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Catalytic autoantibodies against myelin basic protein (MBP) isolated from serum of autistic children impair *in vitro* models of synaptic plasticity in rat hippocampus

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ABSTRACT

Autoantibodies from autistic spectrum disorder (ASD) patients react with multiple proteins expressed in the brain. One such autoantibody targets myelin basic protein (MBP). ASD patients have autoantibodies to MBP of both the IgG and IgA classes in high titers, but no autoantibodies of the IgM class. IgA autoantibodies act as serine proteinases and degrade MBP *in vitro*. They also induce a decrease in long-term potentiation in the hippocampi of rats either perfused with or previously inoculated with this IgA. Because this class of autoantibody causes myelin sheath destruction in multiple sclerosis (MS), we hypothesized a similar pathological role for them in ASD.

1. Introduction

Autism spectrum disorders (ASDs) are a heterogeneous group of neurodevelopmental conditions appearing during early childhood that are characterized by unusual repetitive behaviors and impaired social and communication skills (Volkmar and Pauls, 2003). Although the etiology of ASD is complex and likely involves the interplay of genetic and environmental factors, increasing evidence suggests that immune dysfunction and the presence of aberrant autoimmune responses, including autoantibodies, are associated with behaviors commonly observed in ASD (Careaga and Ashwood, 2012). There is a high correlation between the presence of serum antibodies against brain proteins and the severity of the disease in ASD children (Wills et al., 2011; Goines et al., 2011; Mostafa and Al-Ayadhi, 2012; Mazur-Kolecka et al., 2013; Piras et al., 2014). Furthermore, a similar correlation has been established between the severity of the disease in ASD patients and the presence of autoantibodies against brain proteins in the serum of their mothers (Braunschweig et al., 2012, 2013; Braunschweig and Van de Water, 2012; Fox et al., 2012; Nordahl et al., 2013; Brimberg et al., 2013). These studies used whole immunoglobulin fractions from ASD patients, and the autoantigens responsible for the induction of brain abnormalities were not identified.

The autoantibodies from individuals with autism react with a wide variety of proteins expressed in different regions of the brain, such as the hypothalamus, amygdala, caudate nucleus, cerebellum, brain stem and hippocampus (Mora et al., 2009). One such autoantibody targets myelin basic protein (MBP), a peripheral nervous system and central nervous system (CNS) antigen (Singh et al., 1993; Vojdani et al., 2002; Silva et al., 2004; Connolly et al., 2006; Mostafa and Al-Ayadhi, 2013). In this study, we used serum samples from a population of ASD patients who have autoantibodies against the voltage-dependent anion channel (VDAC) and hexokinase-I, a VDAC protective ligand, showing high reactivity with these antigens in brain tissue lysates from the caudate nucleus, cerebellum and hippocampus (Gonzalez-Gronow et al., 2010). In these patients, we identified both IgA and IgG class

Abbreviations: ASD, autistic spectrum disorder; MBP, myelin basic protein; LTP, long-term potentiation; BSA, bovine serum albumin; PBS, phosphate-buffered saline; ACSF, artificial cerebrospinal fluid; CNS, central nervous system; LTP, long-term potentiation.

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autoantibodies to MBP in high titers, but not IgM class autoantibodies. An evaluation of their specificity revealed that the IgA autoantibodies against MBP exhibit the catalytic activity of a serine proteinase in all ASD patient serum samples analyzed. The purified anti-MBP IgA also degrades MBP *in vitro*. Furthermore, we determined whether this IgA affects synaptic plasticity in rat hippocampal slices, and found that long-term potentiation (LTP) is decreased in the hippocampi of animals either perfused with or previously inoculated with this autoantibody.

2. Materials and methods

2.1. Patients

Thirty-four children with autistic disorder, 32 boys and 2 girls, were included in the study. All patients were recruited through family members of the Chilean Association of Parents with Autistic Children (ASPAUT) and diagnosed following procedures outlined by the American Psychiatric Association in the Diagnostic and Statistical Manual of Mental Disorders, DSM-IV (1994). The instruments used for the screening and diagnosis of autism were the Childhood Autism Rating Scale (CARS) (Schopler et al., 1980), the Autism Diagnostic Interview-Revised (ADI-R) (Lecouteur et al., 1989; Lord et al., 1994), and the Autism Diagnostic Observation Schedule (ADOS) (Lord et al., 2000). These tests were administered by a psychiatrist, Dr. Rina Francos, who is a clinical advisor to ASPAUT. The scores for these patients met the cut-off values for the category designated as "Broad Autistic Spectrum Disorder (ASD)", according to criteria established by the NICHD/NIDCD Collaborative Programs for Excellence in Autism (CPEA) (Lainhart et al., 2006). Clinical assessment included medical and psychiatric history, demographic data, physical examination, and routine laboratory evaluations. Other patients with disorders possibly associated with autism, such as fragile X syndrome, Asperger's syndrome, cerebral palsy, tuberous sclerosis and neurofibromatosis were excluded from this study. Patients with schizophrenia and other psychotic disorders were also excluded.

The patients selected for this study were also assessed for immune disorders, such as juvenile rheumatoid arthritis, rheumatic fever, psoriatic arthritis, ankylosing spondylitis, systemic lupus erythematosus, dermatomyositis, polymyositis, vitiligo, myasthenia gravis, multiple sclerosis (MS), amyothrophic lateral sclerosis, ulcerative colitis, Crohn's disease, hyperthyroidism, hypothyroidism, idiopatic thrombocytopenic purpura, scleroderma, uveitis, polyarteritis nodosa, Wegener's granulomatosis, Takayasu's arteritis, type 1 diabetes, vasculitis, Addison's disease, Sjogren syndrome, pemphigus and Guillain–Barrè syndrome. Clinical assessment procedures included anthropomorphic measurements, dysmorphic examination and neurologic examination. Baseline hematologic parameters, including complete blood count, erythrocyte sedimentation rate (ESR), creatinine, TSH, T3, T4 and cortisol, and urinalysis were also determined. Magnetic resonance imaging (MRI) was also performed in this group of patients. Selected patients showed no significant abnormal features by MRI. Peripheral blood samples were also obtained from age-matched control children admitted to the Clinical Hospital of the University of Chile for minor traumas or elective surgery. Blood samples were collected from the children after written consent was obtained from their parents before admission to the clinics.

2.2. Materials

The chromogenic substrates, D-Ile-Pro-Arg-p-nitroanilide (pNA) (S-2288) and D-Val-Leu-Lys-pNA (S-2251), were obtained from Chromogenix (Bedford, MA). The chromogenic substrates D-Leu-pNA, D-Lys-pNA, D-Arg-pNA and D-Ala-pNA were obtained from Sigma-Aldrich (St. Louis, MO). The affinity resins for proteins A/G, lectin jacalin and the mannan binding lectin cross-linked to Sepharose-CL-6B were purchased from Thermo Scientific-Pierce (Rockford, IL). The other reagents used were of the highest grade available.

2.3. Proteins

Recombinant human MBP and the lectin jacalin-A were obtained from Sigma-Aldrich (St. Louis, MO). Porcine MBP was obtained from Enzo Life Sciences (Farmingdale, NY). Human tissue-type plasminogen activator (t-PA) was purchased from Calbiochem-EMD Chemicals, Inc. (San Diego, CA). The protease inhibitors aprotinin (PTI), leupeptin, plasminogen activator inhibitor 1 (PAI-1) and the alkaline phosphatase-conjugated secondary antibodies against immunoglobulin A (IgA), immunoglobulin G (IgG) and immunoglobulin M (IgM) were purchased from Sigma-Aldrich (St. Louis, MO). The rabbit anti-MBP polyclonal antibody was purchased from Millipore (Temecula, CA). The donkey anti-rabbit IRDye 680 LT-conjugate was purchased from Rockland Immunochemicals, Inc. (Gilbertsville, PA).

2.4. Purification of immunoglobulins from autistic serum

A fraction containing total IgG was purified from a pool of serum (17 ml) from autistic children ($n = 34$) utilizing affinity chromatography on a resin containing equal amounts of proteins A and G cross-linked to Sepharose-CL-6B according to the manufacturer's instructions (Pierce-Thermo Scientific, Rockford, IL). Total IgA was purified from the protein pool not adsorbed to the protein A/G in the previous step, utilizing affinity chromatography on a resin containing the lectin jacalin-A cross-linked to Sepharose-CL-6B as previously described (Roque-Barreira and Campos-Neto, 1985). Total IgM was purified from the pool of proteins not adsorbed during the previous two steps utilizing affinity chromatography on a resin containing mannan binding protein cross-linked to Sepharose-CL-6B as previously described (Nevens et al., 1992).

2.5. Protease activity of purified immunoglobulin fractions from ASD patient serum

Purified IgAs, IgGs or IgMs (10 nM) were tested for proteolytic activity using chromogenic protease substrates at a single concentration (0.3 mM). These substrates include S-2288, S-2251, D-Leu-pNA, D-Lys-pNA, D-Arg-pNA and D-Ala-pNA in 96-well microtiter plates at 37 °C in 0.1 M Tris–HCl and 100 mM NaCl, pH 8.0, in a total volume of 200 μl. The velocity of hydrolysis of the substrates was calculated as the increase of absorbance at 405 nm, caused by released p-NA, measured using a Molecular Devices SPECTRAmax kinetic plate reader (Sunnyvale, CA). The molar extinction coefficient (ε) of p-NA at 405 nm is 10,000 M^{-1} cm^{-1} (Wohl et al., 1980).

2.6. Purification of IgA autoantibodies to MBP by immunoaffinity chromatography

Porcine MBP (2 mg/ml) was covalently attached to Sepharose 4B resin. Total IgA purified from the sera of ASD children was incubated with this resin and the specifically adsorbed anti-MBP IgA was eluted with 1 M guanidine-HCl in PBS at 4 °C. The IgA was then extensively dialyzed against PBS, concentrated to 0.5 mg/ml and stored in small aliquots at −80 °C until further use.

2.7. SDS-PAGE and immunoblotting

Recombinant human MBP was analyzed on 4–12% polyacrylamide gels (1.2 mm thick, 14 × 10 cm) containing 0.1% SDS under reducing conditions. A discontinuous Laemli buffer system was used (Laemli, 1970). The proteins were transferred from the gels to nitrocellulose membranes (Towbin et al., 1979). The molecular weights were assessed

using a set of dye-conjugated Mr markers (Fermentas Life Sciences, Glen Burnie, MD). The membranes were thoroughly rinsed with phosphate-buffered saline (PBS) and then incubated with 3% bovine serum albumin (BSA) in phosphate-buffered saline PBS for 1 h at room temperature to block non-conjugated areas. Then, the membranes were incubated with a rabbit anti-MBP IgG (5 μg/ml) in 3% BSA in Tris-buffered saline (TBS) overnight at room temperature. After incubation with the primary antibody, the membranes were washed 3 times for 5 min each with TBS containing 0.1% Tween 20 (TBST). The membranes were then incubated with a 1:10,000 dilution of an anti-rabbit IRDye 680 LT for 1 h at room temperature in the dark. Then, the membranes were washed twice for 5 min each with TBST and once with TBS for 5 min. The probed membranes were scanned on a Li-Cor Odissey System (Li-Cor Biosciences, Lincoln, NE).

2.8. Enzyme-linked immunosorbent assay (ELISA) procedure

We determined antibody titers against MBP in sera from ASD patients and control subjects. Human MBP (5 μg/ml) in 0.1 M sodium carbonate, pH 9.5, containing 0.01% NaN_3, was used (200 μl/well) to coat 96-well polystyrene flat-bottom ELISA plates. The plates were incubated overnight at room temperature and then washed three times with PBS containing 0.05% Tween 80 (PBS-Tween). Plates were then incubated for 2 h at room temperature with 2% BSA to block non-specific binding. The plates were again washed with PBS-Tween, and then the serum samples diluted 1:500 in PBS-Tween were added to triplicate wells and incubated for 2 h at room temperature. Next, the plates were washed three times with PBS-Tween, and then a 1:1000 dilution (100 ng) of alkaline phosphatase-conjugated goat anti-human IgG, anti-human IgA or anti-human IgM in PBS-Tween was added to each well. The plates were incubated for an additional 2 h at room temperature. After washing three times with PBS-Tween, the plates were incubated with the alkaline phosphatase substrate p-nitrophenylphosphate (1 mg/ml) in 0.1 M glycine, 1 mM $MgCl_2$, and 1 mM $ZnCl_2$, pH 10.4. The absorbance was monitored at 405 nM using a Molecular Devices SPECTRAmax kinetic plate reader (Sunnyvale, CA). Bound IgG, IgA or IgM was expressed as μg/ml serum.

2.9. Amidolytic activity assays

The amidolytic activities of t-PA or the anti-MBP IgA toward S-2288 were analyzed in the presence of a single concentration of t-PA or IgA (10 nM) in 0.1 M Tris–HCl and 100 mM NaCl, pH 8.0. The velocity of S-2288 hydrolysis was calculated as the increase of absorbance at 405 nm measured using a Molecular Devices SPECTRAmax kinetic plate reader (Sunnyvale, CA).

2.10. Analysis of protease activities in autistic serum

Jacalin-A (5 μg/ml) in 0.1 M sodium carbonate, pH 9.5, containing 0.01% NaN_3 was used (200 μl/well) to coat 96-well polystyrene flat-bottom ELISA plates. The plates were incubated overnight at room temperature and then washed three times with PBS-Tween. The plates were then incubated for 2 h at room temperature with 2% BSA to block nonspecific binding. Next, the plates were washed with PBS-Tween, and serum samples (10 μl) in PBS were added to triplicate wells and incubated for 2 h at room temperature. After washing three times with PBS, the plates were incubated with the chromogenic substrate S-2288 (0.3 mM) in 0.1 M Tris–HCl and 100 mM NaCl, pH 8.0. The velocity of S-2288 hydrolysis was calculated as the increase of absorbance at 405 nm measured using a Molecular Devices SPECTRAmax kinetic plate reader (Sunnyvale, CA).

2.11. Animals

Four groups of 4–6 weeks old male Sprague–Dawley rats were used for these experiments. One group consisted of animals (n = 5) injected intraperitoneally for 10 days with a saline solution containing 25 μg/day of anti-MBP IgA purified from autistic serum. A second group (n = 5) was injected with a similar amount of non-immune human IgA in saline. The third group (n = 5), injected with saline alone, was used as a control. A fourth group of animals (n = 14) was not injected and used for experiments in which their hippocampi were exposed directly to a perfusion solution containing either anti-MBP IgA (1 μg/ml) or non-immune IgA (1 μg/ml) in artificial cerebrospinal fluid (ACSF), containing 124 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$ and 10 mM glucose, pH 7.4.

2.12. Hippocampal slice preparation

Transverse hippocampal slices were prepared from all rats in the four groups. The animals were anesthetized with halothane gas and then decapitated. Brains were rapidly removed and immersed in ice-cold dissection buffer containing 212 mM sucrose, 5 mM KCl, 1,25 mM NaH_2PO_4, 3 mM $MgSO_4$, 1 mM $CaCl_2$, 26 mM $NaHCO_3$ and 10 mM glucose, pH 7.4. The hippocampus was dissected and transverse slices (400 μm thick) were obtained from the dorsal portion using a Model HA752 vibratome (Campden Instruments, Leicester, UK). The slices were then transferred to an interface storage chamber saturated with 95% O_2/5% CO_2 in ACSF. For the experiments, single slices were transferred to a recording chamber where they were continually perfused (2 ml/min) with ACSF after submersion.

2.13. Extracellular field recording and long term potentiation (LTP) induction

Field responses were evoked with electrical stimulation (biphasic, constant current, 200 μs stimuli) delivered every 15 s to the Schaffer collateral pathway using bipolar electrodes and recorded in the *stratum radiatum*, to measure the field excitatory postsynaptic potential (fEPSP) slopes (Olmos et al., 2009). Recording electrodes were glass micropipettes (1–3 MΩ) filled with ACSF. At the beginning of each experiment, stimulus/response curves were obtained by increasing the intensity of the stimulus to 50% of the maximum response. LTP was elicited after 10–15 min of a stable baseline by theta burst stimulation (TBS) consisting of 3 trains of stimuli with an inter-train interval of 10 s. Each train consisted of 10 bursts at 5 Hz, each with 4 pulses at 100 Hz. After TBS, data acquisition occurred for 1 h. Data were acquired using an A-M Systems Model 1800, extracellular amplifier from A-M Systems (Sequim, WA) and a data acquisition board from The National Instruments Corporation (Austin, TX) controlled with the IGOR Pro6 software from Wavemetrics Inc. (Lake Oswego, OR). Cumulative probability plots and LTP values were constructed by averaging the percent LTP observed between 30 and 60 min following TBS.

2.14. Statistics

Graph Pad Prism, version 6.0 software from Graph Pad Software, Inc. (San Diego, CA) was used to determine the standard deviation of the experimental data. The significance of differences between the controls and different treatments was determined by a one-way analysis of variance (ANOVA) and unpaired Student's t-tests.

3. Results

3.1. Serum levels of autoantibodies to MBP

Serum levels of autoantibodies to MBP were assessed by ELISA in sera from children with ASD (n = 34) and age-matched control children

(n = 34). Table 1 shows the demographics and baseline characteristics of the patients and their controls. There are no statistically significant differences between the demographic characteristics of the two groups. The average age of the patients when the first autistic symptoms were detected was 19.72 ± 9.17 months and the majority had a very high autistic score according to the CARS, ADI-R and ADOS tests (Table 2). A comparison of the titers for autoantibodies to MBP between ASD children and controls shows statistically significant differences in the titers only for the IgA (Fig. 1A) and IgG classes (Fig. 1B), whereas the IgM class autoantibodies (Fig. 1C) were not different between ASD patients and controls. In ASD patients, the median concentrations of anti-MBP IgA were 5.25 ± 2.43 μg IgA/ml serum compared to 1.87 ± 0.83 μg/ml serum in normal control ($p < 0.005$). The median concentrations of anti-MBP IgG were 6.12 ± 3.51 μg/ml serum and 2.46 ± 1.83 μg/ml serum for children with ASD and normal control, respectively ($p < 0.002$). The median concentrations of anti-MBP IgM were 1.83 ± 0.61 μg/ml serum and 1.28 ± 0.75 μg/ml serum for ASD patients and normal controls, respectively (p = non-significant). No correlation was observed between clinical scores (CARS, ADI-R and ADOS) and IgA and IgG autoantibodies to MBP in these patients, possibly because their high clinical scores show very little dispersion (Table 2).

3.2. Proteolytic activity of autoantibodies to MBP in the sera of autistic children

Autoantibodies to MBP of the IgA, IgG and IgM classes in the sera of MS patients show proteolytic activity and specifically hydrolyze MBP (Polosukhina et al., 2005). Therefore, we evaluated the proteolytic activities of the autoantibodies to MBP in the sera of children with ASD. First, we assessed the amidolytic activities of total IgA, IgG or IgM, purified from a pool of sera containing 0.5 ml of each patients's serum (n = 34). We used a battery of chromogenic protease substrates, including S-2288, S-2251, D-Leu-pNA, D-Lys-pNA, D-Arg-pNA and D-Ala-pNA (Fig. 2A). The ASD IgA shows a significant hydrolytic activity with the substrate S-2288, whereas activity with the other chromogenic substrates is negligible. The proteolytic activity is unique for the IgAs because purified IgGs or IgMs do not show significant activity with any of the substrates. We then evaluated the IgA proteolytic activity in all our ASD patients (n = 34) compared to normal control children (n = 34). The assays were performed in 96-well plates containing immobilized jacalin-A as described under the Section 2.

The results (Fig. 2B) show that IgA with significant proteolytic activity is present in the majority of ASD serum samples analyzed (median: 3.45×10^{-8} mol p-NA/ng IgA) compared to normal control children (median: 0.93×10^{-8} mol p-NA/ng IgA) ($p < 0.005$). To demonstrate the specificity of the IgA protease, we incubated recombinant human MBP with specific IgA purified from a pool of autistic sera, as described under the Section 2. After incubation of MBP (0.5 μg) with this IgA (2 μg) for 3 h at 37 °C, the proteins were separated using gel electrophoresis and analyzed by Western blot. The results (Fig. 3) demonstrate that the specific anti-MBP IgA from ASD children catalyzes the degradation of MBP.

Table 1
Demographics of ASD patients and their controls.

	ASD patients	Controls	p
Age (years). Mean ± SE	9.06 ± 3.07	9.04 ± 3.82	N.S.
Age (years). Median (range)	9.0 (5–14)	8.0 (1–14)	N.S.
Male (n)	32	31	N.S.
Female (n)	2	3	N.S.

N.S. = nonsignificant.

Table 2
Standardized assessment of autistic symptoms of 34 ASD patients.

CARS (number of children)		
Nonautistic, score < 30, n (%)	0	
Mild/moderate, score 31–36, n (%)	4 (9.68)	
Severe, score 37–60, n (%)	30 (90.32)	
ADI-R (mean ± SE)		ADI-R cut-off values for AD
Social interaction	27.42 ± 0.71	10
Verbal communication	13.57 ± 0.78	8
Repetitive behaviors	6.71 ± 2.2	3
ADOS (mean ± SE)		ADOS cut-off values for AD
Social interaction	12.4 ± 1.21	4
Verbal communication	7.26 ± 1.27	3
Stereotyped behaviors	3.8 ± 1.97	Not included in diagnostic algorithm

Abbreviations: CARS, childhood autism rating scale; AD1-R, Autism Diagnostic Interview-Revised; ADOS, Autism Diagnostic Observation Schedule.

3.3. Assessment of the specificity of the protease activity of the ASD anti-MBP IgA pool in the presence of serine-protease inhibitors

The protease activity of the anti-MBP IgA pool was measured with the chromogenic substrate S-2288. Since its discovery, this substrate has been routinely used to evaluate the proteolytic activity of t-PA, but is also used to assess a broad spectrum of serine proteases (Friberger, 1982). The methods we used to purify the anti-MBP IgA minimizes possible contaminations with t-PA or any other plasma serine proteases. To exclude this possibility, we compared the activities of t-PA (0.2 μM) with the protease IgA (0.2 μM) in the presence of several serine protease inhibitors. First, we measured the amidolytic activities of the IgA and t-PA in the presence of increasing concentrations of aprotinin. The results (Fig. 4A) show a progressive inhibition of the IgA activity with aprotinin, whereas t-PA remains unaffected (Lottemberg et al., 1988). This effect of aprotinin distinguishes the IgA from t-PA. Next, we incubated both proteins with increasing amounts of the wide spectrum serine protease inhibitor, leupeptin (Kurinov and Harrison, 1996) and observed inhibition of both their activities (Fig. 4B). Finally, we measured the amidolytic activity of both proteins in the presence of increasing concentrations of the highly specific t-PA inhibitor, PAI-1 (Mottonen et al., 1992), and observed the progressive inhibition of t-PA, whereas the activity of the IgA was not affected (Fig. 4C). These experiments show that the proteolytic activity of the IgA pool, although affected by serine protease inhibitors common to both proteins, is clearly distinguishable from t-PA.

3.4. Effect of anti-MBP IgA exposure on LTP induction in rat hippocampal slices

Three groups of animals (n = 15) were used for experiments in which their hippocampal slices were exposed directly to a perfusion solution containing ASD anti-MBP IgA (1 μg/ml), non-immune IgA (1 μg/ml) or ASD anti-MBP IgA (1 μg/ml) in the presence of recombinant human MBP (2 μg/ml) in ACSF for 3 h at room temperature. Then, the slices (n = 20) were submerged in a recording chamber and continually perfused (2 ml/min) with these solutions. LTP was induced as described under Section 2. The results (Fig. 5A) show a mean potentiation of 157.17 ± 7.93 in hippocampal slices exposed to non-immune IgA in the *stratum radiatum*, whereas slices exposed to ASD anti-MBP IgA show a mean potentiation of 113.3 ± 3.70 (n = 20, squares). Pre-incubation of the hippocampal slices with a mixture of ASD anti-MBP IgA and human recombinant MBP show a mean potentiation of 145.29 ± 19.70. These changes in LTP (Fig. 5B) show a significant decrease in the field potentials, measured in the *stratum radiatum*, ($p < 0.0001$) of hippocampal slices exposed to IgA autoantibodies against MBP compared to the field potentials after

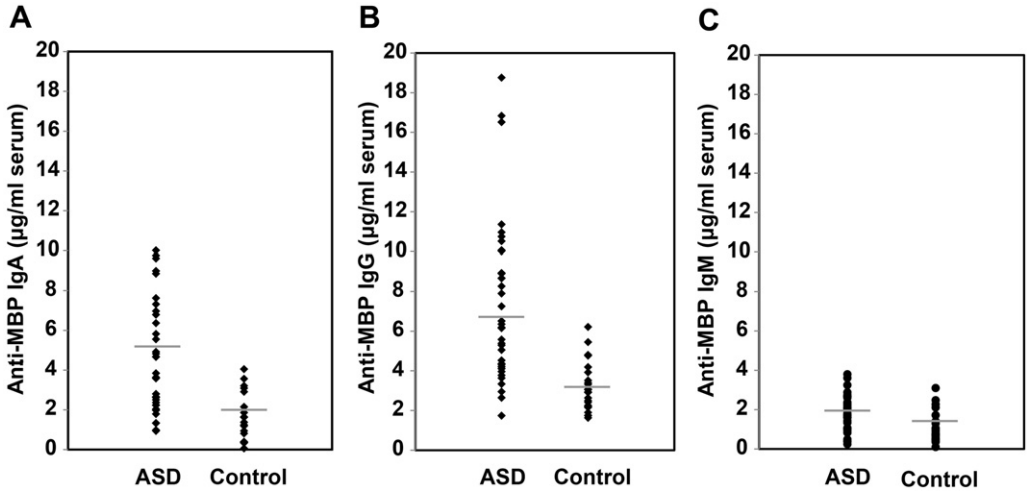

Fig. 1. Serum titers of specific anti-MBP antibodies in ASD patients (n = 34) and age-matched control children (n = 34). (A) Median titers of anti-MBP IgA. (B) Median titers of anti-MBP IgG. (C) Median titers of anti-MBP IgM. Each value represents the mean of experiments performed in triplicate.

exposure to non-immune human IgA or a mixture of anti-MBP IgA and human recombinant MBP. These experiments also demonstrate that pre-incubation of the anti-MBP IgA with its antigen produces an almost complete recovery of LTP in hippocampal slices exposed to this mixture.

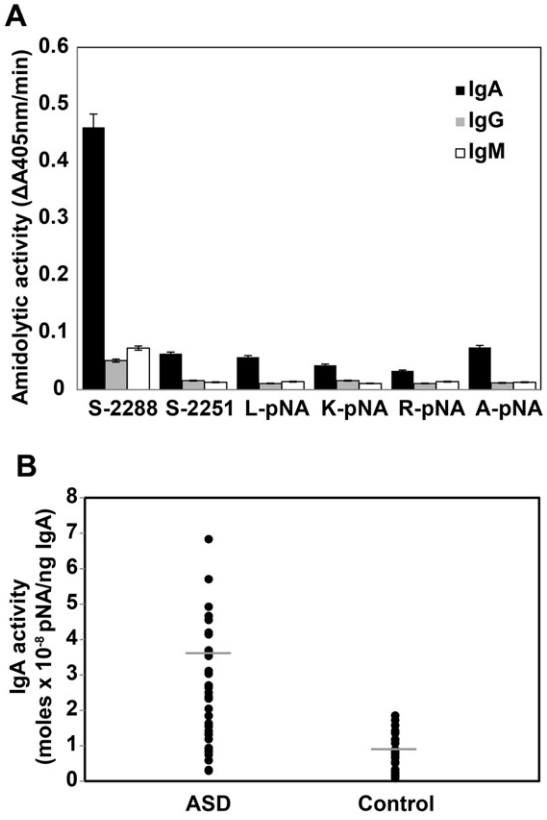

Fig. 2. (A) Proteolytic activity of a single concentration (0.2 μM) of total IgA, IgG and IgM purified from sera of ASD patients at a single concentration (0.3 mM) of the protease substrates IPR-pNA (S-2288), VLK-pNA (S-2251), L-pNA, K-pNA, R-pNA y A-pNA. (B) Proteolytic activity of single ASD patient serum IgA (n = 34) compared with normal control children IgA (n = 34) after immobilization of IgA on jacalin-A plates. The assay was performed as described under Section 2 using S-2288 (0.3 mM) as the substrate. Each value represents the mean from experiments performed in triplicate.

3.5. LTP induction in hippocampal slices of rats previously inoculated with anti-IgA or non-immune human IgA

Young (4–6 weeks old) rats (n = 5) were injected intraperitoneally daily with 25 μg of ASD anti-MBP IgA pool or non-immune control children IgA pool for 10 days. At the end of this period, the animals were anesthetized and decapitated and hippocampal slices from their brains were prepared as described above. The slices were singly transferred and submerged in a recording chamber, continuously perfused (2 ml/min) with ACSF, and LTP was induced as described above. The results (Fig. 6A) show a mean potentiation of 141.62 ± 6.42 in hippocampal slices from rats injected with non-immune human IgA

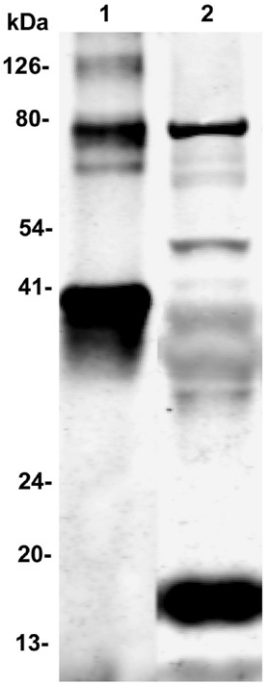

Fig. 3. SDS-PAGE analysis of MBP hydrolysis by purified anti-MBP IgA. Proteins were first separated using gel electrophoresis and then analyzed by Western blot as described under Section 2. Lane 1, recombinant human MBP (0.5 μg). Lane 2, recombinant human MBP (0.5 μg) incubated with anti-MBP IgA (2 μg) for 3 h at 37 °C in PBS.

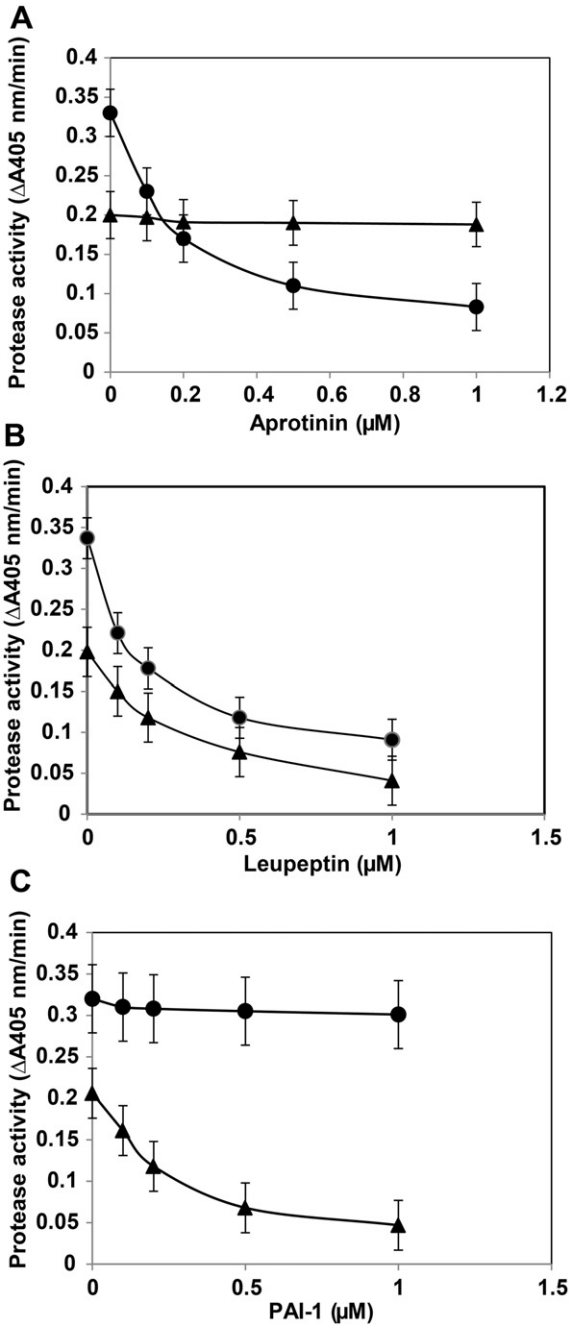

Fig. 4. Comparison of the efficacy of serine-protease inhibitors on the activity of the anti-MBP IgA protease and t-PA. The protease IgA and t-PA at a single concentration (0.2 μM) were singly incubated with increasing concentrations of serine protease inhibitors as described under Section 2. (A) Proteolytic activity of the anti-MBP IgA (●) or t-PA (▲) in the presence of increasing concentrations of aprotinin. (B) Proteolytic activity of the anti-MBP IgA (●) or t-PA (▲) in the presence of increasing concentrations of leupeptin. (C) Proteolytic activity of the anti-MBP IgA (●) or t-PA (▲) in the presence of increasing concentrations of PAI-1. Each value represents the mean of experiments performed in triplicate.

($n = 10$) in the *stratum radiatum*, whereas hippocampal slices from rats injected with the autistic anti-IgA show a mean potentiation of 119.43 ± 5.37 ($n = 10$). The analysis of LTPs (Fig. 6B) shows a significant decrease in the field potentials, measured in the *stratum radiatum*, ($p < 0.05$) of hippocampal slices from rats injected with autoantibodies against MBP compared with the field potentials obtained in hippocampal slices from animals injected with non-immune IgA from normal control children.

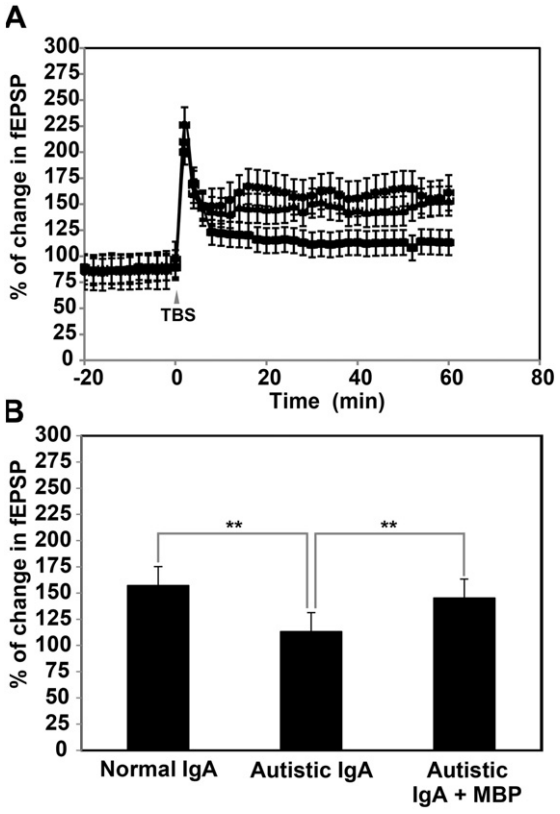

Fig. 5. Effect of anti-MBP exposure on hippoccampal slices LTP. All recordings were obtained from 5 different animals. (A) Effect of exposure time on LTP of hippocampal slices ($n = 20$) continually perfused with 1 μg/ml non-immune control children IgA (●), 1 μg/ml ASD anti-MBP IgA (■) or a mixture of ASD anti-MBP IgA (1 μg/ml) and recombinant human MBP (2 μg/ml) in ACSF (▲). (B) Summary of the data shown above indicating a decrease in the field potentials of hippocampal slices exposed to ASD anti-MBP IgA ($p < 0.0001$) and the recovery of LTP in hippocampal slices exposed to anti-MBP IgA pre-incubated with human recombinant MBP.

4. Discussion

Recently, an increasing number of reports support the hypothesis that immune dysfunction plays a role in ASD (Ashwood et al., 2006). Many of these reports suggest a link between ASD and the presence of maternal brain specific antibodies during early pregnancy (Zimmerman et al., 2007; Braunschweig and Van de Water, 2012), and their capacity to induce autism-like behavior has been validated in animal models including mice (Zhang et al., 2013; Camacho et al., 2014) and monkeys (Bauman et al., 2013). Several additional studies have also reported the presence of autoantibodies to brain antigens in the serum of autistic children (Gonzalez-Gronow et al., 2010; Wills et al., 2011; Goines et al., 2011; Mostafa and Al-Ayadhi, 2012; Mazur-Kolecka et al., 2013; Piras et al., 2014). One study suggested an association between autoimmunity to brain proteins and lower adaptive and cognitive functions, as well as core behaviors associated with autism (Goines et al., 2011). The same study compared data collected from mother and child subject pairs and found no significant familial associations between maternal autoantibodies to fetal brain proteins and the presence of autoantibodies against the cerebellum in their offspring (Goines et al., 2011). These results also show that brain-directed antibodies in maternal plasma have a higher degree of association with ASD than brain-directed antibodies in their offspring. Taken together, this study suggests that exposure to anti-brain antibodies during gestation damages neurodevelopment more than exposure to these brain autoantibodies in early childhood (Goines et al., 2011). To date, no studies have been conducted with autoantibodies from mother–child pairs in animal models.

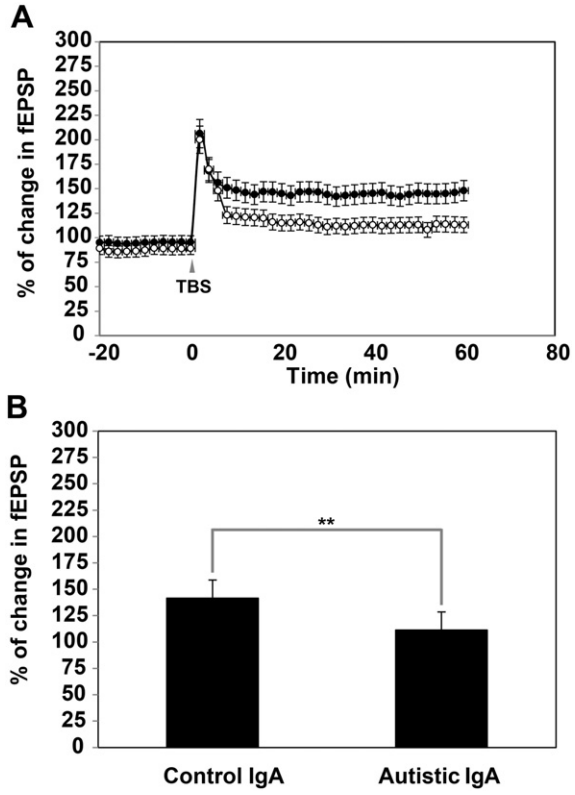

Fig. 6. Effect of the anti-MBP IgA on LTP of hippoccampal slices of an animal previously inoculated with the autoantibody or control animals inoculated with normal control children IgA. All recordings were obtained from 5 different animals. (A) Effect of exposure time on LTP of hippocampal slices ($n = 10$) from animals inoculated with 25 μg/day of normal children IgA (●) for 10 days or 25 μg/day of ASD anti-MBP IgA (■) for 10 days. The slices were continually perfused with ACSF and LTP was induced as described under Section 2. (B) Summary of the data shown above indicating a decrease in the field potentials of hippocampal slices exposed to ASD anti-MBP IgA ($p < 0.005$).

Autoantibodies to MBP have been detected in ASD (Ashwood et al., 2006) and in MS (Weber et al., 2011). We now report that sera from ASD patients contain autoantibodies to MBP both of the IgG and IgA classes in high titers, but not of the IgM class. A thorough analysis of their specificity demonstrated that the IgA autoantibodies against MBP exhibit the catalytic activity of a serine proteinase in all ASD serum samples analyzed. Purified anti-MBP IgA also degrades MBP *in vitro*, similar to that observed with anti-MBP IgA isolated from the sera of MS patients (Polosukhina et al., 2005). We also determined whether this IgA affects the synaptic plasticity of rat hippocampal slices. The results show that LTP decreases are observed in hippocampi isolated from both normal rats perfused with this antibody and hippocampi isolated from animals previously inoculated with this autoantibody and perfused with ACSF alone. The effect of the inoculated IgA on synaptic plasticity suggests that this antibody moves across the blood–brain barrier, as observed in the animal models described above (Zhang et al., 2013; Bauman et al., 2013; Camacho et al., 2014).

In a previous study, it was found that the titers of both anti-MBP IgG and IgM class antibodies were higher in children with ASD compared to normal control children, but the titers of anti-MBP IgA were not determined (Connolly et al., 2006). The role of autoantibodies against MBP has been extensively characterized in MS (Weber et al., 2011), where in addition to its demyelinating activity and complement activation within the CNS, these autoantibodies express enzymatic activity, which produces enhanced axonal damage when transferred to animals (Weber et al., 2011). The catalytic activity of the ASD anti-MBP IgA is affected by aprotinin and leupeptin, indicating a serine-protease with a specificity different from the MS anti-MBP IgA, which is inhibited by leupeptin but not affected by aprotinin (Ponomarenko et al., 2006).

Demyelinating nerve disease in MS is known to be a result of catalytic autoantibodies to MBP present both in the serum (Ponomarenko et al., 2006; Elliot et al., 2012) and cerebrospinal fluid (Doronin et al., 2014). Although, evidence of demyelination in autistic disorders using functional neuroimaging techniques was inconclusive in early studies (Rumsey and Ernst, 2000), more recent studies using diffusion tensor imaging techniques, which measure volumetric changes in the brains of ASD patients, detected abnormalities implicating the myelin component of brain white matter in the pathophysiology of ASD (Catani et al., 2008; Kumar et al., 2009; Shukla et al., 2010). In a recent study using tissue from post-mortem samples (Stoner et al., 2014), children with ASD show a focal disruption of the cortical laminar architecture in their brain cortexes associated with abnormalities in the expression of 63 specific molecular markers for the phenotype cortical microstructure. Several of the samples showed abnormal MBP gene expression, but no mechanisms were proposed for the origin of this dysregulation (Stoner et al., 2014).

Our study is the first to characterize the catalytic anti-MBP autoantibodies of the IgA class in ASD patients, both at the biochemical and physiological levels. All patients in our group ($n = 34$) have this catalytic autoantibody in their serum. Because autoantibodies to MBP cause myelin sheath destruction in MS (Weber et al., 2011), and because the anti-MBP ASD autoantibodies show a similar activity, we hypothesize a similar pathological role for these autoantibodies in ASD, primarily because they degrade MBP *in vitro* and also because they induce a decrease in LTP in models of brain synaptic plasticity in isolated rat hippocampal slices perfused *in vitro* with the antibody or in an *in vivo* model of hippocampal slices from rats injected with the ASD IgA for 10 days. However, it is important to emphasize that until demyelination induced by this type of autoantibody is demonstrated, their role in the neuropathology of autism remains only a hypothesis. In addition to MS, autoimmune disorders, including systemic lupus erythematosus and rheumatoid arthritis, also shows autoantibodies with enzymatic properties (Wootla et al., 2011). Our results support inclusion of ASD in this list.

5. Conclusions

ASD patients have catalytic anti-MBP autoantibodies of the IgA class. These autoantibodies degrade MBP *in vitro* and also induce a decrease in LTP in models of brain synaptic plasticity in isolated rat hippocampal slices perfused *in vitro* with the antibody or in an *in vivo* model of hippocampal slices from rats injected with the ASD IgA for several days.

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References

Ashwood, P., Wills, S., Van de Water, J., 2006. The immune response in autism: a new frontier for autism research. J. Leukoc. Biol. 80, 1–15.
Bauman, M.D., Iosif, A.M., Ashwood, P., Braunschweig, D., Lee, A., Schumann, C.M., Van de Water, J., Amaral, D.G., 2013. Maternal antibodies from mothers of children with autism alter brain growth and social behavior development in the rhesus monkey. Transl. Psychiatry 3, e278.
Braunschweig, D., Van de Water, J., 2012. Maternal autoantibodies in autism. Arch. Neurol. 69, 693–699.
Braunschweig, D., Duncanson, P., Boyce, R., Hansen, R., Ashwood, P., Pessah, I.N., Hertz-Picciotto, I., Van de Water, J., 2012. Behavioral correlates of maternal antibody status among children with autism. J. Autism Dev. Disord. 42, 1435–1445.
Braunschweig, D., Krakowiak, P., Duncanson, P., Boyce, R., Hansen, R.L., Ashwood, P., Hertz-Picciotto, I., Pessah, I.N., Van de Water, J., 2013. Autism-specific maternal autoantibodies recognize critical proteins in developing brain. Transl. Psychiatry 9, e277.
Brimberg, L., Sadig, A., Gregersen, P.K., Diamond, B., 2013. Brain-reactive IgG correlates with autoimmunity in mothers of a child with an autism spectrum disorder. Mol. Psychiatry 18, 1171–1177.

Camacho, J., Jones, K., Miller, E., Ariza, J., Noctor, S., Van de Water, J., Martinez-Cerdeño, V., 2014. Embryonic intraventricular exposure to autism-specific maternal autoantibodies produces alterations in autism-like stereotypical behaviors in offspring mice. Behav. Brain Res. 266, 46–51.
Careaga, M., Ashwood, P., 2012. Autism spectrum disorders: from immunity to behavior. Methods Mol. Biol. 934, 219–240.
Catani, M., Jones, D.K., Daly, E., Embiricos, N., Deeley, Q., Pugliese, L., Curran, S., Robertson, D., Murphy, D.G., 2008. Altered cerebellar feedback projections in Asperger syndrome. Neuroimage 41, 1184–1191.
Connolly, A.M., Chez, M., Streif, E.M., Keeling, R.M., Golumbek, P.T., Kwon, J.M., Riviello, J.J., Robinson, R.G., Neuman, R.J., Deuel, R.M., 2006. Brain-derived neurotropic factor and autoantibodies to neural antigens in sera of children with autistic spectrum disorders, Landau–Kleffner syndrome, and epilepsy. Biol. Psychiatry 59, 354–363.
Diagnostic and Statistical Manual of Mental Disorders 5th edition. American Psychiatric Association, Washington, D.C.
Doronin, V.B., Parkhomenko, T.A., Castellazzi, M., Padroni, M., Pastore, M., Buneva, V.N., Grazniewri, M., Nevinsky, G.A., 2014. Comparison of antibodies hydrolyzing myelin basic protein from the cerebrospinal fluid and serum of patients with multiple sclerosis. PLoS One 9, 1–12.
Elliot, C., Linder, M., Arthur, A., Brennan, K., Jarius, S., Hussey, J., Chan, A., Stroet, A., Olsson, T., Williuson, H., Barnett, S.C., Meinl, E., Linington, C., 2012. Functional identification of pathogenic autoantibody response in patients with multiple sclerosis. Brain 135, 1019–1833.
Fox, E., Amaral, D., Van de Water, J., 2012. Maternal and fetal antibrain antibodies in development and disease. Dev. Neurobiol. 72, 1327–1334.
Friberger, P., 1982. Chromogenic peptide substrates. Their use for the assay of factors in the fibrinolytic and the plasma kallikrein–kinin systems. Scand. J. Clin. Lab. Invest. Suppl. 162, 1–298.
Goines, P., Haapanen, L., Boyce, R., Duncanson, P., Braunschweig, D., Delwiche, L., Hansen, R., Hertz-Picciotto, I., Ashwood, P., Van de Water, J., 2011. Autoantibodies to cerebellum in children with autism associate with behavior. Brain Behav. Immun. 25, 514–523.
Gonzalez-Gronow, M., Cuchacovich, M., Francos, R., Cuchacovich, S., Dernandez, M.P., Blanco, E., Bowers, E.V., Kaczowka, S., Pizzo, S.V., 2010. Antibodies against the voltage-dependent anion channel (VDAC) and its protective ligand hexokinase-I in children with autism. J. Neuroimmunol. 227, 153–161.
Kumar, A., Sundaram, S.K., Sivaswamy, L., Behen, M.E., Makki, M.I., Ager, J., Janisse, J., Chugani, H.T., Chugani, D.C., 2009. Alterations in frontal lobe tracts and corpus callosum in young children with autism spectrum disorder. Cereb. Cortex 20, 2103–2113.
Kurinov, I.V., Harrison, R.W., 1996. Two crystal structures of the leupeptin–trypsin complex. Protein Sci. 5, 752–758.
Laemli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.
Lainhart, J.E., Bigler, E.D., Bocian, M., Dinh, E., Dawson, G., et al., 2006. Head circumference and height in autism: a study by the collaborative program of excellence in autism. Am. J. Med. Genet. A 140, 2257–2274.
Lecouteur, A., Rutter, M., Lord, C., Rios, P., Robertson, S., Holdgrafer, M., McLennan, J., 1989. Autism diagnostic interview: a standardized investigator-based instrument. J. Autism Dev. Disord. 19, 363–387.
Lord, C., Rutter, M., Lecouteur, A., 1994. Autism diagnostic interview for caregivers and individuals with possible developmental disorders. J. Autism Dev. Disord. 24, 659–685.
Lord, C., Risi, S., Lambrecht, L., Cook, E.H., Leventhal, B.L., DiLavore, P.C., Pickles, A., Rutter, M., 2000. The autism diagnostic observation schedule-generic: a standard measure of social and communication deficits associated with the spectrum of autism. J. Autism Dev. Disord. 30, 205–223.
Lottemberg, R., Sjak-Shie, N., Fazleabas, A.T., Roberts, R.M., 1988. Aprotinin inhibits urokinase but not tissue-type plasminogen activator. Thromb. Res. 49, 549–556.
Mazur-Kolecka, B., Cohen, I.L., Gonzalez, M., Jenkins, E.C., Kaczmarski, W., Brown, W.T., Flory, M., Fracowlak, J., 2013. Autoantibodies against neuronal progenitors in sera from children with autism. Brain Dev. 36, 322–329.
Mora, M., Quintero, L., Cardenas, R., Suarez-Roca, H., Zavala, M., Montiel, N., 2009. Association between HSV-2 infection and serum anti-rat brain antibodies in patients with autism. Invest. Clin. 50, 315–326.
Mostafa, G.A., Al-Ayadhi, L.Y., 2012. The relationship between the increased frequency of serum antineuronal antibodies and the severity of autism in children. Eur. J. Paediatr. Neurol. 16, 464–468.
Mostafa, G.A., Al-Ayadhi, L.Y., 2013. The possible relationship between allergic manifestations and elevated serum levels of brain specific auto-antibodies in autistic children. J. Neuroimmunol. 261, 77–81.
Mottonen, J., Strand, A., Symersky, J., Sweet, R.M., Danley, D.E., Geoghegan, K.F., Gerard, R.D., Goldsmith, E.J., 1992. Structural basis of latency in plasminogen activator inhibitor-1. Nature 355, 270–273.
Nevens, J.R., Mallia, A.K., Wendt, M.W., Smith, P.K., 1992. Affinity chromatographic purification of immunoglobulin M antibodies utilizing immobilized mannan binding protein. J. Chromatogr. 597, 247–256.
Nordahl, C.W., Braunschweig, D., Iosif, A.M., Lee, A., Rogers, S., Ashwood, P., Amaral, D.G., Van de Water, J., 2013. Maternal autoantibodies are associated with abnormal brain enlargement in a subgroup of children with autism spectrum disorder. Brain Behav. Immun. 30, 61–65.
Olmos, C., Sandoval, R., Rozas, C., Navarro, S., Wyneken, U., Zeise, M., Morales, B., Pancetti, F., 2009. Effect of short-term exposure to dichlorvos on synaptic plasticity of rat hippocampal slices: Involvement of acylpeptide hydrolase and α_7 nicotinic receptors. Toxicol. Appl. Pharmacol. 238, 37–46.
Piras, I.S., Haapanen, L., Napolioni, V., Sacco, R., Van de Water, J., Persico, A.M., 2014. Antibrain antibodies are associated with more severe cognitive and behavioral profiles in Italian children with autism spectrum. Brain Behav. Immun. 38, 91–99.
Polosukhina, D.I., Buneva, V.N., Doronin, B.M. Tyshkevich, Boiko, A.N., Gusev, E.I., Favorova, O.O., Nevinsky, G.A., 2005. Hydrolysis of myelin basic protein by IgM and IgA antibodies from the sera of patients with multiple sclerosis. Med. Sci. Monit. 11, BR266–BR272.
Ponomarenko, N.A., Durova, O.M., Vorovieb, I.I., Belogurov, A.A., Kurkova, I.N., Petrenko, A.G., Telegin, G.B., Suchkov, S.V., Kiselev, S.L., Lagarkova, M.A., Govorun, V.M., Serebryakova, M.V., Avalle, B., Tornatore, P., Karavanov, A., Morse, H.C., Thomas, D., Friboulet, A., Gabivov, A.G., 2006. Autoantibodies to myelin basic protein catalyze site-specific degradation of their antigen. Proc. Natl. Acad. Sci. U. S. A. 103, 281–286.
Roque-Barreira, M.C., Campos-Neto, A., 1985. Jacalin : an IgA binding lectin. J. Immunol. 134, 1740–1743.
Rumsey, J.M., Ernst, M., 2000. Functional neuroimaging of autistic disorders. Ment. Retard. Dev. Disabil. Res. Rev. 6, 171–179.
Schopler, E., Reichler, R.J., DeVellis, R.F., Daly, K., 1980. Toward objective classification of childhood autism: Childhood Autism Rating Scale (CARS). J. Autism Dev. Disord. 10, 91–103.
Shukla, D.K., Keehn, B., Lincoln, A.J., Müller, R.A., 2010. White matter compromise of callosal and subcortical fiber tracts in children with autism spectrum disorder: a diffusion tensor imaging study. J. Am. Acad. Child Adolesc. Psychiatry 12, 1269–1278.
Silva, S.C., Correia, C., Fesel, C., Barreto, M., Coutinho, A.M., Marques, C., Miguel, T.S., Ataide, A., Bemto, C., Borges, L., Oliveira, G., Vicente, A.M., 2004. Autoantibody repertoires to brain tissue in autism nuclear families. J. Neuroimmunol. 152, 176–182.
Singh, V.K., Warren, R.P., Odell, J.D., Warren, W.L., Cole, P., 1993. Antibodies to myelin basic protein in children with autistic behavior. Brain Behav. Immun. 7, 97–103.
Stoner, R., Chow, M.L., Boyle, M.P., Sunkin, S.M., Mouton, P.R., Roy, S., Wynshaw-Boris, A., Colamarino, S.A., Lein, E.S., Courchesne, E., 2014. Patches of disorganization in the neocortex of children with autism. N. Engl. J. Med. 370, 1209–1219.
Towbin, H., Staehlin, T., Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354.
Vojdani, A., Campbell, A.W., Anyanwu, E., Kashanian, A., Bock, K., Vojdani, E., 2002. Antibodies to neuron-specific antigens in children with autism: possible cross-reaction with encephalitogenic proteins from milk, *Chlamydia pneumoniae* and *Streptococcus* group A. J. Neuroimmunol. 129, 168–177.
Volkmar, F.R., Pauls, D., 2003. Autism. Lancet 362, 1133–1141.
Weber, M.S., Hemmer, B., Cepok, S., 2011. The role of antibodies in multiple sclerosis. Biochim. Biophys. Acta 1812, 239–245.
Wills, S., Rossi, C.C., Bennett, J., Martinez-Cerdeño, V., Ashwood, P., Amaral, D.G., Van de Water, J., 2011. Further characterization of autoantibodies to GABAergic neurons in the central nervous system produced by a subset of children with autism. Mol. Autism 2, 5.
Wohl, R.C., Summaria, L., Robbins, K.C., 1980. Kinetics of activation of human plasminogen by different activator species at pH 7.4 and 37 °C. J. Biol. Chem. 255, 2005–2013.
Wootla, B., Lacroix-Desmazes, S., Warrington, A.E., Bieber, A.J., Kaveri, S.V., Rodriguez, M., 2011. Autoantibodies with enzymatic properties in human autoimmune diseases. J. Autoimmun. 37, 144–150.
Zhang, Y., Gao, D., Kluetzman, K., Mendoza, A., Bolivar, V.J., Reilly, A., Jolly, J.K., Lawrence, D.A., 2013. The maternal autoimmune environment affects the social behavior of offspring. J. Neuroimmunol. 258, 51–60.
Zimmerman, A.W., Connors, S.L., Matteson, K.J., Li-Ching, L., Singer, H.S., Castaneda, J.A., Pearce, D.V., 2007. Maternal antibrain antibodies in autism. Brain Behav. Immun. 21, 351–357.