The brain as 'immunoprecipitator' of serum autoantibodies against NMDAR1

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ABSTRACT

Autoantibodies (AB) against N-methyl-D-aspartate-receptor subunit-NR1 (NMDAR1) are highly seroprevalent in health and disease. Symptomatic relevance may arise upon compromised blood-brain-barrier (BBB). However, it remained unknown whether circulating NMDAR1-AB appear in the cerebrospinal fluid (CSF). Of N=271 subjects with CSF-serum-pairs, 26 were NMDAR1-AB seropositive, but only 1 was CSF-positive. Contrariwise, tetanus-AB (non-brain-binding) were present in serum and CSF of all subjects, with CSF levels higher upon BBB-dysfunction. Translational mouse experiments proved the hypothesis that the brain acts as 'immunoprecipitator': Simultaneous injection of NMDAR1-AB and the non-brain-binding GFP-AB resulted in high detectability of the former in brain and the latter in CSF.

Keywords: NMDAR1, autoantibody, *ApoE* KO mice, blood-CSF-barrier, blood-brain-barrier, immunoglobulin, multiple sclerosis, albumin quotient, tetanus antibodies, anti-GFP antibody, influenza A and B

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INTRODUCTION

Recently, we reported high seroprevalence (age-dependent up to >20%) of autoantibodies (AB) directed against N-methyl-D-aspartate-receptor subunit NR1 (NMDAR1) in both healthy and neuropsychiatrically ill subjects (N=4236)[1]. Neuropsychiatric syndrome relevance was restricted to individuals with compromised blood-brain-barrier (BBB), e.g. *APOE4* carrier status or post-neurotrauma, both clinically and experimentally[2,3]. We now wondered whether NMDAR1-AB would be similarly seroprevalent in multiple sclerosis (MS) patients with their high likelihood of (recurring) BBB disturbance, as indirectly evidenced here as well as in clinical routine by an increased albumin quotient, i.e. a disturbed blood-CSF-barrier (BCB)[4]. In the following, the term BBB/BCB will therefore be used throughout. Importantly, we asked whether NMDAR1-AB would be present also in the CSF, at least of seropositive individuals with BBB/BCB leakage.

METHODS

Participants and AB measurements

MS (N=270) and neuropsychiatric disease control (DC) subjects (N=207) were recruited in Magdeburg and Munich for biomarker studies (Table1). Subject data were collected in accordance with ethical guidelines and the Helsinki Declaration. Sample selection was unbiased, i.e. sera and CSF collection (including determination of albumin quotients; Table1) concluded before analysis of AB was planned.

NMDAR1-AB determination: Commercially available standard procedures for clinical diagnosis, recombinant immunofluorescence tests (Euroimmun, Lübeck, Germany), were used to detect NMDAR-AB in serum and CSF, based on HEK293 cells transfected with NMDAR NR1 subunits[5,6]. Seropositivity for NMDAR1-AB of IgG, IgM, or IgA isotypes (using respective second AB against human Ig; Euroimmun) was assessed by 2 researchers independently (for sample stainings see Figure1)[5,6].

Tetanus-AB determination: Serum and CSF titers of tetanus-AB were measured using a tetanus IgG ELISA kit (Sekisui Diagnostics, LLC, USA) over a BioTek EL808

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ELISA reader (BioTek Instrument GmbH, Bad Friedrichshall, Germany) according to manufacturer's instructions.

Influenza A and B AB determination: Seropositivity for AB (IgG) was determined by ELISA (Novagnost-InfluenzaA-IgG, Novagnost-InfluenzaB-IgG, Siemens Healthcare-Diagnostics GmbH, Eschborn, Germany) and automatically processed on BEP®III (Siemens Healthcare-Diagnostics GmbH).

Mouse experiments

Experiments were approved by the local animal protection committee. Female C57Bl/6N *ApoE*$^{-/-}$ [7] and wildtype littermates (WT; *ApoE*$^{+/+}$) mice (N=6 per group), aged 14–15 weeks, were housed at 4–5 per cage, 12 h light/dark cycle, food/water ad libitum. Mice received an intravenous cocktail of 2 biotinylated monoclonal mouse IgG2b AB (150μg each, Synaptic Systems, Göttingen, Germany): (1) directed against NMDAR1 (NMDAR1-AB) and (2) against the non-brain-antigen, green fluorescent protein (GFP-AB). After 5 days, animals were anesthetized (intraperitoneal injection of 0.25% tribromoethanol, 0.125mg/g body weight, Sigma-Aldrich) and CSF, blood, brain and spinal cord samples collected and processed for AB titer analysis as described in detail in Figure2.

Statistical analysis

Group differences in categorical and continuous variables were assessed using Chi-square and Mann-Whitney U test, respectively. Two-way ANOVA was employed to assess tetanus AB concentrations by BCB leakage and diagnosis. Statistical analyses were performed using SPSS for Windows version 17.0 (IBM-Deutschland GmbH, Munich, Germany).

RESULTS

We confirmed NMDAR1-AB seroprevalence of ~10% (47/477), as expected from mean age (all N=477: 38.85±13.1 years) of the cohort[8] in both MS (N=270) and DC patients (N=207), with an Ig class distribution again similar to that reported earlier[1,2]: 4.8% IgM (N=23), 3.1% IgA (N=15), 0.4% IgG (N=2), 1.3% IgA+IgM (N=6) and 0.2% IgA+IgG (N=1) (Table1). Also serum titer distributions were comparable between MS

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and DC (ranging between 1:10 and 1:320) and did not differ dependent on the albumin quotient as readout of BBB/BCB leakage (Table1, Figure1A,B).

To further validate our previously reported association of NMDAR1-AB with influenza A and B AB seropositivity[2], we additionally performed serological analyses for these AB. We replicated our earlier result on a new cohort of N=1086 individuals, including the subjects of the present study (Table2), strengthening our finding that influenza seropositivity is related to the formation of NMDAR1-AB.

In individuals with serum-CSF pairs available (total N=271: N=119 MS; N=152 DC), altogether N=26 were seropositive for NMDAR1-AB. Surprisingly, only 1 out of the 271 CSF samples was positive for NMDAR1-AB (Figure1A). This positive CSF sample (IgG; 1:10) was from an MS patient with normal albumin quotient and an NMDAR1-AB serum titer of also 1:10 (IgG). In light of the overall low NMDAR1-AB serum titers, the negative CSF results of DC might still be somewhat explained by the predominantly normal albumin quotients (88.2%). The negative CSF results in MS patients with nearly one third confirmed BBB/BCB breakdown (albumin quotient pathological in 30.1%), however, were unexpected. In principle, entry of proteins, including immunoglobulins, from blood into CSF, does not even require BBB breakdown. There are circumventricular organs (organum vasculosum of the lamina terminalis, median eminence, area postrema) that lack a BBB and normally allow proteins to enter CSF and brain, as demonstrated by the classic studies of Broadwell & Brightman using horseradish peroxidase intravenously for tracing[9].

We thus hypothesized that the brain may act as 'immunoprecipitator' of serum AB directed against brain antigens, binding nearly all available AB molecules that pass through the BBB, thereby preventing drainage of a detectable amount of NMDAR1-AB into the CSF. In contrast, AB not expected to specifically bind to brain tissue, should appear in measurable amounts in the CSF, with quantities depending on BBB/BCB function.

In a first approach to prove this hypothesis, we determined in serum and CSF of our cohort the titers of vaccination-induced, abundantly present tetanus-AB (IgG), which should not bind to brain antigens. In healthy individuals, the expected transfer over

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an intact BBB/BCB into the CSF for IgG is 1/500 of the serum concentration[4]. Indeed, Tetanus-AB were detected in all individuals in the CSF, albeit at low levels, dependent on serum concentration (Figure1C-D), and by trend also on BBB/BCB integrity [δ ($r_{BCB\ leakage}$-$r_{BCB\ intact}$): Z=-1.502, p=0.06].

In order to experimentally prove our hypothesis of the brain acting as 'immunoprecipitator', we employed *ApoE*$^{-/-}$ mice with their known BBB disruption and WT littermates[2,7] (Figure2A). In both groups, we simultaneously applied 2 monoclonal mouse IgG AB, NMDAR1-AB and GFP-AB (as non-brain-binding AB), intravenously in concentrations resulting in serum/plasma titers (previously established by pilot experiments) that were theoretically expected to lead to measurable CSF levels even in WT mice (IgG 1:10,000 in serum → 1:20 in CSF[4]).

Five days after application, plasma titers of both AB were lower in *ApoE*$^{-/-}$ mice with their open BBB than in WT littermates (*p=0.004*). CSF was negative for NMDAR1-AB in all mice, but distinctly positive for GFP-AB in 5/6 *ApoE*$^{-/-}$ mice and 1/5 WT mice. All brain regions analyzed in both genotypes were negative for GFP-AB. In contrast, NMDAR1-AB were recovered in prefrontal cortex, hippocampus, cerebellum, brain stem and spinal cord at high titers mainly in ApoE$^{-/-}$ mice, and to a lesser degree and in lower titers in WT mice (Figure2B-C). The recovered titers showed a regionally different pattern. This is interesting with respect to the known widespread distribution of NMDAR1 all over the central nervous system[10-12] and should motivate further study. Hitherto unexplained is the overall negative result for the olfactory bulb, even though NMDAR1 is also highly expressed there in adult mice[12].

DISCUSSION

The present translational study supports the concept of the brain acting as 'immunoprecipitator': Brain-antigen directed AB are strongly bound to brain tissue and thereby prevented from drainage into the CSF whereas non-brain bound AB appear in the CSF in amounts expected from the literature[4]. The BBB/BCB intactness versus leakiness plays a critical role regarding the titers measured in brain and CSF.

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Translating these findings to humans with their high NMDAR1-AB seroprevalence (age-dependent up to >20%)[1,2,8], we may assume that AB crossing over an intact BBB at low rate will not cause major symptoms, whereas in situations of acute or chronic BBB leakage, the concentrations achieved in brain will eventually become symptomatic. The symptoms may then modulate existing disease, e.g. shape the presentation of schizophrenia/schizoaffective disorder[3] or determine disease outcome as shown in stroke, with a reduction of lesion size in individuals with previously intact BBB, but detrimental consequence upon pre-existing BBB disturbance[8]. It is well possible that serum NMDAR1-AB may in conditions of increasing BBB leakiness (as in aging, e.g.[13]) contribute to the slow development of dementia[14].

Importantly, the present data should make clinicians aware that serum NMDAR1-AB may modulate brain functions also in absence of measurable titers in CSF. In cases where pathological significance of circulating NMDAR1-AB is suspected, the BBB/BCB intactness should be tested. This may soon be possible even by routine MRI[13]. In contrast, the high NMDAR1-AB levels reported in CSF of patients with NMDAR encephalitis[5,15], are likely derived from additional intrathecal synthesis, cause high (′oversaturated′) brain levels, severe acute symptoms, and distinct spillover into the CSF.

Regarding factors that predispose to NMDAR1-AB formation, we confirmed here our previously reported association with influenza A and B seropositivity[2]. Considerations of potential molecular mimicry accounting for this association are still speculative. But even though the underlying molecular mechanism is presently unclear, it may help to explain the high seroprevalence of NMDAR1-AB with increasing age.

A limitation of the present study is the fact, that mouse experiments were performed with IgG AB only. Therefore, kinetics of IgA and IgM in brain/CSF remain subject to further work. Nevertheless, in our earlier studies, using IgG, IgA and IgM NMDAR1-AB derived from human serum in the *ApoE*$^{-/-}$ mouse model[2], a comparable consequence of all AB classes could be demonstrated. The same holds true for our findings in ischemic stroke patients where the Ig class did not seem to matter regarding the effects of serum NMDAR1-AB on evolution of lesion size[8].

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To conclude, our translational study may shed new light on the understanding of potential symptomatic consequences of serum AB directed against brain antigens. Whereas leakiness of the BBB has a major role and should be evaluated in cases where pathological relevance of circulating NMDAR1-AB is suspected, negative results regarding AB titers in CSF may not be suitable to exclude brain effects.

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CONFLICTS OF INTEREST

W Stöcker is member of the board of and holds stocks in Euroimmun AG. All other authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Concept and design of the study: HE, ECG

Data acquisition and analysis: ECG, AK, HE, JS, FW, KO, MM, MU, US, BH, CH, GP, AS, VP, ARA, LB, TFS, WS

Drafting the manuscript and figures: HE, ECG, AK

All authors read and approved the final version of the manuscript.

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FIGURE LEGENDS

Figure 1: Human data. (A) NMDAR1-AB titer distribution in serum and CSF presented by diagnosis and BBB/BCB integrity. Titer distribution in serum (IgM purple, IgA yellow, IgG turquois dots) was comparable between multiple sclerosis patients (MS) and neuropsychiatric disease controls (DC) and did not differ dependent on BCB leakage. Only 1 CSF sample of an MS patient was 1:10 positive (IgG, respective serum titer of this patient circled). Please note that numbers in circles plus dots do not always add up to the numbers given for the groups since individual patients may carry AB of 2 Ig classes. **(B)** Representative confocal images of HEK293 cells transfected with NMDAR1 (Euroimmun) as used for serum and CSF tests. NMDAR1-AB seropositivity is illustrated as an example for 3 different patients (using anti-human IgG, IgM or IgA, FITC-labeled as second AB), including the subject with the only positive CSF sample (IgG). All images are Z projections of 10 focal planes located 0.5 μm apart and were taken at 63X magnification under a confocal laser scanning microscope (Leica SP5). **(C)** Intercorrelation of tetanus-AB in CSF and serum in MS and DC, stratified by BBB/BCB integrity. Tetanus-AB were detected in all individuals in the CSF, albeit at low levels, dependent on serum concentration and BBB/BCB integrity. Pearson correlation coefficients are shown. **(D)** Two-way ANOVA, with tetanus-AB levels in serum and CSF as dependent variables and BBB/BCB integrity and diagnosis as factors, resulted in a main effect for BBB/BCB integrity while diagnosis and interaction of both factors were not significant.

Figure 2: Mouse data. (A) Experimental design: Female C57Bl/6N *ApoE*$^{-/-}$ *mice*[7] and their wildtype littermates (WT, *ApoE*$^{+/+}$), received an intravenous cocktail of 2 biotinylated monoclonal mouse IgG2b AB (NMDAR1-AB and, as non-brain-antigen directed AB, GFP-AB; 150μg each). After 5 days, animals were anesthetized and samples were taken. CSF (1-2 μl) was collected from the cisterna magna using a glass capillary. Blood (0.5ml) was drawn from the left ventricle of the heart and then processed as indicated in the figure to obtain the plasma. Brain and spinal cord were freshly extracted and dissected under a stereomicroscope and quickly processed as described in the figure, following a previously described protocol[16] to obtain IgG extracts from tissue. AB titers in CSF, plasma and brain/spinal cord extracts were

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determined using cell-based assays: Samples were tested in different dilutions (from 1:10 to 1:10,000) on HEK293 cells transfected with either NMDAR1 (Euroimmun) or GFP (self-made; pEGFP-N1 4.7 kb plasmid, Lipofectamine 2000) and on HEK293 non-transfected cells (negative controls). Titer was defined as the last dilution in which positivity was observed (green and red co-localizing label, see diagram in A and confocal pictures in B). **(B)** Representative images showing positivity/negativity observed for NMDAR1 and GFP in different samples (abbreviations given refer to those in **C**, too). Note the co-localization of green (anti-mouse IgG-A888) and red (rabbit anti-biotin + anti-rabbit-A594) in all positive cases, demonstrating the high specificity of the test. **(C)** Titer distributions of NMDAR1-AB and GFP-AB dependent on genotype (*ApoE*$^{-/-}$ *mice* with their leaky BBB and *ApoE*$^{+/+}$ with intact barrier) and respective p-values (Mann-Whitney U test). Note that plasma titers of both AB were lower in *ApoE*$^{-/-}$ mice (*p=0.004*). CSF (due to restricted material only tested at 1:10) was negative for NMDAR1-AB in all mice, but distinctly positive for GFP-AB in 5/6 *ApoE*$^{-/-}$ mice and only 1/5 WT mice. All brain regions analyzed in both genotypes were negative for GFP-AB. In contrast, NMDAR1-AB were recovered in prefrontal cortex, hippocampus, cerebellum, brain stem and spinal cord in high titers mainly of ApoE$^{-/-}$ mice, and to a lesser degree and in lower titers in WT mice.

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Table 1. Overview of clinical and laboratory parameters in multiple sclerosis patients and disease controls

	Total sample (only serum available; N=477)			Subsample with serum and cerebrospinal fluid available (N=271)		
	Disease controls[a] N=206-207	Multiple Sclerosis N=263-270	*p* value	Disease controls[b] N=104-152	Multiple Sclerosis N=113-119	*p* value
Gender, *No. (%), women*[c]	150 (72.5)	178 (66.9)	0.194	109 (71.7)	82 (70.7)	0.855
Age at examination, *mean ± SD, years*[d]	38.8 ± 14.5	38.8 ± 11.8	0.559	38.9 ± 15.3	37.1 ± 12.2	0.576
Disease course (Multiple Sclerosis), *No. (%)*						
Clinically isolated syndrome	n/a	56 (21.2)		n/a	37 (31.9)	
Relapsing remitting	n/a	156 (59.1)	n/a	n/a	47 (40.5)	n/a
Primary progressive	n/a	21 (8)		n/a	15 (12.9)	
Secondary progressive	n/a	31 (11.7)		n/a	17 (14.7)	
Duration of disease (MS), *mean ± SD, months*	n/a	96.2 ± 68.6	n/a	n/a	105.5 ± 17.3	n/a
No. of relapses (MS), *No. (%)*						
No relapse	n/a	-		n/a	16 (13.9)	
1 relapse	n/a	-	n/a	n/a	47 (40.9)	n/a
2 or more relapses	n/a	-		n/a	52 (45.2)	
No. of clinical MRI lesions (MS), *No. (%), 2 or more* [e]	n/a	-	n/a	n/a	82 (71.3)	n/a
Expanded Disability Status Scale (MS), *mean ± SD*	n/a	-	n/a	n/a	3.2 ± 1.1	n/a
NMDAR1-AB serostatus, *No. (%), seropositive*[c]	19 (9.2)	28 (10.4)	0.665	14 (9.2)	12 (10.1)	0.809
NMDAR1-AB seroprevalence, *titers, No.* [c, f]						
IgA (1:10; 1:32; 1:100; 1:320)	4; 2; 3; 1	5; 6; 0; 1	0.174	3; 0; 3; 1	2; 4; 0; 0	0.043
IgG (1:10; 1:32; 1:100; 1:320)	0; 0; 0; 0	2; 1; 0; 0	-	0; 0; 0; 0	1; 1; 0; 0	-
IgM (1:10; 1:32; 1:100; 1:320)	2; 5; 3; 0	2; 3; 4; 0	0.606	1; 4; 3; 0	1; 6; 2; 0	0.762
Albumin quotient, *mean ± SD*[d]	n/a	n/a	n/a	5.0 ± 1.7	6.2 ± 3.6	***0.004***
Blood-cerebrospinal fluid barrier, *No. (%), leakage*[c, g]	n/a	n/a	n/a	18 (11.8)	34 (30.1)	***0.0002***
Tetanus titers in serum (IU/ml), *mean ± SD*[d]	n/a	n/a	n/a	2.3 ± 1.3	2.1 ± 1.5	0.198
Tetanus titers in cerebrospinal fluid (IU/ml), *mean ± SD*[d]	n/a	n/a	n/a	0.003 ± 0.003	0.005 ± 0.005	0.032

Multiple testing adjusted significances (Bonferroni: p≤0.008 for the sample with serum available and p≤0.005 for the subsample with serum and cerebrospinal fluid available); [a]this category includes neurodegenerative disorders (12.6%), psychiatric conditions (33.8%), healthy or minor conditions not relevant for cerebrospinal fluid pathology (41%) and others (12.6%); [b]this category includes neurodegenerative disorders other than multiple sclerosis (17.1%), psychiatric conditions (9.9%), healthy or minor conditions not relevant for cerebrospinal fluid pathology (55.9%) and others (17.1%); [c]Chi-square test; [d]Mann-Whitney U test; [e]all other individuals have one MRI lesion; [f]Note that of the total sample N=4 individuals were seropositive for both IgA and IgM; [g]blood-cerebrospinal fluid barrier was diagnosed as 'leakage' when albumin quotient >age/15+4; abbreviations: n/a= not applicable

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Table 2: Replication study testing the association of circulating NMDAR1-AB with influenza A and B seropositivity

Study cohort		MS patients (N=267)		MS controls (DC, N=161)		Stroke patients (N=464)		AD patients (N=81)		Other psychiatric diagnoses (N=71)		Healthy controls (N=42)		Replication Sample Total (N=1086)		Hammer et al 2013 (N=2805)*	
NMDAR1-AB serostatus		-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
		N=241	N=26	N=142	N=19	N=364	N=100	N=69	N=12	N=68	N=3	N=39	N=3	N=923	N=163	N=2522	N=295
Influenza A	N seropositive (%)	194 (80.5)	21 (80.8)	103 (72.5)	17 (89.5)	275 (75.5)	81 (81.0)	53 (76.8)	11 (91.7)	46 (67.6)	3 (100)	23 (59.0)	2 (66.7)	694 (75.2)	135 (82.8)	1672 (66.6)	215 (73.1)
	P value	0.487		0.064		0.128		0.134		0.499		0.397		**0.018**		**0.024**	
	OR (95% CI)	1.02 (0.40-2.84)		3.22 (0.71-14.58)		1.38 (0.79-2.40)		3.32 (0.40-27.72)		n/a		1.38 (0.12-16.68)		1.59 (1.03-2.45)		1.37 (1.04-1.79)	
NMDAR1-AB serostatus		-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
		N=241	N=26	N=142	N=19	N=364	N=100	N=69	N=12	N=68	N=3	N=39	N=3	N=923	N=163	N=2522	N=295
Influenza B	N seropositive (%)	54 (22.4)	9 (34.6)	31 (21.8)	5 (26.3)	62 (17.0)	23 (23.0)	18 (26.1)	4 (33.3)	17 (25.0)	1 (33.3)	6 (15.4)	0 (0.0)	188 (20.4)	42 (25.8)	542 (21.6)	84 (28.6)
	P value	0.085		0.330		0.085		0.302		0.374		0.499		**0.034**		**0.006**	
	OR (95% CI)	1.83 (0.78-4.35)		1.28 (0.43-3.82)		1.46 (0.85-2.5)		1.41 (0.40-5.28)		1.5 (0.13-17.6)		n/a		1.45 (0.97-2.15)		1.45 (1.11-1.90)	

One-sided *P* values are shown for the replication study cohorts; bolded values, p<0.05; +, NMDAR1-AB seropositive; -, NMDAR1-AB seronegative; OR, odds ratio; CI, confidence intervals; n/a, not applicable; * data from the original study (discovery sample) by Hammer et al 2013 for comparison (two-sided *P* values)

Note regarding the replication sample: MS and DC patients are from the present study (numbers smaller due to reduced serum availability), stroke patients from Zerche et al 2015; AD patients, other psychiatric diagnoses and healthy controls from the extended GRAS data collection.

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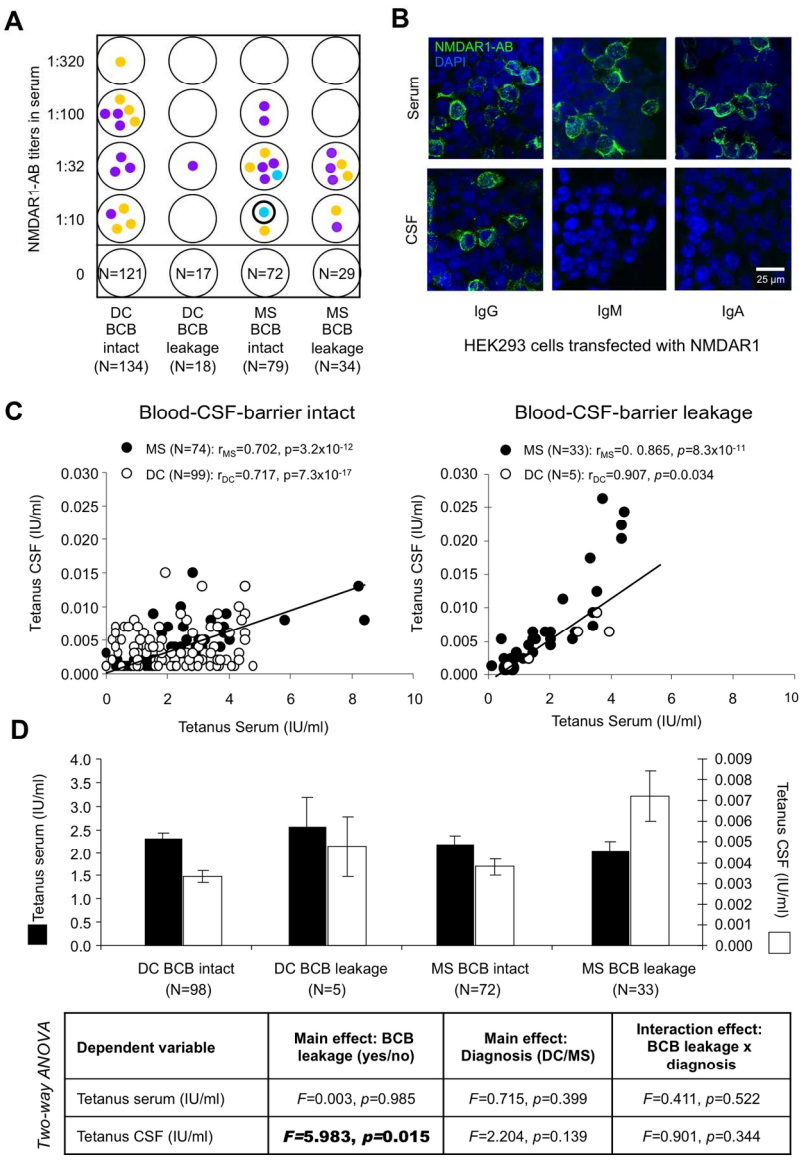
Castillo-Gomez et al Figure 1



Two-way ANOVA

Dependent variable	Main effect: BCB leakage (yes/no)	Main effect: Diagnosis (DC/MS)	Interaction effect: BCB leakage x diagnosis
Tetanus serum (IU/ml)	F=0.003, p=0.985	F=0.715, p=0.399	F=0.411, p=0.522
Tetanus CSF (IU/ml)	***F*=5.983, *p*=0.015**	F=2.204, p=0.139	F=0.901, p=0.344

170x226mm (300 x 300 DPI)

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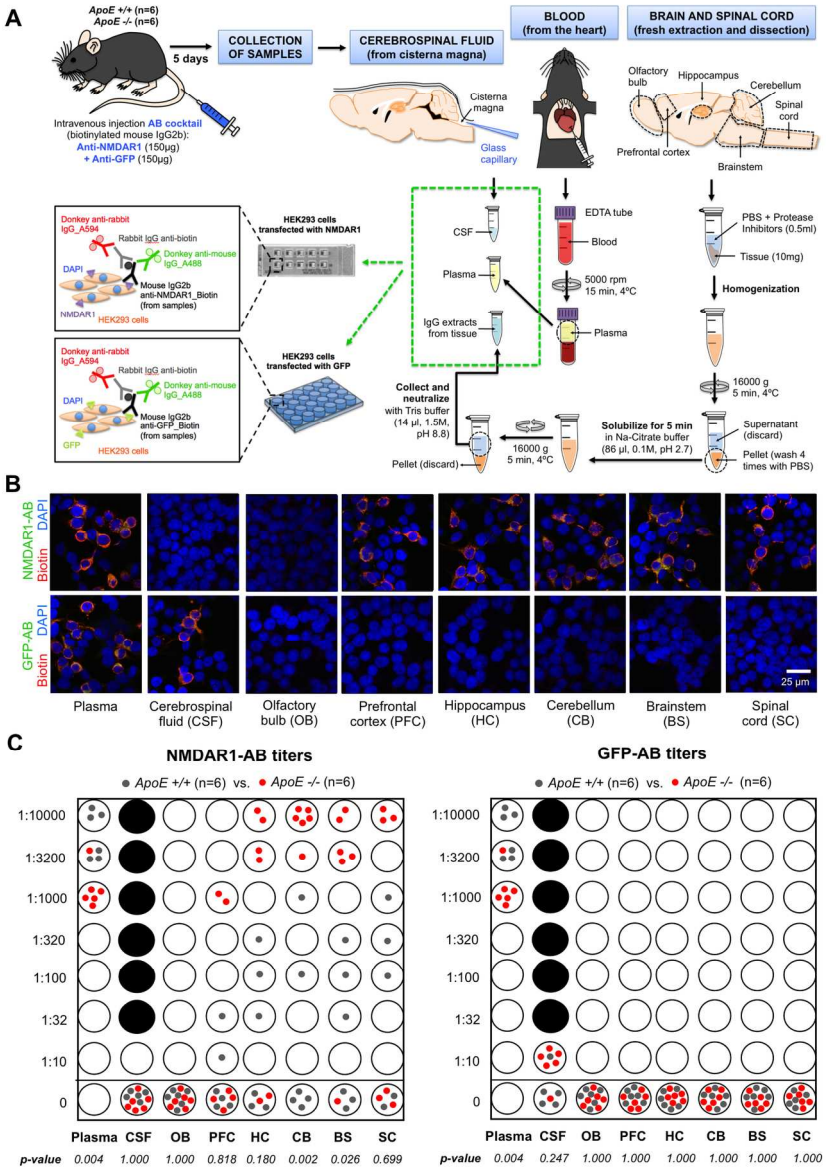
Castillo-Gomez et al Figure 2



170x226mm (300 x 300 DPI)

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