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Autoantibodies to cerebellum in children with autism associate with behavior

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ABSTRACT

Autism is a heterogeneous disorder with a poorly understood biological basis. Some children with autism harbor plasma autoantibodies that target brain proteins. Similarly, some mothers of children with autism produce antibodies specific to autism that target pairs of fetal brain proteins at 37/73 and 39/73 kDa. We explored the relationship between the presence of brain-specific autoantibodies and several behavioral characteristics of autism in 277 children with an autism spectrum disorder and 189 typically developing age-matched controls. Further, we used maternal autoantibody data to investigate potential familial relationships for the production of brain-directed autoantibodies. We demonstrated by Western blot that autoantibodies specific for a 45 kDa cerebellar protein in children were associated with a diagnosis of autism ($p = 0.017$) while autoantibodies directed towards a 62 kDa protein were associated with the broader diagnosis of autism spectrum disorder (ASD) ($p = 0.043$). Children with such autoantibodies had lower adaptive ($p = 0.0008$) and cognitive function ($p = 0.005$), as well as increased aberrant behaviors ($p < 0.05$) compared to children without these antibodies. No correlation was noted for those mothers with the most specific pattern of anti-fetal brain autoantibodies and children with the autoantibodies to either the 45 or 62 kDa bands. Collectively, these data suggest that antibodies towards brain proteins in children are associated with lower adaptive and cognitive function as well as core behaviors associated with autism. It is unclear whether these antibodies have direct pathologic significance, or if they are merely a response to previous injury. Future studies are needed to determine the identities of the protein targets and explore their significance in autism.

1. Introduction

Autism spectrum disorders are a group of psychological conditions that manifest in early childhood. These disorders are characterized by widespread abnormalities of social interactions and communication, as well as restricted interests and repetitive behavior (American Psychiatric Association, 1994; Lord et al., 2000a; World Heath Organization, 2006). The autism phenotype is heterogeneous with regard to behavioral severity and disease onset (Meilleur and Fombonne, 2009; Micali et al., 2004; Ozonoff et al., 2010; Stefanatos, 2008). Autism spectrum disorders are clinically defined, and current diagnosis is based entirely on behavioral testing and analysis of medical and developmental history (Le Couteur et al., 2008; Lord et al., 1994, 2000b). The pathology and etiology of these disorders remain unclear; though emerging evidence suggests that genetic, neurological, environmental, and immune factors are likely involved (Pardo and Eberhart, 2007).

The neurobiology of autism spectrum disorders has been explored through various imaging techniques and examination of post-mortem samples. Data suggest that abnormal brain growth, altered neuronal migration and connectivity, and/or changes in minicolumnar organization may be involved (Pardo and Eberhart, 2007). Subtle differences have been reported in brain regions including the cerebral cortex, limbic structures, and cerebellum (Pardo and Eberhart, 2007). Overall, the neurological basis of autism spectrum disorders remains poorly understood, largely due to difficulties in obtaining quality post-mortem samples and a lack of information on early brain development. Further, the factors that cause neurological abnormalities are largely undefined.

Immune dysregulation has been noted among individuals with an autism spectrum disorder and their family members (Ashwood

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et al., 2006). This includes inflammation in the central nervous system (CNS) and gastrointestinal tract (Ashwood et al., 2004; Vargas et al., 2005), as well as differences in system-wide humoral and cellular immunity (Ashwood et al., 2006; Pardo et al., 2005). There are several reports of altered IgG and cytokine levels in subjects with an autism spectrum disorder compared to typically developing children (Ashwood et al., 2008a,b; Enstrom et al., 2009a; Grigorenko et al., 2008; Heuer et al., 2008). Further, variations in immune parameters often correlate with behavioral severity (Ashwood et al., 2008a,b; Enstrom et al., 2009a, 2010; Grigorenko et al., 2008; Heuer et al., 2008; Onore et al., 2009).

Autoimmune and allergy-associated disorders also appear more frequently in individuals with an autism spectrum disorder and their families compared to control populations (Ashwood and Van de Water, 2004; Ashwood et al., 2006; Cabanlit et al., 2007; Croen et al., 2005; Mostafa and Kitchener, 2009; Silva et al., 2004). Several studies have noted the presence of autoantibodies in peripheral blood that react with components of the central nervous system (CNS) (Enstrom et al., 2009b). The mechanistic role of these antibodies in autism spectrum disorders is not clear, and it remains to be determined whether they are pathogenic or if they are produced as a secondary result of neuronal insult. Similar phenomena have been described in other neurological disorders including Tourette's syndrome, Syndenham's chorea and obsessive compulsive disorder (Church et al., 2002; Swedo et al., 1998). CNS targets reported for autism spectrum disorders include the thalamus, hypothalamus, caudate nucleus, cerebral cortex, putamen, and cerebellum (Cabanlit et al., 2007; Connolly et al., 1999; Silva et al., 2004; Singer et al., 2006; Singh and Rivas, 2004; Vojdani et al., 2004; Wills et al., 2009). Research in our laboratory suggests that the cerebellum is the most consistent target of these antibodies (Cabanlit et al., 2007; Wills et al., 2007), and changes in cerebellum function can result in various behavioral and cognitive issues commonly observed in autism spectrum disorders (Gillig and Sanders, 2010; Steinlin, 2008). However, it is unknown if such autoantibodies to brain proteins coincide with specific behavioral features of the disorder. Although their exact involvement in autism is unknown, these antibodies provide valuable insight into biological mechanisms potentially associated with this behaviorally defined disorder.

In addition to the findings of brain-directed antibodies in children with autism, a subset of mothers of children with autism have been shown to harbor plasma IgG that targets fetal brain proteins (Braunschweig et al., 2008; Croen et al., 2008; Singer et al., 2008; Zimmerman et al., 2007). During pregnancy, maternal IgG is passed across the placenta into fetal circulation (Simister, 2003). Animals exposed to these fetal brain-directed autoantibodies during gestation demonstrate altered behavior, which suggests that maternal antibodies may be of pathologic significance (Martin et al., 2008; Singer et al., 2009). However, it remains unclear whether there is a relationship between the production of fetal brain-directed autoantibodies in mothers of children with autism and the production of autoantibodies specific for mature brain in their children.

The goals of the current study were twofold: (1) to further characterize the occurrence of autoantibodies to mature cerebellum in a large group of children with an autism spectrum disorder, and to determine whether the presence of these autoantibodies relates to specific behavioral outcomes, and (2) to ascertain if there is a familial association for the presence of these autoantibodies in children and the presence of fetal brain-directed antibodies in their respective mothers. The occurrence of cerebellum-specific autoantibodies in children was analyzed in plasma samples from a large, well-characterized, and phenotypically diverse population of subjects with autism (AU), the broader diagnosis of autism spectrum disorder (ASD), and typically developing (TD) age-matched control children. In addition, mothers of the subjects included in the current study were screened for fetal brain-specific autoantibodies to determine possible familial relationships for brain-specific antibodies.

2. Methods

2.1. Subjects

The current study involved 466 children and 439 mothers enrolled through the CHARGE (Childhood Autism Risks from Genetics and the Environment) study at the U.C. Davis M.I.N.D. (Medical Investigations of Neurodevelopmental Disorders) Institute at the University of California, Davis, which has previously been described in detail (Hertz-Picciotto et al., 2006). The CHARGE study is an ongoing population-based case-control study including children with autism (AU) or the broader diagnosis of autism spectrum disorder (ASD), typically developing (TD) control children selected from the general population, and their families. Children enrolled in the study met the following criteria: (1) they were between the ages of 24 and 60 months, (2) lived with at least one biologic parent, (3) had a parent who spoke English or Spanish, (4) were born in the state of California, and (5) resided in the catchment areas of a specified list of Regional Centers in Northern California. This study protocol followed the ethical guidelines of the most recent declaration of Helsinki (Enserink, 2000) and was approved by the institutional review boards at the University of California, Davis and The State of California Department of Developmental Services. Informed consent was obtained prior to participation.

The subject population in the current study consisted of children with a diagnosis of full AU ($n = 207$), the broader phenotype of ASD ($n = 70$), and age-matched TD control children ($n = 189$). Children with an autism disorder were subcategorized into a group of subjects with full autism (AU; met strict diagnostic criteria on ADI-R and ADOS) and a group of subjects with a broader diagnosis of autism spectrum disorder (ASD; met most but not all criteria on the ADI-R and ADOS) (Table 1). For the duration of the paper, autism (AU) will refer to those children with a strict autism diagnosis, autism spectrum disorder (ASD) will refer those children with a broader diagnosis, and autism spectrum disorders (no acronym) will be inclusive for both AU and ASD. AU and ASD diagnoses were confirmed for all cases using the Autism Diagnostic Interview-Revised (ADI-R) (Lord et al., 1994, 1997) and the Autism Diagnostic Observation Schedule, modules 1, 2, and 3 (ADOS) (Lord et al., 2000b). The ADI-R provides a standardized, semi-structured interview and a diagnostic algorithm for the DSM-IV (American Psychiatric Association, 1994) and the ICD-10 definitions of autism (World Heath Organization, 2006). The ADOS is a standardized assessment in which a trained researcher observes the social interaction, communication, play and imaginative use of materials for children suspected of having autism. TD controls were evaluated using the Social Communication Questionnaire (SCQ) (Rutter et al., 2003) to determine whether autism traits were present and if administration of the ADI-R and ADOS was appropriate. In all children, cognitive function was measured using the Mullen Scales of Early Learning (MSEL) (Mullen, 1995) and adaptive function was evaluated using the Vineland Adaptive Behavior Scales (VABS) (Sparrow and Cicchetti, 1984). All children in the current study were assessed by research-reliable clinical faculty for diagnostic consistency. Finally, all participating families completed the Aberrant Behavior Checklist (ABC) prior to their clinical visit. The ABC is a standardized checklist designed to rate inappropriate and maladaptive behaviors in children (Aman MG, 1994). Subjects were diagnosed as AU, ASD, or TD based on the criteria described in Table 1.

Table 1
Subject demographics and diagnosis.

Diagnosis	Diagnostic criteria	*n*	Average age ± SD	Percent males	*n* with maternal data
Autism (AU)	1. Subject meets autism criteria on the ADOS (cutoff for communication + social interaction total) 2. Subject meets autism criteria on the ADI-R (cutoffs for communication, social interaction, stereotyped behavior, and onset of symptoms)	207	3.7 ± 0.9	87%	194
Autism spectrum disorder (ASD)	1. Subject does not meet all criteria for an autism diagnosis (above) 2. Subject meets autism spectrum criteria on the ADOS (cutoff for communication + social interaction total) 3. Subject meets autism spectrum criteria for communication and social interaction sections of the ADI-R (or is within two points of cutoff on one section while meeting criteria for the other)	70	3.8 ± 0.9	83%	65
Typically developing (TD)	1. Subject has a score of 71 + on the Mullen Scales of Early Learning 2. Subject has a score of 70 + on Vineland Adaptive Behavior Scale 3. Subject has, and a score of <15 on Social Communication Questionnaire (SCQ)	189	3.5 ± 0.9	82%	180

2.2. Sample collection and processing

A sample of 8.5 mL of blood was collected from each child and maternal subject into citrate Vacutainer tubes (BD, Franklin Lakes, NJ). Whole blood was centrifuged for 10 min at 2300 rpm, and plasma samples were aliquoted, and stored at −80 °C until use.

2.3. Brain protein preparation

Rhesus macaque cerebellum protein medleys were used to probe child plasma samples for anti-brain IgG reactivity. Though other brain regions are known to be a target of IgG in children with autism, we opted to use cerebellum proteins because they provide the most consistently reliable target protein preparation in children with an autism spectrum disorder (Cabanlit et al., 2007; Wills et al., 2009). Rhesus macaque fetal brain protein medleys were utilized to probe maternal plasma for anti-brain IgG reactivity based upon independent studies by our laboratory and others that demonstrated a high degree of specificity for maternal antibodies to fetal brain in autism (Braunschweig et al., 2008; Zimmerman et al., 2007). Monkey brain specimens were acquired through the University of California, Davis Primate Center and prepared in our laboratory. Whole cerebellum was obtained from two healthy adult male Rhesus monkeys, and a whole fetal brain was obtained from a gestational day 152 Rhesus macaque. To prepare the protein medleys, 1.0 g of fresh brain tissue was suspended in 10 mL of 20 mM HEPES-OH, pH 7.5, containing 320 mM sucrose, 1 mM EDTA, 5 mM DTE, protease inhibitors (1 mM PMSF and Roche Complete™ protease inhibitor), and phosphatase inhibitors (0.2 mM Na_2VO_3 and 1 mM NaF). The suspension was homogenized using a Teflon/potter homogenizer and centrifuged at 800*g* for 10 min to remove nuclei and undissolved material. Protein medleys were then diluted 10-fold with 50 mM Tris–HCl, pH 6.8, containing 25% glycerol and 1% lithium dodecyl sulfate (LDS). The final protein products were reconcentrated to 12.5 mg/mL using Amicon® Ultra-4 centrifugal filter devices (Millipore, Billerica, MA).

A commercially available human cerebellum protein medley (Clontech, Mountain View, CA) was used to demonstrate that the plasma IgG targets in monkey cerebellum are also present in the human cerebellum. Similar comparisons have previously demonstrated

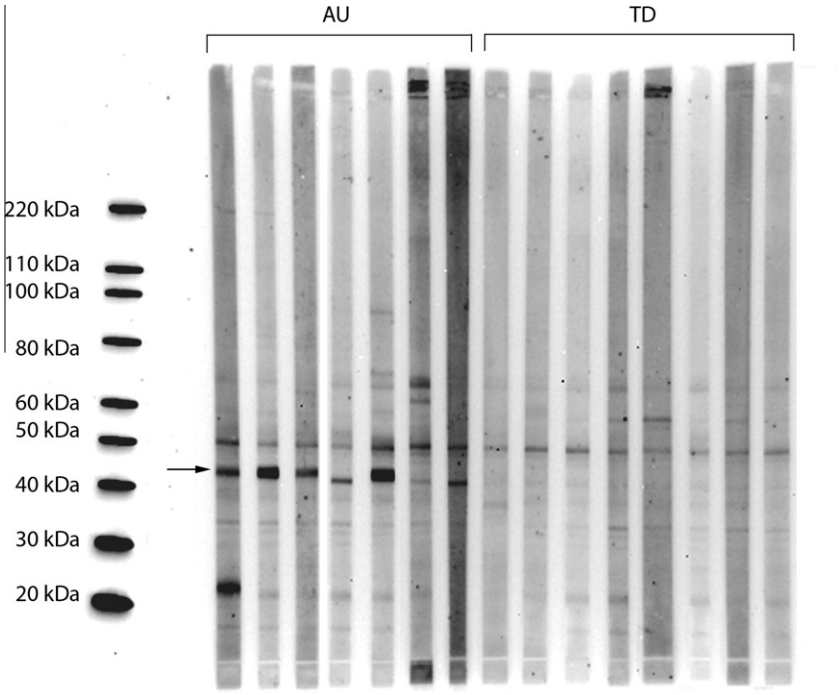

Fig. 1. IgG directed towards a 45 kDa cerebellum protein in children with autism (AU). Examples of plasma IgG reactivity in children with AU positive for the 45 kDa band (7 examples on the left) compared to typically developing (TD) control subjects (8 examples on the right). Each strip is representative of plasma from a single child subject and all samples were run simultaneously on one Western blot.

that the IgG targets in the fetal brain are conserved between monkey and human specimens (Braunschweig et al., submitted for publication).

2.4. Western blotting

Plasma IgG reactivity to brain proteins was measured using Western blot technology. Brain extracts (300 μg/mL) and Magic Mark protein standard (5 μl) (Invitrogen, Carlsbad, CA) were loaded into 4–12% gradient prep-well Nu-PAGE Bis–Tris gels (Invitrogen, Carlsbad, CA) and electrophoresed at 200 V for 1 h. After gel electrophoresis, proteins were transferred at 50 V for 16 h to a nitrocellulose membrane. The membranes were then blocked with casein in PBS (Thermo Scientific, Rockford, IL) for 30 min at room temperature. Membranes were cut into vertical strips, and each strip was incubated with a sample of maternal or child plasma diluted 1:400 in 5% casein in PBS plus 0.05% Tween (PBST) for 2 h at room temperature. Strips were washed five times for 5 min durations with PBST, followed by a 30 min incubation with horseradish peroxidase-conjugated goat anti-human IgG secondary (Zymed, San Francisco, CA) diluted 1:20,000. After washing, the signal was developed using a 5-min incubation with SuperSignal Chemiluminescent Substate (Pierce, Rockford, IL). Bands were visualized using a FluorChem 8900 imager and AlphaEaseFC imaging software. (Alpha Innotech Corporation, San Leandro, CA). Positive and negative control reference standards were run on each blot. Additionally, one strip from each blot was probed with the secondary antibody but not with diluted plasma. This was termed the "secondary only control", and was designed to demonstrate the reactivity that the secondary antibody had against the IgG heavy and light chains present in the protein preparation.

To demonstrate that the autoantibody targets in the Rhesus macaque cerebellum are also present in the human cerebellum, we utilized a 4–12% gradient 10-well Nu-PAGE Bis–Tris gel (Invitrogen, Carlsbad, CA) and analyzed the two protein medleys side-by-side. Plasma IgG reactivity to human and monkey cerebellum proteins was analyzed using the same blotting protocol described above.

The presence of bands and the molecular weights were determined using the AlphaEaseFC software. The relative migration (Rf) of the molecular weight markers was calculated by defining the position of the loading well (start point for protein migration)

Table 2
IgG reactivity towards the cerebellum in children, *n*(%). The incidence of IgG specific for various molecular weight proteins is represented as: total number of subjects with IgG specific for the protein (percentage of subjects with IgG specific for the protein). *p* values for each comparison are represented to the right of the table. There was no significant difference between the total number of cerebellum proteins that were IgG targets in each group (average number of bands: AU = 1.46, ASD = 1.74, TD = 1.47).

Cerebellum protein (kDa)	Child IgG targets in cerebellum				*p* values			
	ASD $n = 70$	AU $n = 207$	AU + ASD $n = 277$	TD $n = 189$	ASD vs. TD	AU vs. TD	AU + ASD vs. TD	AU vs. ASD
33	9 (13%)	21 (10%)	30 (11%)	16 (8.2%)	0.244	0.605	0.431	0.511
38	8 (11.5%)	19 (9%)	27 (10%)	23 (12%)	1.0	0.257	0.452	0.355
45	5 (7.1%)	20 (9.7%)	25 (9%)	7 (3.6%)	**0.07**	**0.017**	**0.025**	0.635
60	10 (15.7%)	36 (17.4%)	46 (17%)	29 (15.4%)	1.0	0.593	0.705	0.855
62	12 (16%)	17 (8.2%)	29 (10%)	16 (8.2%)	**0.043**	1.0	0.524	**0.043**
80	5 (7.1%)	10 (4.8%)	15 (5.4%)	13 (6.7%)	1.0	0.521	0.561	0.541

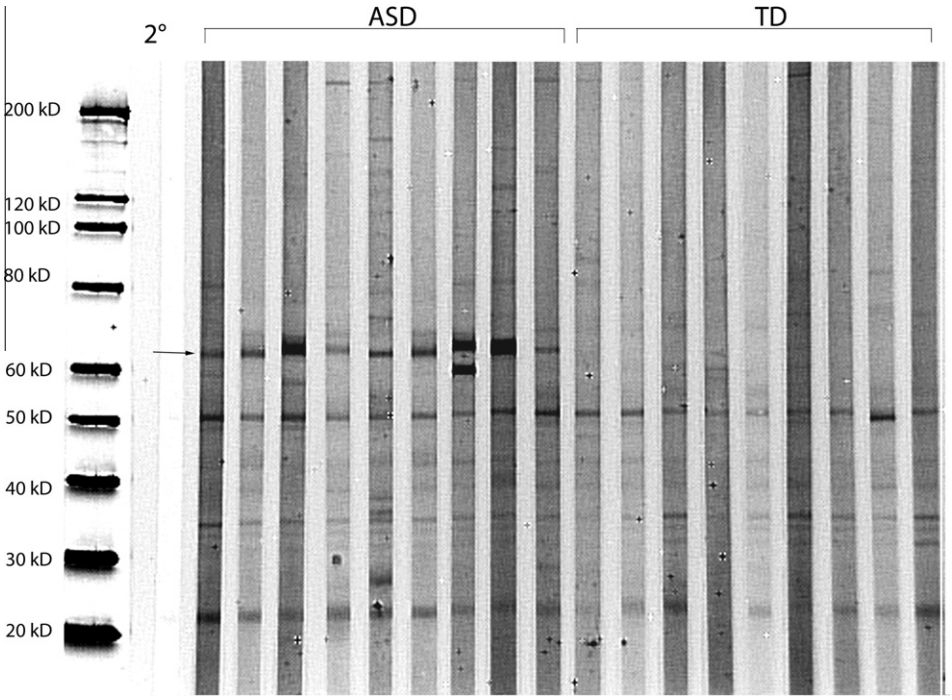

Fig. 2. IgG directed towards a 62 kDa cerebellum protein in children with the broader diagnosis of autism spectrum disorder (ASD). Examples of plasma IgG reactivity in children with ASD positive for the 62 kDa band (9 examples on the left) compared to typically developing (TD) control subjects (9 examples on the right). Each strip is representative of plasma from a single child subject and all samples were run simultaneously on one Western blot.

and the location of the dye front (end-point). The molecular weights of protein bands in plasma samples were determined by applying a point-by-point curve fit to the Rf of the molecular weight markers. Bands were considered to be the same between samples when less than 4% difference was observed in Rf, and the threshold for assigning the presence of a band was a twofold higher densitometry reading above background on the strip.

2.5. Statistical analysis

Data analysis was performed using SAS software. A probability value (p) of less than 0.05 was considered to be significant. Fischer's exact tests were performed to determine significance for IgG reactivity to brain proteins in AU, ASD, and TD child and maternal groups, and non-parametric Spearman rank correlation coefficients with a 95% confidence interval were used to analyze patterns of familial anti-brain IgG reactivity in child/mother pairs. Wilcoxan rank-sum tests were used to determine the association between the presence of brain-directed antibodies and variations in scores on behavioral, cognitive, and adaptive assessments. Bonferroni corrections were made for multiple comparisons.

3. Results

3.1. Cerebellum-targeted autoantibodies in children

Plasma from children with AU, ASD, and TD controls was screened for autoantibodies towards proteins isolated from adult Rhesus macaque cerebellum by Western blot. Two immunoreactive targets within the cerebellum appeared significantly more often among AU and ASD groups compared to controls (Table 2, Figs. 1–3). Children with AU were found to have a significantly higher incidence of autoantibodies directed towards a 45 kDa cerebellum protein compared to the TD group (9.7% in AU, 3.6% in TD, $p = 0.017$) (Fig. 1). When the broader phenotype ASD subjects were combined with the AU group and compared to TD controls, the association remained significant ($p = 0.025$). When the ASD group was considered separately from the AU group, there was a trend for an increased presence of autoantibodies towards the 45 kDa protein in the ASD group (7.1% vs. 3.6%), but this did not quite reach statistical significance ($p = 0.07$). The presence of autoantibodies directed towards an additional protein at 62 kDa was found to be associated with an ASD diagnosis (Fig. 2). 16% of subjects with ASD had IgG reactivity towards the 62 kDa protein, compared to 8.2% of both TD subjects ($p = 0.04$) and AU subjects ($p = 0.04$). The 45 kDa and 62 kDa targets discovered in the monkey cerebellum were also present in human cerebellum (Fig. 3) which demonstrates that these antibodies are autoantibodies that are capable of reacting with human brain antigens. The original study from our group involving cerebellum-directed antibodies in autism described a specific band of interest at 52 kDa (Wills et al., 2009). This original study utilized human cerebellum, a non-gradient 12% gel and a different molecular weight marker system than was used in the current study. After retesting several of the reactive plasma samples included in the original study with our new, more refined Western blot platform, we determined that what was thought to be a band at 52 kDa, was indeed the band described to be 45 kDa herein (Fig. 3).

Several additional cerebellum proteins were found to be targeted by IgG in plasma from AU, ASD, and TD groups. In addition to the 45 and 62 kDa proteins, subjects demonstrated varied

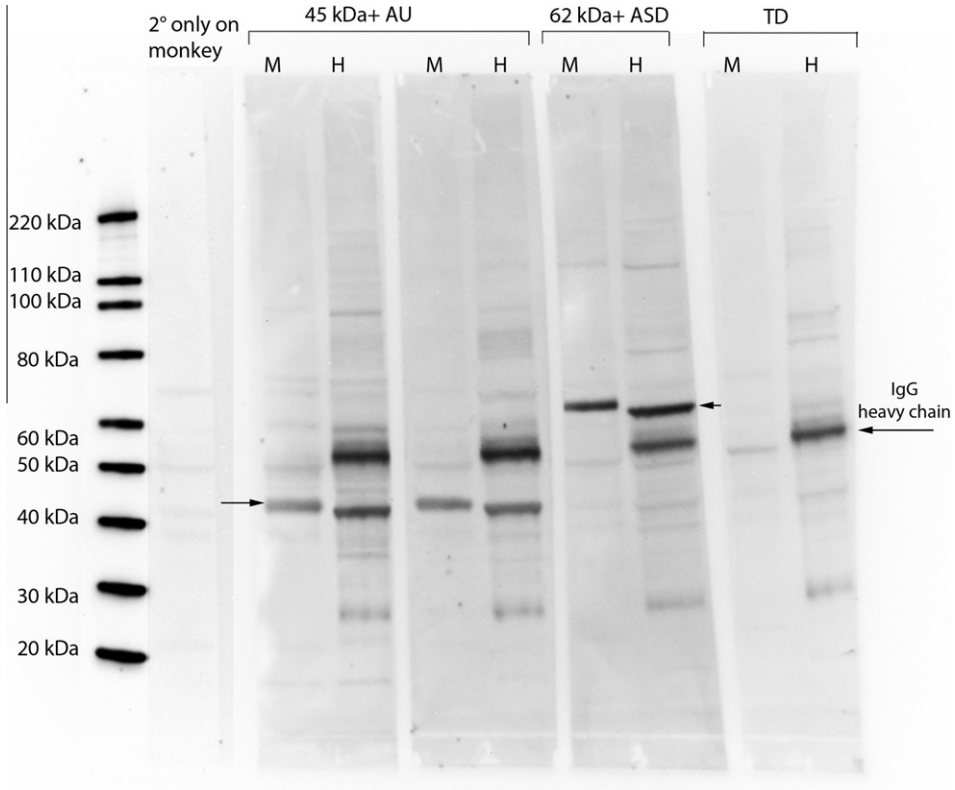

Fig. 3. 45 and 62 kDa cerebellum protein targets are conserved between human and monkey specimens. Representative blot comparing IgG reactivity towards human (H) and Rhesus macaque (M) cerebellum proteins. Plasma IgG reactivity towards human and monkey proteins is compared in two children positive for IgG specific to the 45 kDa protein (left), one child positive for IgG specific to 62 kDa protein (middle) and one typically developing (TD) control child (right). A secondary-only control is depicted on the far left against Rhesus macaque cerebellum. Sample number 2 reactive for the 45 kDa band is a positive control from the previous Wills et al. study (Wills et al., 2009) demonstrating that the previously described 52 kDa band is in fact the same as the 45 kDa band described herein.

Table 3
Significant behavioral associations for children with and without IgG reactivity towards the 45 kDa protein. For the Aberrant Behavior Checklist (ABC), a higher score corresponds to more behavioral impairments. For the Mullen Scales of Early Learning (MSEL) and Vineland Adaptive Behavior Scales (VABS), a lower score corresponds to more cognitive and adaptive impairments. Data displayed as mean test score (standard deviation).

	ASD $n = 70$			AU $n = 207$			TD $n = 189$			All children $n = 466$		
	45^+ $n = 5$	45^- $n = 65$	p	45^+ $n = 20$	45^- $n = 187$	p	45^+ $n = 7$	45^- $n = 182$	p	45^+ $n = 32$	45^- $n = 434$	p
ABC 2: Lethargy	8.2 (7)	9 (7.9)	0.50	12.9 (9.6)	12.3 (7.1)	0.42	0.5 (1.22)	0.47 (1.6)	0.50	9.3 (9.3)	6.7 (7.9)	**0.05**
ABC 3: Stereotypy	3.2 (2.8)	3.3 (3.5)	0.42	7.8 (6.4)	5.7 (4.2)	0.13	0 (0)	0.1 (0.4)	0.26	5.2 (6)	2.9 (4)	**0.01**
MSEL	69 (26.1)	68.4 (17.8)	0.40	53.5 (6.4)	59.5 (17.2)	0.19	108 (13.8)	105.1 (17.7)	0.32	67.1 (24.9)	80.6 (27.8)	**0.005**
VABS	68.5 (19.6)	70.2 (12.7)	0.36	58.6 (7)	62.9 (12)	0.09	100.8 (17.7)	104.9 (14.9)	0.25	68.7 (20.4)	82 (24)	**0.0008**

auto-reactivity towards proteins with the following molecular weights: 31, 33, 38, 40, 47, 48, 55, 60, 68, 70, 80, 100, 120, and 220 kDa. None of these targets were found to associate significantly with an AU, ASD, or TD diagnosis. The total number of cerebellum proteins for which children were seropositive was not found to be significantly different between AU (mean targets: 1.46), ASD (mean targets; 1.74) and TD (mean targets: 1.47) groups.

Finally, we analyzed whether having auto-reactivity towards the 45 or 62 kDa cerebellum protein was associated with scores on behavioral and developmental assessments including the ADI-R, ADOS, ABC, MSEL, and VABS. No significant associations were found between scores on the ADOS or ADI-R and anti-cerebellum autoantibodies. However, in contrast, we found that children with autoantibodies directed against the 45 kDa protein (regardless of AU, ASD, or TD diagnosis) had significantly more impaired scores on the ABC Lethargy subscale ($p = 0.05$), the ABC Stereotypy subscale ($p = 0.01$), the MSEL composite standard score ($p = 0.005$), and the VABS composite standard score ($p = 0.0008$) compared to children without reactivity to the 45 kDa protein (Table 3). Children with reactivity towards the 62 kDa protein had more impaired scores on the ABC Inappropriate Speech subscale compared to children without 62 kDa reactivity ($p = 0.04$) regardless of an AU, ASD, or TD diagnosis. Further, ASD children with reactivity to the 62 kDa protein had more aberrant behavior scores on the total ABC ($p = 0.02$), ABC Lethargy subscale ($p = 0.01$), and ABC Stereotypy subscale ($p = 0.01$) compared to ASD children without reactivity. Finally, TD children with reactivity to the 62 kDa protein scored lower on the VABS composite compared to TD children without IgG ($p = 0.02$) (Table 4).

3.2. Fetal brain-targeted antibodies in mothers of AU, ASD, and TD children

To determine the potential relationship between brain-reactive antibodies in mothers and their offspring, plasma was collected from the mothers of the AU, ASD, and TD children, and probed for IgG reactivity to fetal brain proteins using Western blot. Several targets of maternal IgG were found to be significantly associated with an AU or ASD diagnosis for the child (Table 5).

Maternal IgG reactivity to two fetal brain proteins at 37 and 73 kDa was significantly associated with an AU and ASD diagnosis for the child, where 10.8% of mothers with an AU child, 4.6% of mothers with an ASD child, and 0% of mothers with a TD child demonstrated IgG reactivity towards both proteins (for AU vs. TD, $p < 0.0001$; for ASD vs. TD, $p = 0.01$; and for AU + ASD vs. TD, $p < 0.0001$).

Additionally, maternal IgG reactivity to fetal brain proteins at 39 and 73 kDa was also associated with an AU or ASD diagnosis for the child. where 7.7% of mothers with an AU child, 12.3% of mothers with an ASD child, and 1.7% of mothers with a TD child harbored IgG specific for both proteins, (for AU vs. TD,

Table 4
Significant behavioral associations for children with and without IgG reactivity towards the 62 kDa protein. For the Aberrant Behavior Checklist (ABC), a higher score corresponds to more behavioral impairments. For the Vineland Adaptive Behavior Scales (VABS), a lower score corresponds to more cognitive and adaptive impairments. Data displayed as mean test score (standard deviation).

	ASD $n = 70$			AU $n = 207$			TD $n = 189$			All children $n = 466$		
	62^+ $n = 12$	62^- $n = 58$	p	62^+ $n = 17$	62^- $n = 190$	p	62^+ $n = 16$	62^- $n = 173$	p	62^+ $n = 45$	62^- $n = 421$	p
Total ABC	68.1 (40.9)	38.7 (25.)	**0.02**	48.3 (25.6)	53.1 (26.1)	0.27	10.9 (14.1)	7.1 (10)	0.15	38.9 (35.1)	30.1 (29.5)	0.06
ABC 2: Lethargy	16.6 (11.9)	7.5 (5.9)	**0.01**	10.3 (7.5)	12.6 (7.3)	0.09	0.4 (0.8)	0.5 (1.7)	0.17	8.4 (9.8)	6.7 (7.8)	0.15
ABC 3: Stereotypy	5.6 (3.5)	2.9 (3.3)	**0.01**	5.2 (4)	6 (4.6)	0.32	0.07 (0.3)	0.09 (0.4)	0.46	3.5 (3.9)	3 (4.2)	0.11
ABC 5: Inappropriate Speech	3.5 (3.4)	2.8 (2.8)	0.26	3 (2.9)	2.8 (2.8)	0.33	0.9 (1.5)	0.4 (0.9)	0.14	2.4 (2.9)	1.8 (2.5)	**0.04**
VABS	67.7 (12.3)	70.5 (13.5)	0.2	65.1 (15.8)	62.1 (11.1)	0.23	97.5 (15.4)	105.5 (14.8)	**0.02**	76.9 (20.9)	81.5 (24.3)	0.17

Table 5
IgG reactivity towards the fetal brain in mothers, n(%). The incidence of IgG specific for various molecular weight proteins is represented as: total number of subjects with IgG specific for the protein (percentage of subjects with IgG specific for the protein). The average total number of IgG targets in the fetal brain was higher in mothers of AU and ASD children compared to mothers of TD children (mothers of ASD = 1.63, mothers of AU = 1.6, mothers of TD = 1.15).

Fetal brain protein (kDa)	Maternal IgG targets in fetal brain				p values			
	Mothers of ASD $n = 65$	Mothers of AU $n = 194$	Mothers of ASD + AU $n = 259$	Mothers of TD $n = 180$	ASD vs. TD	AU vs. TD	AU + ASD vs. TD	AU vs. ASD
37 and **73**	3 (4.6%)	21 (10.8%)	24 (9.3%)	0 (0%)	**0.018**	**<0.0001**	**<0.0001**	0.2143
37 (no. 73)	9 (13.8%)	8 (4.2%)	17 (6.6%)	11 (6.1%)	0.0643	0.4814	1	**0.016**
39 and **73**	8 (12.3%)	15 (7.7%)	23 (8.9%)	3 (1.7%)	**0.001**	**0.0069**	**0.0015**	0.3127
39 (no. 73)	8 (12.3%)	19 (9.8%)	27 (10.4%)	29 (16.1%)	0.5479	0.088	0.083	0.6392
73 (no. 37 or 39)	6 (9.2%)	36 (18.6%)	42 (16.2%)	22 (12.2%)	0.6512	0.1154	0.2728	0.083

$p = 0.0069$; for ASD vs. TD, $p = 0.001$; and for AU + ASD vs. TD, $p = 0.0015$).

When reactivity to individual bands was considered, maternal IgG specific for the 37 and 39 kDa proteins was only significantly associated with an AU or ASD diagnosis when there was simultaneous reactivity to the 73 kDa protein. Likewise, mothers who were positive for IgG to the 73 kDa protein, but negative for IgG targeting the 37 and 39 kDa proteins, did not have a significantly higher chance of having a child with AU or ASD. Therefore, similar to previous reports, maternal antibodies targeting the 37, 39, and 73 kDa proteins are only significantly associated with an AU or ASD diagnosis when there is simultaneous IgG reactivity to 37/73 kDa proteins or 39/73 kDa proteins (Braunschweig et al., 2008, 2010) (Table 5).

Finally, mothers demonstrated IgG reactivity to several additional fetal brain proteins that did not correlate with diagnosis. These proteins had molecular weights of 42, 49, 60, 80, and 100 kDa. The total number of proteins that were targets of maternal IgG was also counted for each maternal subject. Mothers of AU and ASD children had significantly more IgG targets in the fetal brain compared to mothers of TD children (1.64 for mothers of AU, 1.59 for mothers of ASD, 1.15 for mothers of TD; AU vs. TD $p < 0.001$; ASD vs. TD $p = 0.003$).

3.3. Familial associations for anti-brain antibodies in mother and child subjects

Data collected from mother and child subject pairs were compared to determine if familial associations exist for antibody reactivity towards brain. No significant familial association was found between the definitive maternal autoantibodies to fetal brain proteins and the presence of autoantibodies to cerebellum in their respective offspring. Namely, mothers with IgG reactivity towards the 37/73 kDa protein pairs or the 39/73 kDa protein pairs (which are significantly associated with an AU and ASD diagnosis) were not more likely to have children with IgG reactivity to the 45 kDa or the 62 kDa proteins (which are also significantly associated with an AU or ASD diagnosis). Some minor familial associations were found for IgG reactivity towards the 62 kDa protein in children and IgG reactivity towards the individual 37 or 39 kDa proteins in mothers (in the absence of IgG reactivity towards the 73 kDa protein). Specifically, ASD children with IgG reactivity towards the 62 kDa protein were more likely to have mothers with IgG reactivity towards the 37 kDa protein ($p = 0.022$), and in AU families, IgG reactivity towards the 62 kDa protein in children was associated with antibodies towards the 39 kDa protein in their mothers ($p = 0.044$). When we considered the overall number of brain proteins that were recognized by IgG, there was a positive association between mothers and children only in the TD population ($p = 0.0036$). Additionally, the total number of IgG targets in TD children correlated significantly with the incidence of having maternal IgG reactivity to the 37 kDa protein alone ($p = 0.029$).

4. Discussion

This study had two primary goals: (1) to further characterize the occurrence of autoantibodies to cerebellum in children with autism spectrum disorders with respect to behavioral outcome, and (2) to ascertain if an association exists between the presence of brain-directed autoantibodies in children and the presence of brain-directed antibodies in their respective mothers. Autoantibody profiles differed between children with autism (AU), the broader phenotype of autism spectrum disorder (ASD), and typically developing (TD) controls. Moreover, we demonstrated for the first time that children harboring these antibodies had more impaired behavioral scores as well as lower cognitive and adaptive function compared to children without the antibodies. In addition, as previously reported, mothers of children with AU and ASD show a unique pattern of antibody reactivity to fetal brain proteins compared to mothers of TD children (Braunschweig et al., 2008, 2010; Croen et al., 2008; Zimmerman et al., 2007). Familial analysis showed a very limited relationship between anti-brain antibodies in plasma from mothers and their children, though this relationship did not extend to the definitive patterns of maternal autoantibodies associated with an AU or ASD diagnosis. This suggests that while there may be some familial propensity for autoantibody production, autism spectrum disorder-associated autoantibodies observed in mothers and children largely occur in different families.

Independent studies have described the presence of autoantibodies directed against various brain proteins in individuals with an autism spectrum disorder (Enstrom et al., 2009b). We previously characterized autoantibodies towards cerebellum proteins in a smaller group of AU subjects (Wills et al., 2009). The results of the present study differ to some extent from the Wills study. First, Wills et al. originally showed that plasma IgG directed towards a 52 kDa cerebellum protein (rather than 45 kDa protein) correlated with an autism diagnosis. This has now been explained by differences in gel systems as noted in the results section. Second, we observed a lower incidence of IgG reactivity to the cerebellum in children with autism in the present study (10% vs. 21%). This difference may be attributable to several factors including (1) an increased sample size, which may have revealed a more accurate estimation of the occurrence of brain-directed antibodies among autism subjects, and/or (2) the use of younger study subjects (mean age of 3.5 years compared to 6 years in Wills et al.). A longitudinal analysis of the same children over time would help to clarify this issue.

This is the first study to examine specific behavioral phenotypes associated with the presence of brain-targeted antibodies in autism spectrum disorders. We demonstrate that children diagnosed with either AU or ASD tend to have divergent IgG targets in the cerebellum; AU children showed significant IgG reactivity towards a 45 kDa protein, while ASD children showed reactivity towards a 62 kDa protein. One explanation for this difference may be that these proteins are involved in a pathway that impacts behavioral traits, and that interfering the 62 kDa protein may lead to a less severe phenotype than interfering with the 45 kDa protein.

In addition to the differences observed between AU and ASD groups, we found that children with these autoantibodies had significantly more impaired behavioral, cognitive and adaptive traits than children without the autoantibodies. In many cases, this difference was observed regardless of diagnosis. This suggests that rather than representing a specific marker of autism, these antibodies might somehow be linked to specific behavioral outcomes associated with, but not specific to, autism spectrum disorders. Future studies should examine the occurrence of these antibodies in neurodevelopmental diseases other than autism disorder. A small number of typically developing children also had the antibodies, and it is possible that additional environmental exposures and/or genetic factors are necessary for the development of full AU or ASD. Previous studies have similarly shown that differences in other immune factors such as cytokines levels and total IgG correlate with behavioral severity among children with an autism spectrum disorder (Ashwood et al., 2008a,b; Grigorenko et al., 2008; Heuer et al., 2008). It is unclear whether these differences in immune measures are responsible for variations in behavioral characteristics, or if they are a secondary manifestation of other factors involved in the disease. Collectively, these studies illustrate that biological factors can be linked to specific behavioral characteristics; an observation that can perhaps help differentiate ontogenic

mechanisms specific to the varying phenotypes within the autism spectrum.

The clinical and mechanistic significance of these brain directed antibodies is not known, and further research is necessary to explore this issue. First, it should be made clear that it is likely that the target antigens for these autoantibodies are not restricted to the cerebellum (as noted in the study by Cabanlit et al. (2007)), and ongoing studies to further examine this possibility are in progress. In addition, it is not entirely clear how a cerebellum-directed immune responses might relate to behavioral abnormalities common in autism spectrum disorders. It is possible that the antibodies observed herein interfere with normal neuronal processes, or are indicative of abnormal cerebellar function. The study by (Wills et al., 2009) described a very particular staining pattern for antibodies reactive to the 52kDa antigen that also reacted against the Golgi interneurons in the Purkinje layer of the cerebellum using immunohistochemistry. These cells act as down-regulators of the excitatory synapses in the granule cell layer of the cerebellum, which impacts the activity of Purkinje cells, and interfering with this pathway could lead to various motor and behavioral abnormalities (Hirano et al., 2002). Other studies have described cerebellar abnormalities in individuals with an autism spectrum disorder, including reduced numbers of Purkinje cells in post-mortem brains (Bailey et al., 1998; Kemper and Bauman, 2002). Further, injury to the cerebellum and alterations in cerebellar development are associated with reduced cognitive function, impaired language, and increased stereotypic behaviors (Gillig and Sanders, 2010; Martin et al., 2010; Steinlin, 2008). For example, mice lacking Purkinje cells demonstrate increased repetitive behaviors (Martin et al., 2010). Stereotypic behavior, cognition, and language were all found to be more severely affected in children harboring the cerebellum-directed antibodies in the present study.

Another critical issue is whether these antibodies are pathogenic on their own or if they are secondary to pathology. In order to be pathogenic, the antibodies must gain access to the central nervous system (CNS). Under normal circumstances, large molecules such as IgG and other immune components are largely excluded from the CNS by the blood–brain-barrier (BBB). However, infectious and environmental factors can increase permeability of the BBB allowing immune components to enter the CNS. Examples of exposures that compromise the integrity of the BBB include pertussis toxin, extreme stress, sub-clinical infection, and exposure to nicotine or epinephrine (Hawkins et al., 2004; Kuang et al., 2004; Kugler et al., 2007; Theoharides and Konstantinidou, 2007). It is possible that TD children with the autoantibodies may not have had the required insult that would allow passage of the autoantibodies to the neuronal targets.

Once in the brain, autoantibodies can act through various pathogenic mechanisms. First, they can mimic receptor ligands and induce excitotoxic death through excessive signaling. This has been demonstrated in individuals with systemic lupus erythematosus (SLE) accompanied by cognitive and neuropsychiatric symptoms (Kowal et al., 2004). In these subjects, DNA-specific antibodies are able to cross-react with the NMDA receptor for glutamate, and administration of these antibodies to mice with a compromised BBB leads to cognitive impairments and apoptotic neuronal death (Huerta et al., 2006). Autoantibodies to neural antigens might also block vital pathways and compromise the development and function of the nervous system. Alternatively, autoantibodies can cause tissue destruction by fixing complement or inducing cell-mediated death. Studies involving passive transfer of these antibodies in animal models will be essential to explore their pathogenic significance.

Alternatively, it is entirely possible that these antibodies were produced as a result of previous neuronal injury, and may not have pathogenic significance on their own. An event in the CNS (perhaps an infection or injury) could have altered the course of neurodevelopment leading to an autism disorder; and simultaneously spurred an immune reaction that caused a break in immune tolerance towards CNS antigens. The antibodies produced in the course of such an event may or may not have pathogenic properties on their own. If these antibodies are not pathogenic, they still represent a potentially valuable biological marker for a subset of autism spectrum disorders and/or autism-associated behaviors. Since the underlying biology of behavioral disorders like autism remains poorly understood, the discovery of any biological connection could be of interest; even one that is secondary to pathology/etiology. Further, if autism behaviors are the result of neuronal injury, and antibodies are produced as a secondary result of this neuronal injury, the antibodies may serve to point researchers towards specific neuronal components/processes that may be involved in the neuropathology. Identification of the 45 and 62 kDa antigens will be an important step towards understanding their role in autism spectrum disorders.

Independent studies have shown that a subset of mothers of children with autism harbor circulating IgG directed towards fetal brain proteins (Braunschweig et al., 2008; Croen et al., 2008; Singer et al., 2008; Zimmerman et al., 2007). This is in contrast to children with AU or ASD, who demonstrate IgG reactivity to the mature brain rather than the fetal brain (Cabanlit et al., 2007; Morris et al., 2009; Singer et al., 2006; Wills et al., 2009). A subset of the mothers included in this study demonstrated IgG reactivity to fetal brain proteins as previously described (Braunschweig et al., 2008, 2010; Croen et al., 2008). Previous primate and murine studies have demonstrated the potential pathogenic significance of maternal anti-fetal brain antibodies, where prenatal exposure to purified IgG from mothers of children with autism resulted in behavioral alterations that were not observed in controls (Martin et al., 2008; Singer et al., 2009). Our final analysis in the current study was designed to determine if there was a familial (maternal/child) relationship for brain-directed antibodies. Our results show very limited relationships between the anti-brain autoantibodies in AU, ASD, and TD mother–child pairs. Interestingly, we found that the overall presence of brain-directed antibodies in maternal plasma has a higher degree of association with an AU or ASD diagnosis than brain directed antibodies in children. It may be that exposure to anti-brain antibodies during gestation is more detrimental to neurodevelopment than exposure to anti-brain antibodies in early childhood. Additionally, the presence of the autoantibodies found in the children may not be sufficient on their own to cause a pathologic insult, or may simply be the result of previous damage through other mechanisms.

In summary, we describe the presence of autoantibodies to proteins in a large well-characterized population of children and their mothers. We further demonstrated that reactivity to specific proteins within the cerebellum was associated with a diagnosis of AU or ASD. Additionally, the presence of these antibodies was linked with more aberrant behaviors and lower cognitive and adaptive function regardless of diagnosis, suggesting a potential association with some features of autism rather than an autism spectrum disorder specifically. Finally, we found limited familial associations for specific patterns of anti-brain antibodies in AU, ASD, and TD mother–child pairs. Future studies will strive to identify the autoantibody targets in the cerebellum and fetal brain, characterize their pathologic significance with respect to autism, and determine whether the development of therapeutic measures would be warranted.

Conflict of interest statement

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