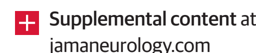
Original Investigation

Neuronal Surface and Glutamic Acid Decarboxylase Autoantibodies in Nonparaneoplastic Stiff Person Syndrome

Thashi Chang, MD, DPhil; Haris Alexopoulos, PhD; Mary McMenamin, DPhil; Alexander Carvajal-González, MD; Sian K. Alexander, MD, DPhil; Robert Deacon, PhD; Ferenc Erdelyi, PhD; Szabó Gabor, PhD; Bethan Lang, PhD; Franz Blaes, MD; Peter Brown, FRCP; Angela Vincent, FRCPath


Supplemental content at jamaneurology.com

IMPORTANCE High titers of autoantibodies to glutamic acid decarboxylase (GAD) are well documented in association with stiff person syndrome (SPS). Glutamic acid decarboxylase is the rate-limiting enzyme in the synthesis of γ-aminobutyric acid (GABA), and impaired function of GABAergic neurons has been implicated in the pathogenesis of SPS. Autoantibodies to GAD might be the causative agent or a disease marker.

OBJECTIVE To investigate the characteristics and potential pathogenicity of GAD autoantibodies in patients with SPS and related disorders.

DESIGN Retrospective cohort study and laboratory investigation.

SETTING Weatherall Institute of Molecular Medicine, University of Oxford.

PARTICIPANTS Twenty-five patients with SPS and related conditions identified from the Neuroimmunology Service.

EXPOSURES Neurological examination, serological characterization and experimental studies.

MAIN OUTCOMES AND MEASURES Characterization of serum GAD antibodies from patients with SPS and evidence for potential pathogenicity.

RESULTS We detected GAD autoantibodies at a very high titer (median, 7500 U/mL) in 19 patients (76%), including all 12 patients with classic SPS. The GAD autoantibodies were high affinity (antibody dissociation constant, 0.06-0.78 nmol) and predominantly IgG1 subclass. The patients' autoantibodies co-localized with GAD on immunohistochemistry and in permeabilized cultured cerebellar GABAergic neurons, as expected, but they also bound to the cell surface of unpermeabilized GABAergic neurons. Adsorption of the highest titer (700 000 U/mL) serum with recombinant GAD indicated that these neuronal surface antibodies were not directed against GAD itself. Although intraperitoneal injection of IgG purified from the 2 available GAD autoantibody–positive purified IgG preparations did not produce clinical or pathological evidence of disease, SPS and control IgG were detected in specific regions of the mouse central nervous system, particularly around the lateral and fourth ventricles.

CONCLUSIONS AND RELEVANCE Autoantibodies to GAD are associated with antibodies that bind to the surface of GABAergic neurons and that could be pathogenic. Moreover, in mice, human IgG from the periphery gained access to relevant areas in the hippocampus and brainstem. Identification of the target of the non-GAD antibodies and peripheral and intrathecal transfer protocols, combined with adsorption studies, should be used to demonstrate the role of the non-GAD IgG in SPS.

JAMA Neurol. doi:10.1001/jamaneurol.2013.3499
Published online July 22, 2013.

Author Affiliations: Author affiliations are listed at the end of this article.

Corresponding Author: Angela Vincent, FRCPath, Nuffield Department of Clinical Neurosciences, Level 6, West Wing, Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, England (angela.vincent@ndcn.ox.ac.uk).

Downloaded From: http://archneur.jamanetwork.com/ by a Ohio State University User on 08/15/2013

High titers (usually >1000 U/mL) of autoantibodies to glutamic acid decarboxylase (GAD) are well documented in association with stiff person syndrome[1] (SPS) and certain forms of cerebellar ataxia, limbic encephalitis, and epilepsy.[2,3] Autoantibodies to GAD are also detected in as many as 80% of patients with type 1 diabetes mellitus (T1DM), but the titers are typically lower (usually <1000 U/mL)[2] than in the neurological syndromes. Differences in reactivity with GAD epitopes have been suggested,[4] although this possibility has since been questioned.[5]

Glutamic acid decarboxylase is the rate-limiting enzyme in the synthesis of γ-aminobutyric acid (GABA), and impaired function of GABAergic neurons has been implicated in the pathogenesis of SPS.[6,7] Although GAD is an intracellular enzyme, some reports have detected pathogenicity. Synthesis of GABA was inhibited in vitro by serum and IgG positive for GAD autoantibodies,[8] cerebrospinal fluid samples positive for GAD antibodies (GAD-Abs) inhibited the activity of cerebellar neurons in brain slices,[9,10] and GABA levels were low in the brain tissue and cerebrospinal fluid of patients with SPS.[11,12] In addition, in vivo injection of SPS monoclonal GAD antibody altered the *N*-methyl-D-aspartate receptor–mediated turnover of glutamate.[13] Recently, intrathecal injection of amphiphysin antibodies induced motor and electrophysiological evidence of SPS,[14] and intraventricular injection of GAD antibodies induced motor changes in rats,[15] although whether GAD antibodies were the causative agent was not clear; GAD autoantibodies might be markers for a disease process that is caused by a T-cell–mediated attack[16] or by other autoantibodies that bind to the neuronal cell surface.

We undertook a comprehensive analysis of GAD-Abs in 25 patients with SPS and SPS-related conditions and compared these characteristics with those of patients with T1DM. We then looked for antibodies binding to the surface of cerebellar neurons and injected purified IgG from 2 SPS patients intraperitoneally into mice to look for clinical and pathological effects and to determine the distribution of human IgG within the mouse brain.

Methods

Patients and Clinical Material

We studied serum samples positive for GAD-Abs from 25 patients with SPS or SPS variants referred for routine analysis from January 1, 1998, through December 31, 2003 (Supplement [eTable]). Clinical data (**Table 1**) were obtained from patient notes or from the neurologists (15 patients examined by P.B.) or both. Serum samples from 5 patients with T1DM and from healthy individuals (controls) were used for comparison. Patients and controls consented to serological testing, and data were retrospectively analyzed.

GAD Autoantibody Assays

Immunohistochemistry studies to detect GAD autoantibodies were performed using free-floating rat brain sections 40 μm thick, as previously described.[18] Radioimmunoprecipitation assays used serial dilutions incubated with 50 μL of iodine 125–labeled GAD65 (RSR Ltd; 18 μCi/μg), with titers expressed in units per milliliter calculated from the standard curve. Subclass and affinity methods are described in the Supplement (eAppendix). To adsorb serum samples against GAD, 10 μL of 4 high-titer SPS serum samples was incubated with 25 μg of recombinant human GAD65 (kind gift of RSR Ltd) overnight at 4°C with constant mixing. After centrifugation at 13 000 rpm for 60 minutes, the supernatants were separated and stored at 4°C until required. Effective adsorption was confirmed by immunoblotting against recombinant human GAD65 on nitrocellulose membranes and comparing with serum samples before adsorption (Supplement [eFigure 1]).

Primary Neuronal Cultures

Primary cultures of cerebellar neurons were prepared from postnatal day 2 pups from mice expressing GAD65 and enhanced green fluorescent protein (GAD-EGFP) in GABAergic neurons (see below), as previously described.[19] Coverslips of cultured cerebellar neurons at 7 days in culture were fixed with 4% paraformaldehyde in phosphate-buffered saline solution (PBS) (Oxoid Limited) for 30 minutes at room temperature, washed, and stored in PBS at 4°C until used. The coverslips were rinsed 3 times for 5 minutes each with PBS and permeabilized with PBS containing biological detergent (0.3% Triton X-100; Sigma-Aldrich) (PBST) for 15 minutes at room temperature. Nonspecific binding was blocked with 5% normal goat serum in PBST (containing 0.1% Triton X-100) for 1 hour at room temperature. Patient serum samples were applied at 1:200 dilution in the blocking solution for 1 hour at room temperature. Fluorochrome-conjugated secondary antibodies (Alexa Fluor; Molecular Probes) were added at dilutions of 1:200 in 5% normal goat serum in 0.1% PBST and incubated at room temperature for 1 hour. Coverslips were mounted using fluorescent mounting medium (DakoCytomation), air dried overnight at 4°C, and visualized using confocal microscopy. All rinses were in PBS. For nonpermeabilized primary cerebellar cultures, permeabilization with 0.3% PBST was omitted and primary and secondary antibodies were prepared in PBS instead of PBST.

For double immunostaining, the diluted serum samples were applied to live cultures, rinsed, and fixed, and fluorophore-conjugated antihuman IgG (647 nm; Alexa Fluor) was applied. The coverslips were then permeabilized as above before addition of the intracellular antibody and a species-appropriate fluorophore-conjugated secondary antibody (568 nm; Alexa Fluor). Coverslips were mounted and examined under the confocal microscope.

Passive Transfer

Transgenic mice on a background of CBA/C57Bl6 that express EGFP under the GAD65 promoter were used to count the number of GABAergic neurons visualized by EGFP expression.[20,21] Mice were housed in groups with water and food ad libitum in a nonspecific pathogen-free environment, and all procedures were performed in accordance with the UK Home Office guidelines.

The mice were injected intraperitoneally with purified IgG, 1 mL/d for 5 days, from the 2 patients (patients 12 and 25) with adequate plasmapheresis material available (Supplement [eAp-

Downloaded From: http://archneur.jamanetwork.com/ by a Ohio State University User on 08/15/2013

Table 1. Clinical Characteristics of the Study Cohort[a]

No./Sex/ Age, y	Diagnosis[b]	Duration, mo	T1DM	OND	Other AI Diseases	Distribution	Sensitivity	Severity Score[c]	Immunotherapy
1/F/32	C-SPS	10	Yes	TLE	None	3	1	4	None
2/F/41	C-SPS	72	No	None	PA	4	3	7	IVIg
3/F/53	C-SPS		Yes	CA	None				IVIg
4/M	C-SPS								
5/F/74	C-SPS	72	Yes	None	PA, hypothyroid	4	2	6	IVIg
6/F/53	C-SPS	11	No	None	None	1	0	1	IVIg
7/M/43	C-SPS	72	Yes	None	None	2	3	5	IVIg
8/F/51	C-SPS	57	Yes	None	Hyperthyroid	2	2	4	IVIg
9/F/55	C-SPS	20	No	None	None	1	2	3	None
10/M/53	C-SPS	132	Yes	None	None	3	4	7	IVIg, rituximab
11/M	C-SPS								
12/F/38	C-SPS	21	No	EPI	None	4	3	7	PE, MMF
13/M/58	SLS	17	Yes	None	None	2	3	5	IVIg
14/M/61	SLS	6	Yes	ON	Hyperthyroid	1	1	2	None
15/F/80	SLS	19	No	None	None	1	0	1	IVIg
16/F/55	SLS	34	No	None	None	2	3	5	IVIg
17/F/62	SLS	60	No	None	None	2	1	3	None
18/F/48	J-SPS	84	No	None	None	1	2	3	IVIg
19/F/52	J-SPS	84	No	TLE	None	2	3	5	IVMP
20/F/50	J-SPS	12	No	None	PA	4	3	7	PE, IVIg
21/F/70	A-SPS	12	Yes	None	Hypothyroid	1	1	2	None
22/M/41	A-SPS	5	No	None	None	1	1	2	None
23/F/57	A-SPS	63	No	None	None	2	2	4	IVMP
24/F/75	A-SPS	1	No	None	None	3	2	5	None
25/F/71	PERM	19	Yes	None	None	2	3	5	PE, IVMP
30/F/46	LADA			PN	None				
31/F/24	T1DM			None	None				
32/F/46	T1DM			None	Vitiligo				
33/ /32	T1DM			None	None				
34/ /36	T1DM			None	None				

Abbreviations: AI, autoimmune; A-SPS, atypical stiff person syndrome (SPS); CA, cerebellar ataxia; C-SPS, classic SPS; EPI, epilepsy; IVIg, intravenous immunoglobulin; IVMP, intravenous methylprednisolone; J-SPS, jerking SPS; LADA, latent autoimmune diabetes in adult; MMF, mycophenolate mofetil; ON, optic neuritis; OND, other neurological diseases; PA, pernicious anemia; PE, plasma exchange; PERM, progressive encephalomyelitis with rigidity and myoclonus; PN, peripheral neuropathy; SLS, stiff limb syndrome; T1DM, type 1 diabetes mellitus; TLE, temporal lobe epilepsy.

[a] Data for 3 patients were incomplete but were included in the study because of clear diagnosis.

[b] Described in the Supplement (eTable).

[c] Severity scores based on Dalakas et al.[17]

pendix]). The IgG preparations contained 7.3 and 3.5 mg/mL of IgG in patients 25 and 12, respectively (GAD autoantibody titers of 9237 U/mL in patient 25 and 1283 U/mL in patient 12), and 14.4 mg/mL of IgG in a control (GAD autoantibody titer <1 U/mL). To increase the chance of the antibodies accessing the central nervous system, lipopolysaccharide was injected intraperitoneally on days 3 (1.5 mg/kg) and 5 (3 mg/kg), and the mouse behavior was tested on days 0, 6, and 9. In addition to observing general well-being and behavior each day, burrowing (a mouse-specific behavior) and results of accelerating rotarod tests (motor power and coordination) and light-dark box and white open-field tests (anxiety and response to stressful environments)[22,23] were measured. All behavior tests were conducted with the investigator blind to the group allocation of the mice. The processing and staining of central nervous system tissue from injected mice and the commercial antibodies used are described in the Supplement (eAppendix).

Results

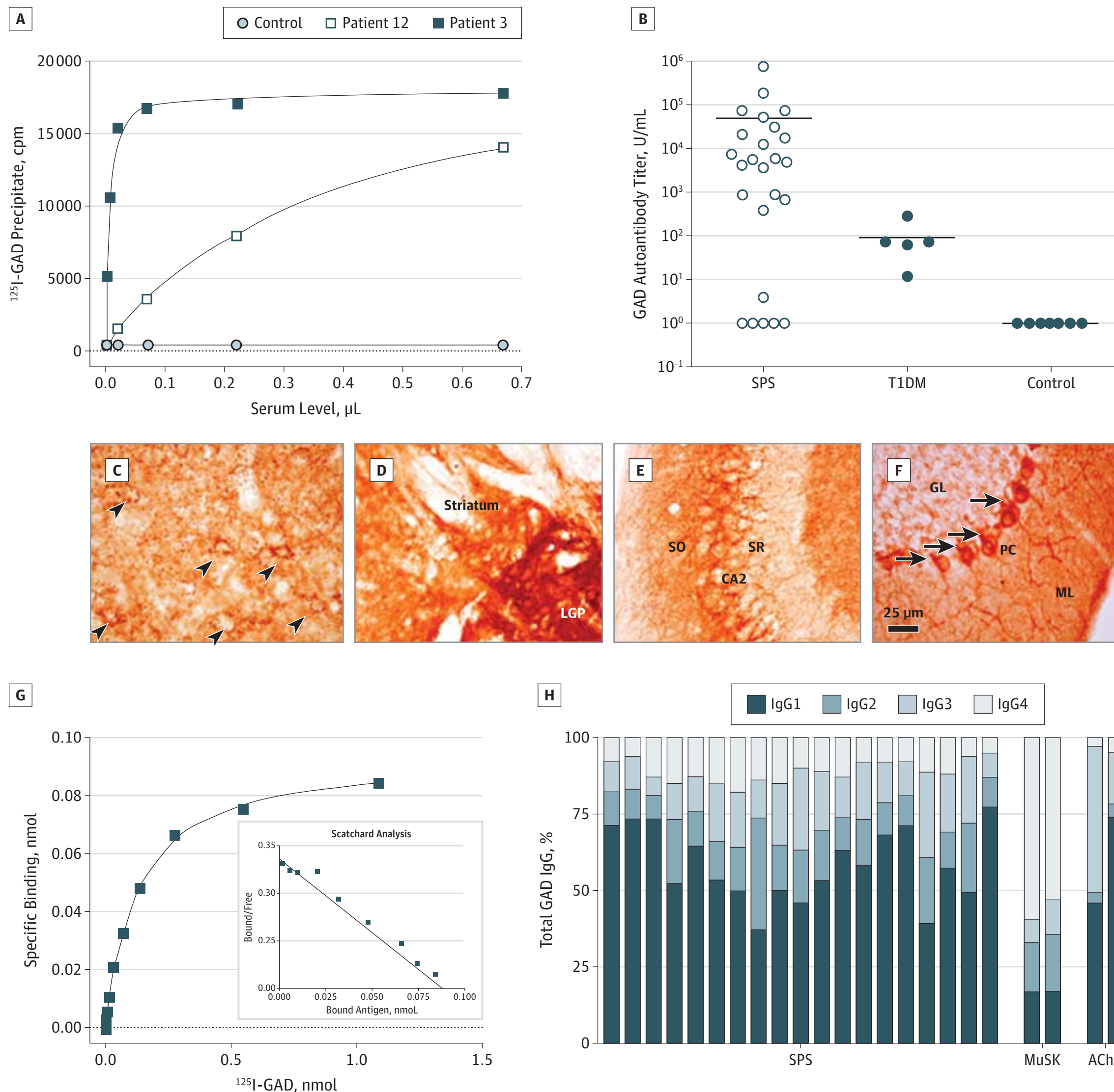
Clinical Characteristics of Patients

Eighteen of the patients with SPS were female (72%) (Table 1), with ages ranging from 32 to 80 years at the time of the study and duration of disease from 1 to 132 months. Nineteen patients (76%) were seropositive for GAD autoantibodies. Only 3 of 11 patients undergoing testing had oligoclonal bands in the cerebrospinal fluid (each seronegative for GAD autoantibodies).

The patients were classified into disease categories based on established clinical criteria[24,25] (Supplement

Downloaded From: http://archneur.jamanetwork.com/ by a Ohio State University User on 08/15/2013

Figure 1. Glutamic Acid Decarboxylase (GAD) Autoantibody Characteristics and Immunohistochemical Staining of Rat Brain Sections With Stiff Person Syndrome (SPS) Serum



A. Typical serum GAD autoantibody titration curves for 2 representative patients with SPS (75 000 U/mL [patient 3] and 836 U/mL [patient 12]) and a healthy control using iodine 125–labeled GAD65 precipitation. B, All GAD autoantibody titers (log scale) in patients with SPS (n = 25), patients with type 1 diabetes mellitus (T1DM) (n = 5), and controls (n = 9). C, Immunoreactive dots outlining pyramidal cells in the cortex of rat brain sections (arrowheads). D, Intense immunoreactivity of lateral globus pallidus (LGP) relative to the striatum of rat brain sections. E, Staining around pyramidal cells in CA2 of the hippocampus of rat brain sections. SO indicates stratum oriens; SR, stratum radiatum. F, Dense immunoreactivity at the axon hillock (arrows) and puncta outlining the perikaryon and dendritic tree of Purkinje cells (PC) with punctate staining in the molecular (ML) and granular (GL) layers of rat brain sections. G, Representative binding curve and Scatchard plot (inset) for a serum sample from a patient with SPS. H, Subclasses of GAD autoantibody IgG in serum samples of patients seropositive for SPS (n = 19), compared with acetylcholine receptor (AChR) and muscle-specific kinase (MuSK) antibodies.

[eTable]): only 12 had classic SPS with high-titer GAD autoantibodies, 5 had stiff limb syndrome, 3 had jerking SPS, 4 had atypical SPS, and 1 had progressive encephalomyelitis with rigidity and myoclonus. Severity scores (computed by adding together the distribution of stiffness score [maximum, 6] and the heightened sensitivity score [maximum, 7])[17] ranged from 1 to 7 at onset. Three patients also had epilepsy, 1 had cerebellar ataxia, and 1 had had optic neuritis. Seven patients (28%) had autonomic symptoms (cardiac arrhythmia/tachycardia, blood pressure fluctuations, or excessive sweating), and 7 had neuropsychiatric disturbances (anxiety, depression, or phobias). Ten patients (40%) had associated diabetes mellitus and 6 (24%) had other autoimmune diseases. Two patients had associated tumors (renal cell carcinoma and cholangiocarcinoma) that were successfully resected, but neither had classic paraneoplastic antibodies. All patients were receiving symptomatic therapy, and 16 had already received immunotherapy (Table 1); 8 (including 6 with GAD autoantibodies) had moderate or substantial improvement.

Downloaded From: http://archneur.jamanetwork.com/ by a Ohio State University User on 08/15/2013

Table 2. Autoantibody Characteristics of Patients With SPS, Epilepsy, and T1DM

Patient No.	Diagnosis	GAD Autoantibody Titer, U/mL	IHC	GAD Autoantibody K_D, nmol	WB rhGAD65, IOD %	IgG Binding, Score[a]		
						EGFP-Expressing Neurons	GABA-Expressing Neurons	EGFP/GABA-Negative Neurons
1	C-SPS	700 000	Positive	0.13	95.3	3	4	1
2	C-SPS	180 000	Positive	0.54	ND	2	4	0
3	C-SPS	75 000	Positive	0.13	ND	1	3	0
4	C-SPS	50 000	Positive	0.33	80.2	2	2	0
5	C-SPS	34 000	Positive	0.43	56.7	1	0	0
6	C-SPS	21 324	Positive	0.13	80.5	2	2	0
7	C-SPS	12 500	Positive	0.28	51.6	2	1	0
8	C-SPS	7500	Positive	0.33	38.1	ND	ND	ND
9	C-SPS	4800	Positive	0.78	40.4	ND	ND	ND
10	C-SPS	3600	Negative	0.33	13.6	0	0	0
11	C-SPS	889	Negative	0.06	3.8	0	0	0
12	C-SPS	836	Negative	0.14	ND	0	0	0
13	SLS	5682	Positive	0.17	59.6	2	2	0
14	SLS	676	Negative	0.14	13.4	ND	ND	ND
15	SLS	405	Negative	0.65	19.2	1	2	1
16	SLS	<1	Negative		ND	0	0	0
17	SLS	<1	Negative		ND	0	1	0
18	J-SPS	4200	Negative	0.28	26.8	0	0	0
19	J-SPS	1	Negative		ND	0	0	0
20	J-SPS	4	Negative		ND	0	0	0
21	A-SPS	80 000	Positive	0.74	88.4	ND	ND	ND
22	A-SPS	17 000	Positive	0.17	14.2	ND	ND	ND
23	A-SPS	<1	Negative		ND	0	0	0
24	A-SPS	<1	Negative		ND	0	0	0
25	PERM	5451	Positive	0.17	ND	0	1	0
30	LADA	285	Negative	0.68	10.3	ND	ND	ND
31	T1DM	72	Negative	0.34	7.3	ND	ND	ND
32	T1DM	72	Negative	0.13	ND	ND	ND	ND
33	T1DM	60	Negative	0.31	5.9	ND	ND	ND
34	T1DM	12	Negative	0.15	6.8	ND	ND	ND

Abbreviations: EGFP, enhanced green fluorescent protein; GABA, γ-aminobutyric acid; GAD, glutamic acid decarboxylase; IHC, immunohistochemistry; IOD, integrated optical densities of immunoreactive bands on Western blotting of recombinant human GAD65 (rhGAD65) expressed as a percentage of the IOD of GAD monoclonal antibody; K_D, antibody dissociation constant; ND, not determined; WB, Western blot. Other abbreviations: See Table 1.

[a] Serum antibodies binding to surface determinants of unpermeabilized primary neurons in culture were scored as 0 (none), 1 (1%-20% of total GABAergic neurons), 2 (21%-40%), 3 (41%-60%), or 4 (>60%).

GAD Autoantibody Characteristics

The GAD autoantibody values were derived from titrations (**Figure 1**A and B). Fifteen of the 19 GAD autoantibody-positive SPS patients had titers greater than 1000 U/mL, with the highest at 700 000 U/mL. On immunohistochemistry, 13 of the 25 serum samples (52%) bound to rat brain tissue (Figure 1C and F), colocalizing with monoclonal antibodies to GAD (Supplement [eFigure 5]). This binding was only observed with GAD autoantibody titers greater than 4000 U/mL (**Table 2**); in 4 samples, staining was essentially abolished after adsorption with recombinant GAD (data not shown). The T1DM samples had lower serum titers and did not show binding to brain tissue. Immunoblotting on recombinant human GAD65 was positivefor binding to GAD in 18 of 19 serum samples undergoing testing, and the binding correlated with the immunoprecipitation titers (Supplement [eFigure 2]).

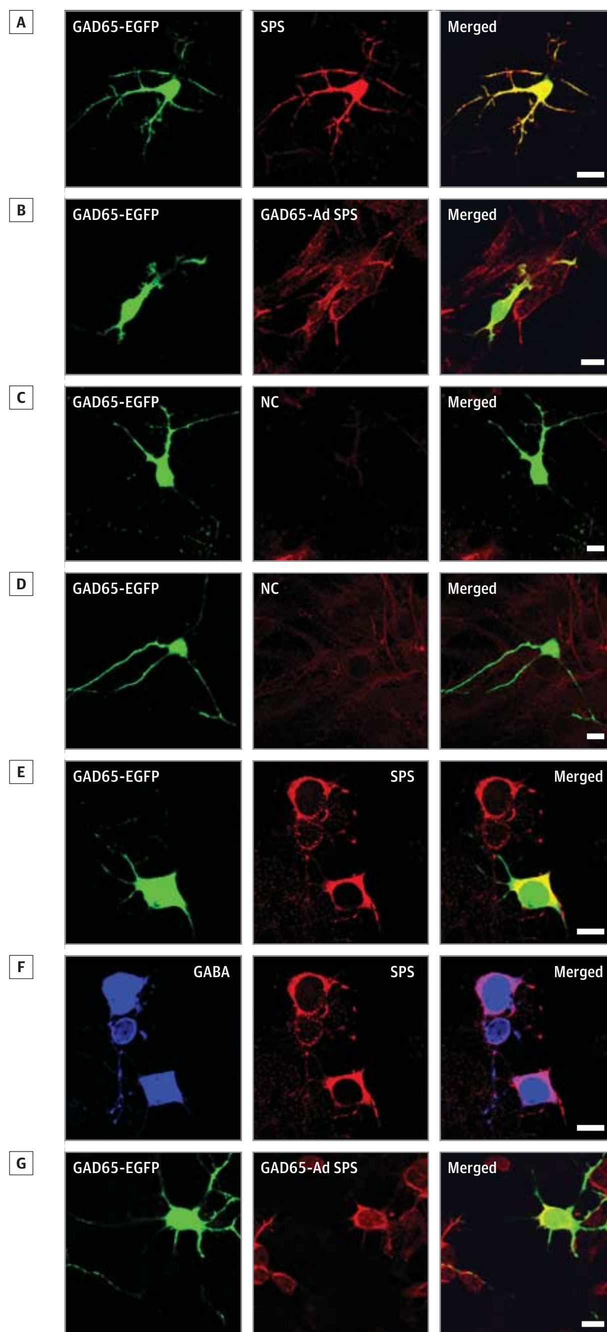
The GAD autoantibodies, including those of the patients with T1DM, were of high affinity, with antibody dissociation constant values ranging from 0.06 to 0.78 nmol (Figure 1G). The GAD autoantibodies were mainly IgG1, similar to the 2 acetylcholine receptor antibody serum samples used for comparison and contrasting with the IgG4 in 2 muscle-specific kinase antibodies (Figure 1H). All results are summarized in Table 2.

Binding of Serum Samples to Primary Neurons in Culture

The SPS serum samples bound strongly to permeabilized GAD-positive neurons, identified by the coexpression of EGFP (**Figure 2**A), and did not colocalize with microglia or astro-

Downloaded From: http://archneur.jamanetwork.com/ by a Ohio State University User on 08/15/2013

Figure 2. Confocal Images of Serum IgG Binding (Red) to Permeabilized Cerebellar Neurons in Primary Cultures Derived From Glutamic Acid Decarboxylase 65–Enhanced Green Fluorescent Protein (GAD65-EGFP)–Expressing (Green) Transgenic Mice



A, Stiff person syndrome (SPS) serum antibodies bind intracellularly to EGFP-expressing neurons. B, After adsorption against GAD65, the same SPS serum (Ad SPS) still binds to the surface of the neurons. C, Normal control (NC) serum samples (red) do not bind to the neurons. D, Some serum samples showed weak nonspecific binding to nonneuronal cells. E, The SPS serum antibodies bind to the surface of EGFP- and non–EGFP-expressing neurons. F, The SPS serum binds to γ-aminobutyric acid (GABA)–expressing (blue) neurons. G, After adsorption against GAD65, the same SPS serum continues to demonstrate surface-binding antibodies. Scale bar indicates 10 μm.

cytes (data not shown). Successful adsorption of the highest serum titer (700 000 U/mL) (Supplement [eFigure 1]) with recombinant human GAD65 substantially reduced the intracellular binding, as expected, but revealed binding to the cell surface of EGFP-positive and some EGFP-negative cells (Figure 2B). Normal healthy serum did not bind to the neurons (Figure 2C), although some showed weak nonspecific binding to nonneuronal cells (Figure 2D).

We then performed a 2-stage immunostaining process. First, unpermeabilized cultures were incubated with SPS serum samples and labeled secondary antibody. Then the cells were fixed, permeabilized, and incubated with a GAD monoclonal antibody or an antibody to GABA, which was detected by a labeled species-specific antibody (Figure 2E and F). The SPS antibodies bound to the surface of EGFP- and non-EGFP-expressing neurons (many GABA-expressing cells are negative for EGFP in this transgenic mouse[21]). All the GAD autoantibody-binding neurons, however, were positive for binding of commercial antibody to GABA (Figure 2F), and surface binding was not obviously reduced by adsorption of serum from patient 1 (Table 2) with recombinant GAD (Figure 2G). Very few EGFP/GABA-negative neurons were bound by antibody. All results were scored on a scale of 0 to 4. The neuronal surface binding was found in 10 of 14 GAD autoantibody-positive SPS serum samples and in only 1 of 6 GAD autoantibody-negative serum samples (Table 2) (Fisher exact test, $P < .05$).

Passive Transfer

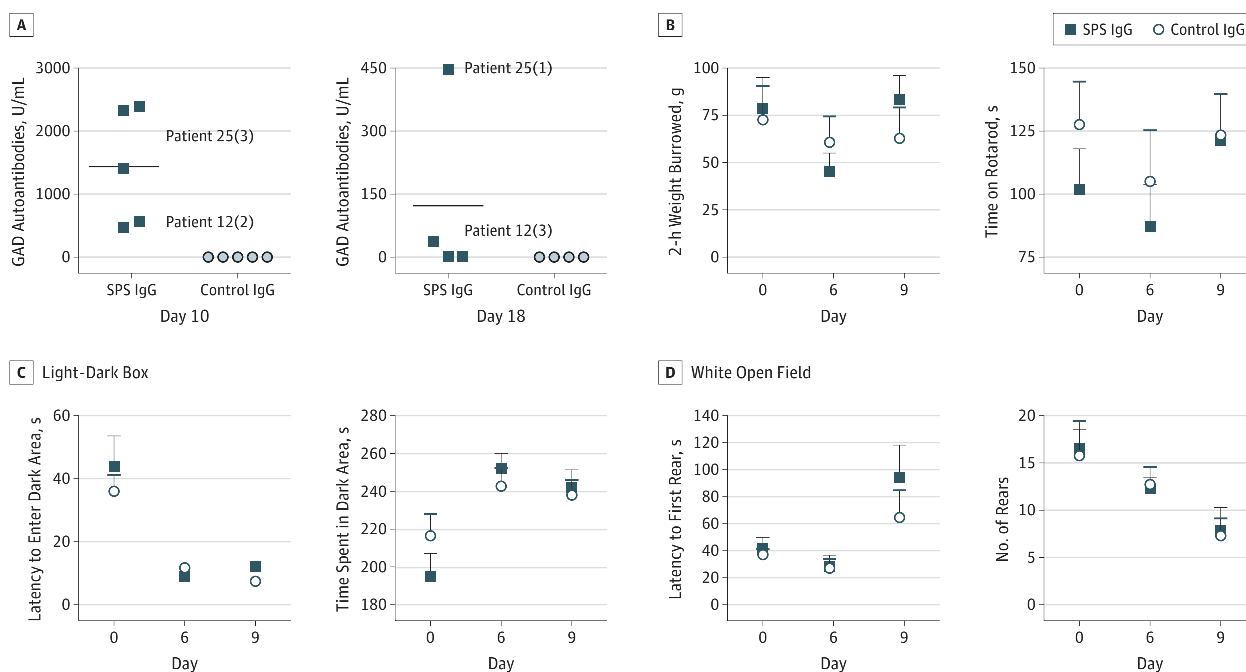
To try to demonstrate pathogenicity of autoantibodies in SPS, we injected mice for 5 days with either purified IgG from 1 patient with progressive encephalomyelitis with rigidity and myoclonus (patient 25; 4 mice) or 1 patient with classic SPS (patient 12; 5 mice), or from pooled healthy controls (9 mice). The protocol is given in the Supplement (eFigure 3). The mice underwent testing on a range of behavioral tests and were humanely killed on day 10 or day 18.

The titers of human GAD autoantibody reached substantial levels in the mouse serum samples as shown at day 10 but had dropped by day 18 (**Figure 3**A). Disappointingly, the behavioral tests in the GAD autoantibody–injected mice did not differ from those of the control IgG-injected mice (2-way repeated-measures analysis of variance; Figure 3B-D). We found a general trend toward reduced activity at day 6, likely the result of the second lipopolysaccharide injection on the preceding day. Nevertheless, IgG was found in several regions of the brain in control and test samples, particularly within the hippocampus and septum and around the lateral and fourth ventricles (**Figure 4**A and B). Although the concentration of IgG was at least twice as high in the control IgG preparation (Supplement [eFigure 4]), the staining appeared stronger in SPS IgG-treated mice compared with control IgG-treated mice, but we did not measure this difference quantitatively.

As expected, test and control mice showed large numbers of neuronal nuclei antibody–positive cells, greater than the EGFP-GAD–expressing cells (Figure 4C and D) in the brainstems. We found no differences between test and control mice in neuronal nuclei antibody- or EGFP-expressing cells (Figure 4E and F). No differences in microglial activation, glial

Downloaded From: http://archneur.jamanetwork.com/ by a Ohio State University User on 08/15/2013

Figure 3. Results of Passive Transfer of Stiff Person Syndrome (SPS) IgG Positive for Glutamic Acid Decarboxylase (GAD) Autoantibody and Control IgG Into Mice



Nine mice were injected with purified SPS IgG (from GAD antibody–positive patient 12 and patient 25) and compared with 9 mice injected with purified control IgG. Measurements were obtained at baseline (day 0) and days 6 (1 day after lipopolysaccharide [LPS] injection) and 9 (4 days after LPS injection). Mice were randomized on day 0 based on the burrowing test results. Lipopolysaccharide was given at days 3 and 5. A, Levels of GAD autoantibodies in the injected mice at days 10 and 18 after injection on days 1 through 5. Numbers in parentheses indicate numbers of mice undergoing sampling and testing for GAD antibodies. B, Results (mean [SEM]) of observed burrowing and accelerating rotarod test measuring mouse-specific behavior and motor power and coordination, respectively. C, Results of light-dark box test measuring anxiety and response to stressful environments. D, Results of white open-field test measuring anxiety and response to stressful environments.

fibrillary acidic protein upregulation, or T-cell infiltration between the test and control mice were detected (data not shown).

Discussion

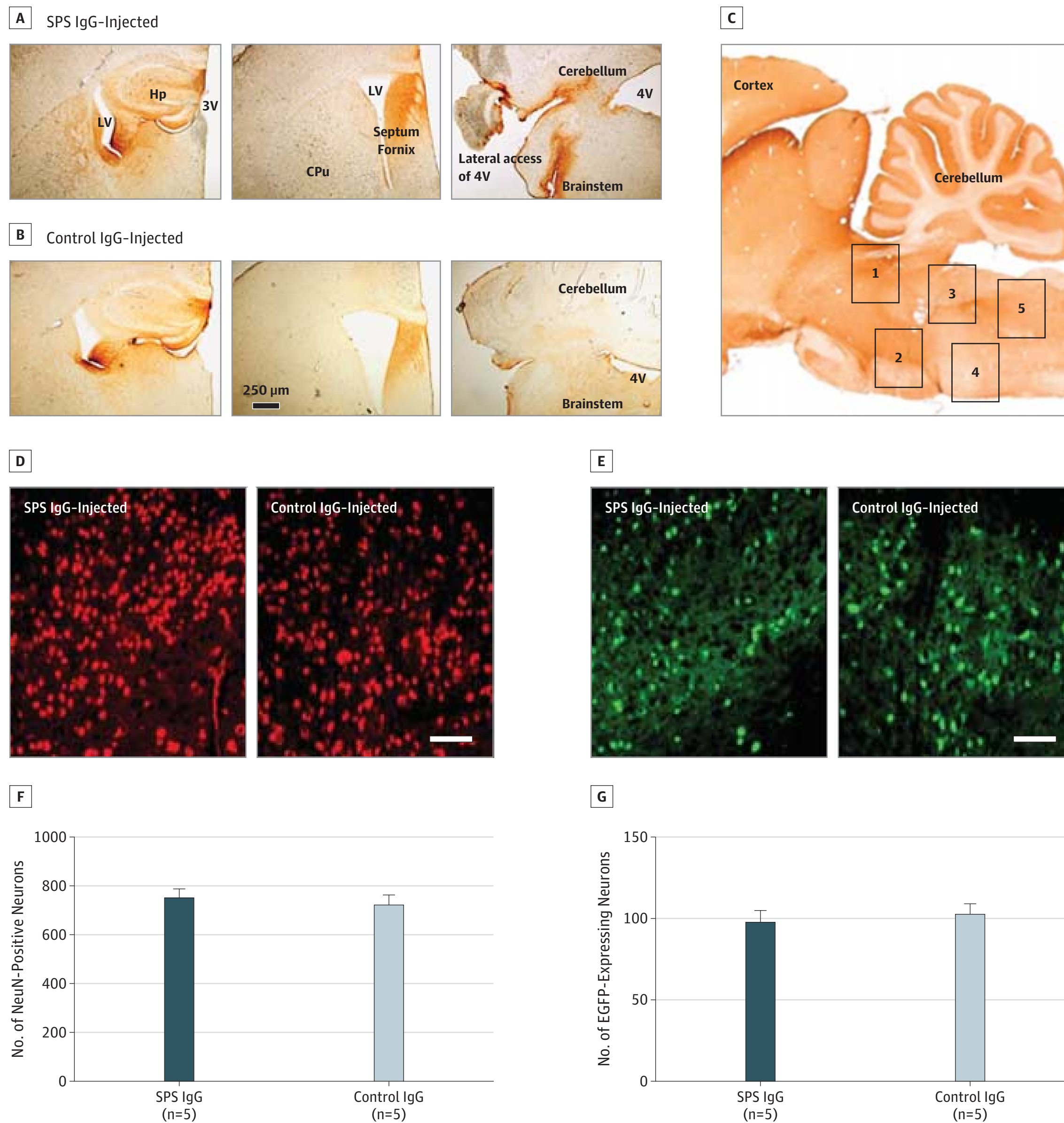
The pathogenicity of GAD autoantibodies in SPS remains unclear despite some improvement after response to immunotherapy.[1,6,7] We found very high titers, high affinity, and a predominantly IgG1 isotype in GAD autoantibodies in all patients with well-defined classic SPS. Although the GAD autoantibodies themselves did not bind to neuronal cell surface epitopes, as evident from the binding to unpermeabilized neurons and the immunoadsorption studies, we found other autoantibodies in SPS serum samples that were capable of binding to the surface of GABAergic neurons. Given the now well-established role of antibodies in the surface neuronal antigens in immune-mediated diseases,[26] these antibodies could contribute to the pathogenesis of the disease. However, a 5-day passive transfer experiment did not induce any dysfunction in the mice or loss of GAD-EGFP neurons in the brainstem.

Our cohort of 25 SPS patients (Table 1) represented all variants of SPS-related syndromes and included classic SPS, stiff limb syndrome, jerking SPS, atypical SPS, and progressive encephalomyelitis with rigidity and myoclonus. The prevalence of T1DM, epilepsy, and other autoimmune diseases or autoantibodies and the association with autonomic features and neuropsychiatric manifestations were consistent with previous reports.[27,28] All 12 patients with classic SPS had high titers of GAD autoantibodies, but some patients with other SPS variants had clearly negative results, as described by others,[24,28] and with 1 exception their serum antibodies did not bind to the cerebellar neurons. Other antibodies may be present in some patients, and this possibility should be examined further. For instance, some patients with SPS or progressive encephalomyelitis with rigidity and myoclonus may have, instead, antibodies against the glycine receptor α subunit[26] and occasionally both autoantibodies.[29]

Titers of GAD autoantibodies in SPS were generally very high and were clearly different from the typically low titers of T1DM. Others have suggested that the antibody characteristics are intrinsically different between patients with SPS and T1DM,[4,30] but our data suggest this difference is largely related to titer. In particular, immunohistochemistry studies of brain sections and immunoblotting correlated well with the GAD autoantibody titers of the serum samples irrespective of the clinical diagnosis (Table 2), and the T1DM serum samples showed the same IgG1 subclass distributions and affinities as the SPS serum samples, confirming a recent report that GAD

Downloaded From: http://archneur.jamanetwork.com/ by a Ohio State University User on 08/15/2013

Figure 4. Diffusion of IgG and Quantification of Enhanced Green Fluorescent Protein (EGFP)-Labeled Neurons After Stiff Person Syndrome (SPS) IgG Injection Into Mice



A, Diffusion of human IgG into the mouse brain after IgG injections. Coronal sections are seen through hippocampus (Hp), striatum (CPu), and the fourth ventricle (4V). The human IgG was found mainly in the Hp and septum, adjacent to the ventricles, and in the brainstem lateral to the 4V. B, Diffusion appeared weaker in control IgG–injected mouse brains. C, Five representative areas in which neurons were counted. D, Examples of neuronal nuclei antibody (NeuN)–positive neurons in the brainstem of mice. E, Examples of EGFP-expressing neurons in the brainstem of mice. F and G, Mean (SEM) total counts for NeuN-positive and EGFP-expressing neurons. LV indicates lateral ventricle; 3V, third ventricle. Scale bar indicates 100 µm.

autoantibody epitope specificities are titer dependent and not disease dependent.[5] The high affinity of the GAD autoantibodies was not much lower than that of acetylcholine receptor or muscle-specific kinase antibodies[31,32] and indicates a specific and affinity-matured B-cell population. Moreover, the predominance of IgG1 subclass antibodies suggests the potential to activate complement. Nonetheless, this subclass was also predominant among patients with T1DM, in whom GAD autoantibodies are considered a biomarker of islet cell destruction rather than causative.

Although recent animal data suggest that amphiphysin antibodies in paraneoplastic SPS can be internalized into neurons by an epitope-specific process,[14] it is still not clear whether other intracellular antigens can be a target for antibody-mediated disease, in contrast to antibodies capable of binding to the surface of target cells.[26] An epitope of GAD has been suggested to be expressed on the cell surface during synaptic vesicle exocytosis in GABAergic neurons, and therefore antibodies binding to this epitope could bind to the cell surface and be directly pathogenic.[7] We

Downloaded From: http://archneur.jamanetwork.com/ by a Ohio State University User on 08/15/2013

found that 71% of high-titer GAD antibodies did bind to unpermeabilized neurons in primary cultures, but the binding was not reduced by adsorption of the serum sample from patient 1 (Table 2) with GAD65, indicating that the serum possesses autoantibodies other than GAD. A previous report of binding to hippocampal neurons was not characterized further.[33] Our findings are unlikely to be related to antibodies against the $GABA_A$-receptor–associated protein earlier reported in SPS, because those antibodies should bind postsynaptically.[34]

Despite the novel evidence presented herein for potentially pathogenic antibodies binding to the surface of GABAergic neurons, passive transfer of SPS IgG to mice failed to demonstrate any evidence of disease. Potentially pathogenic autoantibodies may have impaired GABAergic functions in inhibitory neurons without overt structural damage or detectable cell loss, but no behavioral changes were noted. We were also unable to induce behavioral changes in mice actively immunized against GAD; however, in this case, we found antibodies to the surface of GABAergic neurons and a trend toward reduced numbers of brainstem GAD-EGFP neurons (T.C., Philippa Pettingill, BSc, H.A., A.V.; unpublished data; 2010).

The passive transfer experiments presented herein were limited by availability of plasma samples with high-titer GAD autoantibodies and strong neuronal surface binding and by insufficient material to inject for a longer period. Further work on defining the target of the novel antibodies with techniques now widely used and performing more intensive peripheral and also intraventricular IgG injections, as recently reported,[14,15] is now required. In addition, we should ask whether the neuronal surface binding was present in cerebellar neurons from GAD knockout mice. Nevertheless, human IgG was found in the brainstem and hippocampi of control- and SPS-injected mice. We have also noticed these regions to be "leaky" to mouse IgG in our active immunization model and in unimmunized mice (T.C., Philippa Pettingill, BSc, H.A., A.V.; unpublished data; 2010). Diffusion from the systemic circulation into certain brain regions could prove relevant to other clinical syndromes that are associated with neuronal-specific autoantibodies and helpful in establishing passive transfer models.

ARTICLE INFORMATION

Accepted for Publication: May 13, 2013.

Published Online: July 22, 2013. doi:10.1001/jamaneurol.2013.3499.

Author Affiliations: Nuffield Department of Clinical Neurosciences, Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford, England (Chang, Alexopoulos, Carvajal-González, Alexander, Lang, Brown, Vincent); Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, England (McMenamin); Department of Experimental Psychology, University of Oxford, Oxford, England (Deacon); Department of Gene Technology and Developmental Neurobiology, Institute of Experimental Medicine, Budapest, Hungary (Erdelyi, Gabor); Neurologische Klinik, Kreiskrankenhaus Gummersbach, Gummersbach, Germany (Blaes); currently with Department of Clinical Medicine, University of Colombo, Colombo, Sri Lanka (Chang).

Author Contributions: *Study concept and design:* Chang, Erdelyi, Vincent.
Acquisition of data: Chang, Alexopoulos, McMenamin, Carvajal-González, Alexander, Deacon, Lang, Blaes, Brown.
Analysis and interpretation of data: Chang, Alexopoulos, Carvajal-González, Gabor, Vincent.
Drafting of the manuscript: Chang, Erdelyi.
Critical revision of the manuscript for important intellectual content: Chang, Alexopoulos, McMenamin, Carvajal-González, Alexander, Deacon, Gabor, Lang, Blaes, Brown, Vincent.
Statistical analysis: Chang, Deacon, Blaes, Vincent.
Obtained funding: Chang, Vincent.
Administrative, technical, and material support: Chang, McMenamin, Alexander, Erdelyi, Gabor, Lang, Brown, Vincent.
Study supervision: Deacon, Lang, Vincent.

Conflict of Interest Disclosures: Dr Vincent and the University of Oxford hold patents and receive royalties and payments for antibody tests, but these do not apply to GAD antibodies.

Funding/Support: This study was supported by the Commonwealth Scholarship Commission (Dr Chang); by the Medical Research Council of Great Britain (Dr Alexopoulos); by the DANA Foundation (Dr McMenamin); by the Wellcome Trust OXION initiative (Drs Deacon and Vincent); and by the Oxford National Institute for Health Research Biomedical Research Centre (Dr Vincent).

Additional Contributions: Ming Lim, PhD, of King's College, London, provided guidance with free-floating immunohistochemistry.

REFERENCES

1. Dalakas MC, Fujii M, Li M, McElroy B. The clinical spectrum of anti-GAD antibody–positive patients with stiff-person syndrome. *Neurology*. 2000;55(10):1531-1535.
2. Honnorat J, Saiz A, Giometto B, et al. Cerebellar ataxia with anti-glutamic acid decarboxylase antibodies: study of 14 patients. *Arch Neurol*. 2001;58(2):225-230.
3. Malter MP, Helmstaedter C, Urbach H, Vincent A, Bien CG. Antibodies to glutamic acid decarboxylase define a form of limbic encephalitis. *Ann Neurol*. 2010;67(4):470-478.
4. Vianello M, Keir G, Giometto B, Betterle C, Tavolato B, Thompson EJ. Antigenic differences between neurological and diabetic patients with anti-glutamic acid decarboxylase antibodies. *Eur J Neurol*. 2005;12(4):294-299.
5. Piquer S, Belloni C, Lampasona V, et al. Humoral autoimmune responses to glutamic acid decarboxylase have similar target epitopes and subclass that show titer-dependent disease association. *Clin Immunol*. 2005;117(1):31-35.
6. Alexopoulos H, Dalakas MC. A critical update on the immunopathogenesis of stiff person syndrome. *Eur J Clin Invest*. 2010;40(11):1018-1025.
7. Dalakas MC. Progress and stiff challenges in understanding the role of GAD-antibodies in stiff-person syndrome [published online February 26, 2013]. *Exp Neurol*. doi:10.1016/j.expneurol.2013.02.010.
8. Dinkel K, Meinck HM, Jury KM, Karges W, Richter W. Inhibition of gamma-aminobutyric acid synthesis by glutamic acid decarboxylase autoantibodies in stiff-man syndrome. *Ann Neurol*. 1998;44(2):194-201.
9. Mitoma H, Song SY, Ishida K, Yamakuni T, Kobayashi T, Mizusawa H. Presynaptic impairment of cerebellar inhibitory synapses by an autoantibody to glutamate decarboxylase. *J Neurol Sci*. 2000;175(1):40-44.
10. Takenoshita H, Shizuka-Ikeda M, Mitoma H, et al. Presynaptic inhibition of cerebellar GABAergic transmission by glutamate decarboxylase autoantibodies in progressive cerebellar ataxia. *J Neurol Neurosurg Psychiatry*. 2001;70(3):386-389.
11. Raju R, Foote J, Banga JP, et al. Analysis of GAD65 autoantibodies in stiff-person syndrome patients. *J Immunol*. 2005;175(11):7755-7762.
12. Levy LM, Levy-Reis I, Fujii M, Dalakas MC. Brain gamma-aminobutyric acid changes in stiff-person syndrome. *Arch Neurol*. 2005;62(6):970-974.
13. Manto MU, Hampe CS, Rogemond V, Honnorat J. Respective implications of glutamate decarboxylase antibodies in stiff person syndrome and cerebellar ataxia. *Orphanet J Rare Dis*. 2011;6:3. doi:10.1186/1750-1172-6-3.
14. Geis C, Weishaupt A, Hallermann S, et al. Stiff person syndrome–associated autoantibodies to amphiphysin mediate reduced GABAergic inhibition. *Brain*. 2010;133(11):3166-3180.
15. Hansen N, Grünewald B, Weishaupt A, et al. Human stiff person syndrome IgG–containing high-titer anti-GAD65 autoantibodies induce motor dysfunction in rats. *Exp Neurol*. 2013;239:202-209.
16. Burton AR, Baquet Z, Eisenbarth GS, et al. Central nervous system destruction mediated by glutamic acid decarboxylase-specific CD4+ T cells. *J Immunol*. 2010;184(9):4863-4870.

Downloaded From: http://archneur.jamanetwork.com/ by a Ohio State University User on 08/15/2013

17. Dalakas MC, Fujii M, Li M, Lutfi B, Kyhos J, McElroy B. High-dose intravenous immune globulin for stiff-person syndrome. *N Engl J Med*. 2001;345(26):1870-1876.

18. Lim MJ, Beake J, Bible E, et al. Distinct patterns of serum immunoreactivity as evidence for multiple brain-directed autoantibodies in juvenile neuronal ceroid lipofuscinosis. *Neuropathol Appl Neurobiol*. 2006;32(5):469-482.

19. Huettner JE, Baughman RW. Primary culture of identified neurons from the visual cortex of postnatal rats. *J Neurosci*. 1986;6(10):3044-3060.

20. Brager DH, Luther PW, Erdélyi F, Szabó G, Alger BE. Regulation of exocytosis from single visualized GABAergic boutons in hippocampal slices. *J Neurosci*. 2003;23(33):10475-10486.

21. López-Bendito G, Sturgess K, Erdélyi F, Szabó G, Molnár Z, Paulsen O. Preferential origin and layer destination of GAD65-GFP cortical interneurons. *Cereb Cortex*. 2004;14(10):1122-1133.

22. Deacon RM. Burrowing in rodents: a sensitive method for detecting behavioral dysfunction. *Nat Protoc*. 2006;1(1):118-121.

23. Deacon RM, Brook RC, Meyer D, et al. Behavioral phenotyping of mice lacking the K ATP channel subunit Kir6.2. *Physiol Behav*. 2006;87(4):723-733.

24. Barker RA, Revesz T, Thom M, Marsden CD, Brown P. Review of 23 patients affected by the stiff man syndrome: clinical subdivision into stiff trunk (man) syndrome, stiff limb syndrome, and progressive encephalomyelitis with rigidity. *J Neurol Neurosurg Psychiatry*. 1998;65(5):633-640.

25. Dalakas MC, Li M, Fujii M, Jacobowitz DM. Stiff person syndrome: quantification, specificity, and intrathecal synthesis of GAD65 antibodies. *Neurology*. 2001;57(5):780-784.

26. Vincent A, Bien CG, Irani SR, Waters P. Autoantibodies associated with diseases of the CNS: new developments and future challenges. *Lancet Neurol*. 2011;10(8):759-772.

27. Levy LM, Dalakas MC, Floeter MK. The stiff-person syndrome: an autoimmune disorder affecting neurotransmission of gamma-aminobutyric acid. *Ann Intern Med*. 1999;131(7):522-530.

28. Meinck HM, Thompson PD. Stiff man syndrome and related conditions. *Mov Disord*. 2002;17(5):853-866.

29. McKeon A, Martinez-Hernandez E, Lancaster E, et al. Glycine receptor autoimmune spectrum with stiff-man syndrome phenotype. *JAMA Neurol*. 2013;70(1)44-50.

30. Lohmann T, Londei M, Hawa M, Leslie RD. Humoral and cellular autoimmune responses in stiff person syndrome. *Ann N Y Acad Sci*. September 2003;998:215-222.

31. Vincent A, Newsom-Davis J. Acetylcholine receptor antibody characteristics in myasthenia gravis, I: patients with generalized myasthenia or disease restricted to ocular muscles. *Clin Exp Immunol*. 1982;49(2):257-265.

32. McConville J, Farrugia ME, Beeson D, et al. Detection and characterization of MuSK antibodies in seronegative myasthenia gravis. *Ann Neurol*. 2004;55(4):580-584.

33. Vianello M, Giometto B, Vassanelli S, Canato M, Betterle C, Mucignat C. Peculiar labeling of cultured hippocampal neurons by different sera harboring anti-glutamic acid decarboxylase autoantibodies (GAD-Ab). *Exp Neurol*. 2006;202(2):514-518.

34. Raju R, Rakocevic G, Chen Z, et al. Autoimmunity to GABAA-receptor-associated protein in stiff-person syndrome. *Brain*. 2006;129(pt 12):3270-3276.

Downloaded From: http://archneur.jamanetwork.com/ by a Ohio State University User on 08/15/2013