diaphragms from ALS group was compared individually to the normal group, at 18 and 30°C, it was shown that, at 30°C, 3 of 4 sera became significantly different.

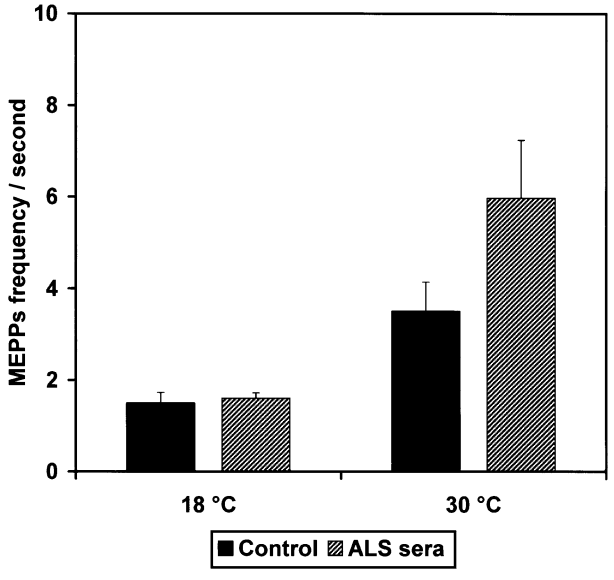


Fig. 3. Effect of temperature change upon spontaneous transmitter release in diaphragms from mice injected with ALS sera and from non-injected mice. MEPPs frequency was recorded at 18 ± 2 and 30 ± 2°C. Errors bars indicate SEM.

## 4. Discussion

Our results provide insights into immunological mechanism proposed to explain the pathogenesis of the sporadic form of ALS. The mouse passive transfer model offered a favorable experimental basis for studying the response of ALS serum upon spontaneous neurotransmitter release in mammalian neuromuscular junctions. In this way, mice passively transferred with ALS sera from 15 patients, keeping bath temperature at 18 ± 2°C, showed different effects on MEPPs frequency depending on the injected serum. The analysis of individual experiments demonstrated that 7 of them have values that were different from control media, 3 of them being higher and other 4 lower. These findings appear to be specific for ALS sera since MEPPs frequency was not affected when injected sera from disease control patients (myasthenia gravis, brain glioma and stroke). These results may be coherent with two types of responses of the synapses due to the action of ALS sera upon different VDCCs. As was demonstrated in a previous paper, spontaneous neurotransmitter release in mammalian neuromuscular junctions is related to $Ca^{2+}$ influx through L- and N-type VDCC (Losavio and Muchnik, 1997) while evoked neurotransmitter release is mediated by $Ca^{2+}$ entry through P/Q-type VDCC. The increase in MEPPs frequency observed in 3 experiments, would be consistent with a stimulatory effect of ALS Ig on N-type VDCC, increasing calcium influx into nerve terminals. The participation of N-type VDCC was already suggested by Appel group, they showed that ALS immunoglobulin mediated cytotoxicity in an hybrid motor neuron line was prevented by ω-Conotoxin-GVIA, selective antagonist of neuronal N-type VDCC (Smith et al., 1994). However, other groups did not detect immunoreactivity with N-type VDCC using immunoprecipitation assay or ELISA (Arsac et al., 1996; Drachman et al., 1995). On the other hand, the presence of synapses with decreased MEPP frequency agree much better with early findings showing a diminished $Ca^{2+}$ current through L-type VDCC (Appel et al., 1993) and with the fact that ALS patients produce antibodies that react with L-type VDCC purified from rabbit skeletal muscle detected by ELISA and it was found that increased antibodies to these channels were able to bind to the purified α1 subunit of the channels on Western blots (Kimura et al., 1994). Nevertheless, these findings were not confirmed by all authors (Arsac et al., 1996).

The L-type VDCC antagonist Nitrendipine (5 μM) affected spontaneous acetylcholine release in different ways: the sensitivity to the action of the dihydropyridine was in the same range as that found in control muscles only in two of the 13 mice injected with ALS sera. In 7 mice, the sensitivity of transmitter release to Nitrendipine was lower than control values. Interestingly, 3 of these experiments were those which had shown a decrease in MEPPs frequency without the drug. This finding may be interpreted as if Nitrendipine could not exert its blocking action because L-type VDCC were previously blocked by

antibodies present in ALS sera. Another possibility to explain the decrease or lack of action of Nitrendipine on spontaneous release could be that the injected sera had induced a change in the density and/or properties of the L-type VDCC. A similar result was observed at newly formed endplates in early stages of development (4–7-day-old mice) where Nitrendipine was ineffective in diminishing spontaneous acetylcholine release (Muchnik et al., 1998), contrary to the 41% decrease in MEPPs frequency observed in adult neuromuscular synapses. Other authors have found that in ALS IgG-treated muscles, L-type VDCC became involved in the evoked transmitter release together with the normally present P/Q-type VDCC, since a significant reduction in quantal content of evoked response was induced after incubation with Nitrendipine, indicating that a novel sensitivity to this channel blocker appears as a result of plastic changes at the nerve terminals (Fratantoni et al., 2000). These findings would suggest that ALS sera could be capable of inducing changes in motor nerve terminals that are typical of immature contacts and/or denervated muscles.

The increased spontaneous neurotransmitter release found in the remaining 4 experiments in presence of Nitrendipine is more difficult to explain. One possible speculation may be that ALS sera could have modified L-type VDCC in such a way that Nitrendipine behaves as an ionophore. Another way to explain this phenomenon might be as that reported by Sugiura and Ko (1997), who have found an increase in EPP amplitude after the addition of Nitrendipine in neonatal neuromuscular junctions, postulating that the drug decreases the release of a neuromodulator which have a tonic inhibition on transmitter release. On the other hand, dihydropyridines have been reported to affect other types of channels (Kamath et al., 1995; Yatani and Brown, 1985).

MEPPs frequency was demonstrated to be temperature-dependent (Elmquist and Feldman, 1965; Hubbard et al., 1971). At temperatures lower than 18°C, MEPPs frequency decreases; between 18 and 30°C, MEPPs frequency does not show important changes, but after 30°C the increase is usually so abrupt that a small change in the bath temperature is associated with a big jump in MEPPs frequency, making the measurements more variable. Our results suggested that at $30 \pm 2$°C, the assay seems to be more sensitive than at $18 \pm 2$°C, since 3 of 4 experiments which had shown MEPPs frequency within the normal range at 18°C, became significantly different when incubating at 30°C. This finding may explain the larger number of cases with increased MEPPs frequency found by other authors (Appel et al., 1993; O'Shaughnessy et al., 1998), since they worked at temperatures higher than 30°C. Uchitel et al. (1988) also found a higher increase in spontaneous acetylcholine release when they studied the effect of ALS Ig in vitro at 23 and 32°C.

In summary, this paper suggests that sera from ALS patients contain factor(s) that are able to modify spontaneous neurotransmitter release in different ways, by altering $Ca^{2+}$ influx to nerve terminal through the normally involved L-type and N-type VDCC, and by even inducing changes in the sensitivity to the L-type VDCC blocker.

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