

ORIGINAL ARTICLE

Neuropsychiatric disease relevance of circulating anti-NMDA receptor autoantibodies depends on blood–brain barrier integrity

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In 2007, a multifaceted syndrome, associated with anti-NMDA receptor autoantibodies (NMDAR-AB) of immunoglobulin-G isotype, has been described, which variably consists of psychosis, epilepsy, cognitive decline and extrapyramidal symptoms. Prevalence and significance of NMDAR-AB in complex neuropsychiatric disease versus health, however, have remained unclear. We tested sera of 2817 subjects (1325 healthy, 1081 schizophrenic, 263 Parkinson and 148 affective-disorder subjects) for presence of NMDAR-AB, conducted a genome-wide genetic association study, comparing AB carriers versus non-carriers, and assessed their influenza AB status. For mechanistic insight and documentation of AB functionality, *in vivo* experiments involving mice with deficient blood–brain barrier (ApoE^{-/-}) and *in vitro* endocytosis assays in primary cortical neurons were performed. In 10.5% of subjects, NMDAR-AB (NR1 subunit) of any immunoglobulin isotype were detected, with no difference in seroprevalence, titer or *in vitro* functionality between patients and healthy controls. Administration of extracted human serum to mice influenced basal and MK-801-induced activity in the open field only in ApoE^{-/-} mice injected with NMDAR-AB-positive serum but not in respective controls. Seropositive schizophrenic patients with a history of neurotrauma or birth complications, indicating an at least temporarily compromised blood–brain barrier, had more neurological abnormalities than seronegative patients with comparable history. A common genetic variant (rs524991, $P = 6.15E - 08$) as well as past influenza A ($P = 0.024$) or B ($P = 0.006$) infection were identified as predisposing factors for NMDAR-AB seropositivity. The > 10% overall seroprevalence of NMDAR-AB of both healthy individuals and patients is unexpectedly high. Clinical significance, however, apparently depends on association with past or present perturbations of blood–brain barrier function.

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INTRODUCTION

N-methyl-D-aspartate receptors (NMDAR) are glutamate-gated ion channels, abundantly expressed in mammalian brain.¹ They form heteromers of NR1, NR2 and NR3 subunits, and are pivotal in regulating synapse function.² In schizophrenia, NMDAR hypofunction has been hypothesized due to induction of psychotic symptoms by antagonists.³ In 2007, Dalmau *et al.*^{4,5} described a paraneoplastic syndrome, based on 12 women with ovarian teratoma, carrying IgG autoantibodies (AB) against the NMDAR NR1/2 subunits. The syndrome, termed ‘anti-NMDAR encephalitis’, variably consisted of psychosis, memory deficits, seizures, dyskinesia, decreased consciousness and autonomic instability. Since its initial description, a flood of publications appeared. The search for anti-NR1 IgG AB in small samples ($N = 46–80$) of schizophrenic patients yielded discordant results.^{6–8} Recently, >400 previously collected cases of anti-NMDAR encephalitis have been reviewed, most without associated tumor.⁹ Similarly, immunomodulatory treatment outcomes of these and

around 100 more cases have been summarized.¹⁰ As a syndrome-pertinent pathophysiological mechanism, an AB-induced decrease of NMDAR-mediated currents, due to enhanced receptor internalization and thus reduced surface expression, has been suggested.¹¹

Few studies explored a role of other classes of immunoglobulins (Ig) in an NMDAR-AB syndrome. In individuals with slow cognitive impairment, anti-NR1 IgA AB were found, which affected synaptic protein expression and decreased NMDAR-mediated currents.¹² Anti-NR1 IgM AB were described in a patient with bipolar disorder¹³ and in patients with herpes simplex encephalitis.¹⁴ In the largest study so far, investigating IgG, IgA and IgM, Steiner *et al.*¹⁵ reported a higher prevalence of AB of all isotypes in 121 schizophrenic patients, compared with healthy controls or patients suffering from affective disorders. Apart from tumors, no sound information is available yet on putative susceptibility factors for the development of anti-NR1 AB.

The present study was designed to (1) systematically screen in an unbiased fashion a large number ($N = 2817$) of healthy

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individuals and subjects suffering from schizophrenia or other brain diseases for presence of NMDAR-AB of IgG, IgA or IgM isotype; (2) specifically address the question of why healthy AB carriers might remain healthy, by extending this work to experiments *in vivo* and *in vitro*; (3) search for genetic and environmental factors predisposing to NMDAR-AB formation.

MATERIALS AND METHODS

Participants

Subject data were collected in accordance with ethical guidelines and the Declaration of Helsinki. Sample selection was unbiased, that is sera collection was concluded before analysis of NMDAR-AB was planned. Schizophrenic patients ($N=1081$) were recruited from 2005–2011 at 23 German sites in the GRAS (Göttingen Research Association for Schizophrenia) study. Patients fulfilling DSM-IV criteria for schizophrenia (81.5%) or schizoaffective disorder (18.5%) were included regardless of disease stage.^{16,17} Healthy GRAS controls were anonymized blood donors ($N=1272$; Transfusion Medicine, Göttingen). Health was ensured by pre-donation screening (questionnaires, interviews, hemoglobin, blood pressure, pulse, temperature). Patients with affective disorders ($N=148$) were also included (ongoing GRAS extension). Parkinson patients ($N=263$) and respective controls ($N=53$) were recruited from 2010–2011 in Italy (Rome area). Of the GRAS patients, three volunteers carrying high titers of anti-NR1 IgG, IgA, or IgM, and three seronegative controls agreed to blood donation for mouse experiments (Supplementary Table S1, Supplementary Appendix).

Phenotypical analyses

On all schizophrenic (GRAS) patients, extensive phenotypical characterization was performed as referenced previously.^{16,17} Frequency and duration of prodrome, age at first psychotic episode, positive and negative syndrome scale (PANSS) scores, chlorpromazine equivalents, neurological symptoms (CNI; Cambridge Neurological Inventory) including fine motor skills (MacQuarrie dotting/tapping), current cognitive functioning (composite score comprising reasoning, executive function, verbal learning and memory), premorbid intelligence and global assessment of functioning (GAF) were employed as disease characteristics. As factors affecting blood–brain barrier (BBB) integrity, past neurotrauma (all severity levels) and birth complications (all pre- and perinatal complications) were carefully and comprehensively assessed. The final judgment regarding experience of birth complications or neurotrauma in the schizophrenic (GRAS) patient cohort was derived from a number of different sources. First, the information from semi-structured interviews about birth and neurotrauma history of each patient was used. To verify the data or increase the amount of detailed information, all discharge letters of each single patient were screened. In the case of neurotrauma, other semi-structured interviews on critical life events, suicidality and aggressive behavior toward others were used to explore whether patients had experienced serious accidents (including brain trauma) or committed suicide attempts that included, for example, falls or jumps from great heights or had been involved in serious fights leading to head injuries. Finally, information from the physical exam of each patient was included to check whether any scars on the head or neck were found indicative of an injury to the head. After collecting all the data, each patient was dichotomously (yes/no) classified as having or not having experienced a neurotrauma or birth complication. In case of contradictory information, the treating physician and even the obstetric hospital were contacted, and in case of still missing information or a high level of uncertainty, patients were excluded from the analysis.

Serological analyses

Commercially available recombinant immunofluorescence tests (Euroimmun, Lübeck, Germany), standard procedures for clinical diagnosis (100% sensitivity and 100% specificity), were used to detect NMDAR-AB, based on HEK293 cells transfected with NR1 or NR1/NR2b NMDAR-subunits.^{5,18} Seropositivity was assessed by two researchers independently. Titers were double-determined in two laboratories (MPI, Euroimmun) by identifying the maximum dilution at which specific fluorescence was still visible. Few samples with discrepant results were re-analyzed, leading to full concordance. The presence of IgG AB against influenza A and B virus was determined by ELISA (Novagnost-InfluenzaA-IgG, Novagnost-InfluenzaB-IgG, Siemens Healthcare Diagnostics GmbH, Eschborn,

Germany), automatically processed on BEPIII (Siemens Healthcare Diagnostics GmbH), and interpreted (manufacturer's instructions) as positive, negative or borderline (the latter negative for statistics).

Immunoglobulin purification

Ammonium sulfate precipitation of a serum fraction containing immunoglobulins (Ig) and dialysis was carried out as described.¹⁹ IgG, IgA, or IgM were quantified by immunodiffusion using NOR partigen immunoplates (Siemens Medical Solutions, Marburg, Germany).

Mouse experiments

Experiments were approved by the local Animal Protection Committee. Male C57Bl/6N ApoE^{-/-} and wild-type (WT) mice, aged 12–16 weeks, were used (housed at 4–5 per cage, 12 h light/dark cycle, food/water *ad libitum*). Groups (4–6 each) received either extracted Ig fractions from NMDAR-AB seropositive (IgG, IgA, or IgM) or seronegative individuals (information on titer/concentration in Supplementary Table S1 and Supplementary Appendix). Daily intravenous (tail) injections (150 μ l each) were performed on 3 consecutive days. Examiners were not aware of group assignment ('blinded'). Spontaneous activity in open field (8 min) was tested in all mice 3–4 days before the first injection (initial group matching). One day after the last injection, spontaneous activity (8 min) was again measured, followed by intraperitoneal injection (0.3 μ g per 10 μ l per gram of body weight) of the non-competitive NMDAR antagonist MK-801 (Dizocilpine; [5R,10S]-(+)-5-methyl-10,11-dihydro-5H-dibenzo[*a,d*]cyclohepten-5,10-imine; Sigma-Aldrich GmbH, Munich, Germany) and extended (120 min) open-field observation.²¹ MK-801 acts as use-dependent ion-channel blocker.

Endocytosis assays and quantification

Primary cortical neurons prepared from mouse embryos (E16) were cultured on poly-L-lysine-coated glass-coverslips in MEM + B27 (Invitrogen, Karlsruhe, Germany) for 14 days. Glass coverslips were washed with cold Hank's balanced salt solution (HBSS), and incubated (20 min, 4 °C) with Ig extracts containing either NMDAR-AB of IgG, IgA or IgM isoforms or seronegative Ig extracts (1:100) in HBSS. The examiner was unaware of the nature of the extract ('blinded'). Unbound AB was removed (3 HBSS washes) before placing cells in pre-warmed (37 °C) growth medium for 15 min to allow internalization. After medium wash-off (cold HBSS), remaining surface NMDAR were labeled with anti-mouse NR1-AB (Abcam, Cambridge, UK) for 15 min on ice. After cold HBSS wash, surface-bound NR1-AB was labeled (4 °C, 15 min) with Alexa-488-coupled 2nd AB (anti-mouse IgG; Invitrogen, Karlsruhe, Germany). After wash-off (HBSS; 4 °C) of unbound AB, cells were fixed in 4% paraformaldehyde. For quantification, confocal images of cell surface staining were taken with identical acquisition parameter on SP2 LSM (Leica, Wetzlar, Germany). Signal intensity was quantified with ImageJ, and ratio of intensity per cell surface area calculated.

Genetic analyses

A semi-custom Axiom myDesign genotyping array (Affymetrix, Santa Clara, CA, USA) was used. For description of array-specifications, quality controls and genome-wide genetic association study (GWAS), see Supplementary Appendix.

Statistical analysis

P -values < 0.05 were considered significant. Data in figures are expressed as mean \pm s.e.m., in tables as mean \pm s.d.

Mouse experiments. Data were compared by analysis of variance, followed by *post-hoc* tests where appropriate using Prism5 (GraphPad-Software Inc., La Jolla, CA, USA) or SPSS (SPSS-Statistics 17.0, IBM-Deutschland GmbH, Munich, Germany). Greenhouse–Geisser correction was applied on violation of sphericity.

Human data. Group differences in categorical and continuous variables were assessed using Chi-square or Mann–Whitney U tests. A generalized linear model was employed upon covariate inclusion. In case of normal distribution of continuous variables, T -tests were performed. To assess main effects and interactions between neurotrauma/birth complications and Ig-positivity regarding CNI scores, two-way independent ANOVA was

Table 1. Prevalence of anti-NR1 AB in patients and controls

Study group	Anti-NR1 seropositivity—N (%)							
	Any	IgG	IgA	IgM	IgG + IgA	IgG + IgM	IgA + IgM	IgG + IgA + IgM
GRAS ^a patients (N = 1081)	93 (8.6)	7 (0.7)	56 (5.2)	46 (4.3)	0 (0)	0 (0)	16 (1.5)	0 (0)
Affective-disorder patients (N = 148)	24 (16.2)	5 (3.4)	15 (10.1)	7 (4.7)	1 (0.7)	0 (0)	2 (1.4)	0 (0)
GRAS ^a controls (N = 1272)	137 (10.8)	5 (0.4)	75 (5.9)	80 (6.3)	2 (0.2)	3 (0.2)	19 (1.5)	1 (0.1)
Parkinson patients (N = 263)	35 (13.3)	1 (0.4)	17 (6.5)	25 (9.5)	1 (0.4)	1 (0.4)	7 (2.7)	1 (0.4)
Parkinson controls (N = 53)	6 (11.3)	0 (0)	3 (5.7)	3 (5.7)	0 (0)	0 (0)	0 (0)	0 (0)
Total (N = 2817)	295 (10.5)	18 (0.6)	166 (5.9)	161 (5.7)	4 (0.1)	4 (0.1)	44 (1.6)	2 (0.1)

^aGRAS patients are schizophrenic individuals of the GRAS data collection (Göttingen Research Association for Schizophrenia). GRAS controls are the respective healthy control collective (see also Materials and methods).

conducted. Corrected values reflect linear regression-based residuals when age, chlorpromazine and PANSS negative scores were independent variables. PLINK (v1.07) was used to test association between single-nucleotide polymorphisms (SNPs) and anti-NR1 serological status (allelic test) and deviations from Hardy–Weinberg equilibrium.²² Principal components were generated using EIGENSTRAT (http://genetics.med.harvard.edu/reich/Reich_Lab/Software.html). Human leukocyte antigen (HLA) types were imputed for seven HLA genes using HIBAG0.9.1 at four-digit resolution, based on a pre-fit European ancestry model (<http://cran.r-project.org/web/packages/HIBAG/index.html>). *P*-values were multiple-testing corrected (Bonferroni) where indicated, but are displayed uncorrected.

RESULTS

NMDAR-AB seroprevalence in 2817 individuals

AB of all here analyzed isotypes (IgG, IgA and IgM), directed against the NMDAR-NR1 subunit, were identified in 10.5% of subjects (Table 1). Importantly, seroprevalence did not differ between schizophrenic (GRAS) patients (8.6%) and GRAS controls (10.8%, *P* = 0.078). An apparently higher incidence in affective-disorder patients (16.2%) is explained by a higher mean age. In fact, seroprevalence increases with age (Supplementary Figure S1, Supplementary Table S2, Supplementary Appendix) and is higher in male than female subjects (Supplementary Table S3, Supplementary Appendix, 11.53% versus 8.68%, *P* = 0.017). Seropositivity between Parkinson patients (13.3%) and respective controls did not differ (11.3%, *P* = 0.694).

Seroprevalence and titer distribution of NMDAR-AB Ig isotypes

Considering each Ig class separately, again no differences in seroprevalence among groups arose (Table 1). Occurrence of IgG anti-NR1 was infrequent (0.6% in total) compared with IgA (5.9%) or IgM (5.7%). A combination of IgA/IgM AB was present in 1.6%, combinations including IgG in only 0.1% each. AB exclusively against the NR1/NR2b heterodimer, that is without reactivity against NR1 alone, were not identified. Titer distributions in patient and control groups as possible explanation of NMDAR-AB pathology did not differ (Supplementary Table S4, Supplementary Figure S2, Supplementary Appendix).

NMDAR-AB functionality in a neuronal endocytosis assay

We next wondered whether AB from controls and patients differ in functionality. Extracts from seropositive subjects, independently of isotype or disease state, resulted in increased endocytosis, compared with seronegative extracts (Figure 1a, Supplementary Table S1, Supplementary Appendix).

Relevance of BBB integrity for NMDAR-AB effects in mice

Having comparable serological (% seropositivity, Ig-isotype, titer distribution) and functional results in controls and patients, we

asked why healthy AB carriers remain healthy. We hypothesized that a compromised BBB might decide on the pathophysiological significance of NMDAR-AB. To approach this hypothesis experimentally, we employed ApoE^{-/-} mice²⁰ (with known BBB leakage)^{23–25} versus WT. Intravenous injection of purified Ig fractions from NMDAR-AB seropositive (IgM, IgG, IgA) subjects led to alterations in spontaneous open-field activity and the response to MK-801 exclusively in ApoE^{-/-} mice. Trends were comparable in groups receiving IgM, IgG or IgA extracts, resulting in significant differences on pooling (Figures 1b and c, Supplementary Figure S3, Supplementary Appendix).

Translating experimental BBB findings to schizophrenic (GRAS) patients

Overall, schizophrenic anti-NR1 carriers and non-carriers do not differ with respect to disease phenotypes, covering the symptom clusters of anti-NMDAR encephalitis (Table 2). Also, occurrence and duration of prodromal phase and age of disease onset are similar between the two groups, arguing against a sudden/atypical syndrome start in AB carriers (Table 2). Following our BBB hypothesis, we compared individuals with birth complications or past brain trauma—conditions known to provoke temporary or persistent (albeit often minor) BBB abnormalities.^{26,27} Indeed, also in humans, a clear impact, that is a more severe neurological phenotype, arises from the combination of compromised BBB function and circulating NMDAR-AB (Figure 1d).

Identification of first genetic susceptibility factors

GWAS have been successful in identifying associations between genomic variants and autoimmune disorders, such as rheumatoid arthritis or systemic lupus erythematosus.²⁸ We performed GWAS to spot SNPs potentially predisposing to formation of NMDAR-AB (Supplementary Appendix). We identified a genome-wide significant SNP, rs524991 (A/G, *P* = 6.15E–08; Bonferroni threshold *P* = 8.62E–08), with an odds ratio (OR) of 2.22 (95% confidence interval (CI) = 1.654–2.991; Supplementary Figure S6, Supplementary Appendix). This variant with a minor allele frequency of 12.45% in seropositive versus 6.01% in seronegative individuals is located in an intergenic region on chromosome1 (Supplementary Figure S7, Supplementary Appendix). Its closest neighboring gene is nuclear factor I/A (*NFIA*, 218.59 kb downstream), a transcription factor reported to mediate neuroprotective effects of NMDAR activation.²⁹ Separate analysis of SNP rs524991 association with NMDAR-AB seropositivity (Table 3) showed a similar tendency for all study groups (except Parkinson) and no gender difference (Supplementary Table S8, Supplementary Appendix). Search for a predisposing role of HLA alleles for NMDAR-AB formation did not deliver hits, apart from a nominally significant association of HLA-A03 with seropositivity (*P* = 0.01; Supplementary Table S9, Supplementary Appendix).

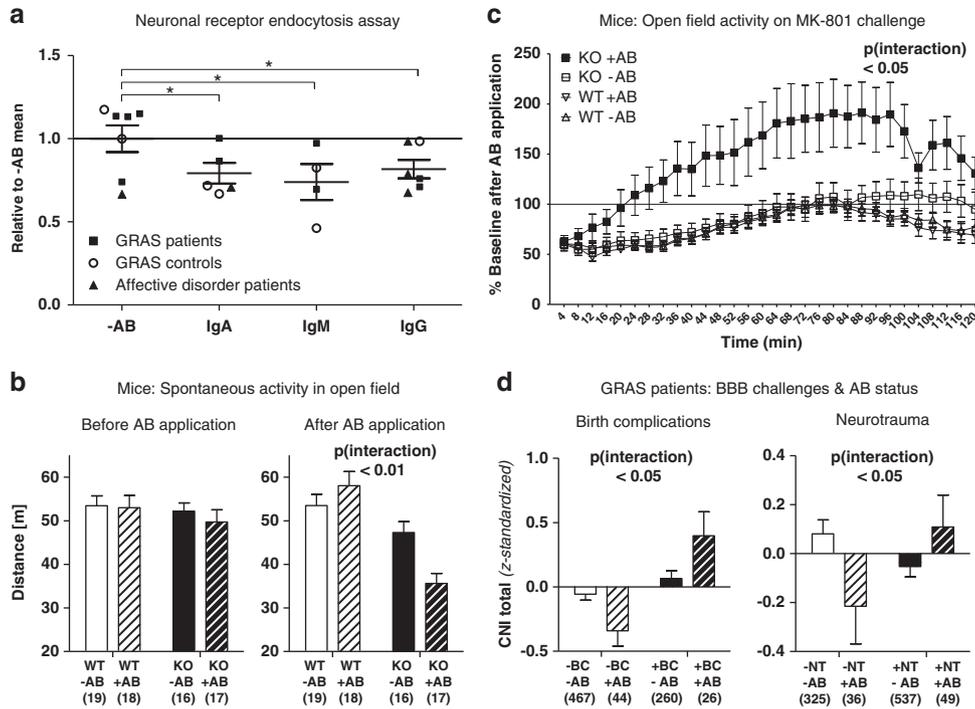


Figure 1. NMDAR-AB functionality and relevance of the blood–brain barrier. **(a)** Reduced AB binding to primary cortical neurons indicates increased endocytosis of NMDAR after incubation with Ig extracts containing either NMDAR-AB or IgG, IgA or IgM isoforms (all $P < 0.05$), or seronegative Ig extracts (one-tailed T -tests). Mean values upon AB extracts (each tested in 1–3 independent experiments, dependent on serum availability) were normalized to the mean of the respective seronegative control extracts. **(b)** ApoE^{-/-} (KO) and WT mice do not differ in spontaneous activity in the open field before AB injection. However, 1 day after the last of three daily injections with seropositive (+ AB) or seronegative (- AB) Ig extracts, a decrease in spontaneous activity was evident exclusively in seropositive ApoE^{-/-} (KO + AB) mice: two-way ANOVA revealed a significant interaction effect of genotype and serotype ($F = 8.96$, $P < 0.01$), as well as a significant main effect of genotype ($F = 27.81$, $P < 0.0001$), but not of serotype. **(c)** Only ApoE^{-/-} (KO) mice with their known compromised BBB respond to intravenous NMDAR-AB extracts with a hypersensitive (psychosis-related) response in the open field to the NMDAR antagonist MK-801. Using a generalized linear model for repeated measures to evaluate results after MK-801 treatment, we obtained significant main effects of time ($F = 36.25$, $P < 0.001$), genotype ($F = 9.54$, $P < 0.01$) and serotype ($F = 4.85$, $P < 0.05$) as well as a significant genotype \times serotype interaction effect ($F = 5.75$, $P < 0.05$). **(d)** In the GRAS sample of schizophrenic patients, birth complications and history of neurotrauma as readouts for temporarily/persistently compromised BBB were examined in NMDAR-AB-positive versus negative subjects with respect to their impact on neurological symptom severity (CNI total score, z-standardized). Birth complications showed an interaction with serotype ($F = 5.80$, $P < 0.05$) regarding CNI and a main effect ($F = 11.24$, $P = 0.001$). Likewise, past neurotrauma showed an interaction with serotype ($F = 4.02$, $P < 0.05$). Group sizes are given as numbers in brackets. * $P < 0.05$; WT, wild type; KO, ApoE^{-/-} knockout; - AB, NMDAR-AB seronegative; + AB, NMDAR-AB seropositive; BC, birth complications; NT, neurotrauma.

Identification of environmental susceptibility factors

As first risk factor for NMDAR-AB formation, the presence of a tumor, preferentially an ovarian teratoma was identified.⁴ Other predisposing factors have remained speculative. Infections have been suggested as triggers of AB formation in autoimmune diseases,³⁰ for example, Epstein-Barr virus in multiple sclerosis.³¹ We hypothesized that a similar role might be attributed to influenza for NMDAR-AB. Anti-NMDAR encephalitis was reported in a patient with influenza H1N1 infection and two subjects after respective vaccination.⁹ NMDAR-AB were described in pediatric cases of encephalitis lethargica, a condition—not unequivocally—associated with influenza.^{32,33} Strikingly, we found an increased anti-NR1 AB prevalence in individuals carrying anti-influenza A ($P = 0.024$, OR = 1.366, CI 95% = 1.042–1.790) and B ($P = 0.006$, OR = 1.453, CI 95% = 1.109–1.904) IgG (Table 3). This association was present in males only (Supplementary Table S10, Supplementary Appendix).

DISCUSSION

The present study provides (1) the first large-scale systematic screen for presence of NMDAR-AB in serum of healthy and

neuropsychiatrically diseased subjects. In >2800 unbiasedly selected individuals, >10% seroprevalence of anti-NR1 AB, independent of group affiliation, was found. (2) From this unexpected observation, the fundamental question arose of why healthy AB carriers have remained healthy, despite comparable distribution of AB isotypes, titers and *in vitro* functionality. An experimental mouse model supports our central hypothesis, that is, the essential role of BBB integrity. Only in ApoE mutant mice, but not in respective controls, we find that human NMDAR-AB cause psychosis-related behavioral perturbation.²¹ The BBB role is further underlined by a hypothesis-driven outcome analysis of schizophrenic (GRAS) patients with history of birth complications or neurotrauma indicating past/present BBB insufficiency. (3) Ultimately, with a genome-wide significant marker, SNP rs524991, and an association of seropositivity with influenza AB status, we provide genetic and environmental risk factors of NMDAR-AB formation.

The most remarkable finding of the present work is the high seroprevalence of NMDAR-AB in healthy individuals. Only one other study included a considerable number of healthy subjects in a screen of psychiatric patients but reported seropositivity for only 1 in 240 controls (0.4%) and 2 in 108 (<2%) affective-disorder patients.¹⁵ Seroprevalence in schizophrenic patients (9.9% of 121)

Table 2. Baseline characteristics, according to study group (means \pm s.d. (range) are displayed, unless indicated otherwise)

Patients and control groups	Total sample	Ig-positive individuals	Ig-negative individuals	P-value (χ^2 , Z, T value) ^a
Schizophrenic (GRAS) patients	N = 774–1081^b	N = 63–93^b	N = 711–988^b	
Age, years	39.37 \pm 12.59 (17–79)	41.15 \pm 11.98 (18–75)	39.20 \pm 12.63 (17–79)	0.115 (Z = -1.58)
Gender, N male (%)	723 (66.9)	68 (73.1)	655 (66.3)	0.181 (χ^2 = 1.79)
Prodrome, N cases (%)	754 (80.6)	64 (79.0)	690 (80.8)	0.698 (χ^2 = 0.15)
Duration of prodrome, years	2.81 \pm 3.57 (0–28.2)	2.64 \pm 3.01 (0–13.0)	2.83 \pm 3.62 (0–28.2)	0.853 (Z = -0.19)
Age at first episode, years	25.88 \pm 8.90 (5–68)	25.85 \pm 8.98 (12–51)	25.89 \pm 8.90 (5–68)	0.890 (Z = -0.11)
PANSS positive score	13.74 \pm 6.25 (7–38)	13.28 \pm 5.71 (7–31)	13.78 \pm 6.31 (7–38)	0.597 (Z = 0.60)
PANSS negative score	18.25 \pm 7.90 (7–46)	17.00 \pm 7.42 (7–37)	18.37 \pm 7.93 (7–46)	0.122 (Z = -1.55)
PANSS general score	33.74 \pm 11.76 (16–82)	32.48 \pm 10.80 (16–75)	33.86 \pm 11.85 (16–82)	0.373 (Z = -0.89)
CPZ	686.53 \pm 697.43 (0–7375)	628.04 \pm 537.82 (0–2620)	691.97 \pm 710.43 (0–7375)	0.580 (Z = -0.55)
CNI ^d	0.00 \pm 1.00 (-2.71–3.07)	-0.03 \pm 0.92 (-2.07–2.11)	0.00 \pm 1.01 (-2.71–3.07)	0.742 (T = 0.33)
MacQuarrie dotting ^e	0.00 \pm 1.00 (-3.61–3.22)	0.14 \pm 1.14 (-2.67–3.07)	-0.01 \pm 0.98 (-3.61–3.22)	0.172 (Z = -1.37)
MacQuarrie tapping ^e	0.00 \pm 1.00 (-4.83–3.14)	0.11 \pm 0.99 (-2.00–3.10)	-0.01 \pm 1.00 (-4.83–3.14)	0.261 (T = -1.13)
Cognitive composite score	-0.02 \pm 0.84 (-2.57–2.98)	-0.01 \pm 0.89 (-2.12–2.03)	-0.02 \pm 0.84 (-2.57–2.98)	0.946 (T = -0.07)
Premorbid IQ (MWT-B ^f)	25.67 \pm 6.36 (4–42)	26.64 \pm 6.28 (6–36)	25.57 \pm 6.36 (4–42)	0.093 (Z = -1.68)
GAF	45.70 \pm 17.18 (5–90)	46.26 \pm 16.54 (10–80)	45.65 \pm 17.25 (5–90)	0.642 (Z = -0.47)
Neurotrauma, N cases (%)	648 (62.4)	55 (62.6)	593 (59.8)	0.592 (χ^2 = 0.29)
Birth complications, N cases (%)	307 (39.7)	27 (42.9)	280 (39.4)	0.589 (χ^2 = 0.29)
Healthy (GRAS) controls	N = 1272	N = 137	N = 1135	
Age, years	37.43 \pm 13.24 (18–69)	40.90 \pm 12.17 (19–68)	37.01 \pm 13.31 (18–69)	< 0.001 (Z = -3.56)
Gender, N male (%)	780 (61.3)	95 (69.3)	685 (60.4)	0.041 (χ^2 = 4.17)
Affective-disorder patients	N = 148	N = 24	N = 124	
Age, years	49.70 \pm 15.49 (20–92)	47.38 \pm 11.87 (25–76)	50.15 \pm 16.09 (20–92)	0.314 (Z = -1.01)
Gender, N male (%)	70 (47.3)	11 (45.8)	59 (47.6)	0.875 (χ^2 = 0.03)
Parkinson patients	N = 253–263^b	N = 33–35^b	N = 220–228^b	
Age, years	66.04 \pm 10.08 (36–86)	69.06 \pm 8.33 (45–81)	65.59 \pm 10.26 (36–86)	0.055 (Z = -1.92)
Gender, N male (%)	175 (66.5)	28 (80.0)	147 (64.5)	0.070 (χ^2 = 3.29)
Parkinson controls	N = 51–53^b	N = 6^b	N = 45–47^b	
Age, years	63.31 \pm 11.68 (22–80)	67.50 \pm 12.58 (44–78)	62.76 \pm 11.59 (22–80)	0.188 (Z = -1.33)
Gender, N male (%)	21 (39.6)	2 (33.3)	19 (40.4)	0.738 (χ^2 = 0.11)

Abbreviations: CPZ, chlorpromazine equivalents; GAF, global assessment of functioning; GRAS, Göttingen Research Association for Schizophrenia; PANSS, positive and negative syndrome scale. Bolded values, $P < 0.05$. ^aFor statistical methods, Mann-Whitney U or χ^2 tests and for normally distributed variables, T-tests were used. ^bDue to missing data, sample sizes vary. ^cCambridge Neurological Inventory mean value if more than 95 items were available. ^dCorrected for age and CPZ. ^eCorrected for age, PANSS negative and CPZ. ^fMehrfach-Wortschatz-Intelligenz test (multiple choice vocabulary test).

was comparable to our study. Reasons for this discrepancy are unclear but perhaps related to the smaller number of controls and their younger age.¹⁵ Importantly, analytical materials/methods of both studies were identical, schizophrenia patients show comparable seroprevalence, and the here randomly selected positive specimens for *in vitro* analyses all confirmed AB functionality.

For exerting pathological effects, NMDAR-AB have to reach NMDAR in the brain. This brain presence may occur via (1) AB transfer over a compromised BBB, which normally restricts large molecules from directly entering the brain in appreciable amounts (expected transfer over an intact BBB, for example, of IgG is only 1/500, of IgA 1/600, and of IgM 1/3000 of the serum concentration); (2) slow accumulation of these large molecules due to reduced cerebrospinal fluid (CSF) flow,³⁴ in which case, however, a retrograde CSF circulation would have to deliver the AB back to brain tissue, or (3) intrathecal synthesis by B lymphocytes.^{34,35} Our seroprevalence data do not allow any conclusion on AB production in brain. Therefore, healthy AB carriers may differ at least in part from seropositive disease groups by lack of intrathecal AB synthesis. Nevertheless, with *in vivo* experiments in mice and a hypothesis-driven human database screening, we underscore the likely critical role of an intact BBB as protective mechanism against circulating AB-mediated pathology in mouse and man.

Wild-type mice were not behaviorally affected after injection of human serum extract containing IgG, IgA or IgM NMDAR-AB.

In contrast, ApoE^{-/-} mice showed differences in behavior on AB injection, that is reduced spontaneous activity in the open field and hyperlocomotion following MK-801. These behavioral phenomena may be explained by the reported receptor internalization and hypofunction after hippocampal infusion of NMDAR-AB,¹¹ as exactly opposite effects were described after NMDA application in rats.³⁶ Stimulation of locomotion by the NMDAR antagonist MK-801 may be caused by hyperexcitability of limbic circuits through NMDAR blockade on inhibitory GABAergic neurons.^{37,38} This consequence of NMDA receptor inhibition would be amplified by NMDAR-AB. Similarly, increased motor cortex excitability in mice was provoked by NMDAR-AB injection into the prefrontal area.³⁹

In the GRAS sample of schizophrenic individuals, well-documented history of birth complications and brain trauma were evaluated as proxy variables of past or present BBB impairment.^{26,27} Indeed, affected individuals show more severe neurological abnormalities when carrying NMDAR-AB. These findings strengthen the hypothesis of BBB involvement in NMDAR-AB pathology, and—replication provided—may even justify recommendations of anti-NR1 serum screening in case of neurotrauma or other conditions with anticipated BBB dysfunction.

Importantly, we did not find any clinically relevant differences when comparing all schizophrenic NMDAR-AB carriers with all non-carriers. Perhaps with information on CSF (which we do not have in our large cohort), an expected 30% of individuals with

Table 3. Susceptibility factors for NMDAR-AB seropositivity, according to study group

Study group	GRAS patients		Affective-disorder patients		GRAS controls		Parkinson patients		Parkinson controls		Total	
	+	-	+	-	+	-	+	-	+	-	+	-
<i>rs524991</i>												
N, GG (%)	65 (70.7)	867 (88.8)	18 (75.0)	104 (87.4)	111 (82.2)	977 (87.6)	31 (88.6)	187 (85.0)	4 (66.7)	41 (91.1)	229 (78.4)	2176 (87.9)
N, GA (%)	27 (29.3)	105 (10.8)	6 (25.0)	15 (12.6)	19 (14.1)	135 (12.1)	4 (11.4)	32 (14.5)	2 (33.3)	4 (8.9)	58 (19.9)	291 (11.8)
N, AA (%)	0 (0.0)	4 (0.4)	0 (0.0)	0 (0.0)	5 (3.7)	3 (0.3)	0 (0.0)	1 (4.6)	0 (0.0)	0 (0.0)	5 (1.7)	8 (0.3)
P (allelic, df. = 1)	3.23E-06	0.133	0.133	0.006	0.006	0.551	0.551	0.091	0.091	0.091	7.48E-07	
OR (CI 95%)	2.80 (1.78–4.39)	2.12 (0.78–5.79)	2.12 (0.78–5.79)	1.78 (1.17–2.72)	1.78 (1.17–2.72)	0.72 (0.25–2.11)	0.72 (0.25–2.11)	4.30 (0.70–26.52)	4.30 (0.70–26.52)	4.30 (0.70–26.52)	1.99 (1.51–2.63)	
<i>Influenza A</i>												
N, seropositive (%)	71 (77.2)	648 (66.2)	16 (66.7)	91 (74.0)	97 (70.8)	764 (67.4)	25 (71.4)	141 (61.8)	6 (100.0)	28 (59.6)	215 (73.1)	1672 (66.6)
P (Pearson's χ^2)	0.032	0.461	0.461	0.417	0.417	0.274	0.274	0.052	0.052	0.052	0.024	
OR (CI 95%)	1.727 (1.043–2.860)	0.703 (0.275–1.799)	0.703 (0.275–1.799)	1.174 (0.796–1.732)	1.174 (0.796–1.732)	1.543 (0.707–3.367)	1.543 (0.707–3.367)	NA	NA	NA	1.366 (1.042–1.790)	
<i>Influenza B</i>												
N, seropositive (%)	30 (32.6)	202 (20.6)	5 (20.8)	32 (26.0)	37 (27.0)	221 (19.5)	12 (34.3)	79 (34.6)	0 (0.0)	8 (17.0)	84 (28.6)	542 (21.6)
P (Pearson's χ^2)	0.008	0.593	0.593	0.039	0.039	0.966	0.966	0.273	0.273	0.273	0.006	
OR (CI 95%)	1.861 (1.172–2.956)	0.748 (0.258–2.169)	0.748 (0.258–2.169)	1.529 (1.020–2.291)	1.529 (1.020–2.291)	0.994 (0.769–1.286)	0.994 (0.769–1.286)	NA	NA	NA	1.453 (1.109–1.904)	

Abbreviations: +, anti-NR1 seropositive; -, anti-NR1 seronegative; CI, confidence intervals; df., degree of freedom; GRAS, Göttingen Research Association for Schizophrenia; NA, not available; OR, odds ratio.
Bolded values, $P < 0.05$.

permanent barrier dysfunction⁴⁰ could have been extracted and would have allowed us to uncover a clinically relevant difference between AB carriers and non-carriers among them. Instead, we found a clinical difference between AB carriers and non-carriers with past birth complication or neurotrauma as a proxy for at least temporary BBB disturbance.²⁶ It is interesting to speculate that the reported 30% of schizophrenic patients with compromised barrier function⁴⁰ and the post-trauma individuals recognized here might represent an (partly) overlapping subpopulation of schizophrenic subjects. Along these lines of thought, future studies may be initiated, analyzing CSF samples of a large number of schizophrenic patients for NMDAR-AB titers and determining the CSF-serum albumin quotient³⁴ as marker of blood-CSF barrier (dys)function.

Our study is the first to investigate putative genetic susceptibility factors for the formation of anti-NR1 AB. A GWAS approach led to the identification of the genome-wide significant risk SNP rs524991. Further experiments providing mechanistic insight as well as replication analyses are warranted. By a hypothesis-driven approach,^{9,32,33} we uncovered an association of influenza A or B AB with anti-NR1 seropositivity, suggesting molecular mimicry. This phenomenon induces generation of AB reacting both against pathogenic elements and autoantigens,³⁰ and has a role in autoimmune diseases.^{41,42} As the influenza A M2 channel and NMDAR share a common ligand, the antiviral compound amantadine,⁴³ a putative structural homology might act as inducer of NMDAR-AB. The observed association was found in males only. Interestingly, males have a higher incidence of influenza,⁴⁴ and male mice exert a more vigorous immune response on influenza infection.⁴⁵ This gender disposition might also explain the higher anti-NR1 AB prevalence in males.

In conclusion, our study draws an increasingly complex picture of NMDAR-AB pathology, with anti-NMDAR encephalitis possibly constituting the extreme end of a broad spectrum of mild to severe phenotypes associated with NMDAR autoimmunity. Beyond the NMDAR-AB studied here, loss of blood-brain barrier integrity may generally constitute a major risk factor for detrimental effects of peripheral AB against central nervous system epitopes.

CONFLICT OF INTEREST

Dr Stöcker is a full-time employee of and holds stocks in Euroimmun AG. Dr Martens is a full-time employee of Synaptic Systems GmbH. All other authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (<http://www.nature.com/mp>)