

## Supplementary Online Content

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This supplementary material has been provided by the authors to give readers additional information about their work.

## eMethods. Supplemental Methods

### 1. *GAD65-ab radioimmunoassay.*

GAD65-ab were measured by radioimmunoassay using a commercial kit (CIS biointernational, France) following the manufacturer's instructions. Briefly, 20  $\mu$ l of standards (SMS serum containing GAD-Ab at different dilutions expressed in arbitrary units U/ml), and serum samples, were incubated with 50  $\mu$ l of  $^{125}$ I-labeled human recombinant GAD65 for 2 hours at room temperature. Then, 50  $\mu$ l of protein A-sepharose was added, and the mixture incubated for 1 hour at room temperature. After centrifugation at 1500 g for 30 minutes at 4°C, the precipitates were counted for  $^{125}$ I with a gamma scintillation counter. The results were interpolated in the standard curve constructed using the dilutions of the positive control serum. For each serum conditions were established in that appropriate dilutions of the serum produced results that were in the straight line portion of the standard curve.

### 2. *Immunohistochemistry.*

Wistar rats were anesthetized and perfused with saline followed with 4% paraformaldehyde in phosphate buffered saline (PBS). The cerebellum was further fixated with 4% paraformaldehyde for 4 hours and cryoprotected with 20% sucrose in PBS overnight. Samples were frozen in isopentane chilled in liquid nitrogen. 10 mm frozen sections were air-dried for 30 minutes and, after inhibition of endogenous peroxidase with 0.3% hydrogen peroxide in PBS for ten minutes, were sequentially incubated with 10% normal goat serum for 20 minutes, patient's serum (screening dilution 1:500) for 3 hours at 37°C, biotinylated goat anti-human IgG for 30 minutes, and the avidin biotin immunoperoxidase complex (Vector Laboratories, Burlingame, CA) for 30 minutes. The reaction was developed with 0.05% diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St.Louis) with 0.01% hydrogen peroxide in PBS with 0.5% Triton X-100. Dilution of antibodies was done in PBS with 0.3% Triton X-100. To study the GAD65-ab IgG subtypes, brain section incubated with the positive GAD65-ab serum were subsequently incubated with secondary biotinylated mouse monoclonal anti-human IgG 1-4 (Sigma-Aldrich, St.Louis) diluted 1:100 for IgG1 and 1:200 IgG2-4.

### 3. *GAD65 immunoblot.*

Human GAD65 recombinant protein (Diamyd, Stockholm, Sweden) was electrophoretically separated in a 4-12% Bis-Tris polyacrylamide gel (Life Technologies, Carlsbad, CA) and transferred to PVDF membrane. After blocking with 5% dry Carnation milk, strips were incubated with the patient's serum (1:1000 dilution) or GAD-6 monoclonal antibody (Hybridoma Bank, Iowa City, IA) overnight at room temperature and incubated with biotinylated goat anti-human IgG, diluted 1:1,000, or horse antimouse IgG (Vector Laboratories, Burlingame, CA), diluted 1:10000 in 5% normal goat serum for 1 hour. Strips were then immunoreacted with an avidin-biotin technique and developed with diaminobenzidine tetrahydrochloride.

### 4. *Immunocytochemistry on HEK293 cells.*

#### Fixed cells

HEK293 cells were transfected with plasmids containing the human GAD65 or GAD67 (OriGene, Rockville, MD). Cells were grown for 24 hours after transfection before assessment. Transfected cells were fixed in 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 and then incubated with patients' serum (1:40) or CSF (1:5) along with a commercial mouse antibody against against the GAD65 (1:4000, GAD-6 Hybridoma Bank, Iowa City, IA), or the GAD67 (1:4000, Abcam, Cambridge), for 1 hour at room temperature, and the corresponding fluorescent secondary antibodies (Alexa Fluor 488 goat anti-human IgG, A11013, diluted 1:1000; and Alexa Fluor 594 goat anti-mouse IgG, A11032, diluted 1:1000, both from Life Technologies, Carlsbad, CA). Results were photographed under a fluorescence microscope using Zeiss Axiovision software.

#### Live cells

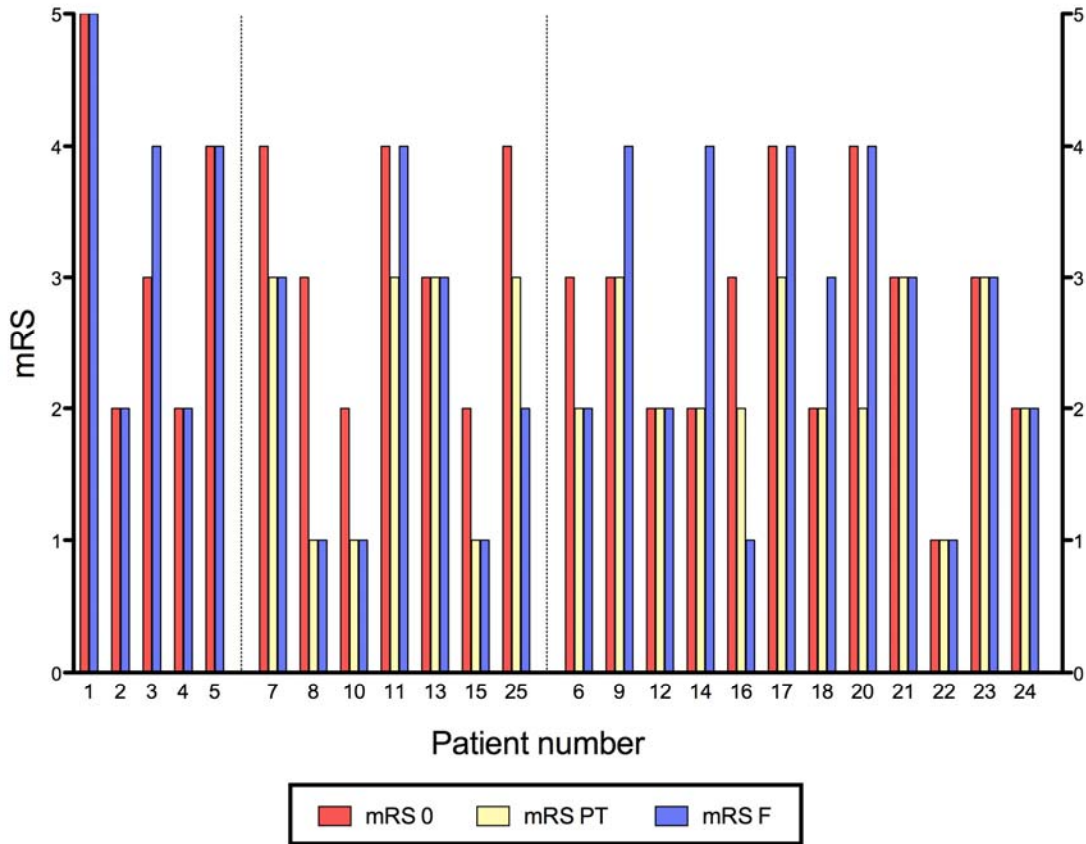
HEK293 cells were transfected with plasmids containing the human  $\alpha$ 1 subunit of the glycine receptor. Live transfected HEK cells were incubated with serum (1:40) or CSF (1:5) of the patient together with the commercial antibody against  $\alpha$ 1 subunit of the glycine receptor (dilution 1:1000, Synaptic Systems Goettingen, Germany) for 1

hour at 37°C, washed, and fixed with 4% paraformaldehyde for 5 minutes. After washing cells were then incubated with the corresponding Alexa Fluor secondary antibodies indicated above.

*5. Primary neuronal cell cultures and in vivo immunocytochemistry.*

Hippocampal neurons were obtained from E18 Wistar rat embryos. Cells were enzymatically and mechanically disrupted and resuspended in Neurobasal medium supplemented with B27 (Life Technologies, Carlsbad, CA).<sup>1</sup> Granular cell cultures were prepared from dissected cerebella of 8-day-old Wistar rats and processed equally as hippocampal neurons but using Neurobasal-A medium containing 25 mM of KCl and supplemented with B27.<sup>2</sup> Cells were plated in poly-L-lysine pre-coated P24 plates and 10 μM cytosine-D-arabino-furanoside (Sigma-Aldrich, St. Louis, MO) was added to the cultures 20 h after plating, to prevent proliferation of non neuronal cells. In the immunocytochemistry experiments, samples (1:5 CSF dilution and 1:200 serum dilution) were incubated on live neurons for 1 hour, then fixed with 4% paraformaldehyde and permeabilized with 0.3% Triton TX-100. Appropriate fluorescent secondary antibodies were applied and the coverslips were mounted with Vectashield with DAPI mounting media (Vector Laboratories, Burlingame, CA) and visualized with an Axio Imager M2 ZEISS microscope.

**eFigure.** Degree of Disability at Diagnosis, at the End of the First Treatment (up to 6 Months), and at the Last Follow-up Visit



Individual outcomes in 25 patients with CA and GAD 65-ab. Bars represent the mRS at onset (mRS 0); at 6 months of treatment (mRS PT), and at last follow-up visit (mRS F). Patients are grouped in non-treated (patient 1 to 5) and treated with subacute (7 to 25) and chronic (6 to 24) onset. Patients' number are the same than in Figure 1.

**eTable.** Case Reports of Patients With Cerebellar Ataxia and GAD65-Ab Treated With Immunotherapy

Reference <sup>a</sup>	Age <sup>b</sup> / sex	Onset CA	Delay <sup>c</sup>	Induction	Maintenance	Outcome (time <sup>d</sup> )
Virgilio <sup>3</sup>	76/M	chronic	7	IV steroids	Steroids	Improved (11)
Planche 1 <sup>4</sup>	72/F	subacute	6	IVIg x2, rituximab	No	Progressed after IVIg, improved after rituximab* (10)
Planche 2 <sup>4</sup>	73/F	unknown	36	IVIg, rituximab	IVIg x3	Improved* (13)
Planche 3 <sup>4</sup>	65/M	unknown	36	IVIg x2, rituximab, Cy	No	Progressed (17)
Pedroso <sup>5</sup>	51/M	chronic	108	IVIg x3	No	Improved (4)
Nociti <sup>6</sup>	42/F	chronic	7	Steroids	AZA	Improved (21)
Nanri 1 <sup>7</sup>	51/F	unknown	120	IVIg x4	No	Improved* (36)
Nanri 2 <sup>7</sup>	72/F	unknown	48	IVIg	No	Improved* (12)
Lauria <sup>8</sup>	66/F	chronic	5	IV steroids, IVIg	Steroids, Cy	Improved (17)
Bonnan 2 <sup>9</sup>	38/F	chronic	12	IV steroids, IVIg, PE	No	Improved* (36)
Bonnan 3 <sup>9</sup>	45/F	chronic	12	IV steroids, IVIg	Periodic IVIg, AZA	Stable (31)
Bonnan 4 <sup>9</sup>	75/F	chronic	10	IV steroids	No	Stable (12)
Bayreuther <sup>10</sup>	47/F	chronic	24	IVIg	No	Improved* (6)
Vulliemoz <sup>11</sup>	58/M	subacute	2	IV steroids	Steroids, AZA	Improved (8)
Abele <sup>12</sup>	50/F	chronic	216	IVIg x2	No	Improved* (2)
McFarland <sup>13</sup>	70/M	chronic	9	IVIg, PE	AZA	Improved (19)

<sup>a</sup>Same references indexed in main text, but in different order. <sup>b</sup>At diagnosis. <sup>c</sup>Months from onset of CA until first treatment. <sup>d</sup>Months from onset of treatment to last follow-up visit. \*Description in text reflects mild improvement. None of them improved more than 12/100 points in the ICARS (International Cooperative Ataxia Rating Scale). These patients probably would not achieve a shift in the mRS and the effect of therapy should be interpreted as stable response when compared with the data of our series.

AZA: azathioprine; CA: cerebellar ataxia; Cy: cyclophosphamide; IV: intravenous; IVIg: intravenous immunoglobulins; PE: plasma exchange

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