Intrathecal immunoglobulin A and G antibodies to synapsin in a patient with limbic encephalitis

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ABSTRACT

Objective: To report on the identification of intrathecally synthesized immunoglobulin A (IgA) and immunoglobulin G (IgG) antibodies to synapsin, a synaptic vesicle-associated protein, in a patient with limbic encephalitis.

Methods: Methods included clinical characterization, indirect immunofluorescence, immunoprecipitation, mass spectrometry, immunoblots of wild-type and synapsin I/II/III knockout mice, and cell-based assays with synapsin Ia, Ib, IIa, and IIb plasmids.

Results: A 69-year-old man presented with confusion, disorientation, seizures, and left hippocampal hyperintensities on MRI. CSF examinations revealed an intrathecal IgA and IgG synthesis. Except for IgG antibodies to voltage-gated potassium channels in CSF, screening for known neuronal autoantibodies in serum and CSF was negative. However, indirect immunofluorescence using the patient's CSF showed binding of IgA to mouse hippocampus, amygdala, and cerebellum. Immunoprecipitation with CSF IgA followed by mass spectrometry identified synapsin as autoantigenic target. Knockout tissues and cell-based assays confirmed that IgA and IgG in the patient's CSF and serum reacted with synapsin Ia, Ib, and IIa. Calculation of antibody indices proved intrathecal synthesis of anti-synapsin IgA and IgG. The patient responded clinically to immunotherapy but developed left hippocampal atrophy. CSF IgA or IgG of the patient did not bind to live, unfixed, and nonpermeabilized mouse hippocampal neurons, compatible with synapsin being an intracellular antigen.

Conclusions: This report identifies isoforms of the synaptic vesicle-associated protein synapsin as targets of intrathecally produced IgA and IgG antibodies in a patient with limbic encephalitis. Future studies should clarify the prevalence and pathogenic relevance of anti-synapsin antibodies in limbic encephalitis. *Neurol Neuroimmunol Neuroinflamm* 2015;2:e169; doi: 10.1212/NXI.0000000000000169

GLOSSARY

AI = antibody index; **CBA** = cell-based assay; **IgA** = immunoglobulin A; **IgG** = immunoglobulin G; **VGKC** = voltage-gated potassium channels.

Limbic encephalitis is characterized by memory dysfunction, seizures, behavioral changes, and mesiotemporal involvement on imaging or neuropathologic studies, and is frequently associated with antibodies to neuronal autoantigens.[1] We report on the identification of synapsin, a synaptic vesicle-associated protein, as an antigenic target of intrathecally synthesized immunoglobulin A (IgA) and immunoglobulin G (IgG) in a patient with limbic encephalitis.

METHODS **Standard protocol approvals, registrations, and patient consents.** The study was approved by the institutional review board of Charité–Universitätsmedizin Berlin (EA1/083/15) and written informed consent was obtained from the patient reported in this study.

Supplemental data at Neurology.org/nn

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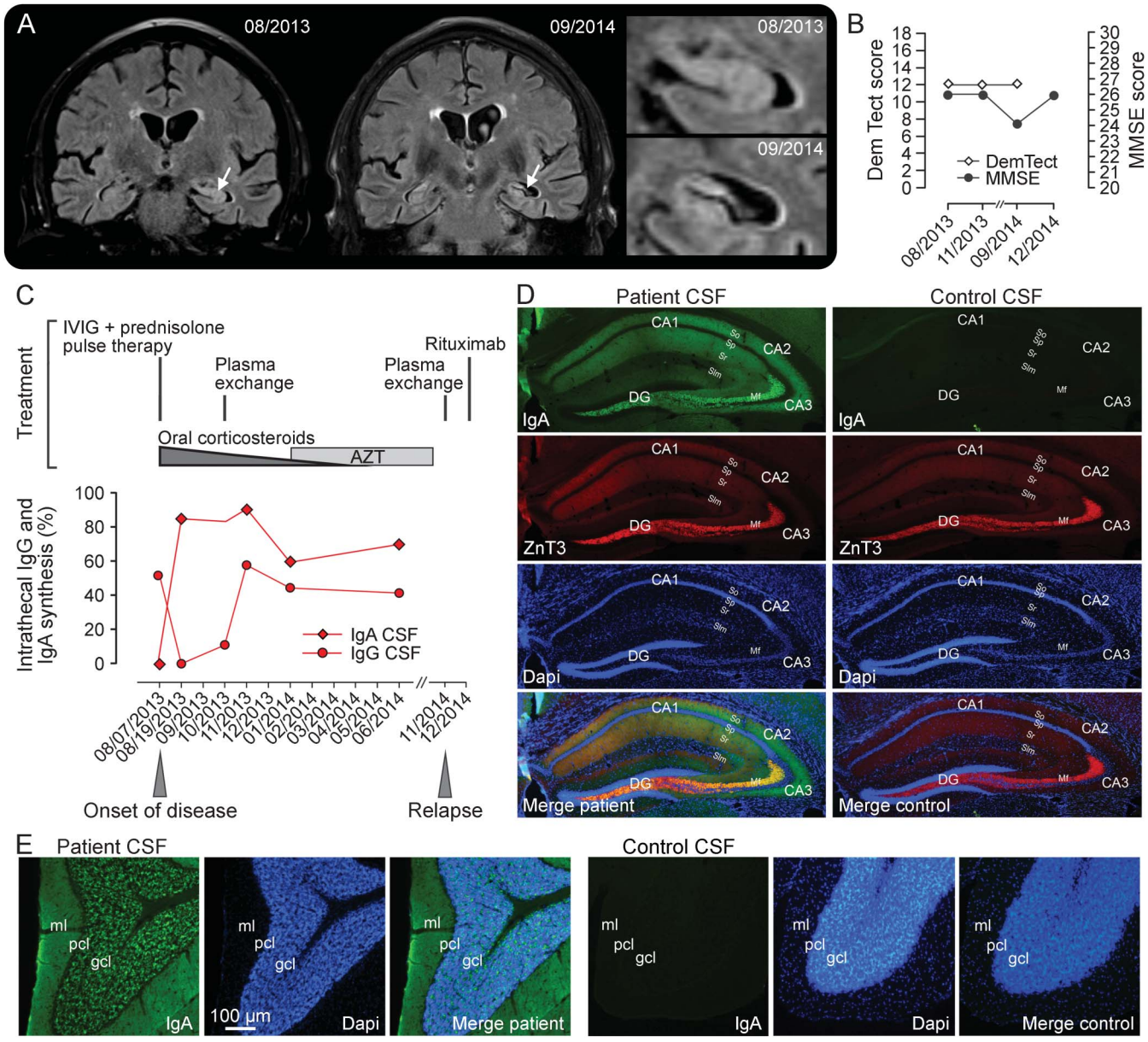
Figure 1 Clinical findings and CNS immunoreactivity of CSF IgA of a patient with limbic encephalitis



(A) Cranial MRI of a 69-year-old man with limbic encephalitis demonstrates left mesiotemporal signal hyperintensities on coronal fluid-attenuated inversion recovery sequences (arrow, see also enlarged region) at first presentation in August 2013, which progressed into hippocampal atrophy (arrow, see also enlarged region) until September 2014. (B) Repeated cognitive screening by DemTect and Mini-Mental State Examination (MMSE) show mild cognitive impairment. (C) Time course of clinical symptoms, immunotherapies, and calculated percentage of intrathecally synthesized immunoglobulin A (IgA) or immunoglobulin G (IgG) of the total IgA or IgG in CSF. (D) Fixed and permeabilized mouse hippocampus sections were stained with patient CSF at a dilution of 1:100 (left panels) and a FITC-coupled antihuman IgA secondary antibody, demonstrating strong immunoreactivity within the neuropilar regions of the cornu ammonis and dentate gyrus including a prominent signal mapping to the mossy fiber tract. Immunoreactivity of the patient's CSF IgA colocalized with that of ZnT3, a marker of glutamatergic mossy fiber terminals expressed in synaptic vesicles. Control CSF adjusted to the same IgA concentration as the patient's CSF revealed no signal (right panels). Dapi staining was used to visualize nuclei. (E) Mouse cerebellum sections were stained with patient and control CSF as described above, demonstrating binding of patient CSF IgA to the molecular layer and the granule cell layer glomeruli, but not to Purkinje cells. AZT = azathioprine; CA = cornu ammonis; Dapi = 4′,6-Diamidin-2-phenylindol; DG = dentate gyrus; gcl = granule cell layer; IVIg = IV immunoglobulin; Mf = mossy fiber tract; ml = molecular layer; pcl = Purkinje cell layer; Slm = stratum lacunosum moleculare; So = stratum oriens; Sp = stratum pyramidale; Sr = stratum radiatum.

Laboratory methods. Details of the laboratory methods used in this work, including indirect immunofluorescence, immunoprecipitation, mass spectrometry, synapsin knockout mice, cell-based assays, and calculation of antibody indices (AIs) are provided in appendix e-1 at Neurology.org/nn.

RESULTS **Case report.** In August 2013, a 69-year-old man with a history of a previous seizure in March 2012 presented with confusion, disorientation, and a generalized epileptic seizure. Cerebral MRI revealed signal hyperintensities in the left mesial temporal lobe

(figure 1A). EEG showed left frontocentral slowing and few epileptiform discharges. Neuropsychological testing revealed mild cognitive impairment (figure 1B). CSF analyses demonstrated a lymphocytic pleocytosis (22 cells/μL), elevated protein (1.43 g/L, reference <0.5 g/L), and CSF-specific oligoclonal IgG bands with 47% of the total IgG in CSF being synthesized intrathecally (figure 1C). Further CSF examinations additionally revealed a strong intrathecal IgA synthesis, persisting until the last follow-up CSF examination in June 2014.

Virologic and bacteriologic studies were normal. Screening of serum and CSF for known antineuronal antibodies (NMDA receptor, AMPA receptor, GABAB receptor, LGI1, CASPR2, glycine receptor, Hu, Ri, Yo, Tr, Ma/Ta, GAD, amphiphysin, aquaporin-4) using cell-based assays (EUROIMMUN, Lübeck, Germany) as well as testing of serum for antinuclear antibodies and antibodies to gliadin was negative. However, IgG antibodies to voltage-gated potassium channels (VGKC) were detected by radioimmunoprecipitation assay in CSF (17 pmol/L, reference <2 pmol/L, see appendix e-2), but not in serum. Intrigued by the patient's strong intrathecal IgA synthesis, we investigated a possible reactivity of IgA in the patient's CSF with fixed and permeabilized mouse hippocampus sections by indirect immunofluorescence. This demonstrated strong binding of IgA in the patient's CSF to the neuropilar regions of the *Cornu ammonis* and dentate gyrus, including a prominent signal in the mossy fiber tract (figure 1D). Immunoreactivity of the patient's CSF IgA colocalized with that of the zinc transporter ZnT3, a marker of glutamatergic mossy fiber terminals expressed in synaptic vesicles. CSF IgA also stained the amygdala (figure e-1) and the molecular and granular layers, but not Purkinje cells, in mouse cerebellum (figure 1E). No immunoreactivity was observed in mouse brain sections stained with control CSF applied at the same IgA concentration as the patient's CSF (figure 1, D and E).

The patient was diagnosed with limbic encephalitis associated with antineuronal antibodies. Whole-body CT, urologic examination, esophagogastroduodenoscopy, and colonoscopy did not reveal an underlying tumor. Immunotherapies (summarized in figure 1C) were associated with clinical improvement, but MRI revealed progressive left hippocampal atrophy (figure 1A). After a relapse in November 2014, the patient was switched to rituximab. Under this therapy, he is currently asymptomatic except for mild cognitive impairment and has had no further relapses.

Synapsin Ia, Ib, and IIa as target antigens. CSF IgA of the patient, but not of a control, detected proteins between 70 and 100 kDa in immunoblots from mouse brain homogenates. Subcellular fractionation showed enrichment of these proteins in synaptic vesicles (figure 2A). CSF IgA of the patient, but not of a control, immunoprecipitated the 70–100 kDa proteins (figure 2B), which were subsequently excised from a Coomassie gel (figure 2C) and analyzed by mass spectrometry, demonstrating sequences from both synapsin I and synapsin II (figure 2D).

To confirm the specificity of the patient's antibodies for synapsin and to identify the targeted synapsin isoforms, we carried out immunoblots with brain homogenates from wild-type, synapsin I, synapsin II, or synapsin I/II/III knockout mice (figure 2E). Reactivity of the patient's CSF IgA with synapsin Ia, Ib, and IIa was abolished in the respective knockout mice, indicating that the patient's antibodies recognize synapsin Ia, Ib, and IIa. While the patient's CSF IgA did not detect synapsin IIb, it reacted with an approximately 80 kDa band in synapsin I/II/III knockout mice, possibly representing an additional autoantigen of unknown identity. Immunohistochemical staining of wild-type mouse brain sections by the patient's CSF IgA was abrogated in synapsin I/II/III knockout tissue (figure 2F).

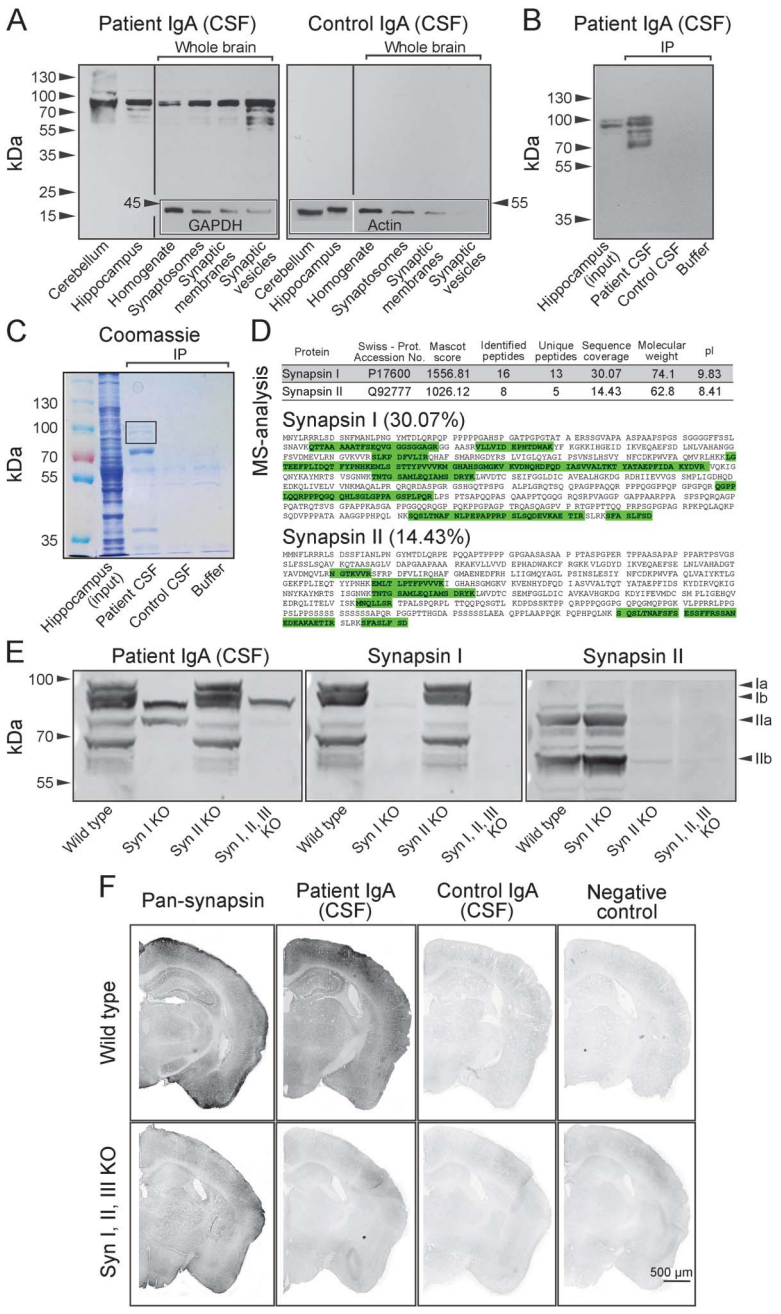
Cell-based assays (CBAs) with fixed and permeabilized HEK293 cells transfected with synapsin Ia, Ib, IIa, or IIb plasmids further confirmed binding of the patient's CSF IgA to synapsin Ia, Ib, and IIa, but not IIb (figure 3A). Staining of fixed and permeabilized mouse hippocampus sections and fixed and permeabilized primary mouse hippocampal neurons with CSF IgA and commercial anti-synapsin I/II antibodies demonstrated colocalization of both signals (figure 3B).

CSF IgG of the patient likewise reacted with synapsin Ia and Ib, and weakly with synapsin IIa, and colocalized with commercial anti-synapsin I/II antibodies and patient CSF IgA in mouse hippocampus sections (figure e-2). Furthermore, IgA and IgG in the patient's serum reacted with synapsin Ia, Ib, and IIa (figure e-3). Screening of sera of 17 healthy controls demonstrated no IgG antibodies to synapsin Ia (figure e-4). IgA antibodies to synapsin were not determined in the healthy controls.

In contrast to the binding of fixed and permeabilized synapsin-transfected HEK293 cells as well as of fixed and permeabilized primary hippocampal neurons by the patient's CSF, staining of live, unfixed, and nonpermeabilized HEK293 cells transfected with synapsin Ia as well as of live, unfixed, and nonpermeabilized primary mouse hippocampal neurons with CSF IgA and IgG and with commercial anti-synapsin I/II antibodies revealed no immunoreactivity (figure e-5).

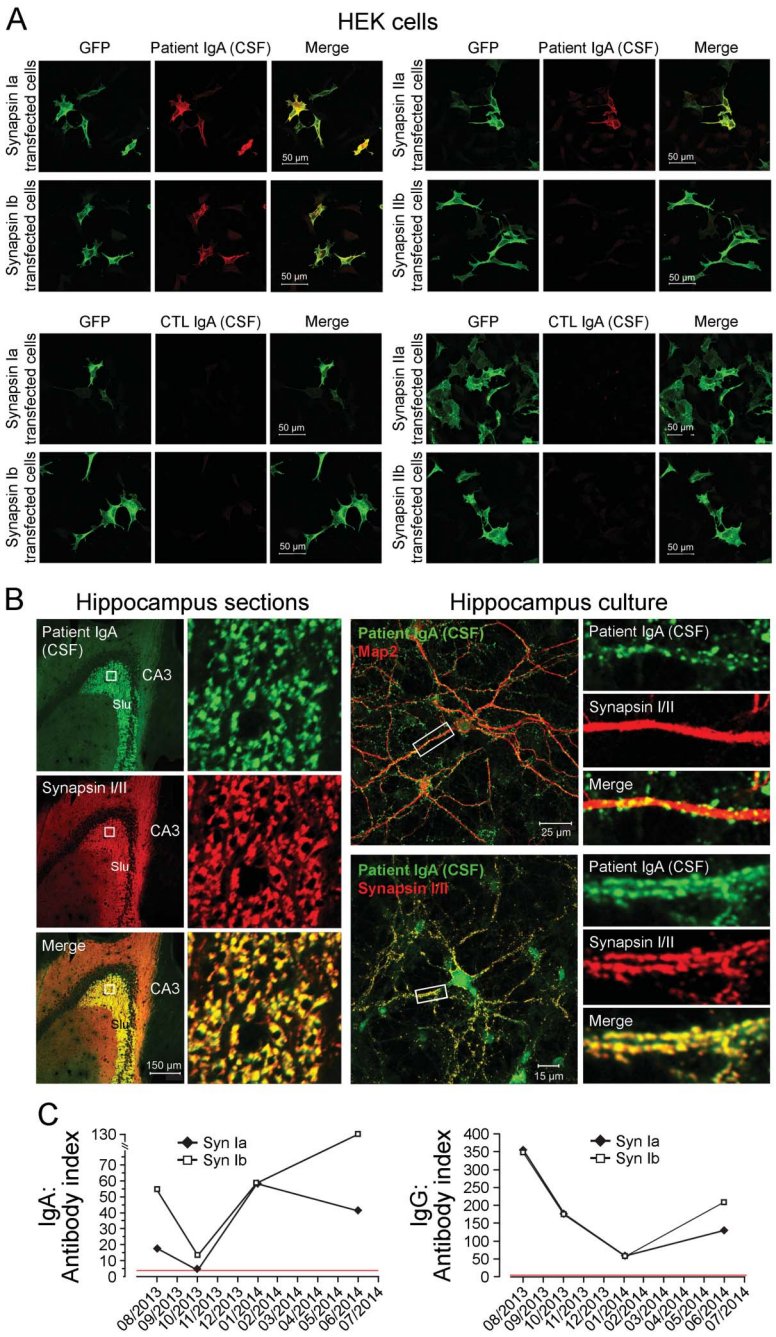
Finally, we determined titers of IgA and IgG to synapsin Ia and Ib in all available CSF/serum pairs of our patient using CBAs (appendix e-2). Subsequent calculation of AIs demonstrated strongly elevated AI values, indicating intrathecal synthesis of IgA and IgG to synapsin Ia and Ib (figure 3C).

Figure 2 **Identification of synapsin Ia, Ib, and IIa as antigenic targets of IgA antibodies in CSF**



(A) Homogenates of mouse cerebellum and hippocampus as well as subcellular fractions from whole brain progressively enriched for synaptic vesicles (homogenate, synaptosomes, synaptic membranes, synaptic vesicles) were blotted and incubated with patient CSF. Bound immunoglobulin A (IgA) was detected using a peroxidase-labeled antihuman IgA secondary antibody and enhanced chemoluminescence (ECL), demonstrating proteins between 70 and 100 kDa with enrichment in synaptic vesicles. Incubation with control CSF yielded no signals. glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and actin served as loading controls. (B) Mouse hippocampus homogenate was immunoprecipitated with antihuman IgA (heavy chain) agarose beads preincubated with patient CSF. Pelleted beads were blotted and membranes incubated with patient CSF and antihuman IgA to detect the precipitated antigens. ECL detection revealed proteins of 70–100 kDa. Beads preincubated with control CSF or buffer did not precipitate proteins. (C) Coomassie-stained gel of the immunoprecipitated proteins. The boxed area, corresponding to the upper 3 bands seen in lane 2 in (B), was excised from the gel and subjected to mass spectrometry. (D) Mass spectrometry (MS) identified synapsin (Syn) I and Syn II as the 2 proteins with the highest Mascot scores. Amino acid sequences of Syn I and Syn II are shown with the identified peptides highlighted in green. (E) Brain extracts from wild-type, Syn I, Syn II, or Syn I/II/III knockout (KO) mice were blotted and incubated with patient CSF. Bound IgA was detected using a FITC-labeled antihuman IgA secondary antibody. Corresponding membranes were developed with commercial Syn I and Syn II antibodies. Syn Ia, Ib, and IIa bands were abolished in the respective KO mice. Syn IIb was not detected by the patient's antibodies. An approximately 80 kDa band visible in Syn I/II/III KO mice could represent an additional autoantigen of unknown identity. (F) Brain sections of wild-type and Syn I/II/III KO mice were incubated with a pan-Syn antibody, patient CSF, control CSF, or no primary antibody (negative control) and developed with a FITC-labeled antihuman IgA secondary antibody. Immunoreactivity of patient CSF in wild-type was abrogated in Syn I/II/III KO mice. IP = immunoprecipitation; pI = isoelectric point.

Figure 3 **Detection of synapsin Ia, Ib, and IIa in cell-based assays, colocalization of patient IgA and commercial synapsin antibodies in hippocampus sections and primary hippocampal neurons, and calculation of antibody indices**



(A) Human embryonal kidney (HEK293) cells transfected with synapsin (Syn) Ia, Ib, IIa, or IIb were fixed, permeabilized, and incubated with patient or control (CTL) CSF. Bound immunoglobulin A (IgA) was detected using an Alexa 594-coupled anti-human IgA secondary antibody. An anti-green fluorescent protein (green fluorescent protein) antibody was used to enhance endogenous fluorescence of transfected cells. CSF IgA-stained cells expressing Syn Ia, Ib, and IIa, but not IIb. Cells incubated with control CSF showed no staining. (B) Left: Murine hippocampus sections were fixed, permeabilized, and incubated with patient CSF (dilution 1:100) and double-stained with a commercial antibody to both Syn I and Syn II, demonstrating colocalization of patient IgA and Syn I/II antibodies at the mossy fiber terminals in the stratum lucidum (Slu) of the CA3 region (boxed regions are depicted at higher magnification). Right: Staining of primary fixed and permeabilized murine hippocampal neurons with patient CSF and antibodies to microtubule-associated protein 2, a somatodendritic marker, showed a punctuate staining of CSF IgA along dendrites, indicative of a synaptic distribution. Double-staining of hippocampal neurons with patient CSF IgA and antibodies to Syn I/II revealed a complete overlap of signals. (C) IgA and immunoglobulin G (IgG) antibodies to Syn Ia/Ib in CSF and serum were titrated using cell-based assays and antibody indices were calculated as described in the Methods. The red line indicates the cutoff of 4, AI values above which indicate an intrathecal antibody synthesis. There was a strong intrathecal synthesis of IgA and IgG to Syn Ia/Ib throughout the course of the disease. CA = cornu ammonis; Map2 = microtubule-associated protein 2.

DISCUSSION We report the identification of synapsin Ia, Ib, and IIa as autoantigenic targets of intrathecally synthesized IgA and IgG in a patient with clinically and radiologically typical limbic encephalitis. This is based on (1) immunoprecipitation of synapsin from mouse brain homogenates by CSF IgA, (2) abrogation of immunoreactivity to synapsin Ia, Ib, and IIa in synapsin knockout mice, (3) detection of synapsin Ia, Ib, and IIa by the patient's antibodies in CBAs, and (4) colocalization of the patient's antibodies with commercial synapsin I/II antibodies in mouse hippocampus sections and primary neurons.

The intrathecal synthesis of synapsin IgA is a remarkable feature of our patient. The majority of known antineuronal antibodies are IgG isotypes and a possible role for antineuronal IgA has only rarely been proposed.[2] We herein identify a neuronal autoantigen using immunoprecipitation with CSF IgA. However, although our results suggest that, besides IgG, also IgA could be relevant in some cases of antineuronal autoimmunity, the significance of synapsin IgA (and IgG) currently remains elusive.

Synapsin isoforms are synaptic vesicle-associated phosphoproteins implicated in neural development and synaptic plasticity.[3] Antibodies to synapsin I were previously identified in serum of a patient with discoid lupus erythematosus, but these antibodies only reacted with denatured synapsin in immunoblots and did not bind to frozen rat brain section or synapsin-transfected cells.[4] In contrast, in addition to the detection of denatured synapsin in immunoblots, our patient's antibodies also recognized conformational synapsin in tissue section and CBAs, strongly suggesting that the previously identified antibodies to synapsin and the anti-synapsin antibodies of our patient target different epitopes. Likewise, the fact that our patient's antibodies detect conformational synapsin indicates a higher likelihood of binding to the target in vivo. Anti-gliadin antibodies, previously shown to cross-react with synapsin I and potentially explaining neurologic deficits associated with celiac disease,[5] were not detected in our patient, excluding such cross-reactivity.

We identified antibodies to synapsin in only a single patient so far and did not detect antibodies to synapsin in sera of 17 healthy controls. Therefore, it is currently impossible to draw any definite conclusions on the syndrome or disease specificity and the prevalence of antibodies to synapsin. We also cannot rule out the possibility that antibodies to synapsin may be an epiphenomenon, e.g., secondary to cell damage. Furthermore, the concomitant presence of antibodies to VGKC and synapsin in the patient's CSF raises the question which autoimmunity may be relevant for the clinical phenotype. However, while VGKC antibodies became undetectable in CSF (appendix e-2), intrathecal synapsin antibody synthesis persisted throughout the course of disease. Lack of synapsin I or synapsin II in mice triggers epileptic seizures[6] and mild cognitive impairment[7] and mutations in the gene encoding synapsin I are associated with epilepsy,[8,9] compatible with the idea that genetic as well as autoimmune ablations of synapsins could result in a similar clinical phenotype.

The absence of staining of live, unfixed, and nonpermeabilized HEK293 cells transfected with synapsin Ia as well as of live, unfixed, and nonpermeabilized primary mouse hippocampal neurons with our patient's CSF IgA and IgG as well as commercial anti-synapsin I/II antibodies was an expected result consistent with the known intracellular expression of synapsin on the cytoplasmic surface of synaptic vesicles.[3] This intracellular expression of synapsin questions the accessibility for autoantibodies and thus their potential pathogenic relevance. Nevertheless, under conditions of high neuronal activity or oxidative stress, synapsin can be extracellularly released from glial-derived exosomes, thereby promoting neurite outgrowth and neuronal survival through modulation of interactions between glia and neurons.[10] Hypothetically, such released synapsin could be accessible for anti-synapsin IgA and IgG.

Altogether, though formal proof is lacking, a potential pathogenic role of synapsin antibodies appears conceivable and possible pathogenic mechanisms of anti-synapsin antibodies should be further investigated. Future studies should also clarify the prevalence of antibodies to synapsin in patients with limbic encephalitis, other neurologic diseases, and the general population.

AUTHOR CONTRIBUTIONS

J.P., M.H., F.B., D.G., A.P., G.A.-H., and K.R. designed the study and analyzed data. C.O., H.H., J.P., and K.R. collected and interpreted clinical data. J.P., M.H., F.B., D.G., A.P., A.M., F.C., and J.-F.Z. carried out experiments. K.R., G.A.-H., J.P., and M.H. drafted the manuscript, which was revised, edited, and finally approved by all authors.

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