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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.

15. SUBJECT TERMS
Fetal brain; Autism spectrum disorder; antibody; B cells; Caspr2

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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.

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) (Fig 1). 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 (Fig 2). In accordance, adult male mice exposed in utero to C6 showed reduced number of neurons in specific area of the cortex (Fig 2). 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 (Fig 3). Adult mice exposed in-utero to C6 showed impairments in social preference, flexible learning, and increased repetitive behavior (Fig 4). Importantly, we found similar changes to the developing cortex in mice exposed in utero to the plasma in which C6 was isolated from (Fig 5). 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.
We 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 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 is binding to.

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 large amounts of 5 more.

Subtask3: To inject all brain-reactive antibodies and isotope control antibodies into 14 pregnant mice each
We have injected one monoclonal D3 into pregnant mice and have begun to inject some others including A7. Before injecting the monoclonals, we rescreen them for binding to mouse brain to ensure that we have not inactivated them during the purification.

Subtaks4: 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 determined that C6 binds Caspr2 (Fig 6). We have begun testing mice exposed to two other monoclonal anti-bodies D3 and A7. A7 appears to be a second anti-Caspr2 antibody. Thus, we will determine if a second anti-Caspr2 antibody yields the same data.

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 the C6 anti-Caspr2 antibody. We have now perfected our methodology to distinguish cortical layers and have data that these antibodies alter mitosis and migration. We are initiating these studies on brains exposed in utero to A7 to see if another anti-Caspr2 antibody causes similar pathology.

Milestone(s) Achieved
We have submitted a manuscript (attached) on the effects of the C6 anti-Caspr2 antibody on the developing mouse brain. We have demonstrated that it leads to an "autism-like" phenotype in male, but not female, mice.

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 live cell based assays for reactivity to Caspr2 and Edil 3. We continue to work on developing ELISAs but this has proven to be quite challenging as we see much non-specific binding. We have obtained plasmids encoding truncated forms of Caspr2 to localize the epitopes seen by C6 and A7 but these hae had aberrant sequences so we are now making our own truncated constructs.

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 have 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 ʎ) 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 invited to present her work at a podium session at the Society for Neuroscience annual meeting.

How were the results disseminated to communities of interest?
A manuscript on these data has been submitted for publication. In addition, the Society of Neuroscience is featuring these data for a press release.

What do you plan to do during the next reporting period to accomplish the goals?
During the next year, we will identify the epitope of Caspr2 recognized by the monoclonal antibody. We will then determine which women have antibodies to this epitope and explore the phenotype of their affected children

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, PT., Volpe,

BT .and Diamond, B. Caspr2-reactive antibody cloned from a mother of an ASD child mediates an ASD like phenotype in mice. (submitted)

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

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?

Dr. Betty Diamond

Complete Research:
1 R01 AR049126-06 (Diamond) 09/15/02 – 08/31/14
NIH/NIAMS - Neuropsychiatric SLE
Antibodies to NR2 in SLE

1P01AI073693-02 (Diamond) 08/01/08 – 07/31/14
NIH/NIAID – **Anti-NMDA receptor antibodies in adult brain dysfunction and fetal brain development: lessons from lupus**
Project 4 (Diamond)
Preventing anti-NMDAR Ab neurotoxicity: Rational Therapies for NPSLE

NIH R01AR057084 (Diamond) 07/01/10 – 06/30/15
Toward a new approach to SLE therapy

Alliance for Lupus Research (Gregersen & Diamond) 07/01/12 – 06/30/14
Functional analysis of CSK: A newly defined risk gene for lupus

R21 AR063929 (Diamond) 07/01/13-06/30/15
Nelfinavir in SLE: A pilot phase IIa clinical trial

NIH R21 AR065199 (Gregersen) 07/01/13 – 06/30/15
(Diamond co-investigator)
TNIP1 risk haplotypes and immune endophenotypes

NIH U34 AR063407 (Mackay) 09/25/13-08/31/15
Treatment of SLE with ajulemic acid, a non-psychoactive cannabinoid derivative
(Diamond co-investigator)

Empire Clinical Research Investigator Program (ECRIP) 01/15/14-02/15/15
An evolutionary approach to SLE therapy
Project Director (Diamond)

Biogen Idec (Diamond & Gregersen) 07/01/14 - -6/30/16
Investigation of Blimp1 in SLE Patients, Unaffected Sisters and Normal Controls
NIH R43 MH106195 (Burch, R) 02/05/15 – 01/31/16
(Diamond PI)

Autism Spectrum Disorder Diagnostic/Therapeutic Agent
The goal of this research project is to translate molecular insights on pathogenic mechanisms in ASD.

This proposal is the study of T_{FH} cells in SLE

Active Research:
1P01AI073693-06 (Diamond) 07/1/14-06/30/19
NIH/NIAID – **Anti-NMDA receptor antibodies in adult brain dysfunction and fetal brain development: lessons from lupus**
Project 3 (Diamond)
Antibody modulation of neural endocrine network
This is a study of prolactin modulation by anti-NMDAR antibodies.

R21 AR063929 (Diamond) 07/01/13 – 06/30/17
Nelfinavir in SLE: A pilot phase IIa clinical trial
This is a study of the ability of nelfinavir to block DNA binding.

NIH U34 AR063407 (Mackay) 09/25/13-08/31/16
Treatment of SLE with ajulemic acid, a non-psychoactive cannabinoid derivative
(Diamond co-investigator)
This Phase IIa clinical trial will test the safety and efficacy of a non-psychoactive cannabinoid derivative, ajulemic acid, on patients with lupus that have mild to moderate disease activity.

Lupus Research Institute (Diamond) 02/01/14 – 01/31/17
Autoantibodies and prolactin participate in a positive feedback loop
This is a study of the role of anti-NMDAR antibodies in triggering autoantibody production. The overlap with P01AI073693 is being addressed.

NIH 1UM1AI110494 (Aranow) 05/01/14 – 04/30/19
Proposal for The Feinstein Center for Clinical Research in Autoimmune Disease
(Diamond-Mechanistic Investigator)
This is a proposal to establish an Autoimmunity Centers of Excellence Basic Science Center.

NIH P01 AI102852 (Diamond & Tracey) 08/01/14 – 07/31/19
NIH/NIAID - **Cognitive and immune impairments in sepsis survivors**
Project 2 (Diamond)
Immune function in sepsis survivors
Study of immune impairment in monocytes following sepsis.

NIH R01 AR065209 (Kim) 09/01/14-08/31/18
(Diamond co-investigator)
The Blimp-1 SLE risk variant regulates inflammatory function in dendritic cells
This grant is to study the function of the PRDM1 SNP rs548234 in dendritic cells for predisposition to SLE.

NIH UH2 AR067688 (Diamond) 09/24/14-05/31/19
PEARL: Pathway exploration and analysis in renal lupus
This a proposal to apply state of the art genomic and immunophenotyping technologies to lupus nephritis.

NIH/NIAID IUM1AI1095650 (Nepom. G) 05/01/14 – 06/30/19
Co-Chair Diamond
CALIBRATE – Rituximab Plus Cyclophosphamide followed by Belimumab
This is a clinical trial to determine if rituximab followed by belimumab educes autoreactive B cell repertoire.

The NLM Family Foundation (Diamond) 04/01/15 – 03/31/18
Maternal antibody as a contributor to Autism Spectrum Disorder
This is a proposal to develop a decoy antigen to protect the fetal brain from autism related antibodies.

NIH R21AR067012-01A1 (Diamond) 06/01/15 – 05/31/17
Function of the IRF5 risk allele for SLE in B cells
This is a study of the functionality of the IRF5 risk allele in B cells of healthy individuals.

Dr. Peter Gregersen

Completed Research:

Biogen/IDEC **Biomarkers of Anti-TNF-α Therapy Efficacy in Rheumatoid Arthritis to Define Unresponsive Patients (BATTER-UP)** 6/1/10-5/31/15

Alliance for Lupus Research- **Functional Analysis of Csk** 8/1/12-7/31/14
Gregersen/Diamond (PIs)

NIH - R21 **TNIP1 risk haplotypes and immune endophenotypes** 8/1/13-7/31/15
Gregersen (PI), Diamond (Co-PI)

NIH-UH2AR067694-01– AMP-**Molecular and Cellular Dissection of Early Rheumatoid Arthritis**

Gregersen (PI) 10/01/14-05/31/16

Active Research:

NIH– R01AR065209
Blimp-1 SLE risk variant regulates inflammatory function in dendritic cells
Kim (PI) 09/01/14 – 08/31/18
This grant is to study how a Blimp-1 variant associated with systemic lupus regulates the function of dendritic cells and promotes lupus pathogenesis.

NIH ACE – 1UM1AI110494-01
The Feinstein Center for Clinical Research in Autoimmune Diseases
Aranow (PI) 05/01/14 – 04/30/19
The goal is to establish an Autoimmunity Centers of Excellence Basic Science Center

Janssen R&D 07/01/15 – 08/31/16
Gregersen (PI)
The goal is to achieve a deeper understanding of the response to biologic therapy, with a view to developing clinically useful predictive biomarkers that can guide current therapies, and enhance the efficiency of clinical trials.

Dr. Patricio Huerta

Completed Research:

5R01AR049126-10 (Diamond) 7/1/2002 – 6/30/2014
NIH/NIAMS
Antibodies to NR2 in SLE
The goal is to determine the brain neurotoxicity of DNA-reactive autoantibodies, produced by patients with SLE, which cross-react with the NR2 subunits of the N-methyl-D-aspartate receptor.

5P01AI073693-05 (Diamond) 8/1/2008 – 7/31/2014
NIH/NIAID
Anti-NMDA receptor antibodies in brain dysfunction: Lessons from lupus
Project 1 (Huerta)
NIH/NIAID
Mechanisms of neurotoxicity of lupus anti-NMDAR antibodies: Electrophysiology to behavior
The goal is to establish the molecular mechanisms of neurotoxicity of NMDAR-reactive antibodies, using ex vivo and in vivo approaches, and their effects on cognitive behaviors.

5P01AI073693-05 (Huerta)
NIH/ NIAID
Behavioral Core
The goal is to develop a core facility to perform behavioral assessments of mice immunized with a diverse panel of NMDAR-reactive antibodies.

Active Research:

5P01AI073693-06 (Diamond) 8/1/2008 – 06/30/2019

NIH/NIAID
Anti-NMDA receptor antibodies in adult brain dysfunction, & fetal brain Development
Project 1 (Co-PI)
"Modeling variable outcomes of antibody exposure"
This is a study of maintaining the integrity of the blood-brain barrier which does not overlap with the current proposal.

NIH P01 AI102852 (Diamond & Tracey) 04/01/14 – 03/31/19
NIH/NIAID – "Cognitive and immune impairments in sepsis survivors"
Project 1 (Co-PI)
"Nervous system alterations in sepsis-surviving mice"
This project investigates the effects of cytokines on neuroanatomy and has no relation to the current efforts
Core C: Behavioral Function (PI)
The goal is to assess animal models of sepsis-related cytokine elevation through behavioral and neural testing.

R01-AG042508 (Marambaud) 5/9/2013 – 6/30/2017
NIH/NIA
Mechanisms of regulation of amyloid beta metabolism by CALHM1
The goal is to determine the role of the CALHM1 protein in the onset of amyloid beta deposition with the use of murine models.

Dr. Bruce Volpe

Completed Research:

NIH, PO1 AI 073693 (Diamond PI) Sept 08- August 2014
"Anti-NMDA receptor antibodies in brain dysfunction, lessons from lupus"
Project 2 – (Volpe PI)
"Developmental Neurotoxicity: Maternal anti-NMDAR antibodies and fetal development"
Multiple families of antibodies that recognize the NMDA receptor are produced in patients with lupus. This project will study the effects of these molecules wit in vivo and in vitro techniques and with clinical experiments. There is no overlap with the current proposal.

NIH, RO1 AR49126-01 (Volpe co-I) Sept 07- August 2014
"Antibodies to NR2 in SLE"
The goal is to develop the scientific foundation for preventative therapies for cognitive decline in SLE. There are no experiments in this proposal that overlap with the current request.

Active Research:

NIH, PO1 AI 073693 (Diamond PI) Sept 2008- August 2019
"Anti-NMDA receptor antibodies in adult brain dysfunction, & fetal brain Development"
Project 1 – (Volpe PI)
"Modeling variable outcomes of antibody exposure"
This is a study of maintaining the integrity of the blood-brain barrier which does not overlap with the current proposal and a study of peptide therapy which includes partial overlap with the current proposal. There is no overlap with the current proposal.

NIH 1 RO1 HD 069776-01 (PI D Edwards) Jan 2012- Dec 2017

"Transcranial direct current stimulation and robotic training in stroke"
This project will focus on a randomized controlled test of tDCS provided before robot therapy, using outcomes measures that will provide insight into mechanisms of the effect of the tDCS via changes in brain physiology and motor control. There is no overlap of the experiments in this proposal.

NIH P01 AI102852 (Diamond & Tracey) April 2014 – March 2019
NIH/NIAID – "Cognitive and immune impairments in sepsis survivors"
Project 1 (Volpe)
"Nervous system alterations in sepsis-surviving mice"
This project investigates the effects of cytokines on neuroanatomy and has no relation to the current efforts.
Core D **Histology** (Volpe)
The histology core will provide services to all the investigators in each of the projects. Quantitative pathological neuroanatomy is important to several of the experiments. Also the pathological correlations are important in animals where behavior is measured.

NIH 1 RO1 HD 069776-01 (PI D Edwards) Jan 12- Dec 2017
"Transcranial direct current stimulation and robotic training in stroke"
This project will focus on a randomized controlled test of tDCS provided before robot therapy, using outcomes measures that will provide insight into mechanisms of the effect of the tDCS via changes in brain physiology and motor control. There is no overlap of the experiments in this proposal.

Appendices:
None

What other organizations were involved as partners? None

8. **SPECIAL REPORTING REQUIREMENTS** – NA

9. **APPENDICES –** Figures &Submitted manuscript

References

1. Singer, H.S.*, et al.* Antibodies against fetal brain in sera of mothers with autistic children. *Journal of neuroimmunology* **194**, 165-172 (2008).
2. Dalton, P.*, et al.* Maternal neuronal antibodies associated with autism and a language disorder. *Annals of neurology* **53**, 533-537 (2003).
3. Braunschweig, D.*, et al.* Autism-specific maternal autoantibodies recognize critical proteins in developing brain. *Transl Psychiatry* **3**, e277 (2013).
4. Brimberg, L., Sadiq, A., Gregersen, P.K. & Diamond, B. Brain-reactive IgG correlates with autoimmunity in mothers of a child with an autism spectrum disorder. *Mol Psychiatry* **18**, 1171-1177 (2013).
5. Bauman, M.D.*, et al.* Maternal antibodies from mothers of children with autism alter brain growth and social behavior development in the rhesus monkey. *Transl Psychiatry* **3**, e278 (2013).
6. Singer, H.S.*, et al.* Prenatal exposure to antibodies from mothers of children with autism produces neurobehavioral alterations: A pregnant dam mouse model. *Journal of neuroimmunology* **211**, 39-48 (2009).
7. Tiller, T.*, et al.* Efficient generation of monoclonal antibodies from single human B cells by single cell RT-PCR and expression vector cloning. *J Immunol Methods* **329**, 112-124 (2008).
8. Zhang, J.*, et al.* Identification of DNA-reactive B cells in patients with systemic lupus erythematosus. *J Immunol Methods* **338**, 79-84 (2008).
9. Lee, J.Y.*, et al.* Neurotoxic autoantibodies mediate congenital cortical impairment of offspring in maternal lupus. *Nature medicine* **15**, 91-96 (2009).
10. Mader, S.*, et al.* Patterns of antibody binding to aquaporin-4 isoforms in neuromyelitis optica. *PLoS One* **5**, e10455 (2010).
11. Wang, L.*, et al.* Female mouse fetal loss mediated by maternal autoantibody. *The Journal of experimental medicine* **209**, 1083-1089 (2012).
12. Chavan, S.S.*, et al.* HMGB1 mediates cognitive impairment in sepsis survivors. *Mol Med* **18**, 930-937 (2012).
13. Thomas, A.*, et al.* Marble burying reflects a repetitive and perseverative behavior more than novelty-induced anxiety. *Psychopharmacology (Berl)* **204**, 361-373 (2009).

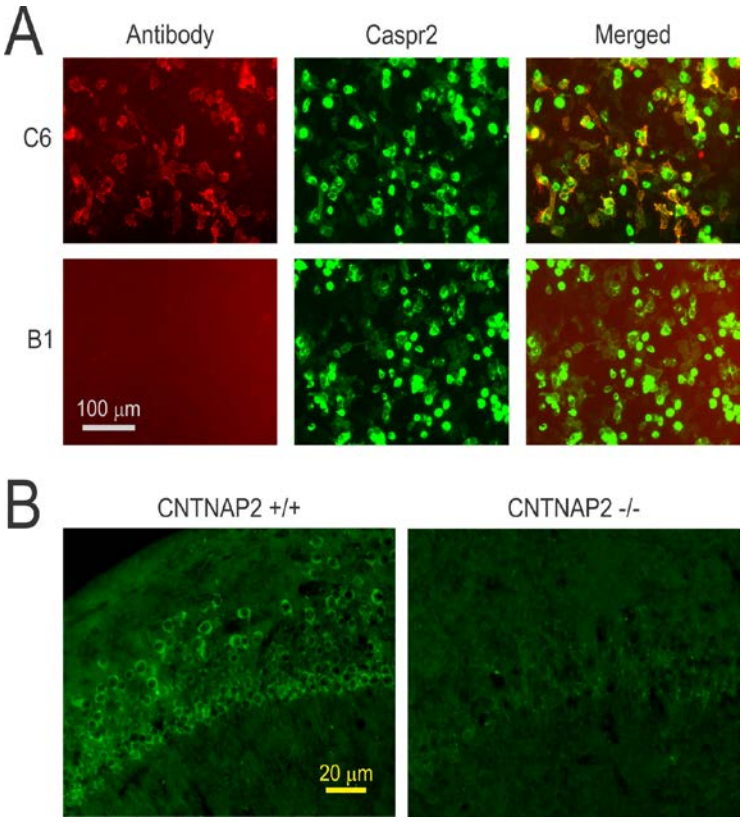

Figure 1: Brain-reactive monoclonal antibody C6 binds to Caspr2.

(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 to wild- type (left) mice.

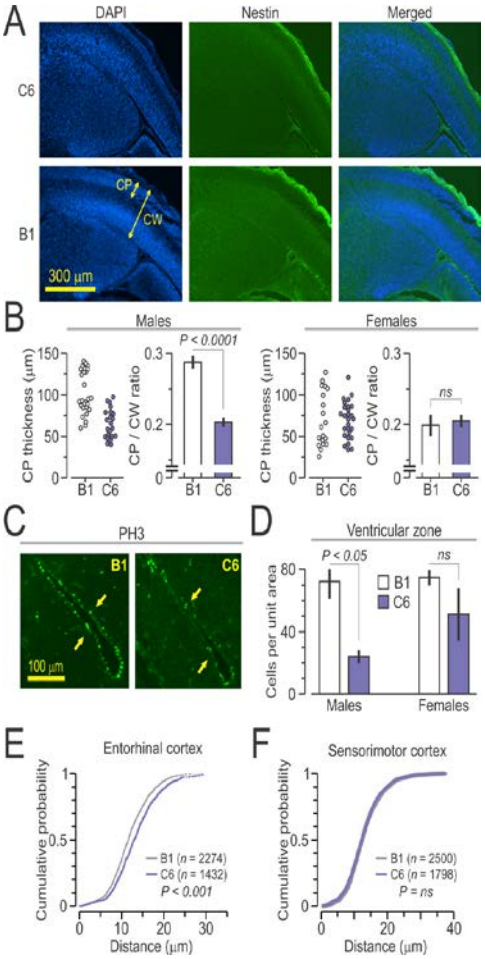

Figure 2: Male mice exposed *in utero* to C6 manifest cortical abnormalities. (A-D) Number of mice included in the analysis : Male: B1=5, C6= 4; Female: B1=5, C6= 5. Mice were derived from 2 litters for each antibody (A)The cortical plate is abnormally thin in C6-exposed (top) relative to control B1-exposed (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 mm2; * *P* < 0.05, Z=2.34, Mann Whitney test..

(E-F) Cumulative probability based on nearest neighbor analysis in 5 adult B1- and 4 adult C6-exposed mice (2 litters each)**. Dots represent the number of cells.**

(E) Entorhinal Cortex (Cell number: B1, n = 2274 , C6, n = 1432), P < 0.001, K-S. (F) Somatosensory Cortex (Cell number : B1, n=2500, C6, n=1798), P= N.S.

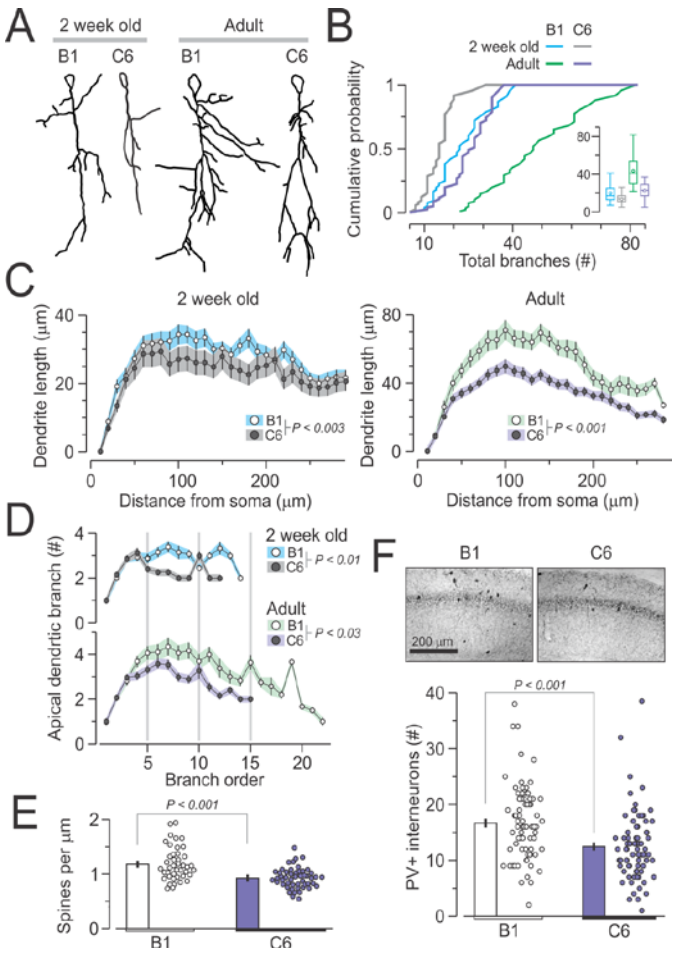

Figure 3: C6-exposed male offspring display significantly reduced hippocampal dendritic complexity. CA1 pyramidal neurons of C6-exposed male mice show reduced dendritic length and spine density. Analysis includes: 2 week old mice (n = 4 per group); neurons: B1 = 45, C6 = 49, and adult animals (n = 4 per group); neurons: B1=70, C6= 83. Each group includes animals from 2 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 represents individual dentrites.** B1=1.18±0.04 spines/µm; C6=0.92±0.02 spines/µm; ** $P < 0.001$, $t = 4.78$, 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, 4 animals per group, 2 litters.

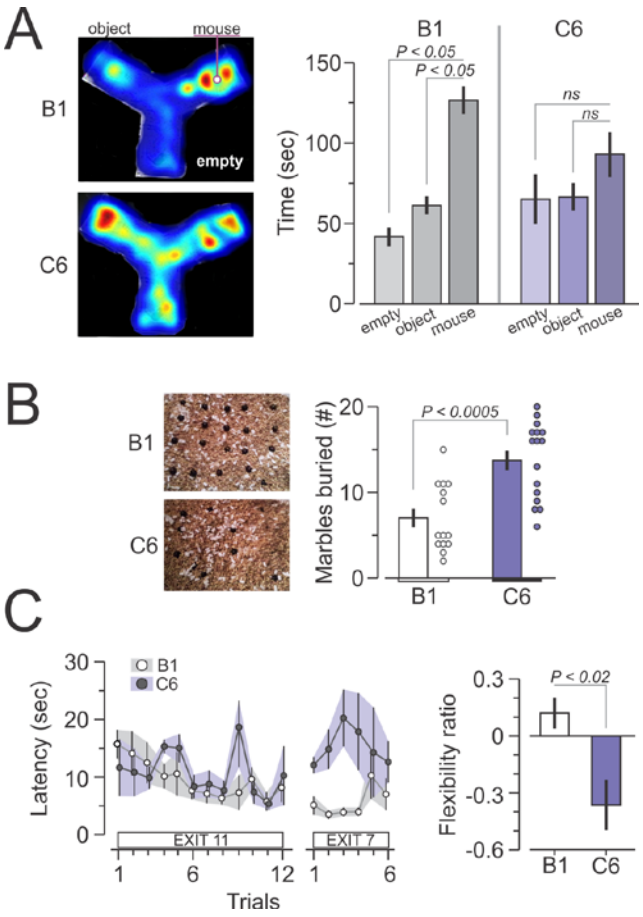

Figure 4: Impaired performance of C6-exposed male offspring in tasks that resemble core ASD symptoms. Maternal antibody-exposed male mice were subjected to behavioral assessment at adulthood (10–14 weeks). (A) Social approach task. (Left) representative heat map showing social patterns in B1- and C6-exposed mice. (Right) B1-exposed male mice (n=15) displayed normal sociability, defined as spending significantly more time with the novel mouse compared to the novel object, whereas C6-exposed male mice (n=13) spent a similar amount of time near the novel object and the novel mouse. 5 litters per group . ANOVA, followed by Bonferroni test, $P < 0.05$. (B) Marble burying task. (Left) representative examples of burying patterns in the two groups. (Right) C6-exposed mice (n = 16) display enhanced stereotypic behavior i.e, they bury more marbles than the B1-exposed mice (n = 14); ** $P < 0.0005$, t = 4.2, *t* test; 5 litters per group. (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.

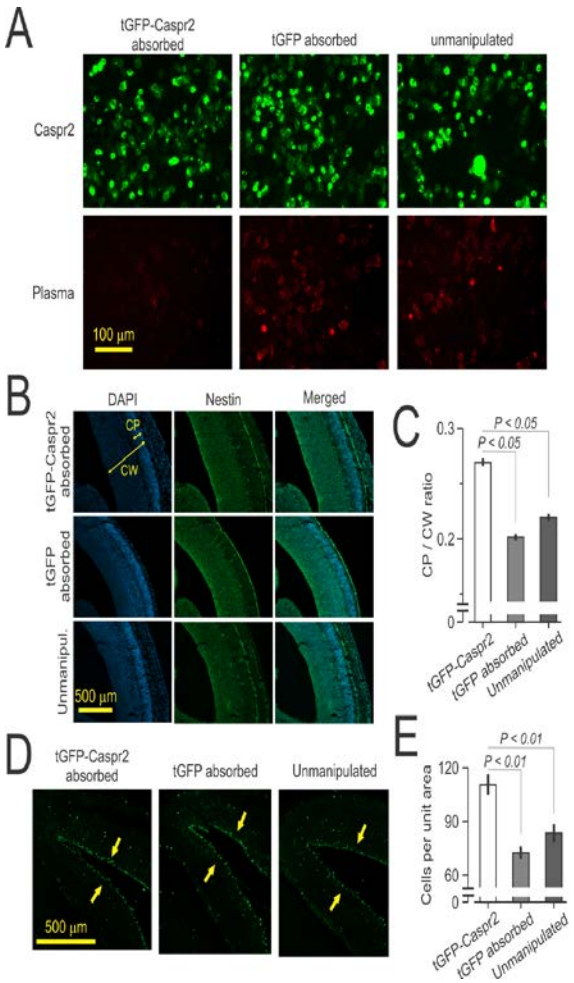

Figure 5: (A) Plasma absorbed on tGFP-Caspr2 expressing cells (left panel); plasma absorbed on tGFP expressing cells (middle panel) and unmanipulated plasma (right panel) binding to t-GFP Caspr2 transfected cells. All plasma contained 60 ug/ml of IgG. tGFP-Caspr2 absorbed plasma show little if any binding HEK 293T cells expressing tGFP-Caspr2. tGFP- absorbed plasma and unmanipulated plasma show binding. No staining was seen on cells expressing only tGFP or non-transfected cells (data not shown). (B) The cortical plate is thinner in male fetal brains exposed to tGFP-absorbed plasma (middle panel) and unmanipulated plasma (bottom panel) relative to tGFP-Caspr2 absorbed plasma (top panel) as revealed by DAPI (left) and nestin (middle) staining (C) Quantification of cortical plate thickness, the ratio of cortical plate to cortical width, * $P < 0.05$, $Z = 2.5$, Mann-Whitney test. (D) Exposure *in utero* to tGFP-absorbed plasma (middle panel) and unmanipulated plasma (right panel) leads to fewer mitotic cells than exposure to tGFP-Caspr2 absorbed plasma (left panel) revealed by PH3 staining in the ventricular zone of male brains. (E) Quantification of mitotic cells (PH3+), ** $P < 0.001$, $t = 5.7$, t test. Unit area = 62.5 mm^2. Mice used: tGFP-Caspr2 absorbed plasma, n = 5; tGFP-absorbed plasma, n = 3; unmanipulated plasma, n = 5.

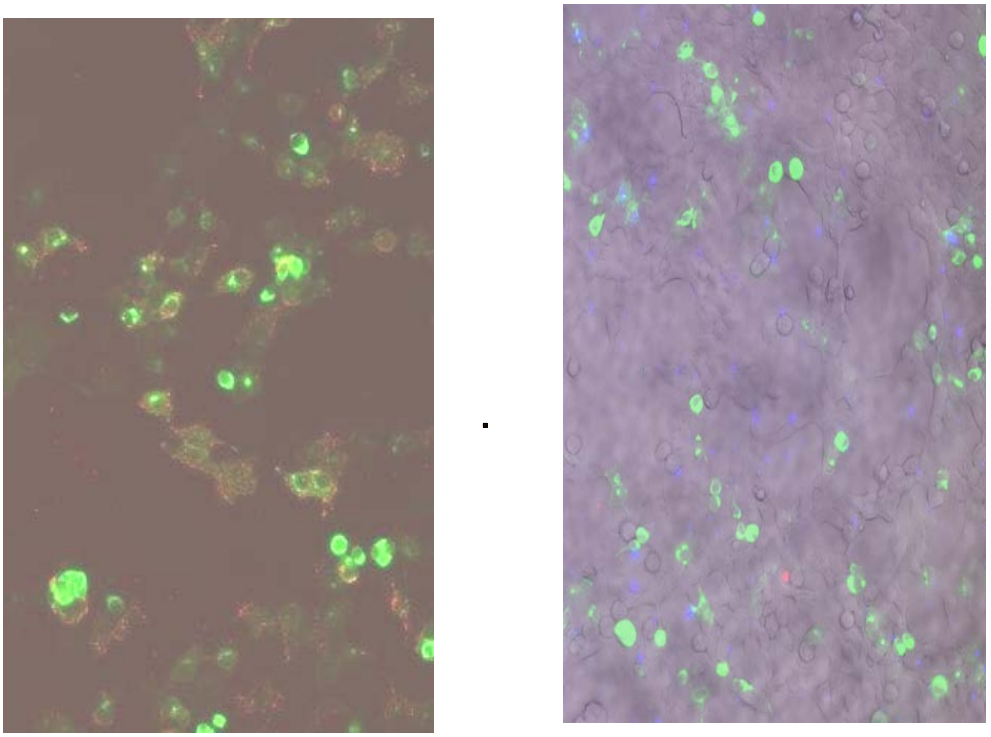

Figure 6. A7 binds Caspr2 transfected cells
Left: A7; right B1 control monoclonal

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

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Running Title: Maternal anti-Caspr2 IgG and ASD.

Abstract

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. While 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.

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 (e.g., infection) has been implicated in ASD in human and murine offspring [4]. In murine models, elevated maternal production of IL-6 [5] or IL-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 (Mabs) 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].

Methods

Research subjects

Plasma from mothers with an ASD child was obtained from the Simons Simplex Collection (SSC, http://sfari.org/resources/simons-simplex-collection) [21]. 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 [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, SD= 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 SSC, previously identified as having brain-reactive antibodies [9]. 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 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). 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 [23].

cDNA Synthesis and RT-PCR

cDNA synthesis of individual IgH (γ only) and IgL chain (κ or λ) was performed as previously described [22, 23]. 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 [23] (See also Supplementary methods). Purified antibody was dialyzed extensively against PBS; integrity was

determined by nonreducing SDS 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 tGFP-Caspr2 or tGFP only in 96 wells plate as described [25]. Seventy-two hours after transfection, 50 ul fractions of plasma were incubated with the cells for one hour at 4ºC. The incubation was repeated 7 times. The plasma was centrifuged for 15 minutes at 12,000 x g. IgG 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, 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. 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 ul containing 300 ug 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 [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 (Sakura) on dry ice and stored at -80˚C. Sagittal sections were cut (12 µm thick) on a Cryostat (Leica) and mounted on gelatin-coated slides and stored at -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 06-570) or anti-nestin antibody (1:200, Millipore MAB353) 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 [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) or CNTNAP2-/- mice (a gift from Dr. Brett S. Abrahams, Albert Einstein College of Medicine, NY).

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.) 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), 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 weeks old mice. Anti-Parvalbumin antibody (Abcam, ab11427) was used. For quantification details see Supplementary Methods.

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. 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 (ANOVA) as well as Student's t-test for datasets that were normally distributed (and with samples larger than 10). For smaller datasets, we performed the Mann-Whitney test. To analyze categorical data, a Chi-squared test for

independence was used. The nonparametric Kolmogorov–Smirnov (K-S) 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 $p < 0.05$. 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

Since 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 to 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]. Since the blood-brain barrier (BBB) 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 BBB will normally exclude IgG [14].

Strikingly, male, but not female, fetuses from dams sacrificed 2 days after C6 administration exhibited a thinned cortical plate (Figure 2A-B). C6-exposed male, but not female, fetuses showed a decrease in proliferating cells in the developing cortex (Figure 2C-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). In contrast, in the somatosensory cortex there was no apparent differences (Figure 2F).

In utero exposure to C6 affects hippocampal neurons

Since 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 (Figure 3A-E). We also examined CA1 neurons in 2 week-old male mice before full development of these cells has occurred [36] to determine if the abnormalities observed in the adult mice represented a failure in maturation, or a late consequence of *in utero* exposure to antibody (Figure 3A-D). We found the 2 week old C6-exposed mice exhibited reduced apical dendritic branches when compared to age-matched B1-exposed mice (Figure 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 (Fig. 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. C6-exposed mice of either sex were indistinguishable from B1-exposed of the same sex (male: n =16, 5 litters each; female: B1 n=16, C6=11, 4 litters each), in body weight, coat, grip strength, body tone, reflexes andsickness behavior **as described in [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; ANOVA center/periphery x group, *P* > 0.8, respectively).**

When subjected to tasks that focus on core ASD symptoms [38], adult 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 (Fig. 4A). **In contrast, B1-exposed mice spent more time in proximity to the unfamiliar mouse than the unfamiliar object (Fig. 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. While this might be interrupted as general motivational impairment, i.e. 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. While this hypothesis has not been formally tested in our study, it should be 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] (Fig. 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 in flexible learning (Fig. 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 to 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 (chi-square 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 (NMDA) 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.

While 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 (TAG1), which co-localizes 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 NMDA 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]. Since 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 if 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 BBB, 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.

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Conflict of interest

The authors have no conflicts of interest to report.

Figure Legends

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 to wild-type (left) mice.

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 2 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 mm2; * $P < 0.05$, Z=2.34, Mann Whitney test..
(E-F) Cumulative probability based on nearest neighbor analysis in 5 adult B1- and 4 adult C6-exposed mice (2 litters each)**. Dots represent the number of cells.**
(E) Entorhinal Cortex (Cell number: B1, n = 2274 , C6, n = 1432), P < 0.001, K-S. (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 2 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 represents individual dentrites.** B1=1.18±0.04 spines/µm; C6=0.92±0.02 spines/µm; ** $P < 0.001$, $t = 4.78$, 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, 4 animals per group, 2 litters.

Figure 4: Impaired performance of C6-exposed male offspring in tasks that resemble core ASD 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 to the novel object, whereas C6-exposed male mice (n=13) spent a similar amount of time near the novel object and the novel mouse. 5 litters per group . ANOVA, followed by Bonferroni test, $P < 0.05$. (B) Marble burying task. (Left) representative examples of burying patterns in the two groups. (Right) C6-exposed mice (n = 16) display enhanced stereotypic behavior i.e, they bury more marbles than the B1-exposed mice (n = 14); ** $P < 0.0005$, t = 4.2, *t* test; 5 litters per group. (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.

Figure 1 ~ Brimberg et al.

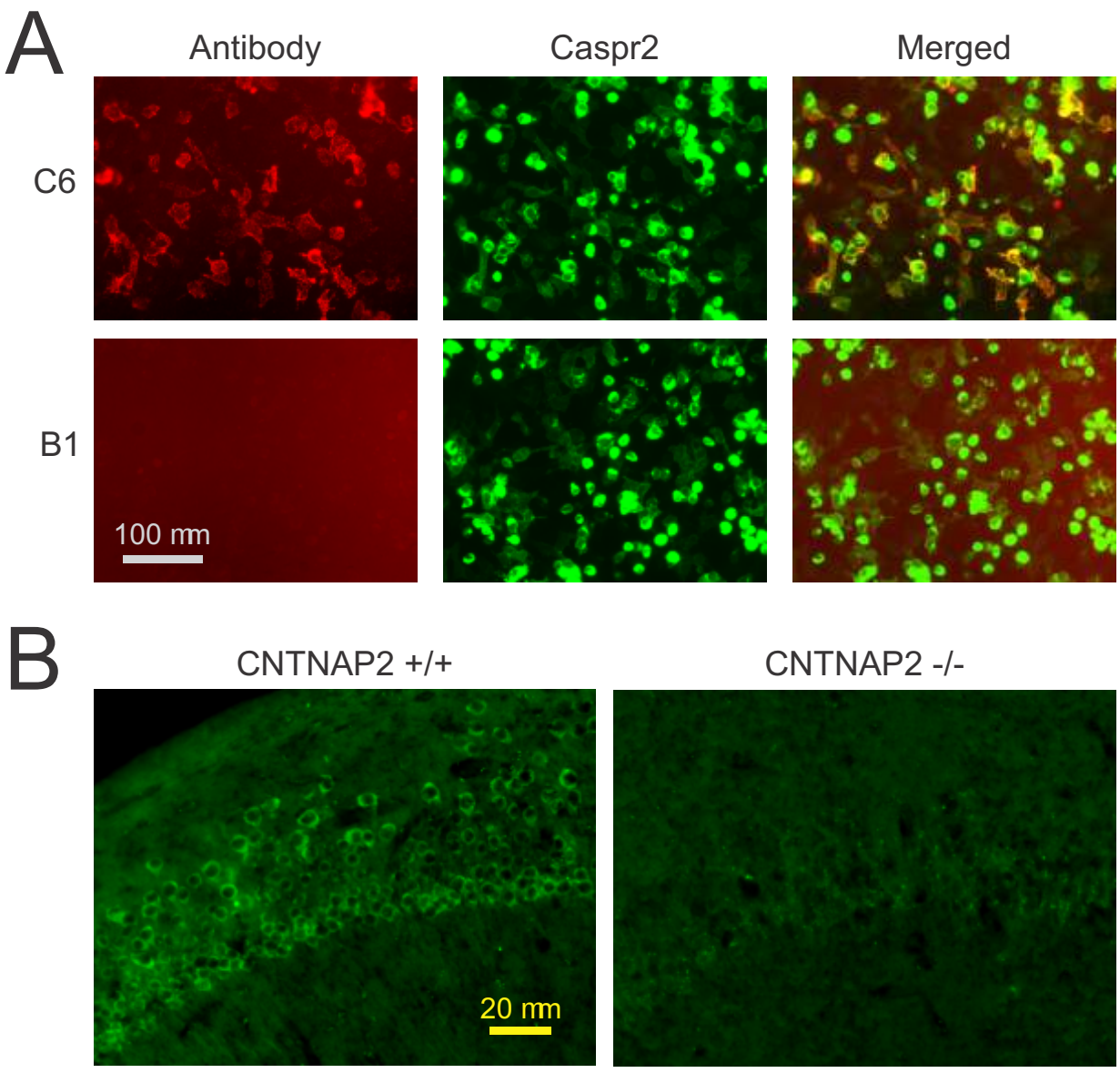


Figure 2 ~ Brimberg et al.

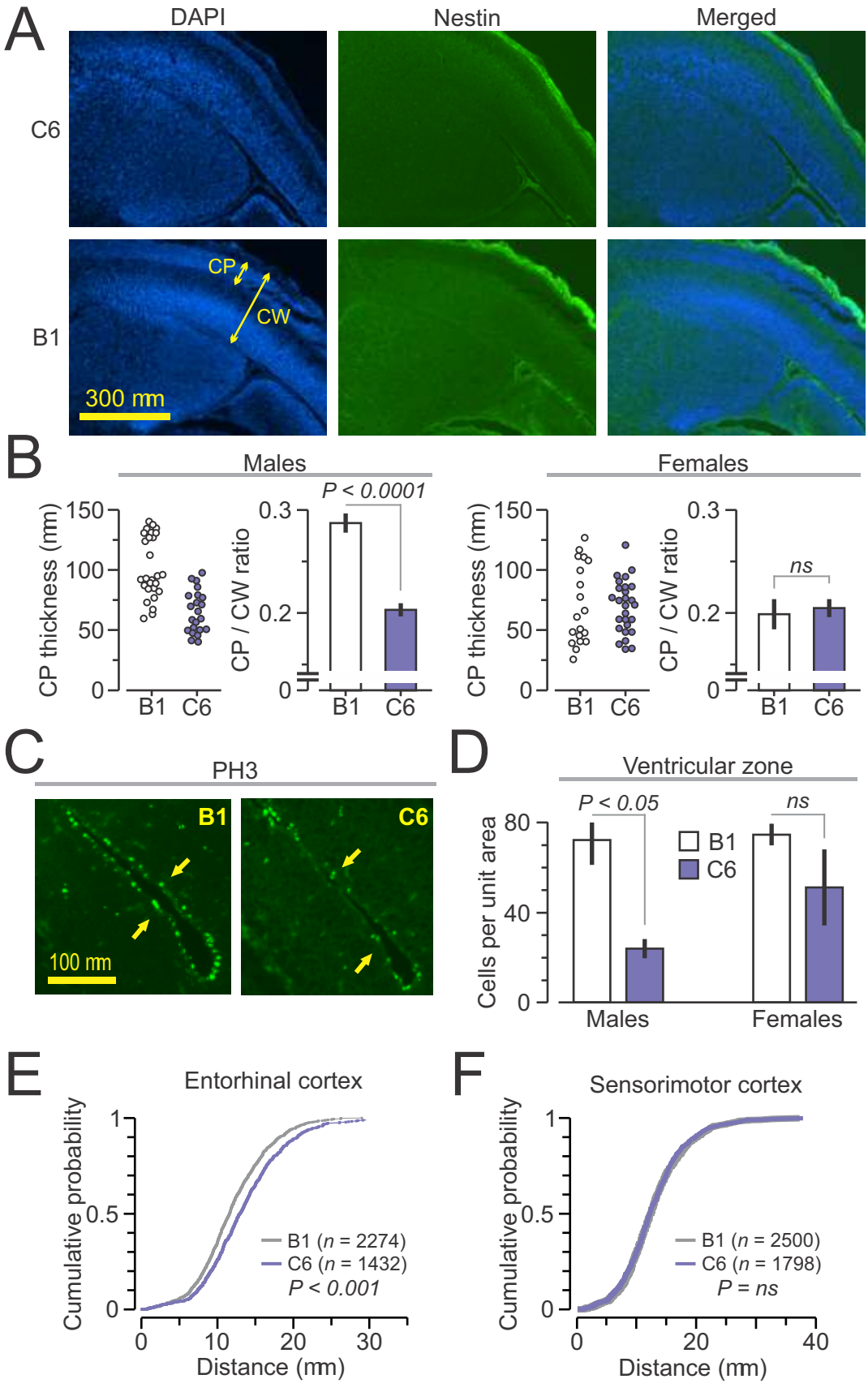


Figure 3 ~ Brimberg et al.

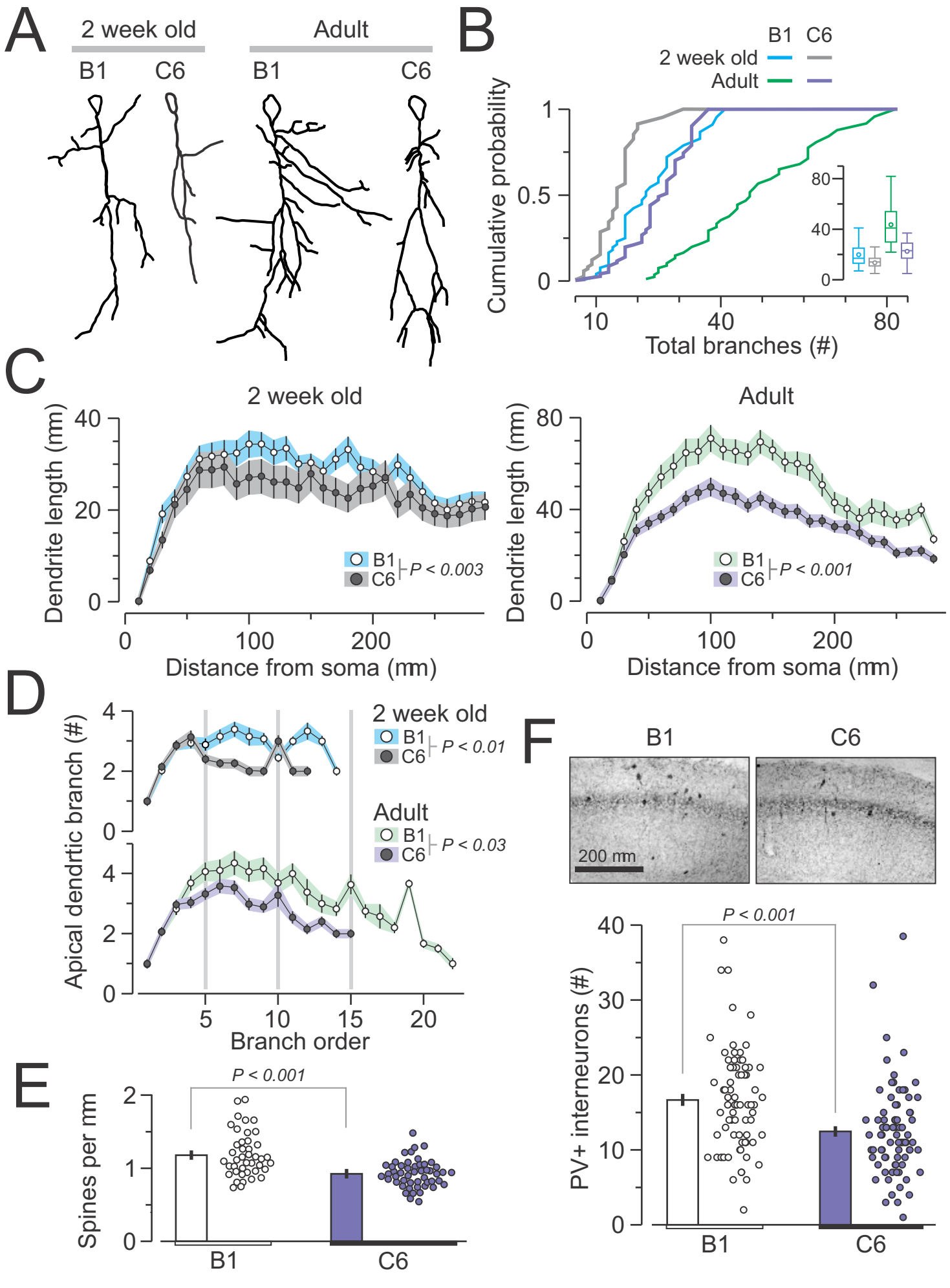
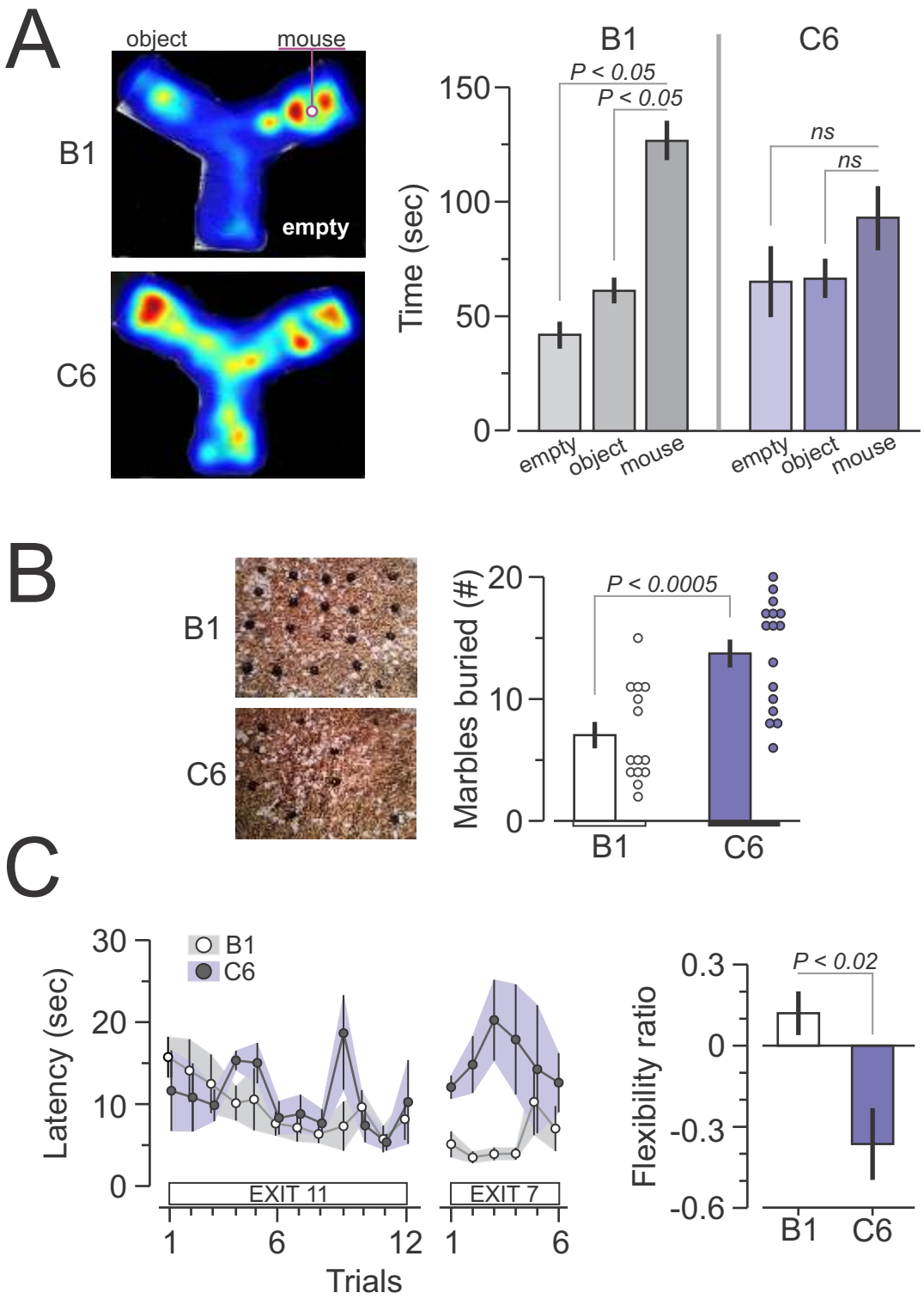


Figure 4 ~ Brimberg et al.



Supplementary Information

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

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Supplementary Methods

Antibody Production

Antibodies were expressed *in vitro* as described previously [1], with several modifications. In brief, 24 h before transfection with both heavy and light chain plasmids, human embryonic kidney fibroblast 293T cells (HEK-293T, ATCC CRL 11268^{TM}) 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).Eight hours before transfection, the medium was changed to SFM4Transfx-293 (HyClone, GE Healthcare) supplemented with glutamine (1%) and penicillin-streptomycin (1%). Cells were cotransfected with plasmids 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). Purified antibody was dialyzed extensively against PBS; integrity was determined by nonreducing SDS gels stained with Coomassie blue and concentration was measured by both anti–human IgG ELISA [1, 2] and Nanodrop.

HEK-293T cells were tested periodically for the presence of mycoplasma and remained negative.

Binding assays using transfected HEK-293T cells

Plasma and the C6 and B1 [3] human monoclonal antibodies, were analyzed for binding to Caspr2 using a live cell-based immunofluorescence assay as previously described [4]. HEK-293T cells (ATCC) 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 FBS (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 IgG binding. The commercially available Anti-Caspr2 antibody directed to an extracellular epitope (NeroMab UC Davis, CA clone K67/25) served as positive control. Anti-Caspr2 antibody directed to a cytoplasmic epitope (Abcam, ab137052) served as negative control. Cells transfected with tGFP vector lacking Caspr2 and non-transfected cell served as additional negative controls. Intensity and localization of reactivity was determined by two independent observers. One observer was blinded to the identity of the samples.

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 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 at -80°C. To determine protein concentration, samples were treated with Compat-Able^{TM} 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 using NuPAGE (Invitrogen) and transferred to PDVF membranes. Membranes were incubating in blocking buffer for 1 h at RT (4% milk, 0.1% PBS-Tween) followed by incubation with anti-Caspr2 monoclonal antibody (Abcam ab137052) (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 (Abcam, ab58475 ,1:10,0000) used as the loading control. Secondary antibody alone showed no binding.

Caspr2 RNA expression

Total RNA was extracted from brain and placenta with an RNeasy kit (Qiagen) and subjected to reverse transcription with an iScript cDNA synthesis kit (Bio-Rad). cDNA was analyzed by quantitative polymerase chain reaction (qPCR) with a LightCycler 480 master

mix with TaqMan probes for mouse CNTNAP2 and Polr2A (Applied Biosystems,Thermo Fisher Scientific). Data were normalized to Polr2a; relative induction was calculated by ΔΔCt .

Immunohistology of fetal brains

Staining of C6 and B1: Timed pregnant C57BL/6 dams were sacrificed and the placental sac containing the embryos was surgically removed. The embryos were kept on ice cold Hank's Balanced Salt Solution (HBSS, ThermoFisher Scientific). Each embryo was kept in its placental sac until ready for perfusion. Cardiac perfusion was performed as described in [5]. Embryonic mice were perfused with heparinized preperfusion buffer followed by paraformaldehyde (4%). The volume used for perfusion was 100 µl for 15.5 days old embryos and the flow rate was 0.001 ml/minute. After perfusion, E15.5 embryos 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 at -80˚C. Sagittal sections were cut (12 µm thick) on a Cryostat (Leica) and mounted on gelatin-coated slides and stored at -80˚C. Prior to staining, sections were thawed to RT, rinsed twice with PBS and blocked for 1 h with PBS (3%) with bovine serum albumin (BSA) and 3% heat inactivated FBS in Triton X100 (0.1%) at RT. B1 or C6 (10ug/ml) were added for 1 hour at RT. After washing in PBS/0.1%Tween, antibody binding was detected using Alexa 488 goat anti-human (Life Technologies) and visualized with an Axio-Imager (Z-1, Axio-Vision 4.7, Zeiss).

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 [3]. Every eighth section was incubated in anti-NeuN antibody (Millipore, Mab 337) at a 1:1000 dilution in 0.1 M PBS/1% BSA overnight at 4°C, and then washed and incubated with biotinylated anti-mouse IgG (1:200, Vector Laboratories) followed by avidin – biotin horseradish peroxidase complex at a 1:200 dilution

for 1 h (Vectastain Elite ABC Kit (Vector, PK-6100), and 3,3′-diaminobenzidine (DAB, 0.05%) with hydrogen peroxide (0.003%). Sections were dehydrated and coverslipped.

An assessment of the nearest neighbor (MBF, Williston, Vt.) generated information about the distribution of neurons within the layers examined. The first neuron that appeared in focus in an optical plane at the top of the physical section was chosen without bias (systematic random sampling grid employed) and the distance to the next neuron in focus was measured. This strategy was repeated to create a series of measurements between neurons in the specified region of interest. This method also generated an unbiased estimate of the number of neurons within the specified region. Neuron number was calculated according to a standard formula, and the nearest neighbor distances were expressed by a cumulative probability plot.

Golgi staining and analysis

Mice exposed *in utero* to antibody were randomly chosen and studied at 2 weeks or at 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), 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 visualized neuron had to have apical dendrites and a cell body and needed to be distinguishable 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) were obtained. Images stacks 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 stacks (2.0-μm separation, N.A.=0.75) were collected, the files were transferred for analysis, and the tracing of the dendritic arbor was quantified.

Parvalbumin staining

Staining was performed similarly to NeuN staining, on the same set of 16-20 weeks old mice. Anti-parvalbumin antibody (Abcam, ab11427) was used at a 1:500 dilution followed by incubation with biotinylated anti-rabbit IgG (1:200, Vector Laboratories). 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 (average±sem: C6=0.154 ± 0.005mm^2, B1=0.145±0.003mm^2, *P*=N.S).

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. Mice undergoing behavioral assessments were analyzed according to their cage number, assigned randomly. **Experiments were conducted at separate times for female and male mice.** Cage numbers did not indicate the antibody the mice were exposed to *in utero* and therefore the testing was performed in a blinded fashion. 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 [6] was conducted to assess autonomic responses and neurological reflexes . An open field test was used to examine locomotion; 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. The behavioral tests resembling the core symptoms of ASD were the marble burying assay, the social approach 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 [7] 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% marbles covered by bedding material) was recorded. The social approach test was performed in a Y-shaped maze [8] (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 **an age and sex matched C57BL/6** mouse (placed inside an inverted strainer cup) that had never been in contact with the test mouse, and 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. **Bedding was changed between each run. The restrained mouse was removed to its home cage between sessions and a longer break was allowed every 30 min.** The time spent exploring the object and the novel mouse were used to analyze sociability. The clockmaze task [6] 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, but not to induce anxiety. 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.

Supplementary Figures

Supplementary Figure 1: Monoclonal antibodies derived from a mother of a child with ASD stain mouse brain. Mouse brain transverse sections were incubated with supernatant of a human monoclonal antibody. Anti-brain IgG was detected using Alexa 488 anti-human IgG. (A-B) Example of a brain-reactive monoclonal antibody generated from a B cell of a mother of an ASD child. (C-D) Example of a monoclonal antibody that does not bind brain.

Supplementary Figure 2: Binding of plasma and C6 to brain tissue. (A-B) Plasma of the woman from whom C6 was isolated shows reduced binding to the hippocampus of CNTNAP2 -/- mice. (5x magnification**). (C-D) C6, but not B1, show staining to embryonic brain on embryonic day 15.5. Staining is greatest in the developing cortex and in the ventricular zone. CT, cortex; VZ, ventricular zone**.

Supplementary Figure 3: Expression of Caspr2 during development. Caspr2 relative gene expression in the (A) brain and (B) placenta. Caspr2 is expressed in early developmental stages in the brain and less in the placenta. (C) Protein expression of Caspr2 in the membrane fraction of brain. Sodium/ Potassium ATPase served as a loading control. (D) Levels of expression of Caspr2 in the brains of female and male mice on embryonic (E) days 13.5 and 17.5, postnatal (P) days 6 and 14 and in adult brains.

Supplementary Figure 4: (A) Plasma absorbed on tGFP-Caspr2 expressing cells (left panel); plasma absorbed on tGFP expressing cells (middle panel) and unmanipulated plasma (right panel) binding to t-GFP Caspr2 transfected cells. All plasma contained 60 ug/ml of IgG. tGFP-Caspr2 absorbed plasma show little if any binding HEK 293T cells expressing tGFP-Caspr2. tGFP- absorbed plasma and unmanipulated plasma show binding. No staining was seen on cells expressing only tGFP or non-transfected cells (data not shown). (B) The cortical plate is thinner in male fetal brains exposed to tGFP-absorbed plasma (middle panel) and unmanipulated plasma (bottom panel) relative to tGFP-Caspr2 absorbed plasma (top panel) as revealed by DAPI (left) and nestin (middle) staining (C) Quantification of cortical plate thickness, the ratio of cortical plate to cortical width, * $P < 0.05$, $Z = 2.5$, Mann-Whitney test. (D) Exposure *in utero* to tGFP-absorbed plasma (middle panel) and unmanipulated plasma (right panel) leads to fewer mitotic cells than exposure to tGFP-Caspr2 absorbed plasma (left panel) revealed by PH3 staining in the ventricular zone of male brains. (E) Quantification of mitotic cells (PH3+), ** $P < 0.001$, $t = 5.7$, t test. Unit area = 62.5 mm^2. Mice used: tGFP-Caspr2 absorbed plasma, n = 5; tGFP-absorbed plasma, n = 3; unmanipulated plasma, n = 5.

Supplementary Figure 5: C6-exposed female offspring display no behavioral phenotype. Behavior of C6- and B1-exposed female mice assessed at 10-14 weeks of

age. Representative behaviors are shown (A) Social approach. B1- and C6-exposed female mice displayed normal sociability, defined as spending significantly more time with the novel mouse compared to the novel object, B1, n=16, C6 n=11, 4 litters (ANOVA, Bonferroni , $P < 0.05$). (B) Marble burying test. B1, n=16, C6 n=11, 4 litters (t test, P=N.S).

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Table 1: Primary screen

A. Male

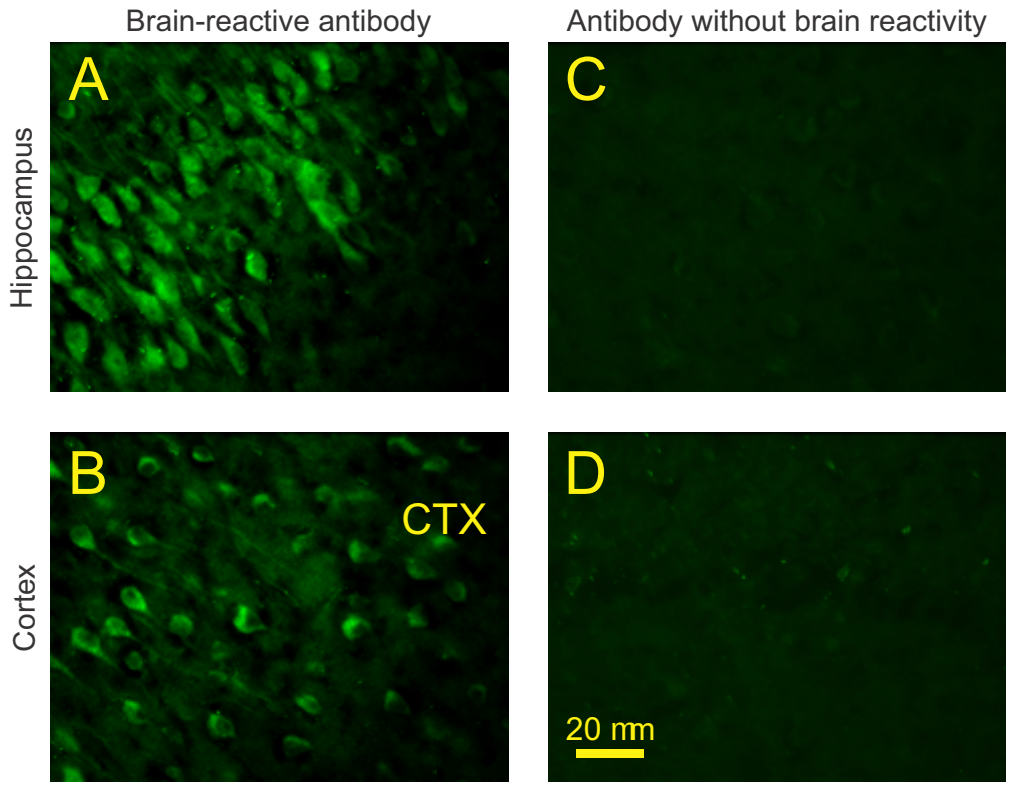
No.	Variable	Scale Range	B1	C6	U	*P*
1	Coat length	0–4	0 (0/0)	0 (0/0)	33	1
2	Hair length	0–3	0 (0/0)	0 (0/0)	33	1
3	Hair morphology	0–2	0 (0/0)	0 (0/0)	33	1
4	Body position	0–8	5 (5/5)	5 (5/5)	40	0.38
5	Spontaneous activity	0–8	5 (5/5)	5 (5/6)	18	0.05
6	Respiration rate	0–3	2 (2/2)	2 (2/2)	33	1
7	Tremor	0–2	0 (0/0)	0 (0/0)	33	1
8	Defecation	0–10	3 (2/6)	1 (0/2)	49	0.11
9	Urination	0–1	0 (0/0)	0 (0/0)	33	1
10	Transfer arousal	0–6	5 (5/5)	5 (5/5)	30	0.54
11	Latency to move	0–30	2 (1/3)	1 (1/1)	51	0.04
12	Locomotion	0–30	17 (14/20)	20 (19/24)	18.5	0.16
13	Piloerection	0–1	0 (0/0)	0 (0/0)	33	1
14	Palpebral closure	0–2	0 (0/0)	0 (0/0)	33	1
15	Startle response	0–3	1 (1/1)	1 (1/1)	33	1
16	Gait	0–3	0 (0/0)	0 (0/0)	33	1
17	Pelvic elevation	0–3	2 (2/2)	2 (2/2)	33	1
18	Tail elevation	0–2	1 (1/1)	1 (1/1)	33	1
19	Touch escape	0–3	2 (0/2)	2 (1/2)	40	0.41
20	Positional passivity	0–4	1 (0/2)	0 (0/1)	40	0.46
21	Trunk curl	0–1	1 (1/1)	1 (1/1)	30	0.54
22	Limb grasping	0–1	1 (1/1)	1 (1/1)	33	1
23	Visual placing	0–4	2 (2/3)	2 (2/2)	42.5	0.28
24	Grip strength	0–4	3 (2/3)	2 (2/3)	37	0.7
25	Body tone	0–2	1 (1/1)	1 (1/1)	22	0.06
26	Pinna reflex	0–2	1 (1/1)	1 (1/1)	33	1
27	Corneal reflex	0–2	1 (1/2)	1 (1/1)	38.5	0.68
28	Toe pinch	0–4	3 (2/3)	3 (2/3)	34.5	0.91
29	Body length	0–10	8 (7.8/8.5)	8.3 (7.3/8.5)	33	1
30	Tail length	0–10	7 (6.8/7.6)	6.8 (6.1/7.8)	39.5	0.54
31	Lacrimation	0–1	0 (0/0)	0 (0/0)	33	1
32	Whisker morphology	0–1	0 (0/0)	0 (0/0)	33	1
33	Provoked biting	0–1	1 (0/1)	0 (0/1)	43	0.27
34	Salivation	0–2	0 (0/0)	0 (0/0)	27.5	0.22
35	Heart rate	0–2	1 (1/1)	1 (1/1)	33	1
36	Abdominal tone	0–2	1 (1/1)	1 (1/1)	33	1
37	Skin color	0–2	1 (1/1)	1 (1/1)	33	1
38	Limb tone	0–4	2 (2/2)	2 (2/2)	30	0.54
39	Wire maneuver	0–4	0 (0/1)	0 (0/1)	34	0.95
40	Righting reflex	0–3	0 (0/0)	0 (0/0)	33	1
41	Contact reflex	0–1	1 (1/1)	1 (1/1)	33	1
42	Negative geotaxis	0–4	0 (0/0)	0 (0/0)	27.5	0.22
43	Fear to experimenter	0–1	0 (0/0)	0 (0/0)	33	1
44	Irritability	0–1	0 (0/0)	0 (0/0)	33	1
45	Aggression	0–1	0 (0/0)	0 (0/0)	30.5	0.72
46	Vocalization	0–1	0 (0/0)	0 (0/0)	30.5	0.72
47	Bizarre behavior	0–1	0 (0/0)	0 (0/0)	33	1
48	Weight	0-30	23 (21/26)	25 (21/29)	23.5	0.37

B. Female

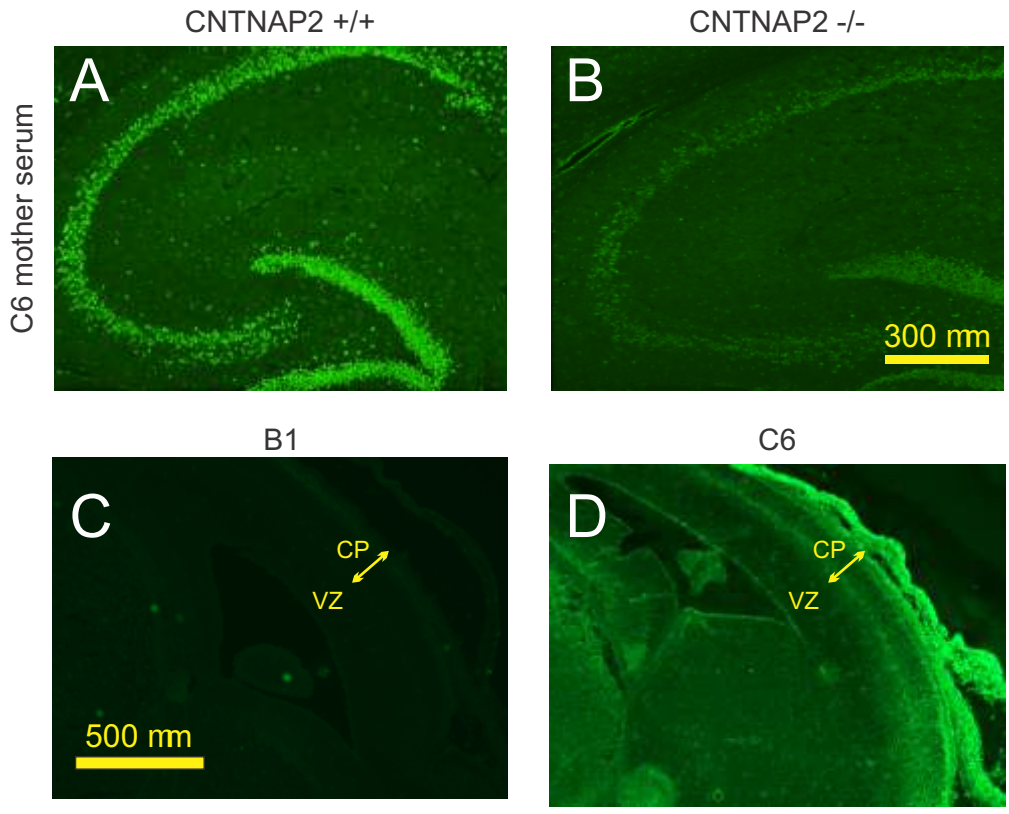
No.	Variable	Scale Range	B1	C6	U	*P*
1	Coat length	0–4	0 (0/0)	0 (0/0)	0	1
2	Hair length	0–3	0 (0/0)	0 (0/0)	0	1
3	Hair morphology	0–2	0 (0/0)	0 (0/0)	0	1
4	Body position	0–8	4 (4/5)	5 (5/5)	1.3	0.18
5	Spontaneous activity	0–8	5 (4/5)	4 (4/4)	1.1	0.25
6	Respiration rate	0–3	2 (2/2)	2 (2/2)	0	1
7	Tremor	0–2	0 (0/0)	0 (0/0)	0	1
8	Defecation	0–10	4 (3/4)	5 (4/6)	1.57	0.12
9	Urination	0–1	0 (0/0)	1 (1/1)	0	1
10	Transfer arousal	0–6	5 (5/5)	5 (5/5)	0.07	0.9
11	Latency to move	0–30	1 (1/4)	3 (2/3)	0.6	0.54
12	Locomotion	0–30	19 (17/19)	15 (13/17)	1.89	0.06
13	Piloerection	0–1	0 (0/0)	0 (0/0)	0	1
14	Palpebral closure	0–2	0 (0/0)	0 (0/0)	0	1
15	Startle response	0–3	1 (1/1)	1 (1/1)	0	1
16	Gait	0–3	0 (0/0)	0 (0/0)	0	1
17	Pelvic elevation	0–3	2 (2/2)	2 (2/2)	0	1
18	Tail elevation	0–2	1 (1/1)	1 (1/1)	0	1
19	Touch escape	0–3	2 (1/2)	2 (2/2)	0.49	0.6
20	Positional passivity	0–4	0 (0/1)	3 (3/3)	2.53	0.01
21	Trunk curl	0–1	1 (1/1)	1 (1/1)	0	1
22	Limb grasping	0–1	1 (1/1)	1 (1/1)	0	1
23	Visual placing	0–4	2 (2/2)	2 (2/2)	1.5	0.13
24	Grip strength	0–4	2 (1/3)	2 (2/2)	1.5	0.13
25	Body tone	0–2	1 (1/1)	1 (1/1)	0	1
26	Pinna reflex	0–2	1 (1/1)	1 (1/1)	0	1
27	Corneal reflex	0–2	1 (1/1)	1 (1/1)	0	1
28	Toe pinch	0–4	3 (2/3)	2 (2/2)	2.1	0.03
29	Body length	0–10	7 (7/7)	7.8 (7.5/8.3)	2.3	0.02
30	Tail length	0–10	7 (6.5/7)	6.8 (6.4/7.1)	0.26	0.8
31	Lacrimation	0–1	0 (0/0)	0 (0/0)	0	1
32	Whisker morphology	0–1	0 (0/0)	0 (0/0)	0	1
33	Provoked biting	0–1	0 (0/0)	1 (0/1)	0.83	0.41
34	Salivation	0–2	0 (0/0)	0 (0/0)	0	1
35	Heart rate	0–2	1 (1/1)	1 (1/1)	0	1
36	Abdominal tone	0–2	1 (1/1)	1 (1/1)	0	1
37	Skin color	0–2	1 (1/1)	1 (1/1)	0	1
38	Limb tone	0–4	2 (2/2)	2 (2/2)	0.18	0.86
39	Wire maneuver	0–4	0 (0/0)	0 (0/0)	0.43	0.67
40	Righting reflex	0–3	0 (0/0)	0 (0/0)	0	1
41	Contact reflex	0–1	1 (1/1)	1 (1/1)	0	1
42	Negative geotaxis	0–4	0 (0/0)	0 (0/0)	0.43	0.67
43	Fear to experimenter	0–1	0 (0/0)	0 (0/0)	0	1
44	Irritability	0–1	0 (0/0)	0 (0/0)	0	1
45	Aggression	0–1	0 (0/0)	0 (0/0)	0	1
46	Vocalization	0–1	0 (0/0)	0 (0/0)	0	1
47	Bizarre behavior	0–1	0 (0/0)	0 (0/0)	0	1
48	Weight	0-30	17 (17/18)	17 (17/19)	0.55	0.58

The variables were compared with the non-parametric Mann-Whitney test, followed by Bonferroni correction. Significant P value, $p<0.001$

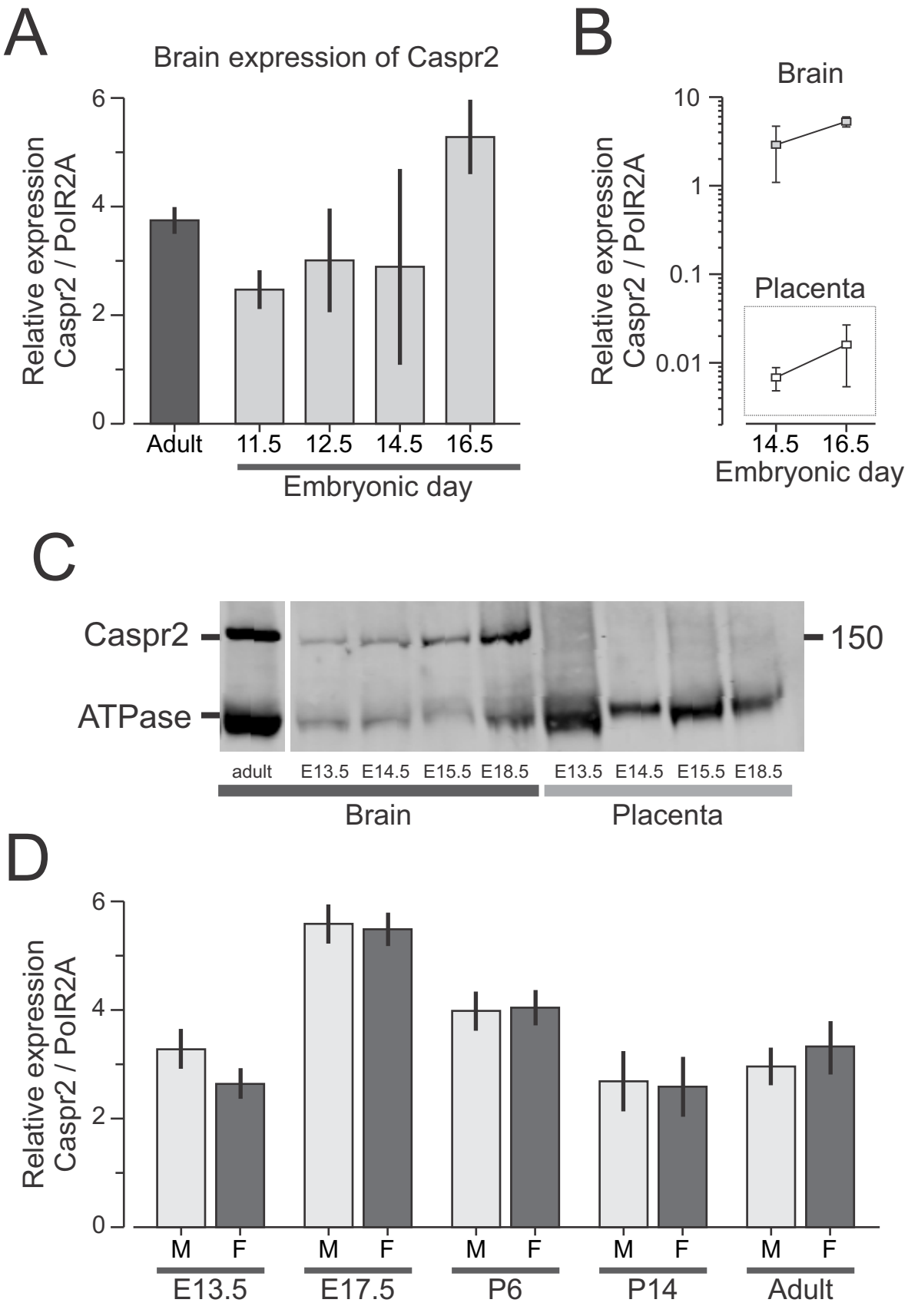
Supplementary Figure 1 ~ Brimberg et al.



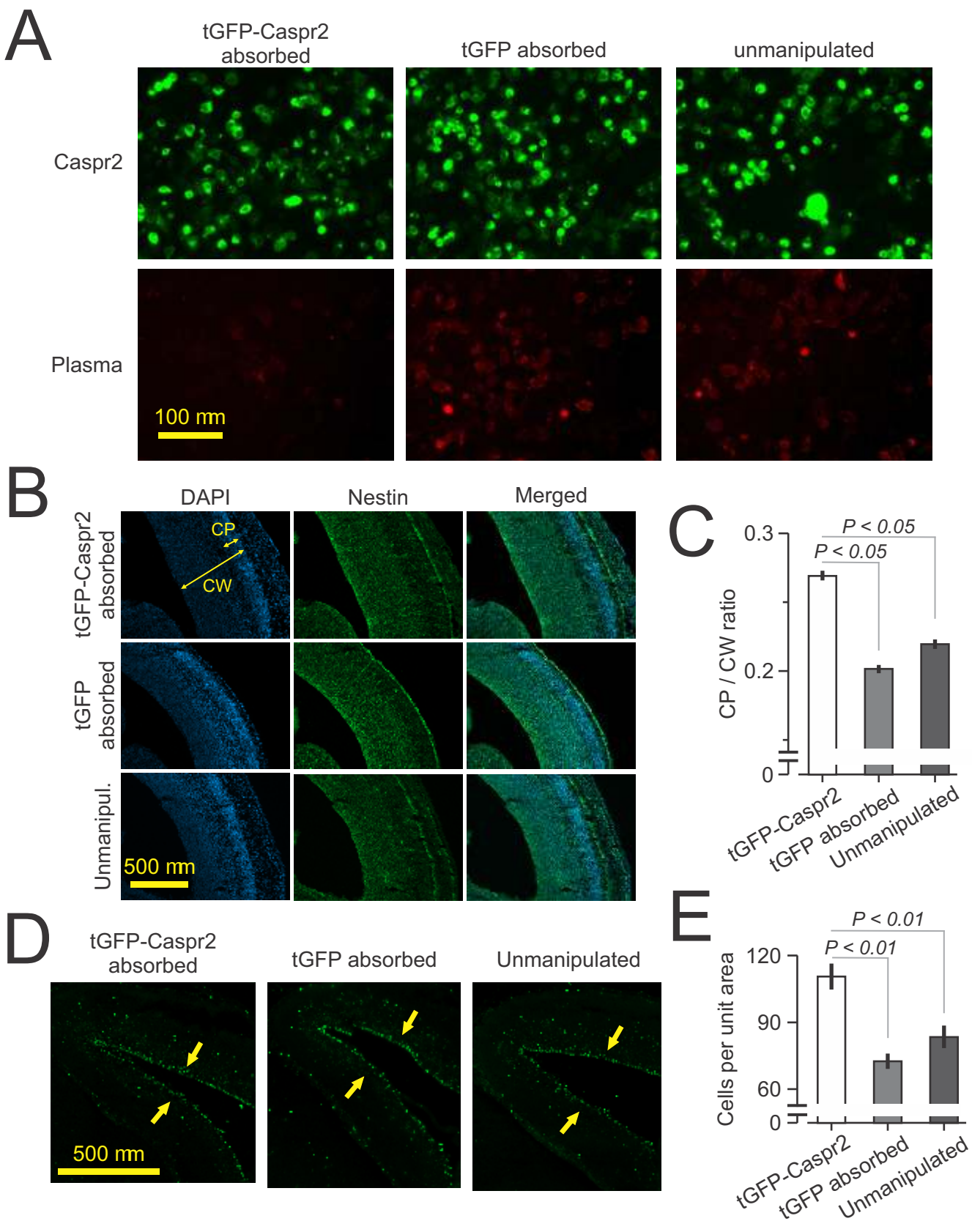
Supplementary Figure 2 ~ Brimberg et al.



Supplementary Figure 3 ~ Brimberg et al.



Supplementary Figure 4 ~ Brimberg et al.



Supplementary Figure 5 ~ Brimberg et al.