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IMMUNOGLOBULINS FROM AMYOTROPHIC LATERAL SCLEROSIS PATIENTS ENHANCE THE FREQUENCY OF GLYCINE-MEDIATED SPONTANEOUS INHIBITORY POSTSYNAPTIC CURRENTS IN RAT HYPOGLOSSAL MOTONEURONS

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Abstract — Amyotrophic lateral sclerosis (ALS) is a devastating, still incurable neurological disorder affecting upper and lower motoneurons. Passive transfer of the disease occurs when immunoglobulins from ALS patients are injected into experimental animals. It is suggested that ALS IgGs cause excitotoxicity by acting on voltage-gated Ca^{2+} channels. We reported previously that ALS IgGs increase spontaneous release of glutamate in hippocampal neurons. Since these cells are not normally affected in ALS, we here studied the effect of ALS IgGs on hypoglossal motoneurons in rat brain-stem slices. The frequency of spontaneous glycine-mediated inhibitory postsynaptic currents (sIPSCs) was augmented, but not that of miniature ones (mIPSCs), thus pointing to an indirect effect on release.

Key words: Amyotrophic lateral sclerosis, IgG, glycinergic synapses, postsynaptic currents, brain-stem slices, patch-clamp

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INTRODUCTION

The most frequently encountered primary form of progressive motoneuron disease is amyotrophic lateral sclerosis (ALS), a devastating neurological disorder affecting upper and lower motoneurons. The only drug currently used to slow down the progression of ALS, although with only modest effect, is riluzole, a putative blocker of glutamate release (Doble and Kennle, 2000; Meininger et al., 2000). Passive transfer of the disease occurs when immunoglobulins (IgGs) from ALS patients are injected into experimental animals (Appel et al., 1991). Neuronal death due to excitotoxicity has been suggested to contribute to ALS etiopathogenesis. Excitotoxicity might be produced by abnormally high levels of glutamate released by nerve terminals following increase of intracellular free calcium ($[Ca^{2+}]_i$) content through an action of IgGs from ALS patients on ligand and/or voltage-gated Ca^{2+} channels, thus suggesting an immunological mechanism involved in this disease. In addition, in ALS autoantibodies against ganglioside GM1, neurofilament proteins, tumor necrosis factor receptor member FAS (CD95), and voltage-dependent calcium channels (Smith et al., 1992; Drachman, 2000) have been reported. In rodents treated with ALS IgGs, a $[Ca^{2+}]_i$ increment associated with degenerative structural alterations in motoneurons and synaptic plasticity during neuromuscular junctions were also observed (Engelhardt et al., 1995; Fratantoni et al., 2000; Pullen and Humphreys, 2000; Pullen et al., 2004; Pagani et al., 2006). However, electrophysiological evidence for ALS IgG modulation of voltage-activated Ca^{2+} currents of central neurons has provided contrasting results, ranging from depression (Zhainazarov et al., 1994) to potentiation (Llinas et al., 1993).

In our previous studies, the effects of ALS IgGs on spontaneous release of glutamate were tested on hippocampal cells in culture. It was reported that ALS IgGs induce a significant increase in frequency but not in amplitude of spontaneous and miniature glutamatergic currents through a mechanism that is independent of external calcium (Andjus et al.,

1997). A specificity of the previous study was the use of hippocampal neurons, not normally affected in ALS. However, although survival of such neurons may simply originate from inadequate exposure to ALS IgGs, testing these immunoglobulins on motoneurons is essential to prove the above hypotheses. We have therefore undertaken a study on a rat brain-stem slice preparation containing hypoglossal motoneurons, which are often one of the first targets in ALS pathology.

MATERIALS AND METHODS

ALS patients with approximately one average year of illness duration provided the sera for the pool of IgGs for experimental applications. Healthy donors of comparable age (around 50 years old) served as control. IgGs were isolated using affinity chromatography (protein A-sepharose). Elution was performed with 1 M acetic acid. The first elution peak contained the IgG-free fraction, while the second peak included IgGs. Samples were dialyzed, lyophilized, resuspended in Hank's balanced salt solution (Sigma) without Ca^{2+} and Mg^{2+} (pH 7.4), pooled, and frozen until used. Aliquots of diluted IgGs (0.1 mg/ml in standard external solution; see below), kept frozen until used, were applied by pressure from a pipette located close to the patched cell.

Experiments were carried out using brain-stem slices obtained from 0- to 9- day-old rats. Thin slices were prepared following a previously published procedure (Viana et al., 1994). The brain-stem was isolated from neonatal rats and immersed in modified, ice-cold Krebs solution (see below). A tissue block containing the lower medulla was then affixed with insect pins to an agar block inside a Vibratome chamber filled with ice-cold Krebs solution (bubbled with O_2/CO_2) to obtain 200-μm-thick slices. Slices were first transferred to an incubation chamber for 1 h at 32°C under continuous oxygenation and subsequently maintained at room temperature for ~1 h before use.

For electrophysiological experiments, brain-stem slices were placed in a small recording chamber, continuously superfused (2–5 ml/min) with Krebs solution (see below) and viewed with a Zeiss Axioscope microscope (Carl Zeiss AG, Germany) connected to an infrared video camera, in order to identify individual motoneurons within the hypoglossal nucleus. All cell recordings were obtained with whole cell patch-clamp electrodes (3–5 MΩ resistance) via an L/M PCA patch clamp amplifier (List Medical, Germany). Data acquisition was achieved with a PC using pClamp 7.1 software (Axon Instruments). All the recorded currents were filtered at 3 kHz and sampled at 5–10 kHz. Spontaneous inhibitory postsynaptic currents (sIPSCs) were mainly glycinergic (strychnine sensitive) while the residual GABAergic component was eliminated by 10 μM bicuculline (Sigma, Italy) (Donato and Nistri, 2000). In order to obtain glycinergic miniature inhibitory postsynaptic currents (mIPSCs), 1 μM tetrodotoxin (TTX, Affiniti Research, UK) was applied in the perfusion.

The solution for slice preparation and maintenance was (in mM): 130 NaCl, 3 KCl, 26 $NaHCO_3$, 1.5 Na_2HPO_4, 1 $CaCl_2$, 5 $MgCl_2$, and 10 glucose (osmolarity 290–310 mOsm). The extracellular solution for electrophysiological recording was (in mM): 130 NaCl, 3 KCl, 26 $NaHCO_3$, 1.5 Na_2HPO_4, 2 $CaCl_2$, 2 $MgCl_2$, and 10 glucose (osmolarity 290–310 mOsm). The patch pipette solution was (in mM): 110 K-gluconate, 20 KCl, 5 NaCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane-sulphonic acid), 10 EGTA, and 2 ATP-Mg (pH 7.2, 260–270 mOsm).

Postsynaptic currents were detected as previously reported (Donato and Nistri, 2000) using AxoGraph 4.6 (Axon Instruments) software, while Sigma Plot (Jandel Scientific, USA) and Clampfit (Axon Instruments) software were used for linear regression analysis of experimental data. Data are presented as means ± SE.

RESULTS

Under whole cell patch clamp conditions, hypoglossal motoneurons in brain-stem slices exhibited spontaneous inhibitory synaptic currents which were Cl^--dependent sIPSCs and mainly mediated by glycine (antagonized by 0.4 μM strychnine; *not shown*). The residual population of GABA-mediated events

was eliminated with bicuculline. The remaining glycine-mediated inhibitory postsynaptic currents were tested for the effect of ALS IgGs. IgGs were applied at a concentration of 0.1 mg/ml by pressure from a pipette located close to the patched cell. Recording of postsynaptic events usually started 400 s before IgGs application, which lasted for 200 s. After IgGs application, the solution flow was stopped and the synaptic activity recorded continuously for about 10 min. Minutes after ALS IgGs application, a rise in the frequency of sIPSCs was already observed (Fig. 1A and D). On average for the period after application of ALS IgGs this frequency enhancement was 2.7 ± 1- fold (n=8) as compared to the period prior to their application (Fig. 2). However, the sIPSC amplitude was not changed (see example in Fig. 1C and average data in Fig. 2). Control IgGs from healthy donors were ineffective in changing either frequency or amplitude of sIPSCs (n= 3; Fig 2). In order to check the effect of ALS IgGs on the release of glycine, hypoglossal slices were pre-treated with 1 μM TTX (in addition to 10 μM bicuculline to knock down residual GABA-mediated responses). However, ALS IgGs did not have any effect on glycinergic miniature currents (mIPSC) recorded in the presence TTX (Fig. 2).

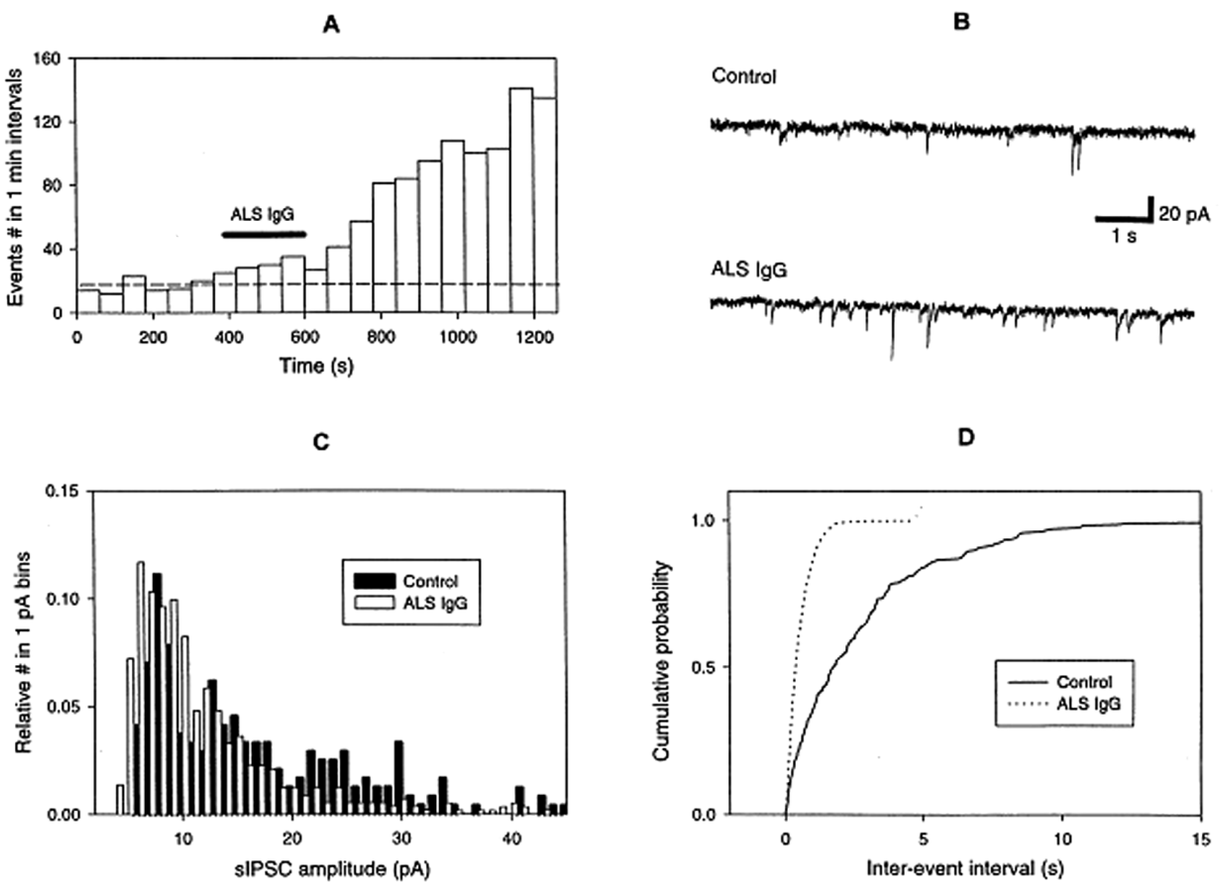

Fig. 1. ALS IgGs induce an increase in frequency but not in amplitude of sIPSCs in hypoglossal motoneurons (example of a single neuron). A - Time course of spontaneous activity. B – Examples of current traces of spontaneous IPSCs, just before (control) and 5 min after application of ALS IgGs. C - Amplitude histograms. The mean amplitude was 16.9±9 pA in the control and 12 ±7 pA after application of ALS IgGs. D – Cumulative distribution of inter-event intervals. The mean frequency rose from 0.27 Hz to 1.59 Hz after application of ALS IgGs.

DISCUSSION

The presented work demonstrates that in addition to lower motoneurons of the spinal cord (Appel

et al., 1991; Engelhardt et al., 1995; Pullen and Humphreys, 2000; Pullen et al., 2004) and neuromuscular junctions (Fratantoni et al., 2000; Pagani et al., 2006), ALS IgGs also affect higher motoneurons in the brain stem. ALS IgGs have been shown to affect transmitter release in motoneurons (Fratantoni et al., 2000; Pagani et al., 2006; Uchitel et al., 1988), as well as in glutamatergic central synapses (Andjus et al., 1997). In hypoglossal neurons, the frequency of sIPSCs was affected by ALS IgGs but not the amplitude, thus

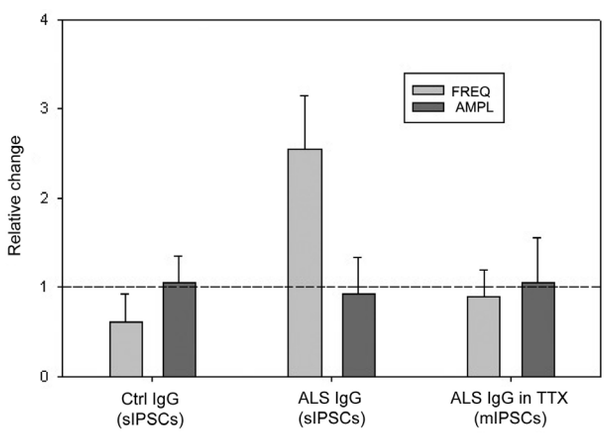

Fig. 2. ALS IgGs induce an increase in frequency of spontaneous IPSCs but not of mIPSCs. ALS IgGs enhance the frequency of spontaneous glycine mediated inhibitory postsynaptic currents without a change in the average amplitude (n=8). On the average (n=7) ALS IgGs did not have an effect on glycinergic miniature currents recorded in TTX (1 μM). Control IgGs from healthy donors were ineffective (n= 3).

pointing to a presynaptic effect. However, although the frequency of sIPCSs was affected, mIPSCs were insensitive to ALS IgGs. A possible explanation may lie in the fact that unlike the glutamatergic synapses and neuromuscular junctions, glycinergic synapses are dually regulated by mechanisms of opposite sign triggered by a rise in $[Ca^{2+}]_i$ (Mukhtarov et al., 2005). These mechanisms are: (i) a decrease of glycinergic inhibitory postsynaptic currents due to a reduction of presynaptic glycine release, predominantly induced by retrograde action of endogenous cannabinoids; and (ii) a potentiation of postsynaptic glycine receptors. Under normal physiological conditions, the postsynaptic effect is masked by powerful presynaptic inhibition. Thus, although ALS IgGs have a potentiating effect on Ca^{2+} channels and Ca^{2+} signaling as demonstrated in several systems (Engelhardt et al., 1995; Fratantoni et al., 2000; Pullen et al., 2004; Pagani et al., 2006; Llinas et al., 1993), this may not have had an influence on glycinergic synapses, where the two opposite effects of $[Ca^{2+}]_i$ rise may have cancelled each other.

The facilitation of spontaneous glycine-mediated events observed in the absence of TTX was probably indirect *via* changes in the basic membrane conductance of glycinergic cells or network-driven through enhanced glutamatergic drive to these neurons. ALS IgGs could have increased the release of glutamate in these neurons, as in the case of hippocampal cells (Andjus et al., 1997). However, further experimental evidence is needed to prove that ALS IgGs act specifically on glutamate release and/or specific membrane conductance in glycinergic cells.

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ИМУНОГЛОБУЛИНИ ALS ПАЦИЈЕНАТА ПОВЕЋАВАЈУ ФРЕКВЕНЦИЈУ ГЛИЦИНОМ ИЗАЗВАНИХ СПОНТАНИХ IPSC У ХИПОГЛОСАЛНИМ МОТОНЕУРОНИМА ПАЦОВА

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Амиотрофна латерална склероза (ALS) је разарајућа, још увек неизлечива неуролошка болест која погађа и горње и доње мотонеуроне. Пасивни трансфер оболења настаје преносом имуноглобулина ALS пацијената у крвоток експерименталне животиње. Сматра се да ALS IgG изазивају ексцитотоксичност дејством на волтажно-зависне Ca^{2+} канале. Наша ранија истраживања показала су да ALS IgG повећавају спонтано ослобађање глутамата у хипокампалним неуронима. Како је чињеница да ове ћелије нису нормално оштећене у ALS овде смо истражили ефекат ALS IgG на хипоглосалне мотонеуроне мождано стабла пацова. Констатовали смо да је дошло је до повећања фреквенције спонтаних глицином изазваних IPSC, али не и минијатурних IPSC, што је указивало на индиректни ефекат.