**Appendix e-1**

**Supplementary Methods**

We re-evaluated 367 archived specimens (serums, 334; CSF specimens [CSFs], 33) from patients referred to the Mayo Clinic Neuroimmunology Laboratory for evaluation of diverse suspected autoimmune neurological diseases (1997-2016) for whom tissue-based immunofluorescence assay (IFA) screening showed a pattern of diffuse synaptic (neuropil) antibody staining appearing like, but not meeting criteria for, amphiphysin-IgG. These archived specimens designated as ‘amphiphysin-IgG-like’ were retested by IFA and grouped according to their patterns of IgG staining. Clinical information was obtained by medical record review.

**Indirect immunofluorescence assay (IFA)**

Patient serum and CSF and commercial monoclonal antibodies were tested on cryosections of adult mouse tissues: cerebellum, midbrain, cerebral cortex, hippocampus, kidney and gut as described previously.1 The following commercial antibodies were used: septin-5 monoclonal antibody (Thermo Fisher Scientific, USA), Septin-5 polyclonal antibody (Sigma, USA).

**Protein purification and Sequencing**

*Antigen Preparation*

Cytosolic and membrane preparations were used. For the cytosolic antigen preparation, adult mouse cerebellum and cerebrum were homogenized with buffer, 2ml/g (10mM Hepes, 1mM MgCl2, 1mM ethylenediaminetetraacetic acid, and complete protease inhibitor cocktail [Roche, Indianapolis, USA]). Homogenate was clarified by centrifugation (50000 x g, 15 minutes) and supernatant was stored at -80°C. For the membrane preparation, pellet from the cytosolic preparation was resuspended in NP-40 buffer (1% NP-40, 0.1%SDS, 0.15M NaCl, 0.01M NaPO4 pH 7.2, 2mM EDTA, and complete protease inhibitor cocktail [Roche, Indianapolis, USA]) for 2 hours then centrifuged at 100000 X g for 45 minutes. Supernatant was stored at -80°C.

*Western blotting, Immunoprecipitation, & Mass Spectrometry*

For western blot, mouse cytosolic or membrane cerebellar extract was separated on 4-15% or 10% polyacrylamide gels, and the membrane was probed with sera from healthy subjects and patients (1:200 dilution), and commercial septin-5-specific IgG (1:5000). Recombinant full length septin-4, septin-5 and septin-7 proteins were used for western blot (Abcam, San Francisco, California, USA).

For immunoprecipitation, serum of 2 patients (patients 2 and 6) was pooled then IgG was purified using protein G sepharose. The IgG were eluted from the protein G complex by acetic acid and dialyzed using a 10000 MCWO membrane (Thermo Scientific, Rockford, IL, USA ) in PBS then HEPES buffer. The collected IgG was bound to Affi-Gel (9:1.5 ratio) rotating at 4°C for 4 hours (Bio-Rad, Hercules, California, USA). The complex was washed in various buffers (NP-406Murea, then again in NP-40) and exposed to cerebellar membrane protein extract (overnight, 4°C). After washing, the antigen was eluted with 6M urea buffer ( 6M urea, 2%Chaps ). The eluted sample was electrophoresed using a 10% polyacrylamide gel, and proteins were located by silver staining and by western blot. The immunoreactive bands were excised from the stained gel and analyzed by high pressure liquid chromatography electrospray tandem mass spectrometry (Mayo Clinic Medical Genome Facility – Proteomics Core).

*Antibody purification*

Mouse cerebellar proteins were separated electrophoretically in 10% polyacrylamide gel (SDS-PAGE), and were transferred electrophoretically to nitrocellulose membrane. Patient IgG bound to the membrane at the target molecular weight were acid-eluted and used for IFA, as previously described.1

**Plasmid constructs**

Human full-length *SEPT5* cDNAs were cloned into pcDNA3.1+/C-(K)DYK + N-eGFP (green fluorescent protein) vectors (GenScript, Piscataway, NJ, USA). All of the plasmid constructs were verified by Sanger DNA sequencing.

**Dual staining by confocal microscopy**

For dual staining of mouse tissue, we applied patient serum (1:960 - 1:1920) and commercial septin-5-IgGs (1:50 – 1:200). Secondary antibodies used were anti-human FITC-conjugated (1:200) and anti-mouse TRITC-conjugated (1:200, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Confocal images were captured using a microscope (20x, 40 × water immersion lens, LSM780; Carl Zeiss AG, Oberkochen, Germany).

**Protein microarray**

This experiment was conducted by ThermoFischer Scientific utilizing the Human ProtoArray v5.0; Invitrogen, based on technology developed by Zhu et al.2, 3 Nine thousand purified human proteins were printed in nitrocellulose-coated microarray slides. The slides were blocked in blocking buffer (50 mM HEPES, 200 mM NaCl, 0.01% Triton X-100, 25% glycerol, 20 mM reduced glutathione, 1.0 mM DTT, 1X synthetic block) at 4°C for 1 hour. The slides were rinsed in PBST buffer then probed with serum from patients or controls (1:500) for 90 minutes at 4°C with gentle agitation. The slides were washed 5 times and incubated (90 minutes, 4°C) with an Alexa Fluor 647-conjugated goat anti-human IgG antibody (1 µg/ml). The slides were washed and dried by spinning. The microarray slides were then scanned using a GenePix 4000B fluorescent microarray scanner.

**Immune absorption assay**

Septin-5 positive patient’s serum was incubated overnight with septin-5 recombinant protein (1:8 ratio) and used for IF assay on mouse tissues.

**Cell based immunofluorescence assay (CBA)**

HEK293 cells stably transfected with GFP-tagged Sept5 cDNA (isoform 1 or isoform 2) were generated and used for CBA.4 Cells were plated in 8-well poly-D-lysine–coated chamber slides (Corning, Corning, NY), fixed in 4% paraformaldehyde and permeabilized with Triton-X-100, as previously described. Patient or control serum (1:200 dilution), CSF (1:10) or commercial septin-5-IgG (1:500) were added to the cells for 45 to 60 minutes at room temperature. Secondary antibody (TRITC–conjugated goat antihuman, anti-rabbit or anti-mouse IgG, 1:400) was applied for 30 minutes.

**Assays for defined neural autoantibodies**

Serum and CSF were tested by standardized indirect immunofluorescence assay on a composite substrate of mouse cerebellum, midbrain, basal ganglia, thalamus, cerebral cortex, hippocampus, stomach and kidney to detect IgG autoantibodies binding selectively to neuronal and glial nuclei (antineuronal nuclear antibodies [ANNA], type‑1 [anti‑Hu], type‑2 [anti‑Ri] and type‑3; antiglial/neuronal nuclear antibody, type‑1 [AGNA or SOX‑1 antibody]), neuronal cytoplasm (Purkinje cell antibodies [PCA, types‑1 (anti‑Yo), ‑2 and -Tr], collapsin response–mediator protein [CRMP]‑5-IgG and amphiphysin-IgG), or to hippocampal and basal ganglionic synapses.

IgGs targeting specific neurotransmitter receptors in hippocampal synapses (NMDA [GluN1], AMPA [GluA1 and GluA2] and GABA-B) were sought by indirect immunofluorescence on HEK293 cells transfected with appropriate cDNAs (EUROIMMUN, Lübeck, Germany).

Antibodies reactive with neural cation channel complexes (neuronal voltage-gated calcium channels [VGCC, P/Q‑type and N‑type], voltage-gated potassium channel-complexes [VGKC-complexes], nicotinic acetylcholine receptors [AChR] of skeletal muscle-type [α1 subunit] and neuronal ganglionic-type [α3 subunit]) and glutamic acid decarboxylase 65 kDa isoform (GAD65) were detected by radioimmunoprecipitation assay. Skeletal muscle striational antibodies were detected by enzyme-linked immunosorbent assay. CRMP-5-IgG and amphiphysin-IgG were additionally sought by recombinant Western blot assays. Sera yielding positive results for VGKC–complex-IgG were tested further for IgG reactive with leucine-rich glioma-inactivated 1 (LGI1) protein or contactin-associated protein-2 (CASPR2) by a cell-based immunofluorescence assay (Euroimmun, Lübeck, Germany).

**References**

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