Appendix e-1

PATIENTS AND METHODS

Patients. In addition to the newly identified patient with anti-DPPX encephalitis described in this work (referred to as patient #1), serum of one previously described patient (case 1 in Balint et al.[1], referred to as patient #2) and sera of two unpublished patients (a 68-year old and a 50 year-old man, both with initial diarrhea followed by neuropsychiatric symptoms, referred to as patient #3 and #4; provided by Josep Dalmau) were included in this study. Sera of all patients (#1-4) contained anti-DPPX antibodies as determined by a cell-based assay.

Serology. DPPX antibodies were detected by a cell-based indirect immunofluorescence assay using HEK293 cells transfected with DPPX (Euroimmun, Lübeck, Germany).

Purification of human antibodies of the IgG subclass. Antibodies of the IgG subclass were purified from sera using HiTrap™ protein G HP columns (GE Healthcare, Buckinghamshire, UK).

Immunofluorescence. *Tissues:* Indirect immunofluorescence was performed with a biochip mosaic containing frozen tissue sections (rat cerebellum, monkey colon; Euroimmun). Furthermore, cryosections (20 µm) obtained from hippocampus or ileum of NMRI mice were incubated overnight at 4°C with patient serum, purified IgG, or control serum. In double-labelling experiments mouse ileum was incubated with purified patient IgG and microtubule associated protein 2 (Map2; Chemicon International, Hofheim, Germany) polyclonal antibodies. An Alexa 594-conjugated

anti-rabbit (Molecular Probes, Eugene, USA) and FITC-coupled anti-human IgG (Dianova GmbH, Hamburg, Germany) served as secondary antibodies. Indirect immunfluorescence was also performed on paraformaldehyde fixed wholemount preparations of guinea pig ileum (submucous and myenteric plexus), which were incubated overnight at room temperature with patient serum (1:1000), followed by Cy3-conjugated donkey anti-human IgG (1:500, Dianova) as secondary antibody. *Primary hippocampal neurons:* Primary hippocampal neurons were incubated for 2 hours in an incubator with patient sera or purified IgG at the indicated dilutions. Subsequently, cells were fixed with 4% formaline and stained for 2 hours at room temperature with secondary antibodies. For double-staining experiments, the following primary antibodies were used: a polyclonal antiserum against Map2, a monoclonal antibody against DPPX (Santa Cruz Biotechnology, St. Cruz, USA), a polyclonal antiserum against Kv4.2 (Alomone Labs, Jerusalem, Israel), polyclonal antisera against synapsin, vesicular glutamate transporters 1 and 2 (VGLUT1 and 2), and vesicular GABA transporter (VGAT; all from Synaptic Systems, Göttingen, Germany). Alexa 594-conjugated anti-rabbit or anti-mouse IgG (Molecular Probes, Eugene, USA) and FITC-coupled anti-human IgG served as secondary antibodies. Images were acquired using a Leica TCS SL confocal laser scanning microscope.

Neuroimaging technique for measurement of enteric nervous system neuron activity. Activity of enteric nervous system neurons was recorded by a previously described technique, which is based on loading of enteric nervous system neurons in guinea pig myenteric or human submucous plexus preparations with a voltage-sensitive fluorescent dye (Di-8-ANEPPS) and detection of changes in fluorescence (indicating enteric neuron membrane potential changes) by photodiodes.[2, 3] Human tissue samples were obtained from surgical specimens from patients undergoing

abdominal surgery (Departments of Surgery, Medical Clinic Freising and Medical Clinic of the Technische Universität München). Samples were taken from macroscopically normal areas as determined by a pathologist. After arrival in the laboratory, the tissue was dissected in cold Krebs solution (containing [mM]: 117 NaCl, 4.7 KCl, 1.2 MgCl2 6H2O, 1.2 NaH2PO4, 25 NaHCO3, 2.5 CaCl2 2H2O and 11 glucose, all from Sigma-Aldrich) to obtain a preparation of the inner submucous plexus that was pinned onto a windowed (5×10 mm²) silicone ring (Sylgard 184, Dow Corning, Midland, USA). Guinea-pig ileum preparations were obtained after sacrificing animals by cervical dislocation. Similarly to human preparations, a piece of ileum was dissected to obtain a longitudinal muscle-myenteric plexus preparation. Preparations were placed in a recording chamber on an inverted microscope (IX50, Olympus, Hamburg, Germany) and continuously perfused with 37°C Krebs solution. Controlled illumination of the preparation was achieved by a green LED (PT39, Luminus Devices, Billerica, USA). Individual ganglia were stained with the fluorescent voltage sensitive dye Di-8-ANEPPS (Life Technologies, Darmstadt, Germany) by local pressure application through a micropipette loaded with 20 µmol/l Di-8-ANEPPS in Krebs solution containing 0.014% Pluronic F-127 (Life technologies) and 0.125% dimethyl sulfoxide extra dry (Acros organics, Geel, Belgium). Stained ganglia were visualized with a 40× oil immersion objective (UAPO/340 Olympus) by using a custom filter cube (exciter: HC 545/30, dichroic 565, emitter: 580LP; AHF analysentechnik, Tübingen, Germany). Signals were acquired at a frame rate of 1.6 kHz by an array of 464 photodiodes (NeuroPDA, RedShirt Imaging, Decatur, GA, USA) and are presented as relative changes in fluorescence (∆F/F) which is linearly related to membrane potential changes.[4] Serum samples were mixed 1:1 with Krebs solution and applied locally by pressure ejection from micropipettes (20 psi, 400 ms). Final dilution at the ganglion was approximately 1:20.[5] In the experiments with human

submucous neurons, patient sera (patient #1 and #2) and control serum were always applied to the same ganglia. In guinea pig myenteric plexus preparations patient sera were tested in more ganglia than the control serum.

Hippocampal cell culture. To prepare hippocampal neurons, fetal NMRI mice were sacrificed at embryonic day 16. Dissected pieces of hippocampi were rinsed with PBS and then with MEM supplemented with 10% fetal calf serum, 100 IE insuline/l, 0.5 mM glutamine, 100 U/ml penicillin/streptomycin, 44 mM glucose and 10 mM HEPES buffer, followed by dissociation. Sedimented cells were resuspended in serum-free neurobasal medium supplemented with B27, 0.5 mM glutamine, 100 U/ml penicillin/streptomycin and 25 µM glutamate and plated at a density of 8×10^4 cells/well on glass cover slips precoated with poly-L-lysine/collagen (all ingredients were from Gibco/BRL Life Technologies, Eggenstein, Germany). Typically, neurons were cultured for 14 days prior to immunocytochemical experiments.

Expression of DPPX and Kv4.2 in hippocampal neuron membranes. Neurons were prepared as described above, seeded in 6-well plates at a density of 6×10^5 cells/well, and allowed to mature for 14 days. Thereafter, they were cultured for 3 further days with daily application of patient or control serum (or purified IgG fractions) at 1:100 dilution. Following removal of medium, cells were washed with PBS, harvested, homogenized, and lysed in a glas/teflon homogenizer under hypoosmotic conditions. Homogenates were centrifuged at 1043×g for 10 min to obtain a postnuclear supernatant. The resulting supernatant was centrifuged at 267008×g for 30 min to obtain highly enriched cellular membranes. Membrane fractions were subjected to immunoblot analysis. A monoclonal antibody against Na^+/K^+ ATPase was obtained from Thermo Scientific (Rockford, USA). The

antibodies against DPPX and Kv4.2 were the same as applied for immunofluorescence. Actin (rabbit polyclonal antibody, SIGMA, St. Louis, USA) and glycerinaldehyd-3-phosphat dehydrogenase (GAPDH; mouse monoclonal antibody, Millipore, Billerica, USA) served as loading controls. Immunoblot quantification was performed using Image Studio software (LI-COR GmbH, Bad Homburg, Germany).

Statistical analysis. Action potential frequencies are given as mean ± standard deviation or median (25% percentile/75% percentile) for normally or non-normally distributed data, respectively. Statistical significance of differences in action potential frequencies was assessed with the t-test for normally distributed data and the Mann-Whitney U test or the Wilcoxon test for non-normally distributed data. Significance of differences in the number of responding neurons was assessed with the χ^2 test (unpaired data) or the McNemars test (paired data). Statistical significance of immunoblot quantifications was assessed by Mann-Whitney U test.

References

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