AWARD NUMBER: W81XWH-14-1-0369

TITLE: Maternal Brain-Reactive Antibodies and Autism Spectrum Disorder

PRINCIPAL INVESTIGATOR: Betty Diamond

CONTRACTING ORGANIZATION: Feinstein Institute for Medical Research
Manhasset, NY 11030-3816

REPORT DATE: December 2017

TYPE OF REPORT: FINAL

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
December 2017	FINAL	09/30/2014 – 09/29/2017

4. TITLE AND SUBTITLE

Maternal Brain-Reactive Antibodies and Autism Spectrum Disorder

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-14-1-0369

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Betty Diamond

E-Mail: bdiamond@northwell.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The Feinstein Institute for Medical Research
350 Community Drive
Manhasset, NY 11030

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

We have determined that one monoclonal antibody cloned from the memory B cell population of the mother of a child with ASD binds Caspr2. This antibody disrupts fetal brain development and leads to an ASD-like phenotype in the offspring. Forty percent of women with brain-reactive serology and a child with ASD exhibit antibodies to Caspr2; thus this antibody may contribute to approximately 5% of cases of ASD.

We have also determined that the pathogenicity of anti-Caspr2 antibodies is determined, in part, by epitope specificity.

15. SUBJECT TERMS

Fetal brain; Autism spectrum disorder; antibody; B cells; Caspr2

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	31	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

Revised Progress Report DOD – Dr. Diamond

1. Introduction:

The goal of this project is to test a series of monoclonal anti-brain antibodies, each cloned from a woman with brain-reactive serology and a child with ASD, for pathogenic potential during fetal brain development.
The etiology of ASD is complex, and includes both genetic and environmental factors. In recent years, the maternal immune contribution has been the focus of studies demonstrating that autoimmune disorders, infections, and maternal brain-reactive antibodies may play a significant role in ASD. In ASD, the concept that maternal autoantibodies potentially affect fetal neurodevelopment and contribute to ASD has been entertained for some time. Several studies [1-3] including our own [4] have demonstrated that mothers of an ASD child are more likely to harbor anti-brain IgG. When serum or purified IgG with brain- reactive antibody derived from the mother of a child with ASD was administered to gestating mice or monkeys, offspring displayed behaviors that are reminiscent some of the symptoms seen in ASD [2,5,6].Yet, the targets of the IgG in these preparations have not been fully identified, and different preparations led to different impairments. Most recently, two papers were published identifying potential antigens recognized by serum of mothers of a child with ASD [3,5]. These studies provide strong evidence that IgG present in some mothers of a child with ASD can affect fetal brain development and lead to behaviors analogous to ASD phenotypes. These studies indeed move the field forward, yet they have some important limitations including the use of polyclonal serum which limits the ability to pin point which fine specificities are those that impair brain development. For this reason and for therapeutic purposes, the generation of monoclonal antibodies is critical.
We devised a novel strategy (Brimberg et al, submitted) and generated monoclonal IgGs from blood samples of mothers with an ASD child previously characterized to have brain-reactive IgG [4]. B cells were incubated with human fetal brain lysate labeled with biotin. B cells that bound to biotinylated fetal brain antigens were isolated with magnetic anti-biotin beads. Because we obtained blood years after the pregnancy that resulted in a child with ASD, we focused exclusively on B cells in the memory compartment. Memory B cells persist in humans for years to decades, and thus can provide a window on autoreactivity that may have been present during a pregnancy occurring years before. We used fluorescent markers to distinguish the memory B cells (CD19+, CD27+) among the brain-reactive B cell population. These were isolated by fluorescence activated single-cell sorting. Using an established technique [7] previously employed in our lab [8], we expressed IgG variable region genes from these B cells in human γ1 and κ chain constructs. These were then co-transfected into 293T cells and intact human IgG1 was harvested from the culture supernatant and purified on a protein G column. Using this approach, we have obtained ~40 monoclonal antibodies from 3 mothers of a child with ASD. So far, 20 of them have been shown to bind mouse brain. As described below we have now extensively characterized one of those monoclonal antibodies, namely C6 and also assessed the frequency of such antibodies in plasma of mothers of an ASD child.

We have, in addition, analyzed two more monoclonal antibodies one binding Caspr2, that were cloned from mothers with brain reactive serology and a child with ASD. These antibodies do not appear to alter fetal brain development in the same experimental paradigm we used to study the C6 antibody. Thus, not all anti-Casp2 antibodies nor all brain-reactive antibodies have pathogenic potential.

2. Keywords:

Fetal brain; Autism spectrum disorder; antibody; B cells; Caspr2

3. Accomplishments:

What were the major goals of the project?

The major goal of this project is to identify antibodies that can perturb fetal brain development and lead to an ASD-like phenotype. The second goal is to determine how frequently such antibodies are present in plasma of a mother of a child with ASD and whether each antibody contributes to a distinct phenotype.

What was accomplished under these goals?

Using our novel strategy to generate a monoclonal IgG from B cells of women with brain-reactive serology and a child with ASD we have identified a monoclonal antibody, namely C6, that targets the membrane protein contact in associated protein-like 2 (Caspr2). Caspr2, encoded by the gene CNTNAP2, is expressed mainly in neurons, and rare variants of CNTNAP2 are associated with neurodevelopmental phenotypes. To demonstrate the contribution to ASD, mice were exposed in utero to C6 transported from the maternal blood or to B1 control antibody (an antibody that does not bind brain). Male fetal brains, but not females, exposed in utero to C6 exhibited abnormal cortical development including thinning of the cortical plate and decreased proliferation of neurons. In accordance, adult male mice exposed in utero to C6 showed reduced number of neurons in specific area of the cortex. In addition, adult males exposed in utero to C6 showed decreased number of parvalbumin inhibitory neurons and decreased dendritic complexity of excitatory neurons in the cortex. Adult mice exposed in-utero to C6 showed impairments in social preference, flexible learning, and increased repetitive behavior. Importantly, we found similar changes to the developing cortex in mice exposed in utero to the plasma in which C6 was isolated from. There were no changes to the developing cortex when mice were exposed in utero to the same plasma after C6 like antibodies were depleted from it.

These data have been published: Brimberg, L., Mader, S., Jeganathan, V., Berlin, R., Coleman, TR., Gregersen, PK., Huerta, P., Volpe, BT., and Diamond, B. Caspr2-reactive antibody cloned from a mother of an ASD child mediates an ASD-like phenotype in mice. Molecular Psych 21:1663-1671(2016).

We have also studied a second monoclonal anti-Caspr2 antibody and determined that in utero exposure to this antibody does not impair fetal brain development. Thus, we learned that epitope specificity contributes to pathogenicity.

We examined how frequently antibodies with Caspr2 specificity are present in women with an ASD child. We analyzed plasma of mothers of a child with ASD that had brain-reactive serology using indirect immunofluorescence on non-permeabilized Caspr2-transfected HEK 293T cells. We found that 37% displayed robust Caspr2 membrane staining, compared to 12% of plasma from mothers of an ASD child lacking brain-reactive antibodies, 12% of plasma from unselected women of child-bearing age, and 7.6% of plasma from mothers of a typically developing child bound Caspr2.

We have started analyzing the specific epitope in Caspr2 to which C6 binds to, and have identified 3 peptides to which it binds and which serve as surrogate antigen. We have determined that maternal serum with anti-Caspr2 reactivity also binds these peptides.

The following task have been accomplished:

Major Task 1: To characterize monoclonal brain reactive antibodies

Subtask 1: To obtain regulatory approval from the DOD – completed

Subtask 2: To produce large amounts of each monoclonal antibody

We have generated large amounts of 5 monoclonal antibodies and are producing more.

Subtask3: To inject brain-reactive antibodies and isotope control antibody into pregnant mice to observe brain development in the offspring.

We have injected two additional monoclonal antibodies D3 and A7 into pregnant mice binding to Caspr2. A7 binds Caspr2 but does not bind the same peptides as C6, demonstrating different epitope specificity. Exposure to A7 in utero is non-pathogenic.

Subtask4: To examine behavior in antibody-exposed offspring (7 litters for each antibody)

We have done an extensive analysis of behavior in offspring exposed in utero to C6 antibody. We have begun testing mice exposed to two other monoclonal antibodies D3 and A7. A7 is a second anti-Caspr2 antibody.

Thus far, we do not observe histologic abnormalities in A7 exposed fetal brains, suggesting that we will not find behavioral changes.

Subtask5: To analyze histology of antibody exposed fetal brain for each antibody (7 litters for each antibody)

We continue to expand our analysis of fetal brains exposed in utero to anti-Caspr2 antibody. We have now perfected our methodology to distinguish cortical layers and have data that these antibodies alter mitosis and migration. We have also shown they bind neural progenitor cells in the ventricular zone.

We have also developed a model in which mice are immunized with Caspr2 and develop polyclonal anti-Caspr2 antibodies. The fetuses of these mice exhibit altered brain histology and live offspring exhibit behavioral abnormalities.

Milestone(s) Achieved

We have published on the effects of the C6 anti-Caspr2 antibody on the developing mouse brain.

Brimberg, L., Mader, S., Jeganathan, V., Berlin, R., Coleman, TR., Gregersen, PK., Huerta, P., Volpe, BT., and Diamond, B. Caspr2-reactive antibody cloned from a mother of an ASD child mediates an ASD-like phenotype in mice. Molecular Psych 21:1663-1671(2016)

Mader, SA., Brimberg, L and Diamond, B. The role of brain-reactive autoantibodies in brain pathology and cognitive impairment. Frontiers In Immunology 8:1101 (2017) PMC5601985.

IACUC Approval – yes

Major Task 2: To characterize maternal serum for reactivity to brain antigens

Subtask1: To develop ELISAs for each of the antigens bound by the monoclonal antibodies

We have developed assays for reactivity to Caspr2 and Edil 3. We continue to work on developing ELISAs but this has proven to be quite challenging. We, therefore, developed a cell based assay. We also obtained plasmids encoding truncated forms of Caspr2 to localize the epitopes seen by C6 and A7 but the Caspr2 protein has several repeat domains. We have, therefore, focused on identifying surrogate antigens (peptides) bound by the antibodies. We now have ELISAs for 2 Caspr2 epitopes, one pathogenic and one nonpathogenic.

Subtask2: To test brain-reactive sera on the ELISAs~300 sera

We have analyzed 60 brain-reactive sera from mothers of a child with ASD, 60 non-brain-reactive sera from mothers of an ASD child, and 60 control sera from mothers of a typically developing child for reactivity to Caspr2 using the cell based assay.

Milestone(s) Achieved

We developed an assay to analyze anti-Caspr2 reactivity. Approximately 40% of women with anti-brain reactivity have antibodies to Caspr2 compared to a significantly smaller percentage in individuals without anti-brain reactivity.

Research subjects

Plasma from mothers with an ASD child was obtained from the Simons Simplex Collection (SSC, http://sfari.org/resources/simons-simplex-collection) 37. Control plasma from women of childbearing age were obtained from the Northwell Health (previously North Shore-LIJ Health System) clinical laboratory and participants in a registry at the Feinstein Institute for Medical Research (http://www.gapregistry.org). Both cohorts were described previously [4].
Plasma of mothers of a typically developing child (determined by the mother report) were obtained from the Genotype and Phenotype registry (http://www.gapregistry.org) at the Feinstein Institute for Medical Research. The age of the mothers at the time the plasma was drawn matched the previous cohorts [4] All individuals provided informed consent through the appropriate institutional review boards.

Sample Collection

Blood was collected into heparinized tubes from consenting mothers enrolled in the SSC, previously identified as having brain-reactive antibodies [4]. The protocol was approved by the SSC as well as by the Feinstein Institute for Medical Research Institute Review Board.

Single cell sorting

Isolation of single human memory brain-reactive B cells was performed as previously described [8] with several modifications. B cells were purified from fresh mononuclear cells by negative selection using a B cell kit (StemCell Technology). They were then incubated for 30 min at room temperature (RT) with human fetal brain lysate (3 μg per ml, Nouvus) labeled with biotin using the EZ-Link Sulfo-NHS-Biotin labeling kit (Life Technologies). Cells bound by biotinylated brain antigens were isolated with a biotin selection kit (StemCell Technologies) and stained with FITC conjugated anti-human CD19, phycoerythrin (PE) conjugated anti- human CD27 and allophycocyanin (APC) streptavidin to allow the separation of $CD19^+$, $CD27^+$, brain $lysate^+$ memory B cells. As a control, the fraction that initially was identified as non brain-reactive was incubated with biotinylated brain antigen and stained as described above. No APC positive cells were detected in this fraction. Finally, $CD19^+$, $CD27^{+,}$ APC^+ single cells were isolated on a BD FACSAria as described in [7].

cDNA Synthesis and RT-PCR

cDNA synthesis of individual IgH (γ only) and IgL chain (κ or ƛ) was performed as previously described [7,8]. Heavy and light chain variable region genes were ligated into IgG1 or κ constant region containing plasmids (a gift from M. Nussenzweig, Rockefeller University, NY).

Antibody Production

Antibodies were expressed in vitro as described previously [7], with few modifications. In brief, 24 h before transfection with both heavy and light chain plasmids, human embryonic kidney fibroblast 293T (HEK-293T) cells were split into a 100 x 20 mm culture dish in high glucose DMEM (HyClone, GE Healthcare), supplemented with heat inactivated fetal bovine serum (FBS, 10%), glutamine (1%) and penicillin- streptomycin (1%, HyClone, GE Healthcare). Subsequently, 8 h before transfection, the medium was changed to SFM4Transfx-293 (HyClone, GE Healthcare) supplemented with glutamine (1%) and penicillin- streptomycin (1%). Cells were then cotransfected with plasmid DNA encoding IgH and IgL chains (5 μg), using Lipofectamine 2000 (Life Technologies). Supernatants were collected after 7 days of culture.

Antibodies were purified on protein G–sepharose (GE Healthcare, Life Technologies), eluted with glycine buffer (0.1 M, pH 3.5) and neutralized in Tris-HCl (1 M, pH 8). Antibody concentrations in supernatants were determined by anti–human IgG ELISA [7,8]. Purified antibodies was dialyzed extensively against PBS; their integrity was determined by nonreducing SDS gels stained with Coomassie blue and their concentration was measured by both anti–human IgG ELISA [7,8] and Nanodrop.

Binding assays using transfected HEK-293T cells

Plasma and the human monoclonal antibodies, C6 and B1, [9] were analyzed for binding to Caspr2 using a live cell-based immunofluorescence assay as previously described [10]. HEK-293T cells were transfected using Tgfp-Caspr2 or Tgfp vector (Origene) and cultured for 72 h. Cells were stained with C6 or B1 (10 µg per ml in PBS/10% FBS). Antibody binding to Caspr2 transfected cells was detected by Alexa 594-conjugated goat anti-human IgG (Life Technologies). To test for the presence of anti-Caspr2 antibodies in plasma, cells were blocked with goat IgG (Sigma-Aldrich) in PBS and FCS (10%), incubated with pre-absorbed (rabbit liver powder, Sigma-Aldrich) plasma samples (dilution 1:100 and 1:200). IgG binding was detected with Alexa 594-conjugated goat anti-human IgG (Life Technologies). Dead cells were visualized with DAPI staining (Sigma-Aldrich) and live cells were analyzed for Caspr2-IgG binding. Commercially available Anti-Caspr2 antibody directed to an extracellular epitope (NeroMab! UC Davis, CA) served as positive control. Anti- Caspr2 antibody directed to a cytoplasmic epitope (Abcam) served as negative control. Cells transfected with tGFP vector and non-transfected cell also served as controls.

Caspr2 protein expression

Embryonic brains and placentas were harvested and immediately frozen in liquid nitrogen and stored (-80°C). Brains and placentas were homogenized in ice cold homogenization buffer containing sucrose (0.32 M), HEPES (10 mM), EDTA (2mM), and protease and phosphatase inhibitors (Fisher Scientific), Homogenized brains were then centrifuged (1000 x g) for 15 min. The supernatant was recovered and centrifuged again at 200,000 x g. The pellet was re-suspended in buffer and centrifuged again at 200,000 x g. Finally, pellets were resuspend in lysis buffer (20mM Tris-HCl pH 8, 137 mM NaCl, 10% glycerol, 1% NP-40, 2 mM EDTA) and stored (-80°C). To determine protein concentration, samples were treated with Compat-Able™ protein assay preparation reagent kit (Thermo scientific) and assayed by BCA protein assay kit (Thermo scientific). Brain and placenta membrane lysates were subjected to SDS–PAGE electrophoresis using NuPAGE (Invitrogen) and transferred to PDVF membranes. Membranes were stained in blocking buffer for 1 h at RT (4% milk, 0.1% PBS-Tween) followed by incubation with anti-Caspr2 monoclonal antibody (Abcam) (1:500 in blocking buffer) overnight at 4°C. Membranes were stained with anti-rabbit IR Dye 680CW and visualized using an Odyssey Infrared Imaging System (LI-COR Biosciences). Sodium potassium ATPase was detected by antibody (Abcam, 1:10,0000) and used as the loading control. Secondary antibody alone showed no binding.

Antibody administration to pregnant dams

C57BL/6 mice (6–8 weeks old) were obtained from the Jackson Laboratory. Animal use was in accordance with institutional guidelines of the Feinstein Institute for Medical Research. For timed pregnancy, 2 females and 1 male were housed together for 14 h. The time when the male mouse was removed from the cage was designated embryonic (E) day 0.5. At E13.5, either C6 antibody (200 µg) or B1 control antibody (200 µg) were administrated by retro-orbital injection to time-pregnant mice under light anesthesia[9]. Embryos were harvested at E15.5 and processed for sex identification (described in [11]) and fetal brain pathology. Additional pregnancies were allowed to reach full term.

Immunohistology of fetal brains

E15.5 brains were fixed in paraformaldehyde (4%) overnight at 4°C followed by sucrose solution (30%) for 48 h at 4°C and then frozen in OCT. compound (Sakura) on dry ice and stored (-80˚C). Sagittal sections were cut (12 µm thick) on a Cryostat (Leica) and mounted on gelatin-coated slides and stored (-80˚C). Prior to staining, sections were thawed to RT, rinsed twice with PBS and blocked for 1 h with PBS (5%) with bovine serum albumin (BSA) in Triton X100 (0.1%) at RT. Anti-$PH3^+$ antibody (1:100, Millipore) or anti-nestin antibody (1:200, Millipore) and DAPI (1 µg per ml, Life Technologies) were added overnight at 4°C. After washing in PBS/0.1%Tween, antibody binding was detected using Alexa 488 goat anti-rabbit or anti-mouse IgG (Life Technologies) and visualized with an Axio-Imager (Z-1, Axio-Vision 4.7, Zeiss). $PH3^+$ cell quantification was performed as described in [9]. Cortical plate and cortical width measurements were obtained from multiple sections of each animal, described in [9]

Immunohistology of adult brains

Brain sections were prepared by anesthetizing mice with isoflurane prior to perfusion. They were perfused with paraformaldehyde (4%), following replacement of blood with heparinized preperfusion buffer.
Immunostaining for brain-reactive antibodies from plasma or cell supernatant was performed as described before

[4] on non-manipulated C57BL/6 (Jackson Laboratories) or CNTNAP2-/- mice (a gift from Dr. Brett S. Abrahams, Albert Einstein College of Medicine, NY).

Golgi staining and analysis

Mice exposed in utero to antibody were studied at 2 weeks or 16-20 weeks of age. Preparation of brains and Golgi staining were done by FD Rapid GolgiStain Kit (Ellicott City, MD), according to the manufacturer's protocol. Coronal sections (100 μm thick) of the CA1 region of the hippocampus were analyzed. To be included in the analysis of spines or dendritic arborization, a neuron had to include apical dendrites and a cell body. The arbor needed to be distinguished visually from nearby neurons. For the spine analysis, Z-stack (0.5-μm separation) photomicrographs of the CA1 pyramidal layer (N.A.=1.4; Axio-Imager. Z-1, Axio-Vision 4.8, Zeiss). Images were transferred to a software program (Neurolucida, MBF) that displayed the Z stack information so that the spines on the dendrites were visualized, identified and counted. For dendrite analysis. Z stack (2.0- μm separation, 345X273-μm tiles, N.A.=0.75) were collected, the files were transferred for analysis, and the tracing of the dendritic arbor was quantified and analyzed. .

Parvalbumin staining

For this analysis, we used 16–20 week old mice that had behavioral assessment. Brains were sectioned by microtome (40 μm thick) and every fourth section was collected and mounted as before (8). Every eighth Sections were incubated in anti-parvalbumin antibody (Abcam) at a 1:500 dilution in PBS (0.1 M, pH 7.4) overnight at 4°C, and . then washed and incubated with biotinylated anti-rabbit IgG (1:200, Vector Laboratories) and avidin–biotin horseradish peroxidase complex at a 1:100 dilution for 1 h followed by a 1:200 solution from Vectastain Elite ABC Kit (Vector, PK-6100), and 3,3′- diaminobenzidine (DAB, 0.05%) with hydrogen peroxide (0.003%). Sections were dehydrated and coverslipped. For quantification, we counted neurons that were positive for the parvalbumin antibody stain from matched coronal sections across the stratum pyramidale of CA1 region of the dorsal hippocampus (Br-1.20 μm to -1.80 μm). (N.A.=0.45, 600X200- μm tiles; Axio-Imager Z1; Zeiss). Comparable volumes were sampled (on average±sem, C6=0.154
± 0.005mm^2, B1=0.145±0.003mm^2, P=0.18).

Behavioral assessment

Mice that were exposed in utero to C6 or B1 were assessed at 10-14 weeks of age. They were maintained on a reverse schedule of darkness (09:00 to 21:00) and light (21:00 to 9:00), with ad libitum access to food and water. One week before testing, mice were handled for 5 days in sessions of 5–10 min during the dark period of their circadian cycle. A behavioral screen [12] was conducted to ascertain that autonomic responses and neurological reflexes were normal in C6-exposed mice (data not shown). An open field test was used to examine that locomotion was normal in C6-exposed mice; each mouse was placed in the center of a square arena (40 cm on the side) with black walls (30 cm high) and was allowed to move freely for 10 min. Animal behavior was recorded with a centrally-placed video camera using video tracking software (EthoVision v8.5, Noldus, Attleboro, MA, USA). We also analyzed the occupancy of the center of the arena (10 x 10 cm square) as a measure of anxiety and found that C6-exposed mice were comparable to B1-exposed mice (data not shown). The behavioral tests resembling the core symptomps of ASD were the marble burying assay, the social preference test, and the clockmaze task. They were performed sequentially, and a resting period of at least 48 h occurred between tests. The marble burying assay [13] was conducted in a cage (38 × 26 cm^2, 18 cm high walls) with soft bedding (4.5 cm deep), in which 20 black glass marbles (1.2 cm diameter) were placed in a 4 × 5 arrangement. The day before the experiment each mouse was familiarized to the cage (without the marbles) for 20 min and the next day, it was placed in the cage (with marbles) for 30 min. The number of marbles buried (> 50% marble covered by bedding material) was recorded. The social preference test was performed in a Y-shaped maze (each arm was 27 x 14 cm^2, 20 cm high walls made of Plexiglas) with a thin layer of bedding placed on the floor. One arm of the Y maze contained a novel object (made of plastic, ~5 cm diameter and 5 cm in height, located at the end of the arm), a second arm had a mouse (placed inside an inverted strainer cup) that had never been in contact with the test mouse, while the third arm was empty. The use of the strainer cup containing the novel mouse ensured that social approach was initiated by the subject mouse only. A day prior to the test, each mouse was placed in the empty Y maze for 10 min, and the next day it was placed for 10 min and its behavior was recorded (Ethovision v8.5). The object was cleaned with ethanol and water between each run. The times spent exploring the object and the novel mouse were used to analyze social preference. The clockmaze task [12] was performed in a circular maze with 12 exits that were located in the wall of the arena like the numbers on

the face of a clock. All exits were blocked (black plugs) except for one that led to a tunnel. Mice learned to escape from the arena, which was filled with water (20°C) to 2 cm depth, sufficient to wet the underside of the belly of mice and provide motivation to find the exit. In the first phase, each animal underwent 6 trials per day, on 2 consecutive days, with an inter-trial interval of at least 20 min. In the second phase, the location of the exit was changed and mice underwent 6 trials on 1 day. The trials were recorded with software (Ethovision 8.5) and the latencies to escape were used to measure learning. A flexibility ratio was computed by the following equation: (L2 – L1) / (L2 + L1), in which L1 was the average inverse of the latency for the last 3 trials in the first phase and L2 was the average inverse of the latency for the first 3 trials on the second phase of the task.

Statistical analysis
We used analysis of variance (ANOVA) as well as Student's t-test for datasets that were normally distributed (and with samples larger than 10). For smaller datasets, we used the Mann-Whitney test. To analyze categorical data, a Chi-squared test for independence was used. The nonparametric Kolmogorov Smirnov test was used for large datasets that were not normally distributed. All tests were performed with the statistical toolbox of Origin (versions 9 and 11), and are indicated in the text. Values were considered significant for $p < 0.05$.

What opportunities for training and professional development has the project provided?
This project has allowed two post doctoral fellows to learn behavior. Lior Brimberg has been promoted to Assistant Professor.

How were the results disseminated to communities of interest?
Publications, abstracts and oral presentations.

What do you plan to do during the next reporting period to accomplish the goals?
Not applicable

4. Impact:
These data demonstrate unequivocally that anti-brain antibody can cause an ASD-like phenotype.

What was the impact on the development of the principal discipline(s) of the project?
Nothing to Report

What was the impact on other disciplines?
Nothing to Report

What was the impact on technology transfer?
Nothing to Report

What was the impact on society beyond science and technology?
Nothing to Report

5. Changes/Problems:
Changes in approach and reasons for change.
Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them.
Nothing to Report

Changes that had a significant impact on expenditures.
Nothing to Report

Significant changes in use or care of human subjects - Nothing to Report

Significant changes in use or care of vertebrate animals - Nothing to Report

Significant changes in use of biohazards and/or select agents - Nothing to Report

6. Products:

Publications, conference papers, and presentations:

International Society of Neuroimmunology (ISNI), The International Meeting for Autism Research (IMFAR), Society for Neuroscience

Journal publications:

Brimberg, L., Mader, S., Jeganathan, V., Berlin, R., Coleman, TR., Gregersen, PK., Huerta, P., Volpe, BT., and Diamond, B. Caspr2-reactive antibody cloned from a mother of an ASD child mediates an ASD-like phenotype in mice. Molecular Psych 21:1663-1671(2016)

Mader, SA., Brimberg, L and Diamond, B. The role of brain-reactive autoantibodies in brain pathology and cognitive impairment. Frontiers In Immunology 8:1101 (2017) PMC5601985.

Books or other non-periodical, one-time publications:

Other publications, conference papers, and presentations

Brimberg, L; Mader, S; Jeganathan, V; Berlin, R; Smith, C; Gregersen P.K; Huerta, P; Volpe, B and Diamond, B. "A monoclonal antibody from a mother of a child with autism resulted in cortical and behavioral alterations in male but not female mice ". International congress of Neuroimmunology (ISNI); 2014; Mintz, Germany. Journal of Neuroimmunology Vol 275 (1-2) p.3; c2014.

Brimberg, L; Mader S; Jeganathan, V; Gregersen P.K; Huerta, P ; Volpe, B and Diamond, B. "Probing the contribution of maternal antibodies to Autism Spectrum Disorder" . Society for Neuroscience, Chicago, 2015

A monoclonal antibody from a mother of a child with autism resulted in cortical and behavioral alterations in male but not female mice Brimberg L, Mader S, Jeganathan V, Gregersen PK, Huerta, Volpe BT, Diamond B. International congress of Neuroimmunology (ISNI); 2014; Mintz, Germany. Journal of Neuroimmunology Vol 275 (1-2) p.3; c2014. Poster

A monoclonal brain-reactive antibody leads to ASD-like phenotype in male mice.
Brimberg, L; Mader S; Jeganathan, V; Huerta, P; Berlin, R; Smith, C; Gregersen P.K; Volpe, BT and Diamond, B.
International Society for Autism Research (INSAR); 2015, Salt Lake City, UT Poster

Probing the contribution of maternal antibodies to Autism Spectrum Disorder
Brimberg, L; Mader S; Jeganathan, V; Gregersen P.K; Huerta, P ; Volpe, B and Diamond, B.
Society for Neuroscience (2015), Chicago. Oral presentation.
Selected presentation for press release, Society for Neuroscience, Chicago, IL

Caspr2-reactive antibody cloned from a mother of an ASD child mediates an ASD-like phenotype in mice
Brimberg, L; Mader S; Jeganathan, V; Huerta, P; Berlin, R; Coleman, TR; Gregersen PK; Volpe, BT and Diamond, B.
International congress of Neuroimmunology (ISNI); 2016, Jerusalem, Israel. Poster.

Maternal antibodies in Autism spectrum disorder: isolation and specificity.
Brimberg, L; Mader S; Jeganathan, V; Huerta, P; Berlin, R; Coleman, TR; Gregersen PK; Volpe, BT and Diamond, B.
International Society for Autism Research (INSAR); 2015, San-Francisco. Oral presentation.

Young Investigator award, International Society for Autism Research

Maternal antibodies in Autism spectrum disorder: toward protection studies.
Brimberg, L; Mader S; Huerta, P; Volpe, BT and Diamond, B
Society for Neuroscience (2017), Washington, D.C. Poster

Website(s) or other Internet site (s)
http://www.feinsteininstitute.org/2015/10/feinstein-institute-researchers-discover-that-maternal-antibodies- are-risk-factors-for-autism-spectrum-disorder/

https://spectrumnews.org/news/maternal-immune-molecule-triggers-autism-symptoms-in-male-mice/

https://www.autismspeaks.org/science/science-news/another-study-implicates-maternal-antibodies-one- cause-autism

Technologies or techniques

Inventions, patent applications, and/or licenses Other Products

7. Participants & other collaborating organizations
What individuals have worked on the project?

Dr. Lior Brimberg – No Changes
Dr. Betty Diamond: No Changes
Dr. Peter Gregersen – No Changes
Dr. Patricio Huerta – No Changes
Dr. Bruce Volpe – No Changes

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period? No Changes

Appendices:
None

What other organizations were involved as partners? None

8. SPECIAL REPORTING REQUIREMENTS – NA

9. APPENDICES –

Molecular Psychiatry (2016) 21, 1663–1671

www.nature.com/mp

IMMEDIATE COMMUNICATION

Caspr2-reactive antibody cloned from a mother of an ASD child mediates an ASD-like phenotype in mice

L Brimberg[1,5], S Mader[1,5], V Jeganathan[1], R Berlin[2], TR Coleman[1], PK Gregersen[3], PT Huerta[4], BT Volpe[2] and B Diamond[1]

Autism spectrum disorder (ASD) occurs in 1 in 68 births, preferentially affecting males. It encompasses a group of neurodevelopmental abnormalities characterized by impaired social interaction and communication, stereotypic behaviors and motor dysfunction. Although recent advances implicate maternal brain-reactive antibodies in a causative role in ASD, a definitive assessment of their pathogenic potential requires cloning of such antibodies. Here, we describe the isolation and characterization of monoclonal brain-reactive antibodies from blood of women with brain-reactive serology and a child with ASD. We further demonstrate that male but not female mice exposed *in utero* to the C6 monoclonal antibody, binding to contactin-associated protein-like 2 (Caspr2), display abnormal cortical development, decreased dendritic complexity of excitatory neurons and reduced numbers of inhibitory neurons in the hippocampus, as well as impairments in sociability, flexible learning and repetitive behavior. Anti-Caspr2 antibodies are frequent in women with brain-reactive serology and a child with ASD. Together these studies provide a methodology for obtaining monclonal brain-reactive antibodies from blood B cells, demonstrate that ASD can result from *in utero* exposure to maternal brain-reactive antibodies of single specificity and point toward the exciting possibility of prognostic and protective strategies.

Molecular Psychiatry (2016) **21,** 1663–1671; doi:10.1038/mp.2016.165; published online 4 October 2016

INTRODUCTION

Autism spectrum disorder (ASD) is a complex neurodevelopmental disorder characterized by impaired communication and social skills, repetitive/stereotypic and inflexible behavior.[1] The incidence of ASD has been increasing sharply worldwide over the last decade and it is now estimated to affect 1 of every 68 children in United States.[2] The etiology of ASD is complex, and includes both genetic and environmental factors.

Several studies suggest that the maternal *in utero* environment can contribute to ASD in offspring. Maternal half-sibs of an ASD-affected individual are more likely to develop ASD than paternal half-sibs.[3] Maternal immune activation (for example, infection) has been implicated in ASD in human and murine offspring.[4] In murine models, elevated maternal production of interleukin-6[5] or interleukin-17[6] can alter brain development and lead to an ASD-like phenotype in the offspring. In epidemiologic studies, maternal influenza infection was associated with increased risk of ASD.[7] Moreover, genetic and environmental risk factors cooperate in determining ASD severity; children who both possess large chromosomal copy number variations (CNVs) and experience *in utero* exposure to maternal infection exhibit more severe symptoms than children who are exposed *in utero* to maternal infection, or have CNVs.[8]

We and others have shown that significantly more mothers of children with ASD have brain-reactive antibodies than unselected women of child-bearing age or mothers of a developmentally normal child.[9–13] These antibodies can enter the fetal brain[14] and disrupt development. When serum or polyclonal antibodies from these mothers were administered to pregnant mice or monkeys, the offspring exhibited neurodevelopmental and behavioral alterations.[13,15,16]

Most recently, potential antigens recognized by serum of mothers of a child with ASD were identified.[15,17] The availability of monoclonal antibodies derived from these mothers permits a clear identification of the fine specificities that impair brain development. In this paper we demonstrate methodology to obtain brain-reactive monoclonal antibodies cloned from B cells from mothers with brain-reactive serology and a child with ASD. We further demonstrate that the presence of a single monoclonal antibody targeting contactin-associated protein-like 2 (Caspr2) in the serum of a healthy pregnant female mouse can cause neurodevelopmental abnormalities in the offspring.

Caspr2, encoded by the gene CNTNAP2, has been linked to ASD.[18] It is a molecule of the neurexin family, initially described to stabilize voltage-gated potassium channels on the myelinated axons.[19] Later studies have indicated a role for Caspr2 much earlier in development. Mice lacking Caspr2 show neuronal migration abnormalities, reduced GABAergic neurons, and ASD-like behavior.[20] Caspr2 is expressed in adult brain in the cerebral cortex and hippocampus. During development, it is highly expressed in proliferating zones, consistent with its reported role in early neuronal development.[20]

[1]Center for Autoimmune and Musculoskeletal Diseases, The Feinstein Institute for Medical Research, Manhasset, NY, USA; [2]Laboratory of Functional Neuroanatomy, The Feinstein Institute for Medical Research, Manhasset, NY, USA; [3]Center for Genomics and Human Genetics, The Feinstein Institute for Medical Research, Manhasset, NY, USA and [4]Laboratory of Immune & Neural Networks, The Feinstein Institute for Medical Research, Manhasset, NY, USA. Correspondence: Dr B Diamond, Center for Autoimmune and Musculoskeletal Diseases, The Feinstein Institute for Medical Research, 350 Community Dr, Manhasset, NY 11030, USA.
E-mail: bdiamond@northwell.edu
[5]Co-first authors.
Received 7 April 2016; revised 3 August 2016; accepted 8 August 2016; published online 4 October 2016

MATERIALS AND METHODS

Research subjects

Plasma from mothers with an ASD child was obtained from the Simons Simplex Collection (http://sfari.org/resources/simons-simplex-collection).[21] Control plasma from women of child-bearing age were obtained from the Northwell Health (previously North Shore-LIJ Health System) clinical laboratory and participants in a registry at the Feinstein Institute for Medical Research (http://www.gapregistry.org). Both cohorts were described previously.[9]

Plasma of mothers of a typically developing child (determined by the mother report) were obtained from the Genotype and Phenotype registry (http://www.gapregistry.org) at the Feinstein Institute for Medical Research. The age of the mothers at the time the plasma was drawn matched the previous cohorts[9] (mean = 36.6, s.d. = 7.4, range = 22–50).

All individuals provided informed consent through the appropriate institutional review boards.

Sample collection

Blood was collected into heparinized tubes from consenting mothers enrolled in the Simons Simplex Collection, previously identified as having brain-reactive antibodies.[9] The protocol was approved by the Simons Simplex Collection as well as by the Feinstein Institute for Medical Research Institute Review Board.

Single-cell sorting

Isolation of single human memory antigenic-specific B cells was performed as previously described[22] with several modifications. B cells were purified from fresh mononuclear cells by negative selection using a B cell kit (StemCell Technology, Vancouver, BC, Canada). They were then incubated for 30 min at room temperature with human fetal brain lysate (3 μg ml^{-1}, Nouvus, Littleton, CO, USA) labeled with biotin using the EZ-Link Sulfo-NHS-Biotin labeling kit (Life Technologies, Carlsbad, CA, USA). Cells bound by biotinylated brain antigens were isolated with a biotin selection kit (StemCell Technologies) and stained with fluoroscein isothiocyanate-conjugated anti-human CD19, phycoerythrin-conjugated anti-human CD27 and allophycocyanin streptavidin to allow the separation of $CD19^+$, $CD27^+$, brain $lysate^+$ memory B cells. As a control, the fraction that initially was identified as non-brain-reactive was incubated with biotinylated brain antigen and stained as described above. No allophycocyanin-positive cells were detected in this fraction. Finally, $CD19^+$, $CD27^+$, $allophycocyanin^+$ single cells were isolated on a BD FACSAria as described in ref. 23.

Complementary DNA synthesis and reverse transcriptase-PCR

Complementary DNA synthesis of individual immunoglobulin H (γ only) and immunoglobulin L chain (κor λ) was performed as previously described.[22,23] Heavy- and light-chain variable region genes were ligated into immunoglobulin G (IgG)1 or κ constant region containing plasmids (a gift from M Nussenzweig, Rockefeller University, New York, NY, USA).

Antibody production

Antibodies were expressed *in vitro* as described previously[23] (See also Supplementary methods). Purified antibody was dialyzed extensively against PBS; integrity was determined by nonreducing sodium dodecyl sulphate gels stained with Coomassie blue and concentration was measured by both anti-human IgG ELISA[22,23] and Nanodrop.

Binding assays using transfected HEK-293T cells

Plasma and the human monoclonal antibodies, C6 and B1,[24] were analyzed for binding to Caspr2 using a live cell-based immunofluorescence assay as previously described.[25] See Supplementary Methods.

Absorption of Caspr2 antibodies from plasma with cells expressing Caspr2

Absorption using live cell-based immunofluorescence assay was described previously.[26] HEK-239T Cells were transiently transfected with turbo-green florescent protein (tGFP)-Caspr2 or tGFP only in 96-well plate as described.[25] Seventy-two hours after transfection, 50 μl fractions of plasma were incubated with the cells for one hour at 4 °C. The incubation was repeated seven times. The plasma was centrifuged for 15 min at 12 000 *g*. Immunoglobulin G concentration in the plasma was determined by ELISA and the presence or absence of Caspr2 antibodies were determined by immunofluorescence using the cell-based assay described above.

Caspr2 protein expression

See Supplementary Methods.

Caspr2 RNA expression

See Supplementary Methods.

Antibody administration to pregnant dams

C57BL/6 mice (6–8 weeks old) were obtained from the Jackson Laboratory. Animal use was in accordance with institutional guidelines of the Feinstein Institute for Medical Research. For timed pregnancy, two females and one male were housed together for 14 h. The time when the male mouse was removed from the cage was designated embryonic (E) day 0.5. Pregnant females were randomly chosen to be injected with C6 antibody or B1 control antibody (200 μg),[24] tGFP-Caspr2-absorbed plasma, or tGFP absorbed plasma or unmanipulated plasma (200 μl containing 300 μg of IgG). IgG or plasma fractions were administered by retro-orbital injection to time-pregnant mice under light anesthesia at E13.5. Embryos were harvested at E15.5 and processed for sex identification (described in ref. 27) and fetal brain pathology. Additional pregnancies were allowed to reach full-term.

Immunohistology of fetal brains

Staining of Phospho-Histone H3 (PH3) and Nestin. E15.5 brains were fixed in paraformaldehyde (4%) overnight at 4 °C followed by sucrose solution (30%) for 48 h at 4 °C and then frozen in OCT compound (VWK, Randor, PA, USA) on dry ice and stored at − 80 °C. Sagittal sections were cut (12 μm thick) on a Cryostat (Leica, Billerica, MA, USA) and mounted on gelatin-coated slides and stored at − 80 °C. Prior to staining, sections were thawed to room temperature, rinsed twice with PBS and blocked for 1 h with PBS (5%) with bovine serum albumin in Triton X100 (0.1%) at room temperature. Anti PH3 antibody (1:100, Millipore 06-570) or anti-nestin antibody (1:200, Millipore MAB353, Wetzlar, Germany) and DAPI (1 μg per ml, Life Technologies) were added overnight at 4 °C. After washing in PBS/0.1%Tween, antibody binding was detected using Alexa 488 goat anti-rabbit or anti-mouse IgG (Life Technologies) and visualized with an Axio-Imager (Z-1, Axio-Vision 4.7, Zeiss, Peabody, MA, USA). $PH3^+$ cell quantification was performed as described in ref. 24. Cortical plate and cortical width measurements were obtained from multiple sections of each animal as described.[24] In all studies, the investigator was blinded to mouse treatment.

Staining of C6 and B1. See Supplementary Methods.

Immunohistology of adult brains

Brain sections were prepared by anesthetizing mice with isoflurane prior to perfusion. They were perfused with paraformaldehyde (4%), following replacement of blood with heparinized

preperfusion buffer. In all studies of mice exposed *in utero* to C6 or B1, the investigator was blinded to group. Immunostaining for brain-reactive antibodies from plasma or cell supernatant was performed as described[9] on non-manipulated C57BL/6 (Jackson Laboratories, Bar Harbor, ME, USA) or CNTNAP2 −/− mice (a gift from Dr Brett S Abrahams, Albert Einstein College of Medicine, Bronx, NY, USA).

Nearest neighbor analysis

For this analysis, we randomly chose 16–20-week-old mice that had undergone behavioral assessment. Brains were sectioned by microtome (40 μm thick) and every fourth section was collected and mounted as before.[24] Sections were stained with anti-NeuN antibody (Millipore, Mab 337). An assessment of the nearest neighbor (MBF, Williston, VT, USA) generated information about the distribution of neurons within the layers examined. The analysis is described in Supplementary methods.

Golgi staining and analysis

Mice exposed *in utero* to antibody were randomly chosen and studied at 2 weeks or 16–20 weeks of age (after the completion of behavioral assessment). Preparation of brains and Golgi staining were done by FD Rapid GolgiStain Kit (Ellicott City, MD, USA), according to the manufacturer's protocol. Details of the analysis are described in Supplementary Methods.

Parvalbumin staining

Staining was performed similarly to NeuN staining described above on the same set of 16–20-week-old mice. Anti-parvalbumin antibody (Abcam, ab11427, Cambridge, UK) was used. For quantification details see Supplementary Methods.

Behavioral assessment

A primary screen was performed on mice that were exposed in utero to C6 or B1 at 5–7 weeks of age, followed by behavioral tests at 10–14 weeks of age. They were maintained on a reverse schedule of darkness (0900 to 2100) and light (2100 to 0900), with *ad libitum* access to food and water. Mice undergoing behavioral assessments were analyzed according to their cage number, assigned randomly. Cage numbers did not indicate the antibody the mice were exposed to *in utero* and therefore the testing was performed in a blinded fashion. Behavioral assays are described in Supplementary Methods.

Statistical analysis

We performed analysis of variance as well as Student's *t*-test for data sets that were normally distributed (and with samples larger than 10). For smaller data sets, we performed the Mann–Whitney test. To analyze categorical data, a χ^2-test for independence was used. The nonparametric Kolmogorov–Smirnov test was used for large data sets that were not normally distributed. All tests were performed with the statistical toolbox of Origin (versions 9 and 11), and are indicated in the text. Values were considered significant for $P < 0.05$. Data are presented as mean and error bars represent standard error. All tests were performed two-sided tailed.

RESULTS

Isolating brain-reactive monoclonal antibodies from a mother of an ASD child

As a full appreciation of the pathology induced by brain-reactive antibodies requires cloning the pathogenic antibodies we established a protocol to identify antigen-specific B cells from mothers with brain-reactive serology and a child with ASD. We biotinylated human fetal brain lysate to tag brain-reactive B cells, and then performed single-cell cloning and expression of IgG. Because blood was obtained number of years after the birth of the affected child, we isolated memory ($CD27^+$) B cells with reactivity to the fetal brain lysate.

Immunofluorescence revealed that the derived monoclonal antibodies stained mouse brain (Supplementary Figure 1). Here, we focus on the C6 monoclonal antibody that was found to bind the extracellular domain of Caspr2 by assessing its reactivity to ASD candidate antigens (Figure 1a). Caspr2 is a transmembrane protein in the soma, axon, dendrites and spines of neurons.[19,28,29] It stabilizes surface expression of voltage-gated potassium channels along axons,[30] glutamate receptors on dendrites[29] and the formation of new synaptic spines.[31]

We chose to focus on an anti-Caspr2 antibody as deletion of Caspr2 has been shown to lead to neurodevelopmental abnormalities,[20] and Caspr2 mutations in human pedigrees associate with neurologic abnormalities including ASD.[32–34] Thus, our choice permitted a proof-of-principle study of the potential impact of a monoclonal brain-reactive antibody on fetal brain development.

Immunohistochemical analysis revealed diminished binding of C6 to brains of mice lacking Caspr2 compared with brains of wild-type mice (Figure 1b), confirming its specificity for Caspr2. Importantly, plasma from the woman from whom C6 was derived also displayed diminished binding to Caspr2-deficient mouse brain (Supplementary Figure 2A-B). Caspr2 expression in the brain begins during fetal development, and is similar between females and males (Supplementary Figure 3). At E14, Caspr2 mRNA expression was localized to the developing cortex and to proliferating zones including the ventricular zone where excitatory projection neurons arise.[19,20] Immunohistochemistry of fetal brain (E15.5) with C6 demonstrated staining in the cortex and the ventricular zone (Supplementary Figure 2C-D).

In utero exposure to C6 affects cortex integrity

To assess the pathogenic potential of C6, we intravenously administered either non-brain-reactive control antibody, B1[24] or C6 to timed-pregnant mice on day E13.5. Maternal antibody begins to cross the placenta and enter the fetal circulation at the beginning of the second trimester of pregnancy.[35] As the blood–brain barrier is not fully formed in the developing fetus, maternal antibody present in fetal circulation can penetrate brain parenchyma starting at embryonic day E12.5. By E16.5-E17.5 the blood–brain barrier will normally exclude IgG.[14]

Strikingly, male, but not female, fetuses from dams killed 2 days after C6 administration exhibited a thinned cortical plate (Figures 2a and b). C6-exposed male, but not female, fetuses showed a decrease in proliferating cells in the developing cortex (Figures 2c and d). To confirm that the anti-Caspr2 antibodies were responsible for the alterations in the developing cortex, we performed additional experiments using plasma of the mother from whom C6 was isolated. For these studies we used: (i) unmanipulated plasma, (ii) plasma preabsorbed with cells expressing tGFP or (iii) plasma preabsorbed with cells expressing tGFP-Caspr2 (Supplementary Figure 4). Each of these preparations was administered intravenously to timed-pregnant mice on day E13.5 and the developing fetuses were analyzed 2 days later. Male fetuses exposed *in utero* to either unmanipulated plasma or plasma preabsorbed with cells expressing tGFP alone displayed a thinner cortex and a decreased number of proliferating cells (Supplementary Figure 4) than fetuses exposed to Caspr2-absorbed plasma, confirming the effects of anti-Caspr2 antibodies on brain development.

In adulthood, male offspring exposed *in utero* to C6 showed focal cortical changes. Based on nearest neighbor analysis the distance between neurons was significantly different in the entorhinal cortex between C6- and B1-exposed mice (Figure 2e).

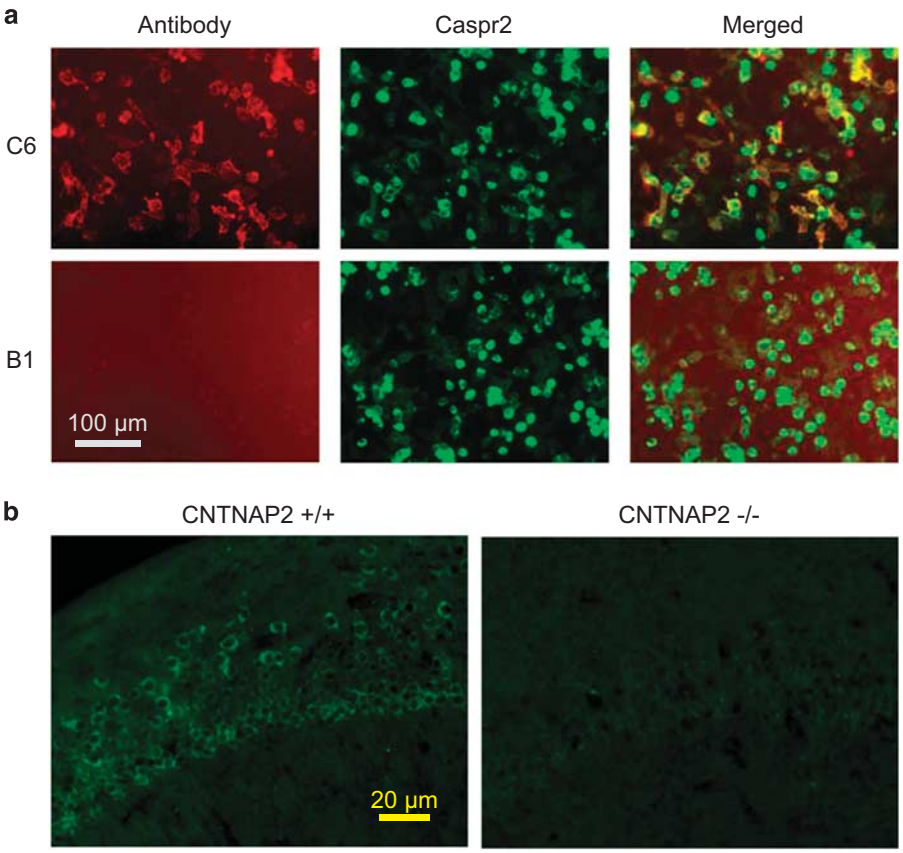

Figure 1. Brain-reactive monoclonal antibody C6 binds to Caspr2. (**a**) C6 (top panels), but not control B1 (bottom panels) antibody co-localize with Caspr2 on HEK-293T cells, expressing tGFP-Caspr2. No staining was seen on cells expressing only tGFP or non-transfected cells (data not shown). (**b**) C6 antibodies show reduced staining to the CA1 region in the hippocampus of CNTNAP2$^{-/-}$ (right) compared with wild-type (left) mice. Caspr2, contactin-associated protein-like 2; tGFP, turbo-green florescent protein.

In contrast, in the somatosensory cortex there was no apparent differences (Figure 2f).

In utero exposure to C6 affects hippocampal neurons

As the entorhinal cortex represents a major input and output structure for the hippocampus, we assessed pyramidal hippocampal neurons and observed fewer dendritic arbors and dendritic spines, and less-dendritic branching in the neurons of C6-exposed male mice compared to B1-exposed male mice (Figures 3a-e). We also examined CA1 neurons in 2-week-old male mice before full development of these cells has occurred[36] to determine whether the abnormalities observed in the adult mice represented a failure in maturation, or a late consequence of *in utero* exposure to antibody (Figures 3a–d). We found the 2-week-old C6-exposed mice exhibited reduced apical dendritic branches when compared with age-matched B1-exposed mice (Figures 3b-d), demonstrating a maturational defect.

Finally, we stained for GABAergic parvalbumin positive (PV+) neurons in adult male mice exposed *in utero* to C6 or B1, as mice with genetic deletion of Caspr2 display fewer GABAergic neurons. Fewer PV+ neurons were present in the hippocampus in C6-exposed male mice (Figure 3f), consistent with the data from Caspr2-deficient mice, which exhibit a 20% reduction in PV+ hippocampal interneurons.[20]

Behavioral abnormalities in male mice exposed to C6 *in utero*

To examine C6-mediated effects on behavior, adult offspring were studied. In total, 5–7-week-old C6-exposed mice of either sex were indistinguishable from B1-exposed of the same sex and age (B1 and C6 male: $n = 16$, five litters each; female: B1 $n = 16$, C6 = 11, five litters each), in body weight, coat, grip strength, body tone, reflexes and sickness behavior as described in ref. 37 (Supplementary Table 1). Moreover, in the open-field test, mobility and time spent in the center of the arena (suggestive of anxiety-like behavior) were not different (*t*-test, $P > 0.4$; analysis of variance center/periphery × group, $P > 0.8$, respectively).

When subjected to tasks that focus on core ASD symptoms,[38] 10–14-week-old C6-exposed male mice displayed behavioral abnormalities.

C6-exposed mice showed abnormal sociability; they spent equal time in proximity to the unfamiliar mouse and an unfamiliar object in the social approach test (Figure 4a). In contrast, B1-exposed mice spent more time in proximity to the unfamiliar mouse than the unfamiliar object (Figure 4a).

Previous ASD models defined impaired sociability as the lack of difference between time spent with the unfamiliar mouse and the unfamiliar object.[38,39] Time spent in the non-occupied area is not included in the definition of impaired sociability; C6-exposed mice spent equal time with the unfamiliar object and in the empty space. Although this might be interrupted as general motivational impairment, that is, lack of interest regardless of the stimulus, it is important to note that C6-exposed male mice spent as much time as B1-exposed male mice near the unfamiliar object (Figure 4a). This suggests that the diminished interest of C6-exposed mice in the unfamiliar mouse was specific to that stimulus and less likely to reflect a general motivational problem.

It is possible that C6-exposed mice are impaired in olfaction and therefore less likely to initiate social behavior. Although this hypothesis has not been formally tested in our study, it should be

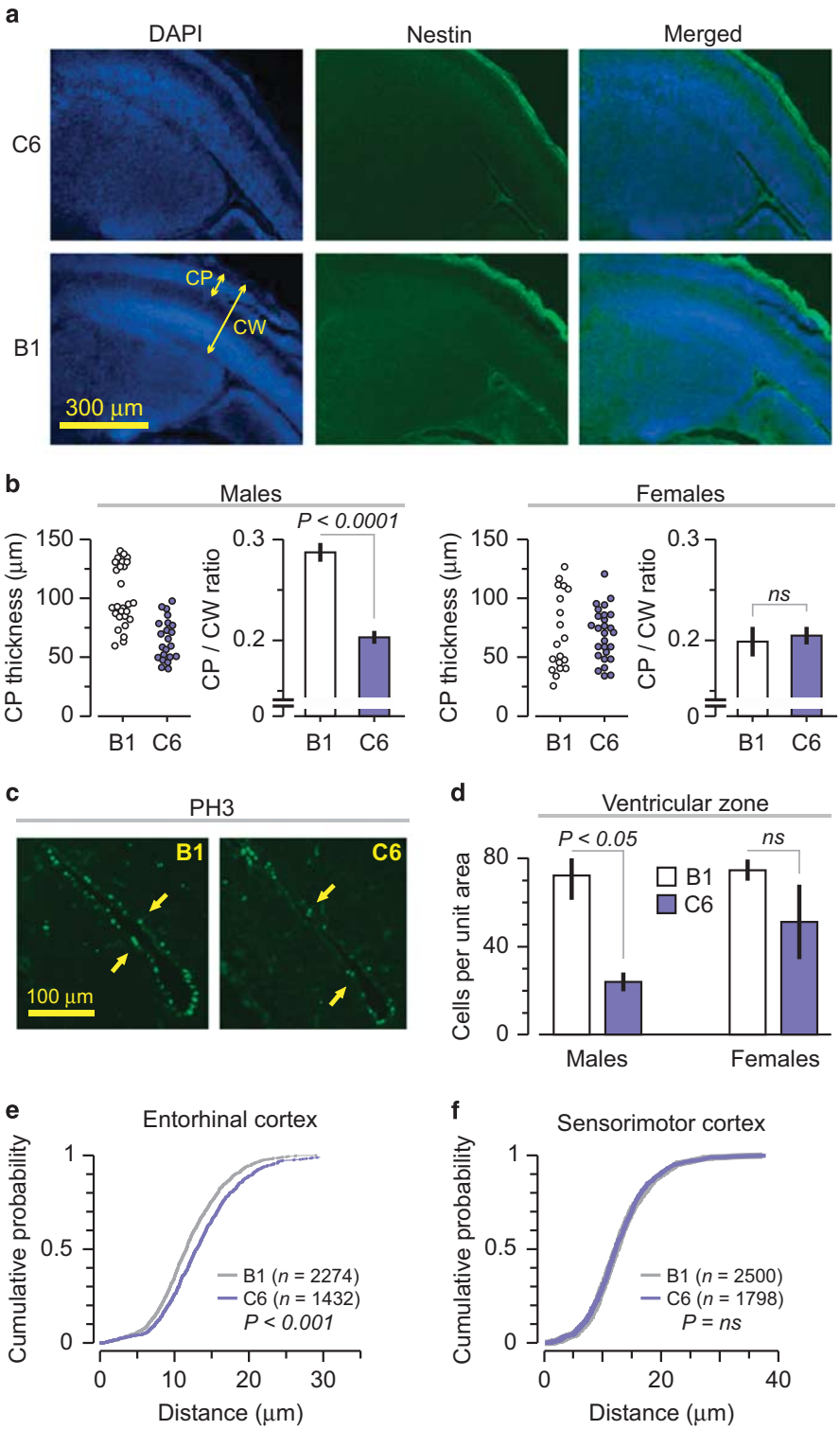

Figure 2. Male mice exposed *in utero* to C6 manifest cortical abnormalities. (**a–d**) Number of mice included in the analysis: Male: B1 = 5, C6 = 4; Female: B1 = 5, C6 = 5. Mice were derived from two litters for each antibody. (**a**) The cortical plate is abnormally thin in C6-exposed (top) relative to control B1-exposed (bottom) male brains as revealed by DAPI (left) and nestin (middle) staining; CP, cortical plate; CW, cortical width. (**b**) (Left) Quantification of cortical plate thickness. Dots correspond to number of measurements performed (Right). The ratio of cortical plate to cortical width in male and female fetal brain; $P < 0.0001$, $t = 7.15$, *t*-test. (**c**) C6-exposed male brains (right) display fewer mitotic cells than B1-exposed male brains (left) as revealed by PH3 staining. Arrows identify regions of neurogenesis in the ventricular zone (VZ). (**d**) Quantification of mitotic cells (PH3+) in the VZ, Intermediate zone and sub-plate for both males and females, as indicated. Unit area = 62.5 mm^2; $P < 0.05$, $Z = 2.34$, Mann–Whitney test. (**e–f**) Cumulative probability based on nearest neighbor analysis in five adult B1- and four adult C6-exposed mice (two litters each). Dots represent the number of cells. (**e**) Entorhinal cortex (Cell number: B1, $n = 2274$, C6, $n = 1432$), $P < 0.001$, KS. (**f**) Somatosensory cortex (Cell number: B1, $n = 2500$, C6, $n = 1798$), $P =$ N.S.

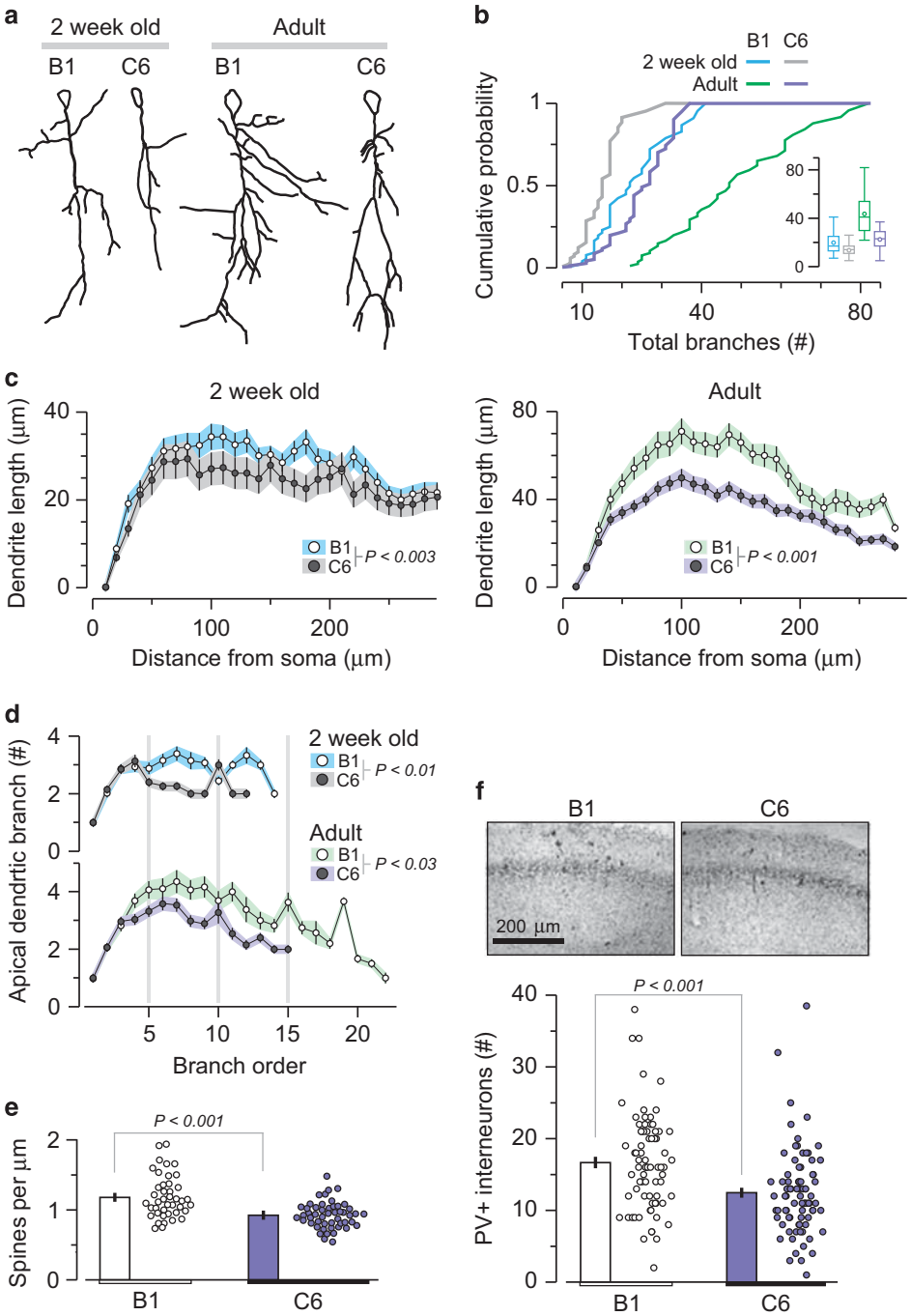

Figure 3. C6-exposed male offspring display significantly reduced hippocampal dendritic complexity. CA1 pyramidal neurons of C6-exposed male mice show reduced dendritic length and spine density. Analysis includes: 2-week-old mice ($n=4$ per group); neurons: B1 = 45, C6 = 49 and adult animals ($n=4$ per group); neurons: B1 = 70, C6 = 83. Each group includes animals from two litters. (**a**) Traced drawings of representative Golgi-impregnated CA1 pyramidal neurons from 2-week-old (left) and adult (16–20-week old) (right) B1- and C6-exposed mice. (**b**) Cumulative probability of total number of branches. 2-week-old B1 vs C6, $P<0.003$, $D=0.35$, Kolmogorov–Smirnov (KS) test; adult, $P<0.001$, $D=0.64$, KS test. Insert, box plot, represents total number of branches, with data presented as mean and quartiles. (**c**) Scholl analysis depicts dendritic length as a function of distance from the soma. Left: 2-week-old mice, $P<0.005$, $D=0.44$, KS. Right: adult mice, $P<0.001$, $D=0.97$, KS test. (**d**) Number of dendritic branches as a function of branch order, centrifugally defined to start at the origin of the tree and continue out towards the termination, and as the number of segments traversed from the origin. The C6- and B1-exposed mice differ significantly at both ages: 2-week-old, $P<0.01$, $D=0.6$, KS test; adult, $P<0.03$, $D=0.45$, KS test. (**e**) Reduced density of synaptic dendritic spines in CA1 neurons in C6-exposed male mice. Dots represent individual dendrites. B1 = 1.18 ± 0.04 spines μm^{-1}; C6 = 0.92 ± 0.02 spines μm^{-1}; $P<0.001$, $t=4.78$, t-test. (**f**) Top, representative photomicrographs of the CA1 field showing labeled PV+ interneurons. Bottom, quantification of PV+ neurons in adult B1 ($n=1251$) and C6 ($n=998$) groups, $P<0.001$, $Z=4.25$, Mann–Whitney, four animals per group, two litters.

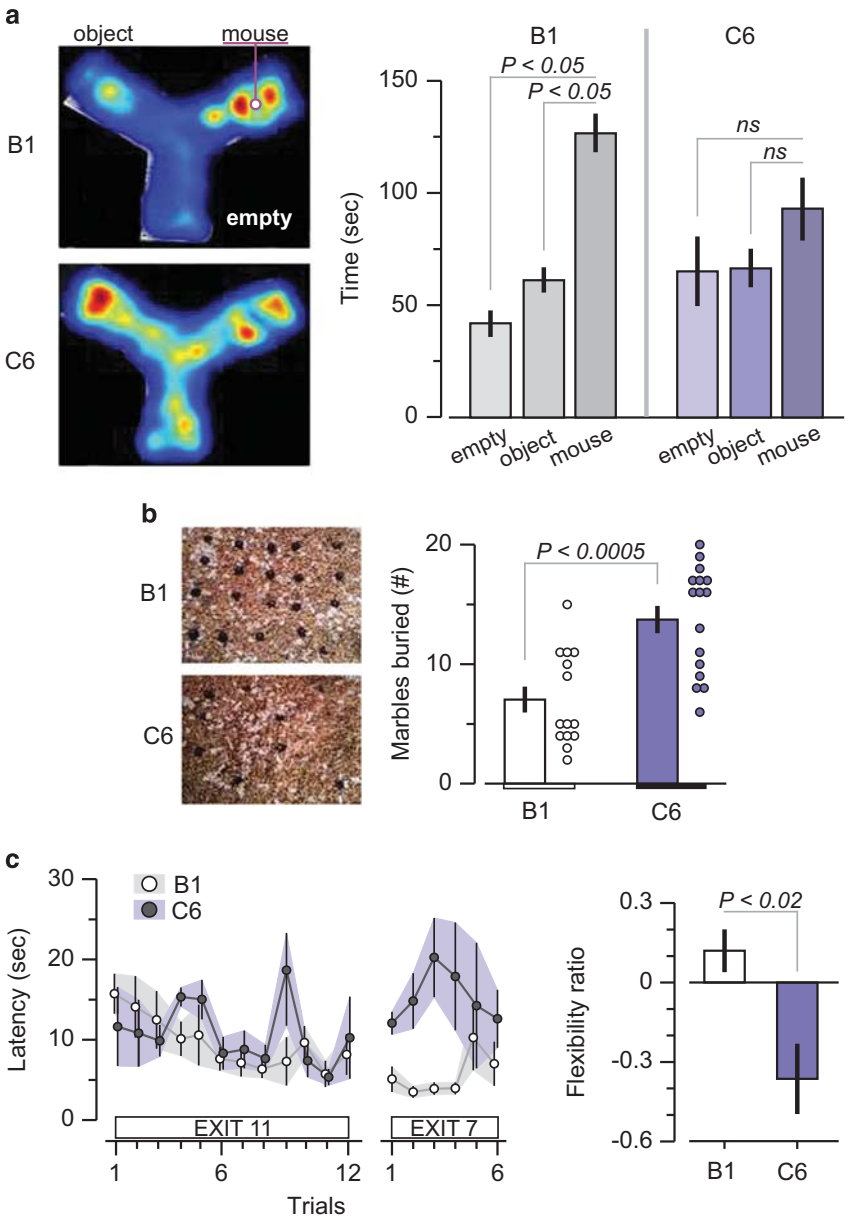

Figure 4. Impaired performance of C6-exposed male offspring in tasks that resemble core autism spectrum disorder symptoms. Maternal antibody-exposed male mice were subjected to behavioral assessment at adulthood (10–14 weeks). (**a**) Social approach task. (Left) representative heat map showing social patterns in B1- and C6-exposed mice. (Right) B1-exposed male mice ($n = 15$) displayed normal sociability, defined as spending significantly more time with the novel mouse compared with the novel object, whereas C6-exposed male mice ($n = 14$) spent a similar amount of time near the novel object and the novel mouse. Five litters per group. ANOVA, followed by Bonferroni test, $P < 0.05$. Two C6 and one B1 mouse were excluded due to a technical failure (**b**) Marble-burying task. (Left) representative examples of burying patterns in the two groups. (Right) C6-exposed mice ($n = 16$) display enhanced stereotypic behavior, that is, they bury more marbles than the B1-exposed mice ($n = 14$); $P < 0.0005$, $t = 4.2$, t-test; five litters per group. One B1 mouse was excluded because it scored more than three s.d.s above the group mean (**c**) Clock maze task. (Left) the graph shows the latency to escape from the center of the maze to a peripherally located exit. C6-exposed male mice ($n = 6$) perform similarly to B1-exposed male mice ($n = 11$) in the initial phase (Exit 11) but are impaired in the second phase, when learning a different location (Exit 7). (Right) the flexibility ratio (defined in Methods) shows that C6-exposed mice are significantly less likely to switch from a familiar exit to a novel one, showing impaired flexible learning; $P < 0.02$, $Z = 2.36$, Mann–Whitney test; 2–3 litters per group.

noted the C6-exposed mice did initiate sniffing in proximity to the unfamiliar mouse (data not shown).

C6-exposed mice buried significantly more marbles than B1-exposed mice in a test that measures stereotypic and/or compulsive behavior[40,41] (Figure 4b). Although they learned the initial exit in the clock maze test with similar efficiency as B1-exposed mice, they were impaired in learning a new exit, demonstrating a specific defect inflexible learning (Figure 4c). C6- and B1-exposed female offspring performed equivalently in these tasks, confirming the absence of an effect of C6 on the developing female fetal brain (Supplementary Figure 5).

Mothers with anti-brain antibodies and with an ASD child are more likely to harbor anti-Caspr2 antibodies

We next examined how frequently antibodies with this specificity are present in women with an ASD child. We analyzed plasma of

mothers of a child with ASD that had brain-reactive serology[9] using indirect immunofluorescence on non-permeabilized Caspr2-transfected HEK-293T cells. We found that 37% (20 of 53) displayed robust Caspr2 membrane staining, compared with 12% (8 of 63) of plasma from mothers of an ASD child lacking brain-reactive antibodies, 12% (6 of 51) of plasma from unselected women of child-bearing age and 7.6% (4 of 52) of plasma from mothers of a typically developing child bound Caspr2 (χ^2-test, $P < 0.001$).

DISCUSSION

In this study we have report a protocol for cloning brain-reactive antibody from mothers with an ASD child. Using this methodology we have demonstrated that a single monoclonal antibody can elicit profound structural brain abnormalities and lead to core ASD behavioral impairments in offspring. There is a period of time when the fetal blood–brain barrier is porous to antibodies;[14] thus potentially pathogenic autoantibodies present in the mother's blood can affect the developing brain even in the absence of detectable disease in the mother. The antibody we studied has specificity for Caspr2, a protein critical to normal brain development. We found that antibodies with Caspr2 specificity are significantly more frequent in the plasma of mothers with anti-brain antibodies and a child with ASD.

This paradigm, in which maternal antibodies affect fetal brain development with long-term cognitive effects, has already been reported in systemic lupus.[24,27,42] *In utero* exposure at day E13.5 to a subset of lupus anti-DNA monoclonal antibody that cross-react with the GluN2 A/B subunit of the *N*-methyl-D-aspartate cause long-term and selective cognitive impairments in male offspring[24] and death of female fetuses.[27] In the course of these studies, it was confirmed that maternal IgG penetrates equally male and female fetal brain parenchyma,[27] thereby setting the foundation for the current study.

The pathogenic antibody, C6, targets Caspr2. In humans, a rare variant of *CNTNAP2*, the gene that encodes Caspr2, is associated with ASD.[32] Families with mutant *CNTNAP2* display epilepsy,[18] obsessive compulsive behaviors[34] and variable, gender-dependent structural abnormalities in the brain.[43] Similarly, mice with a deletion of *CNTNAP2* display disrupted neuronal migration, a loss of inhibitory GABAergic neurons and behavioral characteristics that are considered to represent an ASD-like phenotype in mice, such as hyperactivity, stereotypic behaviors, reduced flexible learning in the Morris water maze and social behavior abnormalities.[20] *In vitro* downregulation of Caspr2 leads to a decrease in dendritic processes[28] and spine density.[31] In these studies no data are provided on whether male and female mice are equally or differentially affected. In our model exposure to C6 led to gender effects as in the human pedigrees and to histopathologic and behavioral similarities to Caspr2 deficiency *in vivo*[20] and *in vitro*,[28,31] including loss of inhibitory GABAergic neurons, reduced dendrites and spines arborization and an ASD-like phenotype.

Although the parallels between the CNTNAP2$^{-/-}$ mice and our study are striking, there are also some differences. The mechanisms leading to neurodevelopmental abnormalities in either Caspr2-deficient mice or in mice exposed *in utero* to anti-Caspr2 antibody are not fully understood. It interesting to speculate that C6 may create a functional hypomorph of Caspr2 at a critical moment in brain development. This hypothesis requires further studies.

Changes in cortical thickness as well as decreased neuronal packing[44] have been observed in ASD. It is possible that cortical thinning may reflect aberrant migration of developing neurons that is mediated by transient axonal glycoprotein-1, which colocalizes with Caspr2[30] and has a role in the migration of proliferating neurons in the cortex.[45,46] Alternatively, Caspr2 may regulate neuronal migration through its effects on glutamate receptor density,[28,29] as the *N*-methyl-D-aspartate receptor is known to regulate neuronal migration during fetal brain development.

Both neuroanatomical and behavioral alterations in C6-exposed mice involved the cortico-hippocampal circuits.[40,47] Particularly strong expression of Caspr2 has been documented in the cortex and hippocampus.[48] Further studies should examine whether increased expression explains the preferentially effect on those area, or whether particular neurons are preferentially affected by the antibody.

It is of interest that only male mice exposed *in utero* to C6 antibody exhibited neurodevelopmental abnormalities given the strong male bias in ASD.[2] Antibody penetration to the placenta and to the fetal brain is not sex dependent.[14,24,27] We also did not observe differences in the expression of Caspr2 between male and female fetuses, or at any time postpartum, and we are not aware of published data on the phenotype of CNTNAP2 $-/-$ female mice. Male gender is a factor in the linkage association between *CNTNAP2* and impaired language in a human genealogy.[32] The basis for gender preference requires further exploration; however a recent paper showed that estrogen agonist can reverse the behavioral Phenotype in Zebrafish CNTNAP2 Mutants.[49] As by day E13.5, there are hormonal differences in male and female fetuses, estrogen may extract a protective effect in C6-exposed female fetuses.

Our data suggest that anti-Caspr2 antibodies are more frequent in mothers with anti-brain antibodies and a child with ASD. It remains to be determined whether these antibodies target the same epitope on Caspr2 as the C6 antibody, and whether epitope specificity is critical to pathogenicity. It is documented that patients with encephalitis, neuromyotonia and Morvan's syndrome have antibodies against Caspr2.[50,51] It is not known how these antibodies penetrate the adult blood–brain barrier, or whether they might lead to fetal brain abnormalities.

The method for deriving human monoclonal brain-reactive antibodies from mothers with an ASD child, and the demonstration that a monclonal maternal anti-brain antibody can mediate structural brain abnormalities and behavioral changes provides the technical and conceptual basis to explore a model of ASD pathogenesis with significant diagnostic and therapeutic implications.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We thank the Simons Foundation and Elena Kowalsky at the Feinstein Institute for help obtaining blood samples. We also thank Dr Kevin J Tracey for helpful comments. The research was funded by the Department of Defense (AR130137), NIH (R43 MH106195), and The Nancy Lurie Marks Foundation and The Simons Foundation. LB is a recipient of a Brain and Behavior NARSAD Young Investigator Foundation Grant. We thank Dr. Czeslawa Kowal for her assistance in perfusing embryos.

REFERENCES

1 *Diagnostic and Statistical Manual of Mental Disorders,* 5th edn. American Psychiatric Publishing: Arlington, VA, USA, 2013.
2 Developmental Disabilities Monitoring Network Surveillance Year 2010 Principal Investigators; Centers for Disease Control and Prevention (CDC). Prevalence of autism spectrum disorder among children aged 8 years - autism and developmental disabilities monitoring network, 11 sites, United States, 2010. *MMWR Surveill Summ* 2014; **63**: 1–21.
3 Risch N, Hoffmann TJ, Anderson M, Croen LA, Grether JK, Windham GC. Familial recurrence of autism spectrum disorder: evaluating genetic and environmental contributions. *Am J Psychiatry* 2014; **171**: 1206–1213.
4 Patterson PH. Maternal infection and autism. *Brain Behav Immun* 2012; **26**: 393.

5 Smith SE, Li J, Garbett K, Mirnics K, Patterson PH. Maternal immune activation alters fetal brain development through interleukin-6. *J Neurosci* 2007; **27**: 10695–10702.
6 Choi GB, Yim YS, Wong H, Kim S, Kim H, Kim SV *et al.* The maternal interleukin-17a pathway in mice promotes autism-like phenotypes in offspring. *Science* 2016; **351**: 933–939.
7 Atladottir HO, Henriksen TB, Schendel DE, Parner ET. Autism after infection, febrile episodes, and antibiotic use during pregnancy: an exploratory study. *Pediatrics* 2012; **130**: e1447–e1454.
8 Mazina V, Gerdts J, Trinh S, Ankenman K, Ward T, Dennis MY *et al.* Epigenetics of autism-related impairment: copy number variation and maternal infection. *J Dev Behav Pediatr* 2015; **36**: 61–67.
9 Brimberg L, Sadiq A, Gregersen PK, Diamond B. Brain-reactive IgG correlates with autoimmunity in mothers of a child with an autism spectrum disorder. *Mol Psychiatry* 2013; **18**: 1171–1177.
10 Croen LA, Braunschweig D, Haapanen L, Yoshida CK, Fireman B, Grether JK *et al.* Maternal mid-pregnancy autoantibodies to fetal brain protein: the early markers for autism study. *Biol Psychiatry* 2008; **64**: 583–588.
11 Braunschweig D, Ashwood P, Krakowiak P, Hertz-Picciotto I, Hansen R, Croen LA *et al.* Autism: maternally derived antibodies specific for fetal brain proteins. *Neurotoxicology* 2008; **29**: 226–231.
12 Singer HS, Morris CM, Gause CD, Gillin PK, Crawford S, Zimmerman AW. Antibodies against fetal brain in sera of mothers with autistic children. *J Neuroimmunol* 2008; **194**: 165–172.
13 Dalton P, Deacon R, Blamire A, Pike M, McKinlay I, Stein J *et al.* Maternal neuronal antibodies associated with autism and a language disorder. *Ann Neurol* 2003; **53**: 533–537.
14 Braniste V, Al-Asmakh M, Kowal C, Anuar F, Abbaspour A, Toth M *et al.* The gut microbiota influences blood-brain barrier permeability in mice. *Sci Transl Med* 2014; **6**: 263ra158.
15 Bauman MD, Iosif AM, Ashwood P, Braunschweig D, Lee A, Schumann CM *et al.* Maternal antibodies from mothers of children with autism alter brain growth and social behavior development in the rhesus monkey. *Transl Psychiatry* 2013; **3**: e278.
16 Singer HS, Morris C, Gause C, Pollard M, Zimmerman AW, Pletnikov M. Prenatal exposure to antibodies from mothers of children with autism produces neurobehavioral alterations: a pregnant dam mouse model. *J Neuroimmunol* 2009; **211**: 39–48.
17 Braunschweig D, Krakowiak P, Duncanson P, Boyce R, Hansen RL, Ashwood P *et al.* Autism-specific maternal autoantibodies recognize critical proteins in developing brain. *Transl Psychiatry* 2013; **3**: e277.
18 Strauss KA, Puffenberger EG, Huentelman MJ, Gottlieb S, Dobrin SE, Parod JM *et al.* Recessive symptomatic focal epilepsy and mutant contactin-associated protein-like 2. *N Engl J Med* 2006; **354**: 1370–1377.
19 Poliak S, Gollan L, Martinez R, Custer A, Einheber S, Salzer JL *et al.* Caspr2, a new member of the neurexin superfamily, is localized at the juxtaparanodes of myelinated axons and associates with K+ channels. *Neuron* 1999; **24**: 1037–1047.
20 Penagarikano O, Abrahams BS, Herman EI, Winden KD, Gdalyahu A, Dong H *et al.* Absence of CNTNAP2 leads to epilepsy, neuronal migration abnormalities, and core autism-related deficits. *Cell* 2011; **147**: 235–246.
21 Fischbach GD, Lord C. The Simons Simplex Collection: a resource for identification of autism genetic risk factors. *Neuron* 2010; **68**: 192–195.
22 Zhang J, Jacobi AM, Mackay M, Aranow C, Wang T, Chinnasamy P *et al.* Identification of DNA-reactive B cells in patients with systemic lupus erythematosus. *J Immunol Methods* 2008; **338**: 79–84.
23 Tiller T, Meffre E, Yurasov S, Tsuiji M, Nussenzweig MC, Wardemann H. Efficient generation of monoclonal antibodies from single human B cells by single cell RT-PCR and expression vector cloning. *J Immunol Methods* 2008; **329**: 112–124.
24 Lee JY, Huerta PT, Zhang J, Kowal C, Bertini E, Volpe BT *et al.* Neurotoxic autoantibodies mediate congenital cortical impairment of offspring in maternal lupus. *Nat Med* 2009; **15**: 91–96.
25 Mader S, Lutterotti A, Di Pauli F, Kuenz B, Schanda K, Aboul-Enein F *et al.* Patterns of antibody binding to aquaporin-4 isoforms in neuromyelitis optica. *PLoS One* 2010; **5**: e10455.
26 Bradl M, Misu T, Takahashi T, Watanabe M, Mader S, Reindl M *et al.* Neuromyelitis optica: pathogenicity of patient immunoglobulin in vivo. *Ann Neurol* 2009; **66**: 630–643.
27 Wang L, Zhou D, Lee J, Niu H, Faust TW, Frattini S *et al.* Female mouse fetal loss mediated by maternal autoantibody. *The J Exp Med* 2012; **209**: 1083–1089.
28 Anderson GR, Galfin T, Xu W, Aoto J, Malenka RC, Sudhof TC. Candidate autism gene screen identifies critical role for cell-adhesion molecule CASPR2 in dendritic arborization and spine development. *Proc Natl Acad Sci USA* 2012; **109**: 18120–18125.
29 Varea O, Martin-de-Saavedra MD, Kopeikina KJ, Schurmann B, Fleming HJ, Fawcett-Patel JM *et al.* Synaptic abnormalities and cytoplasmic glutamate receptor aggregates in contactin associated protein-like 2/Caspr2 knockout neurons. *Proc Natl Acad Sci USA* 2015; **112**: 6176–6181.
30 Poliak S, Salomon D, Elhanany H, Sabanay H, Kiernan B, Pevny L *et al.* Juxtaparanodal clustering of Shaker-like K+ channels in myelinated axons depends on Caspr2 and TAG-1. *J Cell Biol* 2003; **162**: 1149–1160.
31 Gdalyahu A, Lazaro M, Penagarikano O, Golshani P, Trachtenberg JT, Geschwind DH. The autism related protein contactin-associated protein-like 2 (CNTNAP2) stabilizes new spines: an in vivo mouse study. *PLoS One* 2015; **10**: e0125633.
32 Alarcon M, Abrahams BS, Stone JL, Duvall JA, Perederiy JV, Bomar JM *et al.* Linkage, association, and gene-expression analyses identify CNTNAP2 as an autism-susceptibility gene. *Am J Hum Genet* 2008; **82**: 150–159.
33 Falivelli G, De Jaco A, Favaloro FL, Kim H, Wilson J, Dubi N *et al.* Inherited genetic variants in autism-related CNTNAP2 show perturbed trafficking and ATF6 activation. *Hum Mol Genet* 2012; **21**: 4761–4773.
34 Verkerk AJ, Mathews CA, Joosse M, Eussen BH, Heutink P, Oostra BA. CNTNAP2 is disrupted in a family with Gilles de la Tourette syndrome and obsessive compulsive disorder. *Genomics* 2003; **82**: 1–9.
35 Simister NE. Placental transport of immunoglobulin G. *Vaccine* 2003; **21**: 3365–3369.
36 Casanova JR, Nishimura M, Swann JW. The effects of early-life seizures on hippocampal dendrite development and later-life learning and memory. *Brain Res Bull* 2014; **103**: 39–48.
37 Chavan SS, Huerta PT, Robbiati S, Valdes-Ferrer SI, Ochani M, Dancho M *et al.* HMGB1 mediates cognitive impairment in sepsis survivors. *Mol Med* 2012; **18**: 930–937.
38 Silverman JL, Yang M, Lord C, Crawley JN. Behavioural phenotyping assays for mouse models of autism. *Nat Rev Neurosci* 2010; **11**: 490–502.
39 McFarlane HG, Kusek GK, Yang M, Phoenix JL, Bolivar VJ, Crawley JN. Autism-like behavioral phenotypes in BTBR T+tf/J mice. *Genes Brain Behav* 2008; **7**: 152–163.
40 Deacon RM. Digging and marble burying in mice: simple methods for in vivo identification of biological impacts. *Nat Protoc* 2006; **1**: 122–124.
41 Thomas A, Burant A, Bui N, Graham D, Yuva-Paylor LA, Paylor R. Marble burying reflects a repetitive and perseverative behavior more than novelty-induced anxiety. *Psychopharmacology (Berl)* 2009; **204**: 361–373.
42 Chang EH, Volpe BT, Mackay M, Aranow C, Watson P, Kowal C *et al.* Selective impairment of spatial cognition caused by autoantibodies to the N-methyl-d-aspartate receptor. *EBioMed* 2015; **2**: 755–764.
43 Tan GC, Doke TF, Ashburner J, Wood NW, Frackowiak RS. Normal variation in fronto-occipital circuitry and cerebellar structure with an autism-associated polymorphism of CNTNAP2. *Neuroimage* 2010; **53**: 1030–1042.
44 Simms ML, Kemper TL, Timbie CM, Bauman ML, Blatt GJ. The anterior cingulate cortex in autism: heterogeneity of qualitative and quantitative cytoarchitectonic features suggests possible subgroups. *Acta Neuropathol* 2009; **118**: 673–684.
45 Denaxa M, Chan CH, Schachner M, Parnavelas JG, Karagogeos D. The adhesion molecule TAG-1 mediates the migration of cortical interneurons from the ganglionic eminence along the corticofugal fiber system. *Development* 2001; **128**: 4635–4644.
46 Furley AJ, Morton SB, Manalo D, Karagogeos D, Dodd J, Jessell TM. The axonal glycoprotein TAG-1 is an immunoglobulin superfamily member with neurite outgrowth-promoting activity. *Cell* 1990; **61**: 157–170.
47 Kogan JH, Frankland PW, Silva AJ. Long-term memory underlying hippocampus-dependent social recognition in mice. *Hippocampus* 2000; **10**: 47–56.
48 Gordon A, Salomon D, Barak N, Pen Y, Tsoory M, Kimchi T *et al.* Expression of Cntnap2 (Caspr2) in multiple levels of sensory systems. *Mol Cell Neurosci* 2016; **70**: 42–53.
49 Hoffman EJ, Turner KJ, Fernandez JM, Cifuentes D, Ghosh M, Ijaz S *et al.* Estrogens suppress a behavioral phenotype in zebrafish mutants of the autism risk gene, CNTNAP2. *Neuron* 2016; **89**: 725–733.
50 Lancaster E, Huijbers MG, Bar V, Boronat A, Wong A, Martinez-Hernandez E *et al.* Investigations of caspr2, an autoantigen of encephalitis and neuromyotonia. *Ann Neurol* 2011; **69**: 303–311.
51 Irani SR, Alexander S, Waters P, Kleopa KA, Pettingill P, Zuliani L *et al.* Antibodies to Kv1 potassium channel-complex proteins leucine-rich, glioma inactivated 1 protein and contactin-associated protein-2 in limbic encephalitis, Morvan's syndrome and acquired neuromyotonia. *Brain* 2010; **133**: 2734–2748.

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

frontiers in Immunology

REVIEW
published: 11 September 2017
doi: 10.3389/fimmu.2017.01101

The Role of Brain-Reactive Autoantibodies in Brain Pathology and Cognitive Impairment

*Simone Mader, Lior Brimberg and Betty Diamond**

The Feinstein Institute for Medical Research, The Center for Autoimmune, Musculoskeletal and Hematopoietic Diseases, Northwell Health System, Manhasset, NY, United States

Antibodies to different brain proteins have been recently found to be associated with an increasing number of different autoimmune diseases. They need to penetrate the blood–brain barrier (BBB) in order to bind antigens within the central nervous system (CNS). They can target either neuronal or non-neuronal antigen and result in damage either by themselves or in synergy with other inflammatory mediators. Antibodies can lead to acute brain pathology, which may be reversible; alternatively, they may trigger irreversible damage that persists even though the antibodies are no longer present. In this review, we will describe two different autoimmune conditions and the role of their antibodies in causing brain pathology. In systemic lupus erythematosus (SLE), patients can have double stranded DNA antibodies that cross react with the neuronal *N*-methyl-D-aspartate receptor (NMDAR), which have been recently linked to neurocognitive dysfunction. In neuromyelitis optica (NMO), antibodies to astrocytic aquaporin-4 (AQP4) are diagnostic of disease. There is emerging evidence that pathogenic T cells also play an important role for the disease pathogenesis in NMO since they infiltrate in the CNS. In order to enable appropriate and less invasive treatment for antibody-mediated diseases, we need to understand the mechanisms of antibody-mediated pathology, the acute and chronic effects of antibody exposure, if the antibodies are produced intrathecally or systemically, their target antigen, and what triggers their production. Emerging data also show that *in utero* exposure to some brain-reactive antibodies, such as those found in SLE, can cause neurodevelopmental impairment since they can penetrate the embryonic BBB. If the antibody exposure occurs at a critical time of development, this can result in irreversible damage of the offspring that persists throughout adulthood.

Keywords: autoantibodies, brain, systemic lupus erythematosus, neuromyelitis optica, cognition, blood–brain barrier, maternal antibodies

OPEN ACCESS

Edited by:
Niccolo Terrando, Duke University, United States

Reviewed by:
Patrick Joseph Waters, University of Oxford, United Kingdom
Angelo A. Manfredi, Vita-Salute San Raffaele University, Italy

***Correspondence:**
Betty Diamond
bdiamond@northwell.edu

Specialty section:
This article was submitted to Inflammation, a section of the journal Frontiers in Immunology

Received: *14 July 2017*
Accepted: *22 August 2017*
Published: *11 September 2017*

Citation:
Mader S, Brimberg L and Diamond B (2017) The Role of Brain-Reactive Autoantibodies in Brain Pathology and Cognitive Impairment. Front. Immunol. 8:1101. doi: 10.3389/fimmu.2017.01101

INTRODUCTION

Over the last several years, many different anti-brain antibodies have been associated with various autoimmune diseases (1). They can be classified as binding either neuronal or non-neuronal antigen and binding extracellular or intracellular antigen (2). Most importantly, the emerging questions are if they can be used for assessment of disease risk, severity, prognosis, and whether they contribute to brain pathology.

Some autoantibodies, such as those present in paraneoplastic disorders of the central nervous system (CNS) or in celiac disease, have been consistently reported to contribute to brain pathology and to cause neurological and cognitive impairment (3–7). For other anti-brain antibodies, such as those present in multiple sclerosis (MS) or narcolepsy, pathogenicity has not been established (8–10).

Given that some autoantibodies can be found also in a subset of healthy individuals (11), it is essential to determine if the antibodies can be used for diagnostic biomarkers of disease or if the autoantibodies are indeed pathogenic. In classic paraneoplastic disorders, where patients have antibodies against intracellular antigens, such as anti-Hu antibodies, it is believed that irreversible killing of neuronal cells is mediated by T cells (12), and the antibodies appear to be a secondary event. In contrast, in some diseases, such as neuromyelitis optica (NMO), the antibodies are pathogenic (13). When pathogenic antibodies enter the CNS, there are two possible outcomes. The pathological consequence of antibody exposure can be reversible. In limbic encephalitis associated with antibodies against cell surface receptors, such as antibodies against extracellular parts of the voltage-gated potassium channel (VGKC) complex, predominantly LGI1, or *N*-methyl-D-aspartate receptor (NMDAR) subunit GluN1, removal of pathogenic antibodies often results in complete remission of severe neuropsychiatric symptoms (12, 14). Alternatively, antibodies may trigger irreversible mechanisms that may continue even when antibody is no longer present in the brain. In patients with systemic lupus erythematosus (SLE) with cognitive dysfunction, pathology can be caused by acute antibody exposure to the NMDA receptor subunits GluN2A/GluN2B and proceed to chronic damage of surviving neurons even when brain-antibodies are no longer present (15).

While brain imaging continues to advance, it remains difficult to study human tissue in those brain diseases. Thus, animal models are needed to study transient and/or permanent tissue injury and to understand which pathology is the consequence of repeated exposure to antibodies and which pathology progresses even in the absence of continued exposure to brain-antibodies. Understanding the contribution of antibodies to disease pathogenesis is essential for the development of efficient and less invasive treatment options and for disease prevention.

INTRATHECAL IgG-SYNTHESIS OR SYSTEMIC IMMUNE REACTION

Brain-reactive antibodies can be produced intrathecally or can be passively transferred from the circulation to the CNS. For example, there is a growing body of evidence that autoantibodies in MS are produced intrathecally and that the presence of antibodies in the cerebrospinal fluid (CSF) is linked to oligoclonal antigen-specific B cells, which infiltrate the impaired blood–brain barrier (BBB) (16, 17). It has been suggested that the antibodies in the CSF of MS patients target ubiquitous intracellular antigens released as cellular debris (18), consequent to neuronal damage and, therefore, constitute a secondary process in disease progression. It may commonly be the case that intrathecal production of antibody is oligoclonal, as the only B cells to take up residence in the CNS may be those that have a B cell receptor for a brain antigen.

Antibodies can also reach the brain from the systemic circulation. Two main questions arise when a disease is caused by circulating anti-brain antibodies. The first question is what triggers their production. One possibility is that the antibodies are triggered by a bacterial or viral infection and cross-react with brain antigens that share structural similarities with the microbial target, a phenomenon defined as molecular mimicry, and was described for example in Sydenham's chorea (19). Lack of negative selection against CNS antigens as the repertoire of immunocompetent B cells is established might enable activation of B cells with cross-reactivity to brain. In paraneoplastic diseases, antibodies can be produced as a response to a tumor in a non-CNS site, such as lung or ovary, which expresses brain antigens (20).

The second question is how antibodies cross the BBB. BBB endothelial cells express tight junction proteins, which allow only strictly regulated transport into and out of the brain (21). To date, there is limited information available regarding the establishment of the BBB during development; however, there appears to be a time window during which antibodies can penetrate the fetal brain before an intact BBB is established (22). Anti-brain antibodies affecting the developing brain have been suggested to be the cause, for example, in a subset of Autism spectrum disorders, as well as lead to intellectual and cognitive impairments in children born to mothers with SLE (11, 23). In adulthood, certain insults to BBB integrity allow antibodies to penetrate CNS tissue. Different insults to the functionally established BBB lead to different regions of antibody penetration in the CNS. Depending on the location where antibodies gain access to the CNS tissue, various neurological symptoms might occur. Indeed in animal models, region-dependent effects are observed (24, 25). Moreover, circumventricular organs, such as the area postrema, the subfornical organ, and the vascular organ of lamina terminalis, lack tight junction proteins and might be an area for autoantibody entry in some autoimmune diseases (26). Some antibodies might even be able to cause BBB impairment by themselves. In NMO, antibodies to glucose-regulated protein 78 have been associated with BBB disruption (27). In experimental systems, most commonly, the BBB is breached by using either bacterial lipopolysaccharides (LPS) (25), epinephrine (24), and similar agents or by using different pathogenic CNS reactive T cells (28). These manipulations may all result in additional inflammation and make it more difficult to identify the antibody-mediated effect itself.

BRAIN ANTIBODIES AND THEIR PATHOGENICITY

Pathogenicity of brain-reactive antibodies depends on the accessibility of their target epitopes, the density of their presence in tissue and, if required, the presence of effector mechanisms in the brain in sufficient amounts (28). Antibodies from patients are often injected into rodent models and must result in a phenotype

similar to the one observed in the human disease to conclude that the antibodies themselves are pathogenic. However, not all patient-derived brain-reactive antibodies bind to rodent tissue; thus, a negative outcome regarding antibody pathogenicity in rodents has to be interpreted cautiously. This is the case in patients with different inflammatory CNS diseases associated with antibodies to myelin oligodendrocyte glycoprotein (MOG), where the majority of human anti-MOG antibodies do not recognize rodent MOG (29). In addition, pathogenicity of brain-reactive antibodies requires breach of the BBB when antibodies are injected systemically into the rodent blood stream. As stated above, insults to the BBB may add confounding factors to the study of antibody pathogenicity and will direct antibody penetration to certain brain regions, which may or may not be those brain regions most often targeted in patients. Alternatively, antibodies can be directly injected into the brain by stereotactic injection, which bypasses the need to breach the BBB. In order to investigate if the antibody results in cognitive impairment, a recognized and sometimes subtle consequence of antibody-mediated pathology, a battery of behavioral assays is performed using *in vivo* models. As more and more brain antibodies are discovered, we need to extend our *in vivo* studies to address whether pathological damage is caused by direct exposure to brain antibodies or if pathology persists even when the antibody is no longer present. Most studies have focused on the effect of acute antibody exposure; only a limited number of studies addressed a possible secondary stage of damage even when the antibody is no longer present in the brain. This secondary stage could be caused through inflammation caused by infiltrating T cells, microglial activation with secretion of proinflammatory cytokines.

For example, in a model of neurocognitive SLE, it is documented that anti-DNA/anti-NMDA receptor antibodies (DNRAbs) lead to persisting neuronal damage even after the antibodies are no longer present (15). It has been recently entertained that the surviving neurons are compromised as a secondary effect mediated by microglia (15). These extended *in vivo* studies are very important for future therapeutic targeting in disease, since removal of antibodies might prevent acute tissue damage, but may not address a subsequent disease phase.

Whereas *in vivo* models to study antibody-mediated brain disease in adults all require a BBB breach, pathogenicity of maternal anti-brain antibodies can be determined without BBB impairment since the fetal BBB allows penetration of antibodies for a period of time (23, 30). Thus, injection of antibodies into pregnant rodents or immunization of rodents with the antigen prior to pregnancy permits a subsequent investigation of the offspring for behavioral impairment and/or histological abnormality. Injecting antibodies into pregnant rodents enables the study of the effect of maternal antibody exposure at one particular time point, whereas immunization with the antigen results in exposure to maternal antibody throughout pregnancy. The binding of maternal anti-brain antibodies to embryonic brain will depend on the expression level of the antigen, which can vary from expression in the adult brain. Furthermore, some antigens exhibit distinct posttranslational modification in the embryonic brain; for example, there may be differences in glycosylation patterns of the antigen (31), which may affect the binding of the antibodies.

BRAIN ANTIBODIES AND THEIR MECHANISM OF ACTION

Following the proof of a pathogenic effect of brain-reactive autoantibodies, it is of central importance to investigate the pathogenic mechanism(s) in order to develop therapeutic interventions.

In some cases, preexisting inflammation may be required to reveal an antigenic epitope or antibody binding may lead to inflammation giving rise to inflammatory mediators that lead to pathology. Alternatively, complement-dependent cellular cytotoxicity (CDCC) or antibody-dependent cytotoxicity (ADCC) can cause target cell lysis, a possible mechanism of pathogenicity of some autoantibodies (32). Some antibodies can also result in cell death or dysfunction in the absence of inflammatory cell infiltration, CDCC, and ADCC, through altering cell signaling (32). Cell signaling alterations can also activate or impede cellular processes. Finally, antibodies can also cause internalization of membrane receptors, creating functional hypomorphs (2, 32).

The mechanisms of pathogenicity will determine the degree of recovery of brain function. Whereas CDCC and ADCC are more likely to result in irreversible tissue destruction, a pathogenic effect caused by internalization of membrane receptors can be reversed upon removal of antibodies, such as occurs in limbic encephalitis (6). In some autoimmune diseases, therefore, recovery of patients may be linked to the reestablishment of a functionally intact BBB, which prevents further antibody exposure in the CNS. In other autoimmune diseases, brain-antibodies result in a chronic condition, which may be due to constant antibody exposure or to pathology that is no longer dependent on the presence of antibodies (15). Similarly, *in utero* exposure to maternal brain antibodies can cause neurodevelopmental impairments in the offspring that persist throughout adulthood due to irreversible damage at a critical time of development (23, 30).

ILLUSTRATIVE EXAMPLES

In this review, we will describe two autoimmune conditions. First, SLE was discovered to be an autoimmune disease in the 1940s, but antibodies against defined neuronal antigens have been only recently described and linked to neurocognitive dysfunction (33–35). In SLE, pathology may be caused by acute exposure to brain-antibodies, but may persist even upon antibody removal due to irreversible damage and death of neurons and secondary pruning of healthy neurons (15). The role of microglial activation in this secondary disease phase remains to be investigated.

Second, NMO was initially described as a severe variant of MS but due to the discovery of anti-astrocytic antibodies and dramatically different responses to treatment (36, 37) NMO was segregated from MS and defined as a separate disease (38). In order to enable appropriate treatment, we need to understand the reversible and irreversible effects of aquaporin-4 (AQP4)-IgG-mediated tissue damage. In addition, it is important to understand the role of pathogenic T cells for disease initiation as well as for disease progression. Removal of antibodies or blocking of antibody-mediated mechanisms might not be sufficient to address possible disease progression even when the antibody is no longer present.

Neuron-Directed Antibodies in SLE

Systemic lupus erythematosus is a chronic autoimmune disease that is characterized by inflammation, pain, and tissue damage. SLE can affect any organ, including the brain (39). Since neuropsychiatric manifestations of SLE (NPSLE) are difficult to diagnose due to the diversity of clinical presentations, which include seizures, psychosis, cognitive dysfunction, and more (40), it is difficult to estimate the frequency of neuropsychiatric SLE (NPSLE). Many symptoms, such as headache or demyelination are not unique to NPSLE but can also be found in other autoimmune diseases. Studies claim that as few as 10% to as many as 90% of SLE patients suffer from neuropsychiatric symptoms (41). Cognitive impairment manifested as memory deficit is one of the most commonly observed symptoms in NPSLE patients (42), but is still poorly understood. It may be caused by a variety of mechanisms, both antibody and non-antibody mediated. Hypertension and accelerated atherosclerosis can also lead to cognitive impairment and confound the assessment of diseases-specific mechanisms.

To date, over 100 autoantibodies have been associated with SLE, of which, some associate with neuropsychiatric symptoms (43). Certain autoantibodies, such as anti-ribosomal P, anti-neurofilament, anti-endothelial, anti-Ro, or anti-Smith antibodies have been associated with neuropsychiatric manifestations other than cognitive impairment, whereas anti-neuronal, antiphospholipid, and anti-double stranded DNA (dsDNA) antibodies cross-reactive with the *N*-methyl-D-aspartate receptor (NMDAR) subunits GluN2A or GluN2B (anti-NR2) have been linked to neurocognitive impairment in SLE (44–48). Here, we describe in more detail the contribution of anti-dsDNA–NMDAR antibodies to cognitive impairment in SLE patients.

dsDNA–NMDAR CROSS-REACTIVE ANTIBODIES RESULT IN COGNITIVE IMPAIRMENT IN SLE

Anti-double stranded (ds) DNA antibodies are diagnostic of SLE. Previously, our group has shown that some SLE patients harbor anti-dsDNA antibody, which cross-react with a peptide sequence DWEYS present in the extracellular domain of the GluN2A and GluN2B subunits of the NMDAR. This cross-reactivity was first detected using the murine monoclonal anti-DNA antibody R4A. DNA-GluN2 cross-reactive antibodies (DNRAbs) bind to the extracellular part of GluN2 (49). DNRAbs can be detected either by ELISA or by a cell-based assay using human embryonic kidney (HEK) cells expressing the subunits GluN2A or GluN2B in combination with GluN1 (15, 50). They bind preferentially to the active configuration of the NMDAR and enhance the influx of calcium into the cell (51). They are found in approximately 40% of SLE patients (52). It remains to be investigated how the systemically produced DNRAbs gain access to the CNS. It has been suggested that they are able to breach the BBB by themselves (53), or other factors such as cytokines/chemokines or complement activation may be needed.

The pathogenicity of these antibodies was first demonstrated by injecting R4A into mouse brain, leading to apoptosis of neuronal cells. At lower concentrations, the antibody augments NMDAR-mediated synaptic potentials; at higher concentrations, it triggers mitochondrial stress and apoptosis through binding specifically to GluN2A-containing NMDARs (**Figure 1**). DNRAbs were eluted from the brain of a SLE patient and also caused neuronal apoptosis and cognitive impairment when injected into mice (25). Mice immunized with the DWEYS sequence multimerized on a polylysine backbone (termed MAP-DWEYS) develop DNRAbs, which cause loss of hippocampal neurons after LPS-induced compromise of BBB integrity (54). This occurs in the absence of inflammatory cell infiltration, CDCC, or ADCC. DNRAb-induced neuronal cell death results in cognitive dysfunction and spatial memory impairment associated with structural abnormalities in the surviving pyramidal neurons in the hippocampus (15). The change in spatial memory that occurred after LPS-facilitated DNRAb penetration into the hippocampus is accompanied by expansion in place field size of CA1 place cells in the hippocampus and shortened dendritic processes and spines of surviving hippocampal pyramidal cells (15). Remarkably, the functional and structural changes, which cause alterations in spatial cognition occur at a time when the antibodies are no longer present in the hippocampus and BBB integrity has been restored. Currently, we are investigating the role of microglial activation in the pathology. We believe that there is a two hit model in SLE. Our animal model showed that exposure of neurons to DNRAbs results in neuronal cell death. However, surviving neurons in the hippocampus show structural abnormalities, which are likely to be caused through secondary pruning of the surviving neurons by activated microglial cells (15). In contrast, removal of anti-brain antibodies in limbic encephalitis, which also target the NMDAR, mostly reverses disease symptoms, as these antibodies do not cause cell death.

In the animal model, neuronal cell death can be abolished through administration of the NMDAR antagonist memantine prior to BBB breach by LPS (24). Memantine has no effect on antibody binding, but blocks the triggering of NMDAR activation by DNRAbs.

Studies in patients show that NPSLE is associated with increased levels of GluN2A/GluN2B antibodies in the blood (55), and CSF titers of GluN2A/GluN2B antibodies correlate with the severity of NPSLE (56). Some studies have also associated cognitive impairments in NPSLE with the presence of anti-GluN2A/GluN2B antibodies (57, 58). Certainly, cognitive impairments in NPSLE will not be solely caused by those antibodies; other antibodies or cytokines likely also contribute to cognitive problems in NPSLE (55).

MATERNAL DNRAbs ARE NEUROTOXIC AND HAVE A GENDER-SPECIFIC EFFECT

During fetal development, pathogenic antibodies such as DNRAbs can penetrate the embryonic brain before the BBB is functionally established (59). Studies suggest an increased incidence of learning disabilities, fetal loss, and altered sex ratio in children of SLE mothers (60–63). It was, therefore, of interest to investigate the effect of DNRAbs on the fetal brain. We established a mouse

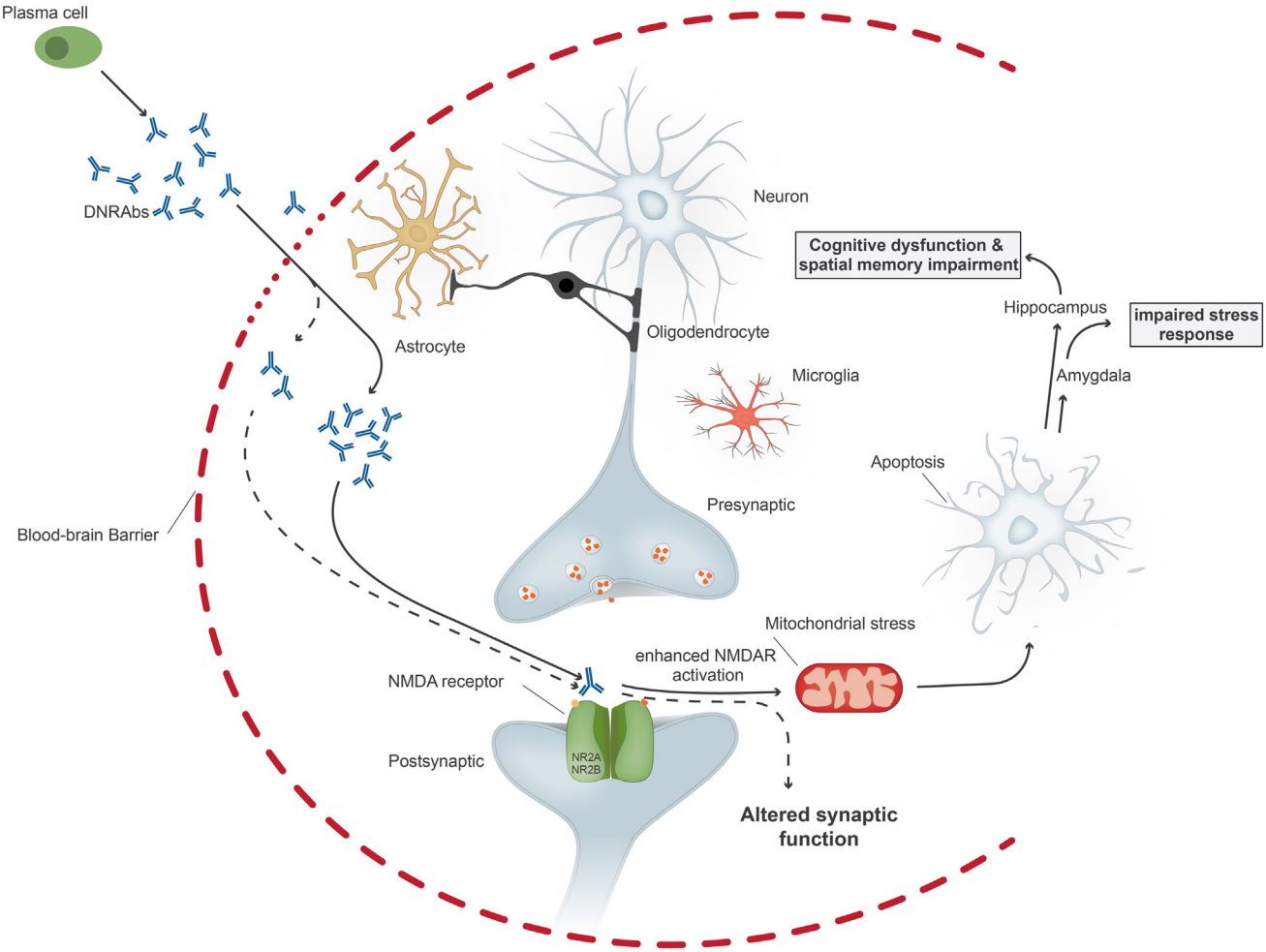

FIGURE 1 | Upon penetration of anti-DNA/anti-NMDA receptor antibodies (DNRAbs) into the central nervous system through the impaired blood–brain barrier, the antibodies trigger two different pathomechanisms dependent on the antibody concentration. At high concentrations (continuous line), the antibodies lead to mitochondrial stress and leads to neuronal apoptosis through receptors containing GluN2A. If the neuronal loss occurs in the hippocampus, the antibody binding results in spatial memory impairment and cognitive dysfunction. Binding of antibodies in the amygdala affects the stress response. At lower concentrations (dotted line), antibody binding results in altered synaptic function.

model in which pregnant mice expressed DNRAbs throughout gestation (30, 64). Maternal DNRAbs antibodies caused neuronal death in the fetal neocortex and resulted in cortical abnormalities and cognitive impairment in the adult male offspring. In contrast to the cognitive impairment observed in male mice, maternal DNRAbs resulted in increased death of female fetuses, thereby skewing the gender ratio of living offspring (30, 64). We showed that there was no difference in transplacental transfer of the pathogenic antibodies to male or female fetal brain. The gender-dependent effect may be explained by an increased expression of GluN2A in the fetal female brainstem during development compared to male littermates, or to gender-dependent differences in the vulnerability of fetal neurons to GluN2A signaling (64). Neutralization of pathogenic antibodies during pregnancies, perhaps by decoy antigen, may prevent neurodevelopmental impairment.

It should be noted that other antibodies present in SLE patients may affect fetal neurodevelopment. For example, antiphospholipid antibodies can lead to placental problems affecting fetal growth or fetal loss. Moreover, a study suggested that learning disabilities in children born to a mother with SLE were associated with high titers of maternal antiphospholipid antibody (65).

Astrocyte-Directed Antibodies in NMO

While most anti-brain antibodies target epitopes expressed on neuronal cells, anti-brain antibodies can also be directed to antigens expressed on non-neuronal cells, and thereby cause different brain pathology. In NMO, anti-brain antibodies bind to a protein expressed on astrocytes. NMO is a neurological autoimmune disease that is characterized by the presence of antibodies that bind to the water channel protein AQP4 (66), which is expressed on astrocytic endfeet that surround blood vessels. AQP4 is

particularly expressed at the BBB interface. Approximately 80% of NMO patients harbor AQP4 antibodies and the presence of AQP4 antibodies has important diagnostic and prognostic significance (67, 68). These antibodies are conformation dependent and can be detected with highest sensitivity using a cell-based assay with HEK cells expressing AQP4 on their cell surface (67, 69). The presence of AQP4 antibodies differentiates NMO from MS, which have overlapping clinical symptoms, particularly at disease onset. It is of high importance to differentiate MS from NMO since they benefit from different treatment choices (70). Several studies consistently showed that AQP4 antibodies are not present in MS patients or healthy controls and if found they predict development of NMO (68). Thus, AQP4-IgG serostatus has been included in the diagnostic criteria for the disease (38).

Neuromyelitis optica patients have lesions in areas of high AQP4 expression, such as the brain, optic nerve, and spinal cord (71). Histological findings show antibody deposition around blood vessels in the brain of patients (72). The disease primarily presents with astrocyte loss, inflammation with infiltration of granulocytes, macrophages and T cells, deposition of antibodies

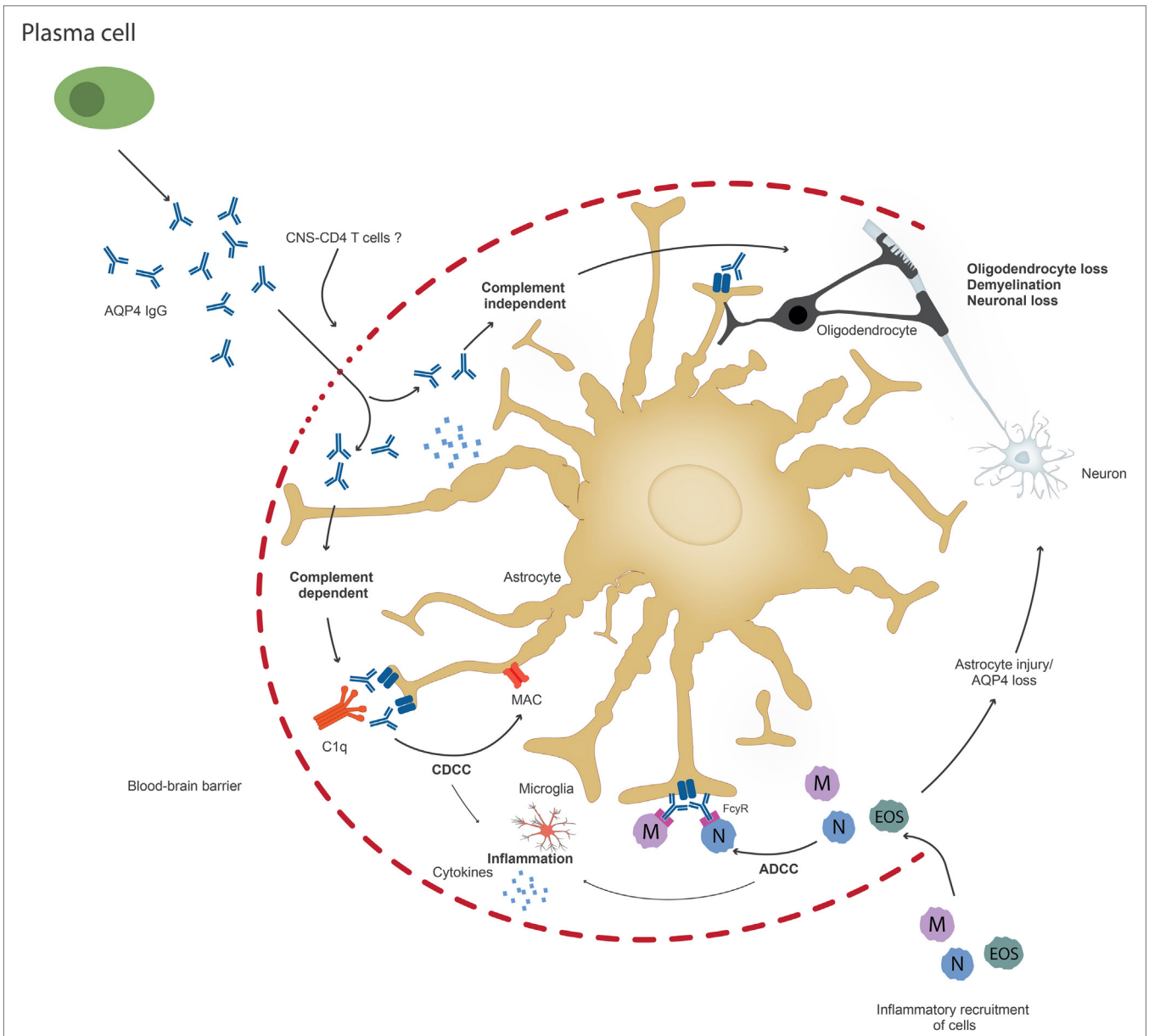

FIGURE 2 | Upon penetration of aquaporin-4 (AQP4) antibodies to the central nervous system, possibly through the help of pathogenic T cells, AQP4 antibodies can lead to astrocyte impairment either through complement-dependent cellular cytotoxicity (CDCC), antibody-dependent cytotoxicity (ADCC), or through downregulation of AQP4. Inflammation might occur prior to or secondary to antibody penetration. Ultimately, oligodendrocytes are affected resulting in demyelination and neuronal loss.

and complement around blood vessels and, in a later stage of the disease, demyelination, neuronal loss, and scar formation (72, 73). It remains to be investigated how AQP4-IgG that binds to astrocytes can damage oligodendrocytes and how the demyelination observed in NMO occurs. AQP4 antibodies are produced in the systemic circulation of patients and can be found at high serum titers in the CSF (74, 75). It has been suggested that AQP4 antibodies are produced through molecular mimicry to certain microbes (76), a hypothesis, which needs to be further investigated.

Several *in vitro* and *in vivo* models show a pathogenic effect of the AQP4 antibody either by itself, in association with pathogenic T cells, complement or different cytokines and chemokines (28, 77–81) It is possible that AQP4-IgG acts through multiple mechanisms, as suggested by pathological findings showing that, within the same patient, complement deposition is present in some active NMO lesions, while other lesions lack complement deposition (82). In current rodent models, either AQP4-IgG is injected directly into the brain or the BBB is breached prior to antibody injection, often by autoimmune encephalitis (EAE), administering activated autoreactive T cells directed to different CNS antigens (79, 83). In the human disease, we do not know how antibodies enter the brain. It has been suggested that circumventricular organs might be a possible route of entry, supported by findings of NMO lesions in these areas particularly at disease onset (84). Antibodies directed to glucose-regulated protein 78 were recently associated with BBB disruption in NMO and might facilitate penetration of AQP4-IgG antibodies into the CNS (27).

Once AQP4 antibodies penetrate the brain, they bind to astrocytes and trigger CDCC or ADCC (37) (**Figure 2**). It has been suggested that these two mechanisms result in astrocyte loss and inflammation and cause or increase BBB damage, which leads to further oligodendrocyte injury and demyelination, which finally results in neuronal loss (37). However, it is also possible that inflammation occurs prior to AQP4 IgG infiltration. It is possible that pathogenic T cells, maybe AQP4 specific or directed to other CNS antigens, are important not only to facilitate antibody production, but also for BBB disruption and may be required for astrocyte and neuronal damage. Current animal models do not closely resemble human patients with respect to the size and location of NMO lesions (13). This discrepancy could be caused by the choice of the target antigen of pathogenic T cells in animal models (85). It is also possible that NMO patients harbor not only AQP4 antibodies but also antibodies to neuronal antigens, which may or may not contribute to disease pathology. More studies are needed that address the role of other antibodies, microglial activation, and proinflammatory cytokine secretion, which could also be responsible for irreversible disease damage observed in NMO, possibly even when the AQP4-IgG antibodies are no longer present in the brain. There is evidence that AQP4-IgG also affects AQP4 function (86).

Cognitive dysfunction has only been recently assessed in NMO patients and needs further investigation (87–89). NMO patients have a different frequency and pattern of cognitive impairment compared to MS patients, suggesting different mechanisms of brain injury (90). Further animal models are needed to study if AQP4 antibodies contribute to cortical neuronal loss and if they can lead to cognitive impairment.

Current studies are trying to develop less invasive treatment options for NMO patients to bypass the highly immunosuppressive treatment (37). One possibility would be to block the AQP4 antibodies or their mode of action. Recently, Eculizumab, a monoclonal antibody that inhibits the classical complement pathway, has been shown to be an effective treatment in an open-label study, suggesting an important role for CDC in NMO (91). However, other effector pathways cannot be ruled out. Thus, it is very important to understand the direct effect of AQP4-IgG on tissue pathology, whether there are other pathogenic effector mechanisms and what processes may be initiated by antibody but continue even when antibody is no longer present. Targeting acute exposure of AQP4-IgG through inhibiting CDCC or/and ADCC might not be sufficient to prevent tissue destruction.

MATERNAL AQP4 ANTIBODIES IN NMO PREGNANCIES

Neuromyelitis optica patients have only recently been shown to have an increased frequency of miscarriages (92). Larger follow-up studies are needed to investigate the long-term effect of *in utero* exposure to AQP4-IgG on children of NMO patients, but there are case studies suggesting that maternal AQP4 IgG might result in birth defects (92, 93). Since astrocytes are expressed rather late in development, it is possible that AQP4 is expressed on astrocyte precursor cells during embryonic development.

CONCLUSION

Determining the mechanism of action and pathogenicity of several brain-reactive autoantibodies could facilitate more accurate and rapid diagnosis and enable novel treatment options. Here, we describe two examples of autoimmune diseases, which are mediated, at least in part, by autoantibodies and their pathology is well characterized. Both antibodies in those diseases are targeting extracellular antigens on brain cells, either neurons or astrocytes, but differ in their mechanism(s) of action, and hence their pathology.

Anti-DNA/anti-NMDA receptor antibodies in SLE are targeting neurons, resulting in neuronal cell death by enhancing NMDAR activation. Depending on the localization of BBB impairment, DNRAbs result in different neurocognitive or neurobehavioral phenotypes. There is no CNS inflammation following acute exposure to DNRAbs. The sustained, chronic state of neuronal damage secondary to neuronal death that persists after antibody exposure is no longer present in the CNS may reflect either neuron intrinsic effects secondary to antibody exposure or microglial activation.

Brain antibodies in NMO bind to the astrocyte water channel protein AQP4 and result in irreversible astrocyte damage due to CDCC or ADCC. There is increasing evidence that AQP4-IgG can also act by themselves and result in reversible internalization of the AQP4-IgG complex, which is coupled to the excitatory amino acid transporter (EAAT2) endocytosis (94). Different mechanisms might contribute to reversible and irreversible tissue damage of NMO patients [**Figure 2**, modified from Ref. (37)]. NMO is an example

of an antibody-mediated disease where brain pathology of patients shows an inflammatory infiltrate in the CNS, yet, the role of pathogenic T cells in the disease pathogenesis remains to be investigated.

In order to enable appropriate and less invasive treatment, we need to understand the acute and chronic effects of antibody exposure.

AUTHOR CONTRIBUTIONS

All authors contributed to writing the review.

ACKNOWLEDGMENTS

The authors thank Benjamin Obholzer for the image design.

FUNDING

This work was supported by the National Institutes of Health grant NIH 1P01 AI073693 (BD), a following grant from the SLE Lupus Foundation (SM), and a Brain and Behavior NARSAD Young Investigator Foundation Grant (LB).

REFERENCES

1. Brimberg L, Mader S, Fujieda Y, Arinuma Y, Kowal C, Volpe BT, et al. Antibodies as mediators of brain pathology. *Trends Immunol* (2015) 36:709–24. doi:10.1016/j.it.2015.09.008
2. Diamond B, Honig G, Mader S, Brimberg L, Volpe BT. Brain-reactive antibodies and disease. *Annu Rev Immunol* (2013) 31:345–85. doi:10.1146/annurev-immunol-020711-075041
3. Hadjivassiliou M, Grünewald RA, Chattopadhyay AK, Davies-Jones GA, Gibson A, Jarratt JA, et al. Clinical, radiological, neurophysiological, and neuropathological characteristics of gluten ataxia. *Lancet* (1998) 352:1582–5. doi:10.1016/S0140-6736(98)05342-2
4. Hadjivassiliou M, Boscolo S, Davies-Jones GA, Grünewald RA, Not T, Sanders DS, et al. The humoral response in the pathogenesis of gluten ataxia. *Neurology* (2002) 58:1221–6. doi:10.1212/WNL.58.8.1221
5. Boscolo S, Sarich A, Lorenzon A, Passoni M, Rui V, Stebel M, et al. Gluten ataxia: passive transfer in a mouse model. *Ann N Y Acad Sci* (2007) 1107:319–28. doi:10.1196/annals.1381.034
6. Dalmau J, Gleichman AJ, Hughes EG, Rossi JE, Peng X, Lai M, et al. Anti-NMDA-receptor encephalitis: case series and analysis of the effects of antibodies. *Lancet Neurol* (2008) 7:1091–8. doi:10.1016/S1474-4422(08)70224-2
7. Dalmau J, Lancaster E, Martinez-Hernandez E, Rosenfeld MR, Balice-Gordon R. Clinical experience and laboratory investigations in patients with anti-NMDAR encephalitis. *Lancet Neurol* (2011) 10:63–74. doi:10.1016/S1474-4422(10)70253-2
8. Hohlfeld R, Dornmair K, Meinl E, Wekerle H. The search for the target antigens of multiple sclerosis, part 2: CD8+ T cells, B cells, and antibodies in the focus of reverse-translational research. *Lancet Neurol* (2016) 15:317–31. doi:10.1016/S1474-4422(15)00313-0
9. Ahmed SS, Volkmuth W, Duca J, Corti L, Pallaoro M, Pezzicoli A, et al. Antibodies to influenza nucleoprotein cross-react with human hypocretin receptor 2. *Sci Transl Med* (2015) 7:294ra105. doi:10.1126/scitranslmed.aab2354
10. Bergman P, Adori C, Vas S, Kai-Larsen Y, Sarkanen T, Cederlund A, et al. Narcolepsy patients have antibodies that stain distinct cell populations in rat brain and influence sleep patterns. *Proc Natl Acad Sci U S A* (2014) 111:E3735–44. doi:10.1073/pnas.1412189111
11. Brimberg L, Sadiq A, Gregersen PK, Diamond B. Brain-reactive IgG correlates with autoimmunity in mothers of a child with an autism spectrum disorder. *Mol Psychiatry* (2013) 18:1171–7. doi:10.1038/mp.2013.101
12. Lancaster E. The diagnosis and treatment of autoimmune encephalitis. *J Clin Neurol* (2016) 12:1–13. doi:10.3988/jcn.2016.12.1.1
13. Bradl M, Lassmann H. Experimental models of neuromyelitis optica. *Brain Pathol* (2014) 24:74–82. doi:10.1111/bpa.12098
14. Hughes EG, Peng X, Gleichman AJ, Lai M, Zhou L, Tsou R, et al. Cellular and synaptic mechanisms of anti-NMDA receptor encephalitis. *J Neurosci* (2010) 30:5866–75. doi:10.1523/JNEUROSCI.0167-10.2010
15. Chang EH, Volpe BT, Mackay M, Aranow C, Watson P, Kowal C, et al. Selective impairment of spatial cognition caused by autoantibodies to the *N*-methyl-D-aspartate receptor. *EBioMedicine* (2015) 2:755–64. doi:10.1016/j.ebiom.2015.05.027
16. Obermeier B, Lovato L, Mentele R, Brück W, Forne I, Imhof A, et al. Related B cell clones that populate the CSF and CNS of patients with multiple sclerosis produce CSF immunoglobulin. *J Neuroimmunol* (2011) 233:245–8. doi:10.1016/j.jneuroim.2011.01.010
17. Stern JN, Yaari G, Vander Heiden JA, Church G, Donahue WF, Hintzen RQ, et al. B cells populating the multiple sclerosis brain mature in the draining cervical lymph nodes. *Sci Transl Med* (2014) 6:248ra107. doi:10.1126/scitranslmed.3008879
18. Brändle SM, Obermeier B, Senel M, Bruder J, Mentele R, Khademi M, et al. Distinct oligoclonal band antibodies in multiple sclerosis recognize ubiquitous self-proteins. *Proc Natl Acad Sci U S A* (2016) 113:7864–9. doi:10.1073/pnas.1522730113
19. Kirvan CA, Swedo SE, Heuser JS, Cunningham MW. Mimicry and autoantibody-mediated neuronal cell signaling in Sydenham chorea. *Nat Med* (2003) 9:914–20. doi:10.1038/nm892
20. Dalmau J, Rosenfeld MR. Paraneoplastic syndromes of the CNS. *Lancet Neurol* (2008) 7:327–40. doi:10.1016/S1474-4422(08)70060-7
21. Engelhardt B. Neuroscience. Blood-brain barrier differentiation. *Science* (2011) 334:1652–3. doi:10.1126/science.1216853
22. Kowal C, Athanassiou A, Chen H, Diamond B. Maternal antibodies and developing blood-brain barrier. *Immunol Res* (2015) 63:18–25. doi:10.1007/s12026-015-8714-5
23. Brimberg L, Mader S, Jeganathan V, Berlin R, Coleman TR, Gregersen PK, et al. Caspr2-reactive antibody cloned from a mother of an ASD child mediates an ASD-like phenotype in mice. *Mol Psychiatry* (2016) 21:1663–71. doi:10.1038/mp.2016.165
24. Huerta PT, Kowal C, DeGiorgio LA, Volpe BT, Diamond B. Immunity and behavior: antibodies alter emotion. *Proc Natl Acad Sci U S A* (2006) 103:678–83. doi:10.1073/pnas.0510055103
25. Kowal C, Degiorgio LA, Lee JY, Edgar MA, Huerta PT, Volpe BT, et al. Human lupus autoantibodies against NMDA receptors mediate cognitive impairment. *Proc Natl Acad Sci U S A* (2006) 103:19854–9. doi:10.1073/pnas.0608397104
26. Papadopoulos MC, Verkman AS. Aquaporin water channels in the nervous system. *Nat Rev Neurosci* (2013) 14:265–77. doi:10.1038/nrn3468
27. Shimizu F, Schaller KL, Owens GP, Cotleur AC, Kellner D, Takeshita Y, et al. Glucose-regulated protein 78 autoantibody associates with blood-brain barrier disruption in neuromyelitis optica. *Sci Transl Med* (2017) 9:eaai9111. doi:10.1126/scitranslmed.aai9111
28. Bradl M, Misu T, Takahashi T, Watanabe M, Mader S, Reindl M, et al. Neuromyelitis optica: pathogenicity of patient immunoglobulin in vivo. *Ann Neurol* (2009) 66:630–43. doi:10.1002/ana.21837
29. Mayer MC, Breithaupt C, Reindl M, Schanda K, Rostásy K, Berger T, et al. Distinction and temporal stability of conformational epitopes on myelin oligodendrocyte glycoprotein recognized by patients with different inflammatory central nervous system diseases. *J Immunol* (2013) 191:3594–604. doi:10.4049/jimmunol.1301296
30. Lee JY, Huerta PT, Zhang J, Kowal C, Bertini E, Volpe BT, et al. Neurotoxic autoantibodies mediate congenital cortical impairment of offspring in maternal lupus. *Nat Med* (2009) 15:91–6. doi:10.1038/nm.1892
31. Laitinen L, Virtanen I, Saxen L. Changes in the glycosylation pattern during embryonic development of mouse kidney as revealed with lectin conjugates. *J Histochem Cytochem* (1987) 35:55–65. doi:10.1177/35.1.3794309
32. Ludwig RJ, Vanhoorelbeke K, Leypoldt F, Kaya Z, Bieber K, McLachlan SM, et al. Mechanisms of autoantibody-induced pathology. *Front Immunol* (2017) 8:603. doi:10.3389/fimmu.2017.00603
33. Diamond B, Huerta PT, Mina-Osorio P, Kowal C, Volpe BT. Losing your nerves? Maybe it's the antibodies. *Nat Rev Immunol* (2009) 9:449–56. doi:10.1038/nri2529

34. Segovia-Miranda F, Serrano F, Dyrda A, Ampuero E, Retamal C, Bravo-Zehnder M, et al. Pathogenicity of lupus anti-ribosomal P antibodies: role of cross-reacting neuronal surface P antigen in glutamatergic transmission and plasticity in a mouse model. *Arthritis Rheumatol* (2015) 67:1598–610. doi:10.1002/art.39081
35. Bravo-Zehnder M, Toledo EM, Segovia-Miranda F, Serrano FG, Benito MJ, Metz C, et al. Anti-ribosomal P protein autoantibodies from patients with neuropsychiatric lupus impair memory in mice. *Arthritis Rheumatol* (2015) 67:204–14. doi:10.1002/art.38900
36. Trebst C, Jarius S, Berthele A, Paul F, Schippling S, Wildemann B, et al. Update on the diagnosis and treatment of neuromyelitis optica: recommendations of the Neuromyelitis Optica Study Group (NEMOS). *J Neurol* (2014) 261:1–16. doi:10.1007/s00415-013-7169-7
37. Papadopoulos MC, Bennett JL, Verkman AS. Treatment of neuromyelitis optica: state-of-the-art and emerging therapies. *Nat Rev Neurol* (2014) 10: 493–506. doi:10.1038/nrneurol.2014.141
38. Wingerchuk DM, Banwell B, Bennett JL, Cabre P, Carroll W, Chitnis T, et al. International consensus diagnostic criteria for neuromyelitis optica spectrum disorders. *Neurology* (2015) 85:177–89. doi:10.1212/WNL.0000000000001729
39. Hanly JG, Urowitz MB, Su L, Bae SC, Gordon C, Wallace DJ, et al. Prospective analysis of neuropsychiatric events in an international disease inception cohort of patients with systemic lupus erythematosus. *Ann Rheum Dis* (2010) 69:529–35. doi:10.1136/ard.2008.106351
40. The American College of Rheumatology nomenclature and case definitions for neuropsychiatric lupus syndromes. *Arthritis Rheum* (1999) 42:599–608. doi:10.1002/1529-0131(199904)42:4<599::AID-ANR2>3.0.CO;2-F
41. Hanly JG. Diagnosis and management of neuropsychiatric SLE. *Nat Rev Rheumatol* (2014) 10:338–47. doi:10.1038/nrrheum.2014.15
42. McLaurin EY, Holliday SL, Williams P, Brey RL. Predictors of cognitive dysfunction in patients with systemic lupus erythematosus. *Neurology* (2005) 64:297–303. doi:10.1212/01.WNL.0000149640.78684.EA
43. Sherer Y, Gorstein A, Fritzler MJ, Shoenfeld Y. Autoantibody explosion in systemic lupus erythematosus: more than 100 different antibodies found in SLE patients. *Semin Arthritis Rheum* (2004) 34:501–37. doi:10.1016/j.semarthrit.2004.07.002
44. Levy DM, Ardoin SP, Schanberg LE. Neurocognitive impairment in children and adolescents with systemic lupus erythematosus. *Nat Clin Pract Rheumatol* (2009) 5:106–14. doi:10.1038/ncprheum0988
45. Arnett FC, Reveille JD, Moutsopoulos HM, Georgescu L, Elkon KB. Ribosomal P autoantibodies in systemic lupus erythematosus. Frequencies in different ethnic groups and clinical and immunogenetic associations. *Arthritis Rheum* (1996) 39:1833–9. doi:10.1002/art.1780391109
46. Isshi K, Hirohata S. Differential roles of the anti-ribosomal P antibody and antineuronal antibody in the pathogenesis of central nervous system involvement in systemic lupus erythematosus. *Arthritis Rheum* (1998) 41:1819–27. doi:10.1002/1529-0131(199810)41:10<1819::AID-ART14>3.3.CO;2-P
47. Hirohata S, Sakuma Y, Yanagida T, Yoshio T. Association of cerebrospinal fluid anti-Sm antibodies with acute confusional state in systemic lupus erythematosus. *Arthritis Res Ther* (2014) 16:450. doi:10.1186/s13075-014-0450-z
48. Sanna G, Bertolaccini ML, Cuadrado MJ, Laing H, Khamashta MA, Mathieu A, et al. Neuropsychiatric manifestations in systemic lupus erythematosus: prevalence and association with antiphospholipid antibodies. *J Rheumatol* (2003) 30:985–92.
49. Gaynor B, Putterman C, Valadon P, Spatz L, Scharff MD, Diamond B. Peptide inhibition of glomerular deposition of an anti-DNA antibody. *Proc Natl Acad Sci U S A* (1997) 94:1955–60. doi:10.1073/pnas.94.5.1955
50. DeGiorgio LA, Konstantinov KN, Lee SC, Hardin JA, Volpe BT, Diamond B. A subset of lupus anti-DNA antibodies cross-reacts with the NR2 glutamate receptor in systemic lupus erythematosus. *Nat Med* (2001) 7:1189–93. doi:10.1038/nm1101-1189
51. Faust TW, Chang EH, Kowal C, Berlin R, Gazaryan IG, Bertini E, et al. Neurotoxic lupus autoantibodies alter brain function through two distinct mechanisms. *Proc Natl Acad Sci U S A* (2010) 107:18569–74. doi:10.1073/pnas.1006980107
52. Diamond B, Volpe BT. A model for lupus brain disease. *Immunol Rev* (2012) 248:56–67. doi:10.1111/j.1600-065X.2012.01137.x
53. Yoshio T, Okamoto H, Hirohata S, Minota S. IgG anti-NR2 glutamate receptor autoantibodies from patients with systemic lupus erythematosus activate endothelial cells. *Arthritis Rheum* (2013) 65:457–63. doi:10.1002/art.37745
54. Kowal C, DeGiorgio LA, Nakaoka T, Hetherington H, Huerta PT, Diamond B, et al. Cognition and immunity; antibody impairs memory. *Immunity* (2004) 21:179–88. doi:10.1016/j.immuni.2004.07.011
55. Tay SH, Fairhurst AM, Mak A. Clinical utility of circulating anti-*N*-methyl-D-aspartate receptor subunits NR2A/B antibody for the diagnosis of neuropsychiatric syndromes in systemic lupus erythematosus and Sjogren's syndrome: an updated meta-analysis. *Autoimmun Rev* (2017) 16:114–22. doi:10.1016/j.autrev.2016.12.002
56. Arinuma Y, Yanagida T, Hirohata S. Association of cerebrospinal fluid anti-NR2 glutamate receptor antibodies with diffuse neuropsychiatric systemic lupus erythematosus. *Arthritis Rheum* (2008) 58:1130–5. doi:10.1002/art.23399
57. Lapteva L, Nowak M, Yarboro CH, Takada K, Roebuck-Spencer T, Weickert T, et al. Anti-*N*-methyl-D-aspartate receptor antibodies, cognitive dysfunction, and depression in systemic lupus erythematosus. *Arthritis Rheum* (2006) 54:2505–14. doi:10.1002/art.22031
58. Omdal R, Brokstad K, Waterloo K, Koldingsnes W, Jonsson R, Mellgren SI. Neuropsychiatric disturbances in SLE are associated with antibodies against NMDA receptors. *Eur J Neurol* (2005) 12:392–8. doi:10.1111/j.1468-1331.2004.00976.x
59. Braniste V, Al-Asmakh M, Kowal C, Anuar F, Abbaspour A, Tóth M, et al. The gut microbiota influences blood-brain barrier permeability in mice. *Sci Transl Med* (2014) 6:263ra158. doi:10.1126/scitranslmed.3009759
60. Novack V, Erez O, Novack L, Jotkowitz A, Meir A, Mazor M. Sex distribution of newborns to mothers with systemic lupus erythematosus. *Epidemiology* (2006) 17:341–2. doi:10.1097/01.ede.0000209417.14819.44
61. James WH. The sex ratio of offspring of patients with systemic lupus erythematosus. *Lupus* (2007) 16:65–6. doi:10.1177/0961203306073365
62. Lahita RG. Systemic lupus erythematosus: learning disability in the male offspring of female patients and relationship to laterality. *Psychoneuroendocrinology* (1988) 13:385–96. doi:10.1016/0306-4530(88)90045-5
63. Urowitz MB, Gladman DD, MacKinnon A, Ibañez D, Bruto V, Rovet J, et al. Neurocognitive abnormalities in offspring of mothers with systemic lupus erythematosus. *Lupus* (2008) 17:555–60. doi:10.1177/0961203308089326
64. Wang L, Zhou D, Lee J, Niu H, Faust TW, Frattini S, et al. Female mouse fetal loss mediated by maternal autoantibody. *J Exp Med* (2012) 209:1083–9. doi:10.1084/jem.20111986
65. Nalli C, Iodice A, Andreoli L, Galli J, Lojacono A, Motta M, et al. Long-term neurodevelopmental outcome of children born to prospectively followed pregnancies of women with systemic lupus erythematosus and/or antiphospholipid syndrome. *Lupus* (2017) 26:552–8. doi:10.1177/0961203317694960
66. Lennon VA, Kryzer TJ, Pittock SJ, Verkman AS, Hinson SR. IgG marker of optic-spinal multiple sclerosis binds to the aquaporin-4 water channel. *J Exp Med* (2005) 202:473–7. doi:10.1084/jem.20050304
67. Mader S, Lutterotti A, Di Pauli F, Kuenz B, Schanda K, Aboul-Enein F, et al. Patterns of antibody binding to aquaporin-4 isoforms in neuromyelitis optica. *PLoS One* (2010) 5:e10455. doi:10.1371/journal.pone.0010455
68. Jarius S, Wildemann B. AQP4 antibodies in neuromyelitis optica: diagnostic and pathogenetic relevance. *Nat Rev Neurol* (2010) 6:383–92. doi:10.1038/nrneurol.2010.72
69. Waters P, Reindl M, Saiz A, Schanda K, Tuller F, Kral V, et al. Multicentre comparison of a diagnostic assay: aquaporin-4 antibodies in neuromyelitis optica. *J Neurol Neurosurg Psychiatry* (2016) 87:1005–15. doi:10.1136/jnnp-2015-312601
70. Saida T, Tashiro K, Itoyama Y, Sato T, Ohashi Y, Zhao Z, et al. Interferon beta-1b is effective in Japanese RRMS patients: a randomized, multicenter study. *Neurology* (2005) 64:621–30. doi:10.1212/01.WNL.0000151856.10387.E2
71. Pittock SJ, Weinshenker BG, Lucchinetti CF, Wingerchuk DM, Corboy JR, Lennon VA. Neuromyelitis optica brain lesions localized at sites of high aquaporin 4 expression. *Arch Neurol* (2006) 63:964–8. doi:10.1001/archneur.63.7.964
72. Lucchinetti CF, Mandler RN, McGavern D, Bruck W, Gleich G, Ransohoff RM, et al. A role for humoral mechanisms in the pathogenesis of Devic's neuromyelitis optica. *Brain* (2002) 125:1450–61. doi:10.1093/brain/awf151

73. Lucchinetti CF, Guo Y, Popescu BF, Fujihara K, Itoyama Y, Misu T. The pathology of an autoimmune astrocytopathy: lessons learned from neuromyelitis optica. *Brain Pathol* (2014) 24:83–97. doi:10.1111/bpa.12099
74. Jarius S, Franciotta D, Paul F, Ruprecht K, Bergamaschi R, Rommer PS, et al. Cerebrospinal fluid antibodies to aquaporin-4 in neuromyelitis optica and related disorders: frequency, origin, and diagnostic relevance. *J Neuroinflammation* (2010) 7:52. doi:10.1186/1742-2094-7-52
75. Dujmovic I, Mader S, Schanda K, Deisenhammer F, Stojsavljevic N, Kostic J, et al. Temporal dynamics of cerebrospinal fluid anti-aquaporin-4 antibodies in patients with neuromyelitis optica spectrum disorders. *J Neuroimmunol* (2011) 234:124–30. doi:10.1016/j.jneuroim.2011.01.007
76. Vaishnav RA, Liu R, Chapman J, Roberts AM, Ye H, Rebolledo-Mendez JD, et al. Aquaporin 4 molecular mimicry and implications for neuromyelitis optica. *J Neuroimmunol* (2013) 260:92–8. doi:10.1016/j.jneuroim.2013.04.015
77. Saadoun S, Waters P, Bell BA, Vincent A, Verkman AS, Papadopoulos MC. Intra-cerebral injection of neuromyelitis optica immunoglobulin G and human complement produces neuromyelitis optica lesions in mice. *Brain* (2010) 133:349–61. doi:10.1093/brain/awp309
78. Bennett JL, Lam C, Kalluri SR, Saikali P, Bautista K, Dupree C, et al. Intrathecal pathogenic anti-aquaporin-4 antibodies in early neuromyelitis optica. *Ann Neurol* (2009) 66:617–29. doi:10.1002/ana.21802
79. Pohl M, Fischer MT, Mader S, Schanda K, Kitic M, Sharma R, et al. Pathogenic T cell responses against aquaporin 4. *Acta Neuropathol* (2011) 122:21–34. doi:10.1007/s00401-011-0824-0
80. Kinoshita M, Nakatsuji Y, Kimura T, Moriya M, Takata K, Okuno T, et al. Neuromyelitis optica: passive transfer to rats by human immunoglobulin. *Biochem Biophys Res Commun* (2009) 386:623–7. doi:10.1016/j.bbrc.2009.06.085
81. Kitic M, Hochmeister S, Wimmer I, Bauer J, Misu T, Mader S, et al. Intrastriatal injection of interleukin-1 beta triggers the formation of neuromyelitis optica-like lesions in NMO-IgG seropositive rats. *Acta Neuropathol Commun* (2013) 1:5. doi:10.1186/2051-5960-1-5
82. Misu T, Höftberger R, Fujihara K, Wimmer I, Takai Y, Nishiyama S, et al. Presence of six different lesion types suggests diverse mechanisms of tissue injury in neuromyelitis optica. *Acta Neuropathol* (2013) 125:815–27. doi:10.1007/s00401-013-1116-7
83. Zeka B, Hastermann M, Hochmeister S, Kögl N, Kaufmann N, Schanda K, et al. Highly encephalitogenic aquaporin 4-specific T cells and NMO-IgG jointly orchestrate lesion location and tissue damage in the CNS. *Acta Neuropathol* (2015) 130:783–98. doi:10.1007/s00401-015-1501-5
84. Popescu BF, Lennon VA, Parisi JE, Howe CL, Weigand SD, Cabrera-Gómez JA, et al. Neuromyelitis optica unique area postrema lesions: nausea, vomiting, and pathogenic implications. *Neurology* (2011) 76:1229–37. doi:10.1212/WNL.0b013e318214332c
85. Pohl M, Kawakami N, Kitic M, Bauer J, Martins R, Fischer MT, et al. T cell-activation in neuromyelitis optica lesions plays a role in their formation. *Acta Neuropathol Commun* (2013) 1:85. doi:10.1186/2051-5960-1-85
86. Hinson SR, Roemer SF, Lucchinetti CF, Fryer JP, Kryzer TJ, Chamberlain JL, et al. Aquaporin-4-binding autoantibodies in patients with neuromyelitis optica impair glutamate transport by down-regulating EAAT2. *J Exp Med* (2008) 205:2473–81. doi:10.1084/jem.20081241
87. Blanc F, Zéphir H, Lebrun C, Labauge P, Castelnovo G, Fleury M, et al. Cognitive functions in neuromyelitis optica. *Arch Neurol* (2008) 65:84–8. doi:10.1001/archneurol.2007.16
88. Zhang N, Li YJ, Fu Y, Shao JH, Luo LL, Yang L, et al. Cognitive impairment in Chinese neuromyelitis optica. *Mult Scler* (2015) 21:1839–46. doi:10.1177/1352458515576982
89. Meng H, Xu J, Pan C, Cheng J, Hu Y, Hong Y, et al. Cognitive dysfunction in adult patients with neuromyelitis optica: a systematic review and meta-analysis. *J Neurol* (2017) 264:1549–58. doi:10.1007/s00415-016-8345-3
90. Vanotti S, Cores EV, Eizaguirre B, Melamud L, Rey R, Villa A. Cognitive performance of neuromyelitis optica patients: comparison with multiple sclerosis. *Arq Neuropsiquiatr* (2013) 71:357–61. doi:10.1590/0004-282X20130038
91. Pittock SJ, Lennon VA, McKeon A, Mandrekar J, Weinshenker BG, Lucchinetti CF, et al. Eculizumab in AQP4-IgG-positive relapsing neuromyelitis optica spectrum disorders: an open-label pilot study. *Lancet Neurol* (2013) 12:554–62. doi:10.1016/S1474-4422(13)70076-0
92. Nour MM, Nakashima I, Coutinho E, Woodhall M, Sousa F, Revis J, et al. Pregnancy outcomes in aquaporin-4-positive neuromyelitis optica spectrum disorder. *Neurology* (2016) 86:79–87. doi:10.1212/WNL.0000000000002208
93. Saadoun S, Waters P, Leite MI, Bennett JL, Vincent A, Papadopoulos MC. Neuromyelitis optica IgG causes placental inflammation and fetal death. *J Immunol* (2013) 191:2999–3005. doi:10.4049/jimmunol.1301483
94. Hinson SR, Clift IC, Luo N, Kryzer TJ, Lennon VA. Autoantibody-induced internalization of CNS AQP4 water channel and EAAT2 glutamate transporter requires astrocytic Fc receptor. *Proc Natl Acad Sci U S A* (2017) 114:5491–6. doi:10.1073/pnas.1701960114

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.