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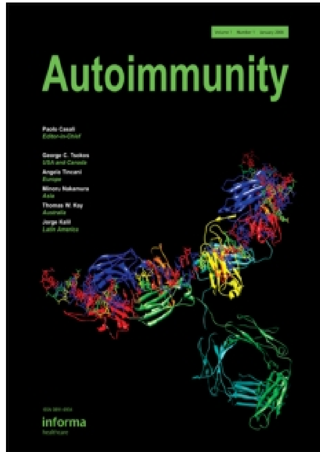

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Marika Vianello [a]; Giacomo Bisson [b]; Marco Dal Maschio [b]; Stefano Vassanelli [b]; Stefano Girardi [b]; Carla Mucignat [b]; Kostantinos Fountzoulas [b]; Bruno Giometto [a]
[a] Department of Neurology, "Ca' Foncello" General Hospital, Treviso, Italy
[b] Section of Physiology, Department of Human Anatomy and Physiology, University of Padova, Pauda, Italy

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Increased spontaneous activity of a network of hippocampal neurons in culture caused by suppression of inhibitory potentials mediated by anti-gad antibodies

MARIKA VIANELLO[1†], GIACOMO BISSON[2†], MARCO DAL MASCHIO[2], STEFANO VASSANELLI[2], STEFANO GIRARDI[2], CARLA MUCIGNAT[2], KOSTANTINOS FOUNTZOULAS[2], & BRUNO GIOMETTO[1]

[1]*Department of Neurology, "Ca' Foncello" General Hospital, Treviso, Italy, and* [2]*Section of Physiology, Department of Human Anatomy and Physiology, University of Padova, Pauda, Italy*

Abstract

Introduction: Anti-glutamic acid decarboxylase autoantibodies (GAD-Ab) are commonly considered the marker of autoimmune diabetes; they were first described in patients affected by stiff-person syndrome and recently, in ataxic or epileptic patients. The pathogenetic role of GAD-Ab remains unclear but inhibition of GABA synthesis or interference with GABA exocytosis are hypothesized. The aim of the study was to assess whether GAD-Ab interfere with neuronal transmission.

Patients and methods: Serum from a GAD-Ab positive epileptic patient (by IHC and RIA), serum from a GAD-positive (only by RIA) diabetic case, sera from two epileptic GAD-Ab negative patients and a normal control were selected. Post-synaptic inhibitory potentials (IPSPs) were registered on hippocampal neurons in culture before and after the application of diluted sera in a patch clamp study.

Results: A significant increase in the frequency of IPSPs was observed after application of GAD-positive epileptic serum, while no effect was noted using sera from negative controls.

Conclusion: The inhibition in neuronal transmission only after application of GAD-positive epileptic serum, suggests an interference with GABA function and consequently with neuronal inhibition supporting a pathogenetic role of GAD-Ab in the development of epilepsy.

Keywords: *GAD-antibodies, epilepsy, diabetes, hippocampal neurons, patch clamp*

Introduction

Glutamic acid decarboxylase (GAD) is the enzyme that catalyses the conversion of glutamic acid to the inhibitory neurotransmitter gamma-aminobutyric acid (GABA), diffusely present in the central nervous system [1].

Autoantibodies (Ab) directed against the enzyme (GAD-Ab) are commonly considered the marker of type 1 diabetes since they are present from the first phase and predict the development of the disease [2–4]. However they were first described in a patient affected by stiff-person syndrome (SPS) [5,6] and were recently found in a few cases of chronic cerebellar ataxia [7–10], drug-resistant epilepsy [11,12] and myoclonus [13,14].

GAD-Ab are usually detected by immunohistochemistry (IHC) on frozen sections of rat cerebellum where they recognize GABAergic structures [15]. The reactivity might be confirmed by radio-immunoassay with recombinant protein [16]. Western Blot developed both by neuronal proteins or recombinant molecules and ELISA are less sensitive [17].

The pathogenetic role of GAD-Ab is still unknown. One hypothesis is inhibition of GAD synaptic activity, causing a decrease in GABA synthesis in the nerve terminals; an alternative assumption is interference with exocytosis of GABA by the terminals [18–21].

Correspondence: M. Vianello, Department of Neurology, Piazza Ospedale 1, 31100 Treviso, Italy. Tel: 39 422 322627. Fax: 39 422 322533. E-mail: marikavianello@libero.it

[†]The two authors contributed equally to this work.

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Functional studies have now been developed using sera from patients affected by SPS or ataxia; but GAD-Ab have also recently been reported in patients affected by drug-resistant epilepsy, suggesting that the disease has autoimmune origin [11,12]. This is also supported by the frequent occurrence of epilepsy in patients affected by SPS [5] and by the frequent co-occurrence of other autoimmune disorders in GAD-Ab-positive epileptic patients [12].

The first step to assess the possible pathogenetic role of GAD-Ab is to verify the reactivity of positive sera on the target cells. We recently analysed the morphological effect of GAD-Ab-positive sera from epileptic patients on hippocampal neurons in culture where GABAergic pathways are well represented [22]. We selected neurological sera that were positive on IHC and RIA and observed strong immunoreactivity when sera from GAD-Ab positive epileptic patients were applied. In addition, control sera from GAD-Ab positive neurological patients affected by ataxia and SPS also showed reactivity, but with a distinct pattern of labelling. No specific reactivity was observed using sera from diabetic patients, who were GAD-Ab positive on RIA but negative on IHC. Two epileptic cases, which were negative on both IHC and RIA and a normal control, showed no reactivity in the same cells. This finding suggests different GABAergic recognition that could, in part, explain the distinct clinical phenotypes [22].

In this study we investigated whether the same sera interfered with the GABAergic pathway by using a patch clamp method on the same cells in culture. We compared the results among a diabetic, two epileptic GAD-Ab negative patients and a normal control.

Patients and methods

Selection of patients

We tested the serum from the first GAD-Ab positive epileptic case reported in the literature [11]. He was a 21-year-old, previously healthy male who began to complain of complex partial seizures, which recurred many times a day with hallucinations and interfered with social activity. Neurological examinations revealed temporal-lobe epilepsy and CSF analysis excluded bacterial or viral causes, confirming an inflammatory profile with pleiocytosis and oligoclonal bands. Serological analysis revealed positivity for GAD-Ab and anti-nuclear Ab, but systemic diseases were ruled out. He was resistant to anti-epileptic drugs or intravenous administration of immunoglobulins and was initially responsive to corticosteroids and finally to plasmapheresis. At the time of writing, after 8-years of follow-up, he has remained asymptomatic and concluded his degree course.

For control purposes, we selected sera from two drug-resistant epileptic patients testing negative for GAD-Ab by IHC and RIA and from a diabetic case, testing positive for GAD-Ab by RIA but negative by IHC on neural tissue (Type 1 diabetes was defined according to National Diabetes Data Group criteria).

Finally, a normal control was used to determine aspecific background.

IHC, RIA and WB

Sera were analysed by IHC on frozen sections of rat cerebellum to detect antineuronal antibodies by standardized procedures [15].

To reveal the presence of GAD-Ab, RIA was performed with a commercially available kit (RSR Ltd, Cardiff, Wales, UK), based on ^{125}I-labelled recombinant human GAD65, following the manufacturer's instructions [16].

WB was performed using recombinant human GAD65 (rGAD65, kindly supplied by Dr Tree, Department of Immunology, King's College Hospital, London UK). The proteins were separated by sodium dodecylsulphate gel electrophoresis (SDS-PAGE) according to the manufacturer's instructions and then transferred to nitrocellulose. Each lane was loaded with 600 ng of the recombinant protein. Nitrocellulose strips were blocked with 2% Tween 20 in PBS and then incubated overnight at 4°C with sera diluted 1:500. Membranes were rinsed and incubated with a solution of peroxidase-conjugated Fab fragments of rabbit anti-human IgG. Then, after further rinses, they were developed using diaminobenzidine tetrahydrochloride (0.05%) and H_2O_2 (0.01%) in PBS.

All immunoblots had a positive (polyclonal rabbit anti-GAD 65, Santa Cruz Biotechnology), and a negative (normal human serum) control lane. A positive result was scored when a band was seen at 65 kDa.

Cell preparation

Hippocampi from E19 rats (Wistar outbred) were harvested and digested in a solution containing 0.5% bovine serum albumin (BSA, Sigma-Aldrich, A-8806, fraction V powder, fatty-acid free, low endotoxin) and 0.5 mg ml^{-1} papain (Sigma-Aldrich, P4762), in phosphate buffered saline (PBS, Gibco Invitrogen, 14040-091), for 20 min, in an incubator at 37°C. Excess medium was removed and cells resuspended in 2.5 ml Dulbecco-modified Eagle's medium (DMEM, Gibco Invitrogen, 61965-059) with 10% fetal bovine serum (FBS, Gibco Invitrogen, 10270-098, batch 40F6414K). After centrifugation at 1200 rpm for 10 min, excess medium was removed and cells were preplated in 10 ml DMEM on a 10-cm-diameter NUNC plate with a Nucleon surface (Nalge Nunc International, 1,50,350), for 60 min in an incubator. After preplating a second time for 30 min on a fresh

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plate, cells were centrifuged and resuspended in 1 ml DMEM. Cell density was determined and adjusted with DMEM to 1,00,000 and 3,00,000 per ml for low-density and high-density cultures, respectively. About 0.5 ml of adjusted cell suspension was applied to the chip and supplemented with 1 ml of Leibovitz-15 (Gibco Invitrogen, 31415-086) with 5% FBS. After 1 h in the incubator, the medium was changed to 1.5 ml neurobasal medium (NB, Gibco Invitrogen, 21103-049) with B27 supplement (Gibco Invitrogen, 17504-044) and glutamaxI (Gibco Invitrogen, 35050-038) and placed in the incubator at 37°C with 10% CO_2. Once a week, 200 ml of medium was removed and 400 ml NB medium was added.

Electrophysiological studies

Measurements: Recordings were made with patch clamp electrodes (tip resistance 3–8 MΩ) filled with a solution containing (in mM): KCl (20), $MgCl_2$ (2), EGTA (10), HEPES(5), K-Gluconate (120), adjusted to pH 7.4. Extracellular solution was composed as follows (in mM): NaCl (135), KCl (5.40), $MgCl_2$ (1.00), $CaCl_2$ (1.00), Glucose (10), HEPES (5), adjusted to pH 7.4. Membrane potential was recorded from hippocampal neurons with a patch clamp amplifier (AXOPATCH 200A, Axon Instr. Co.) using the whole-cell configuration in the current clamp mode, and then digitized. During the experiments, particular care was taken to minimize the contribution of excitatory postsynaptic potentials (EPSPs) by holding the membrane potential at 0 mV. Our experimental protocol envisaged serum perfusion about 100 s after the start of recording at a distance from the recorded neuron (3–5 mm). Subsequently, perfusion and recording lasted over 100 s. Typically, 2 min after the start of perfusion, serum was observed to reach the recorded neuron and the post-synaptic inhibitory potential (IPSPs) amplitude consequently decreased.

Data analysis

Digitized traces were then processed by a detection algorithm based on a modification of Franaszczuk et al.'s method [23], which rendered IPSP detection faster and more robust. All algorithms were implemented in MATLAB 7.0 language. In order to describe IPSPs kinetics, a weighted nonlinear least square algorithm, based on the trust region method [24], using a two-exponential model [25], was used to fit the IPSPs detected in the experiments with GAD-Ab positive serum perfusion:

$V(t) = A1 \times \exp(a1 \times t) - A2 \times \exp(a2 \times t) + ss$ with $A1, A2 > 0$ and $a1, a2 < 0$. IPSPs selected for the fit task were such that other IPSPs were at least 80 ms from it, thus preventing an overlap that could compromise parameter estimation.

Parameter estimations relating to IPSPs detected before perfusion were compared to the ones relating to IPSPs detected after perfusion. A *t*-test was then performed assuming that the two samples came from normal distributions with unknown and possibly unequal variances. Firing rate estimates were obtained by means of a convolution of IPSPs, represented as a Dirac's Delta train, with a Gaussain kernel [26]. This in turn gives a smooth firing rate estimate and the kernel variance, which reflects time resolution, was set to 6.25 s^2. Inter-spike intervals (ISI) were calculated between one IPSP and the next. A histogram relating to ISIs between IPSPs detected before serum perfusion was built and visually compared to the one relating to IPSPs detected after serum perfusion.

Results

IHC, RIA and WB

On IHC on rat cerebellum, the GAD-positive serum stained the axon hillocks of Purkinje cells and diffuse nerve terminals in the molecular and granular layers (data not shown). The reactivity was confirmed using CSF. This pattern is consistent with GABAergic neuron terminal expression. No reactivity was detected on IHC with diabetic and epileptic controls. Serum and CSF from the epileptic patient—which were GAD-positive by IHC—and the diabetic control showed detectable values using a RIA method.

Using WB on rGAD65, the serum from the epileptic patient positive for GAD-Ab on IHC and RIA, stained the antigen (data not shown). No diabetic nor GAD-negative epileptic nor normal control sera were positive.

Electrophysiological studies: IPSP kinetics study

We studied the properties of IPSPs generated in the recorded neuron, due to the activity of the net of cells afferent to the neuron. This enabled us to characterize first the kinetics based on a theoretical model and then their temporal recurrence.

The effect of GAD-Ab positive serum perfusion has been evaluated on the features of the recorded IPSPs. Using an algorithm to automatically detect events, before and after serum perfusion, more than a hundred properly spaced IPSPs have been identified, and a two-exponential mathematical model used to determine fit [25], by estimating its five descriptive parameters A1, a1, A2, a2, ss. Figure 1A B show two intracellular potential recordings (cyan) with the respective fit superimposed (in red).

Fit quality, evaluated in terms of the weighted residual sum of squares, is similar for all IPSPs considered. If we compare the set of average parameters estimated from the IPSPs recorded in the absence of serum, with the ones obtained in its

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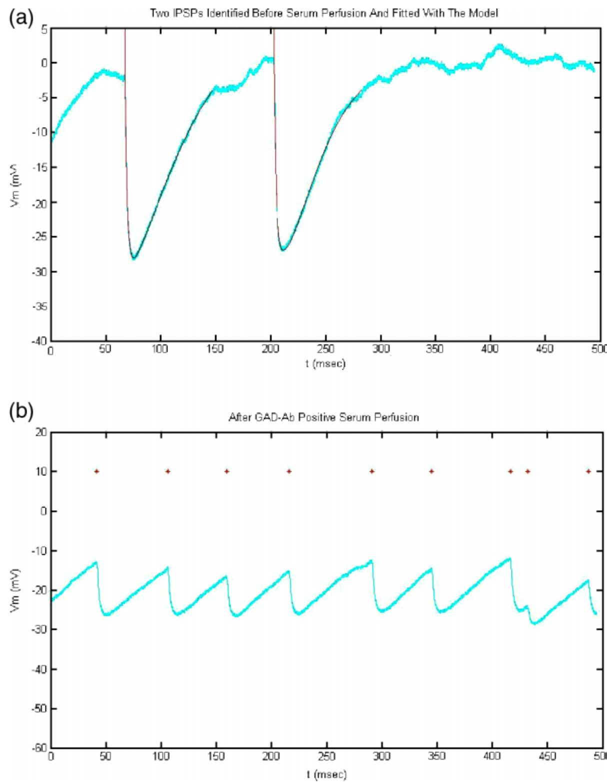

Figure 1. Intracellular potential Vm recorded before (A) and after (B) GAD-Ab positive serum application (cyan) and superimposed fit on detected IPSPs. Note sufficient time lag between IPSPs in order to reject superimposed events.

presence (as reported in Table I), we find that the temporal features expressed in terms of rate constants a1, a2, are not significantly different (p-value (a1) $= 0.49$, p-value (a2) $= 0.18$).

On comparison of the amplitude parameters A1, A2, A1 did not change (p-value (A1) $= 0.14$), while A2 was significantly different (p-value (A2) $= 8.4 \times 10^{-7}$). Underlying this finding is unavoidable temporal superposition of events, which, as we will see later, characterizes the sustained network activity in the presence of GAD-Ab positive serum. This effect is not ascribable to a direct effect of serum on the recording cell, which would imply a progressive fading of IPSPs and the settling of the membrane potential towards the holding value (0 mV). The latter effect, as documented elsewhere [20], is indeed confirmed in our experiments performed over a time spread of several minutes (data not shown), in concomitance with the visually detected arrival of serum in the recording cell zone. As a result, the A2 parameter becomes smaller not because the serum has an effect on GABA release, but because activity becomes more frequent and each IPSP onset is on the tail of the previous, not completely faded one. Amplitude consequently "seems" smaller.

This suggests that during the time spread under examination, lasting no longer than 2 min, the recording depends only on serum action on cells present in the peripheral zone where perfusion was applied.

After determining the nature of the IPSPs and the measurement conditions, we focused on characterizing cultured network activity in the presence of GAD-Ab positive serum, compared with those from GAD-Ab negative patients and healthy ones. Figures 2A and B represent the recorded intracellular potential before and after GAD-Ab positive serum perfusion, respectively, where we can observe the high number of IPSPs that feature the serum effect.

A firing rate analysis (Figure 3A and B) showed that the number of recorded events per time unit was increased at least twofold compared to baseline, only after GAD-Ab positive serum perfusion. Conversely, no change was observed with the sera of GAD-Ab negative and healthy patients. This is probably due to the removal of inhibitory signals from peripheral network levels, which in turn translates into hyper-activity that spreads throughout the network and reaches the recorded neuron.

The other parameter estimated from our recordings is the ISI, which is a measurement of time intervals that separate one IPSP from the next. Histograms of this quantity have been built in order to describe ISI distribution before and after serum application.

Compared to control conditions with the sera of GAD-Ab negative and healthy patients, where distribution features do not change significantly, the histogram shows a single, more concentrated mode after application of GAD-Ab positive serum, whereas it was relatively spread before, with more than one mode (Figure 4A–F). When we tested serum from the diabetic control, results were similar to negative epileptic cases (data not shown).

Discussion

An autoimmune origin for drug-resistant epilepsy has recently been suggested by the detection of GAD-Ab in the serum and CSF of a subgroup of these patients [11,12].

Table I. Table containing estimation of average fit parameters and relative standard deviation in the two recording conditions.

	A1(mV)	SD	a1 (ms-1)	SD	A2(mV)	SD	a2 (ms-1)	SD	ss(mV)	SD
Before perfusion	48.50	13.31	−0.0114	0.007	31.24	9.00	−0.32	0.11	16.75	8.73
After perfusion	45.37	14.54	−0.0106	0.008	24.34	8.60	−0.30	0.07	16.40	13.386

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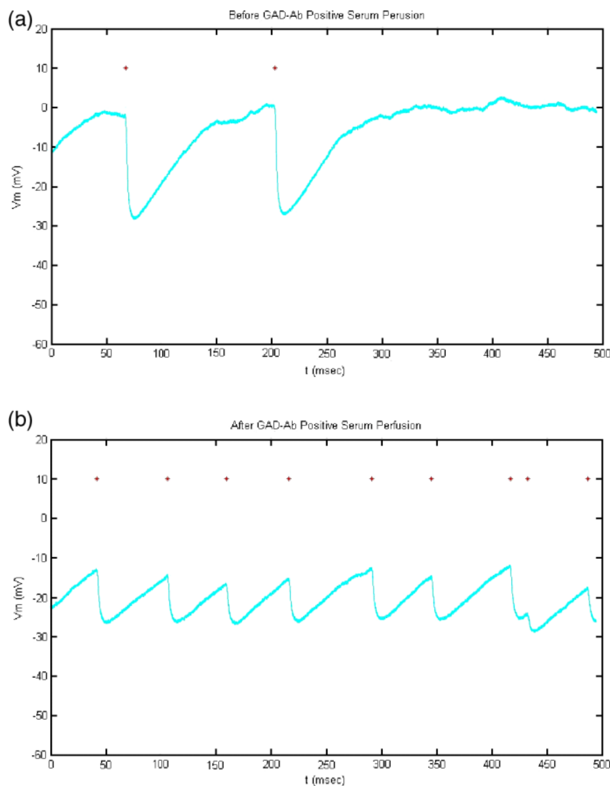

Figure 2. Intracellular potential Vm recorded before (A) and after (B) GAD-Ab positive serum application.

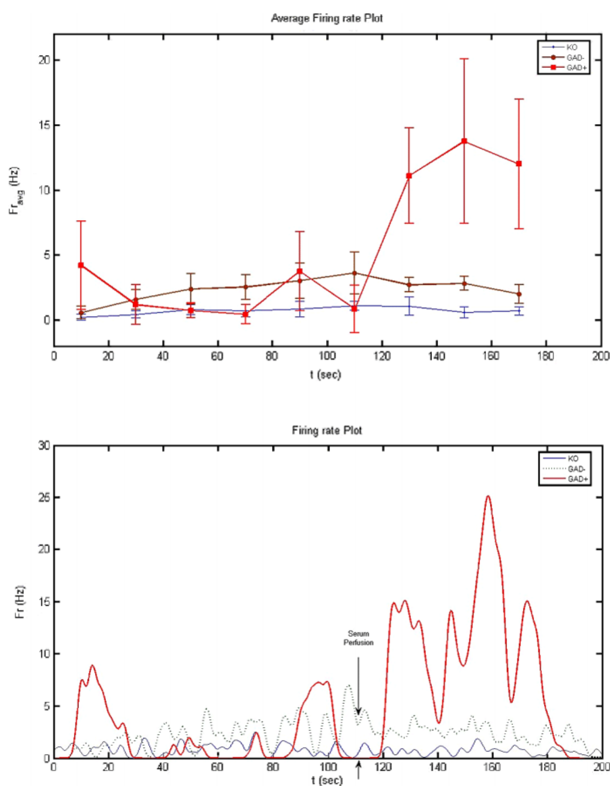

Figure 3. (A) Comparison of three different recordings relative to GAD-Ab positive, GAD-Ab negative and healthy patient's sera. (B) Average values and standard deviations evaluated every 20 s.

The mechanism of neuronal damage in these cases is unclear and the hypothesis that GAD-Ab could have a pathogenetic role should follow preliminary demonstration of their interaction with the target cells. We previously demonstrated a specific reactivity of sera from two GAD-positive epileptics on cultured foetal rat hippocampal neurons where GABAergic synapses are well represented [27,28]. In addition, these neurons are the target site of damage in drug-resistant temporal epilepsy.

The results indicate specific recognition and peculiar labelling of GABAergic neuronal structures by the sera from GAD-positive epileptic patients when tested on their target cells in culture [22].

However, the pathogenetic mechanism of action of GAD-Ab remains under debate. Since GAD is a intracytoplasmic enzyme localised in nerve terminals, the first pathogenetic hypothesis was inhibition in GABA synthesis deriving from a decrease in the enzyme activity caused by Ab [18]. This Ab activity proved to be epitope-specific distinguishing neurological from diabetic cases [18,19].

The first possibility was suggested by Dinkel et al. [18] who demonstrated that IgGs in the sera of SPS patients reduced GAD enzyme activity, leading to reduction in GABA synthesis in crude rat cerebellar extracts. The enzyme activity was preserved using purified IgG from diabetic patients, positive for GAD-Ab, suggesting that a specific epitope recognition of GAD-Ab mediates inhibition of GAD activity.

Following Dinkel et al. hypothesis, in 1999 Ishida and colleagues used a patch clamp method to demonstrate that cerebrospinal fluid (CSF) IgGs from an ataxic patient, positive for GAD-Ab by IHC and WB, suppressed inhibitory transmission registered on Purkinje cells in rat cerebellar slices. This was not observed using sera and CSF from autoimmune diabetic patients [19] and suggests that the autoimmune response to GAD down-regulated GABA synthesis in the terminals of basket cells (GABAergic interneurons in the cerebellar cortex), resulting in the reduction of GABA release on the post-synaptic Purkinje cells. This may have contributed to the manifestation of ataxia [19]. A year later, Mitoma et al., using IHC and whole-cell recording in rat cerebellar slices, demonstrated that the same CSF IgGs prepared from an ataxic patient acted on the pre-synaptic terminals of GABAergic interneurons, causing a decrease in GABAergic release onto Purkinje cells [20].

Similar results were subsequently confirmed in a patient with progressive cerebellar ataxia, unresponsive to intravenous administration of immunoglobulins, suggesting irreversible damage to cerebellar neurotransmission [21].

GAD is an intracytoplasmic enzyme both in neuronal and pancreatic cells, which is never exposed on cytoplasmic membrane during GABA exocytosis. On the other hand, serum and CSF GAD-Ab never reach

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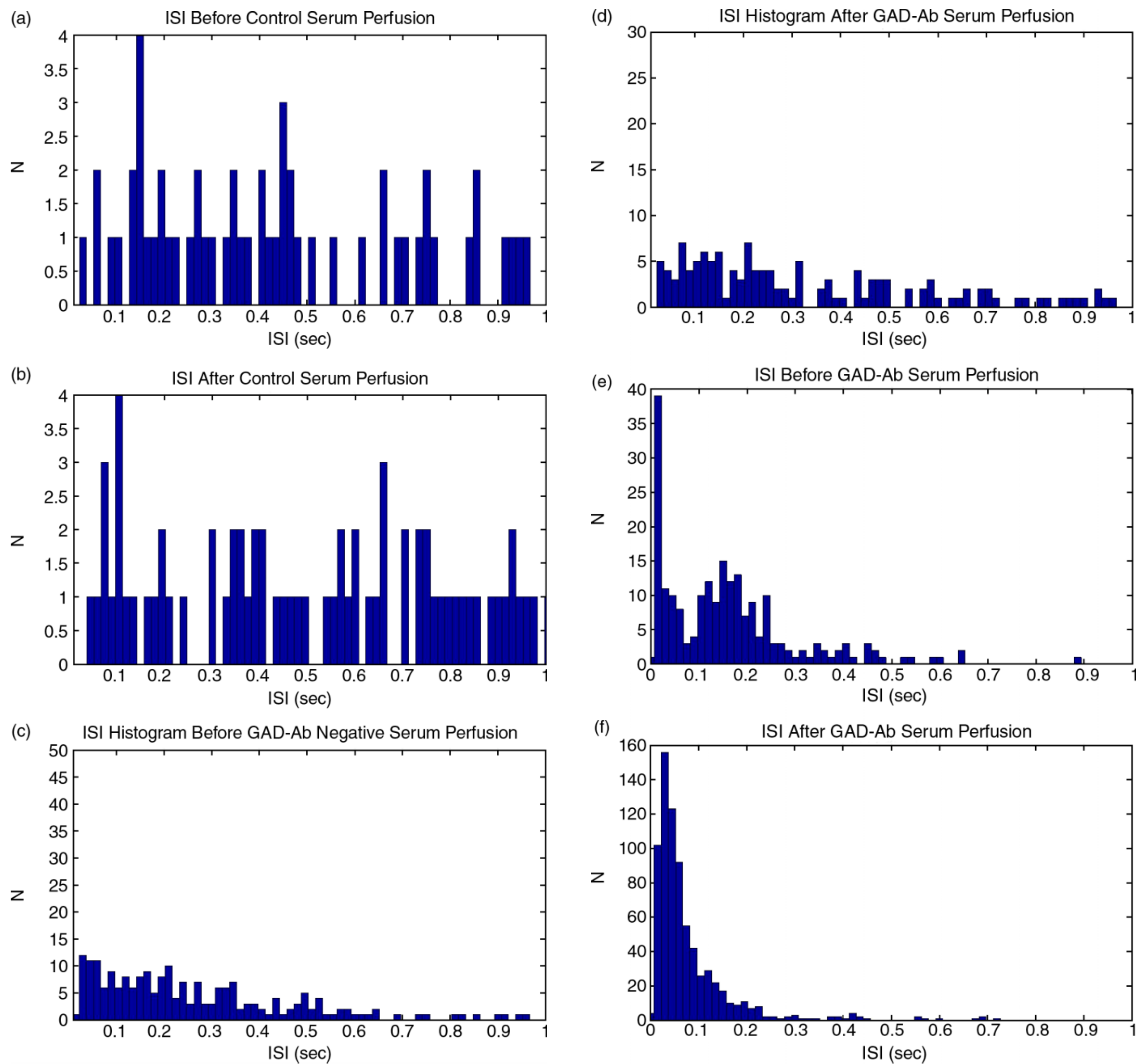

Figure 4. Comparison of ISI distributions of different recordings related to GAD-Ab positive serum before (A) and after (B) perfusion, GAD-Ab negative before (C) and after (D) perfusion, and healthy patient's sera (E and F).

their intracellular target. These data contrast with the idea of inhibition of GABA synthesis caused by GAD-Ab. Nevertheless, it could be that Ab enter neuronal cells [29]; but it may also be that GAD-Ab directed against enzymes act without entering the cells. They are able to mimic GABA and cross-react with the GAD "place-of-link", which is approachable on the neuronal surface, in the same way as anti-nuclear Ab [30].

The pathogenetic role of an auto-Ab can only be hypothesized if we record a functional modification on neuronal transmission.

In order to assess this theory we tested serum from a GAD-Ab positive epileptic patient on cultured rat hippocampal neurons, their target of action, using a patch clamp method and we recorded the IPSPs.

In SPS patients, hyperexcitability in motor cortex neurons was confirmed by brain magnetic stimulation [31], supporting the idea of interference in cortical GABAergic transmission causing clinical manifestation of disease.

GAD-Ab decrease GABA synthesis and its inhibitory effect. Hyperexcitability of cortical neurons in SPS cases could be correlated with a high prevalence of epilepsy in this group of GAD-Ab positive patients, compared to the healthy population.

In experimental animals, hyperexcitability of cortical neurons is described in association with GAD derangement: GAD65 "knockout" mice develop seizures [32] and mice immunized against GAD65 easily develop seizures after kainite stimulation [33].

We observed that the transition from basal activity to hyperactivity takes only a few seconds from the beginning of perfusion. This contrasts with the results of Mitoma et al. [20], where a change in IPSPs amplitude was observed after a time interval of about

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5 min. The latter effect could be ascribable to diffusion processes, while the effective suppression of GABA release could be mediated over a briefer time range. ISI distribution becomes more compact around its unique modal value, once GAD-Ab positive serum has been applied: this reflects the fact that the recorded activity becomes more regular rather than more frequent.

The effect on frequency could derive from and increase in action potentials by GABAergic neurons, not inhibited by serum, surrounding the area of inhibition.

No interference was observed in amplitude or frequency of IPSPs when we applied serum from diabetic, epileptic and normal controls.

These results again support the hypothesis that specific epitope recognition of GAD-Ab mediates inhibition of GAD enzymatic activity in neurological cases. In addition, this Ab-induced suppression of GABA synapses on hippocampal neurons by serum from an epileptic case, may have contributed to the pathogenesis of disease.

Further investigations are needed to verify whether the inhibition is pre- or post-synaptic.

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