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DETECTION OF AUTOANTIBODIES TO NEURAL CELLS OF THE CEREBELLUM IN THE PLASMA OF SUBJECTS WITH AUTISM SPECTRUM DISORDERS

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Abstract

Autism Spectrum Disorders (ASD) are a group of heterogeneous, behaviorally defined disorders characterized by disturbances in social interaction and communication, often with repetitive and stereotyped behavior. Previous studies have described the presence of antibodies to various neural proteins in autistic individuals as well as post-mortem evidence of neuropathology in the cerebellum. We examined plasma from children with ASD, as well as age-matched typically developing controls, for antibodies directed against human cerebellar protein extracts using western blot analysis. In addition, the presence of cerebellar specific antibodies was assessed by immunohistochemical staining of sections from *Macaca fascicularis* monkey cerebellum. Western blot analysis revealed that 13/63 (21%) of subjects with ASD possessed antibodies that demonstrated specific reactivity to a cerebellar protein with an apparent molecular weight of approximately 52kD compared with only 1/63 (2%) of the typically developing controls ($p=0.0010$). Intense immunoreactivity, to what was determined morphologically to be the Golgi cell of the cerebellum, was noted for 7/34 (21%) of subjects with ASD, compared with 0/23 of the typically developing controls. Furthermore, there was a strong association between the presence of antibodies reactive to the 52 kDa protein by western blot with positive immunohistochemical staining of cerebellar Golgi cells in the ASD group ($r=0.76$; $p=0.001$) but not controls. These studies suggest that when compared with age-matched typically developing controls, children with ASD exhibit a differential antibody response to specific cells located in the cerebellum and this response may be associated with a protein of approximately 52 kDa.

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Keywords

Autism Spectrum Disorders (ASD); brain; immune; autoantibody; cerebellum; Golgi cell; immunohistochemistry

Introduction

Autism Spectrum Disorders (ASD) are a group of heterogeneous, behaviorally defined disorders characterized by disturbances in social interaction and communication (verbal and nonverbal) and often with repetitive and stereotyped behavior which is typically apparent between 2–3 years-of-age. The prevalence of ASD has been estimated at 1 in 150 in the total population and affects approximately four times as many males as females (MMWR 2007). The potential exists for numerous etiologies, including those of a genetic and/or environmental nature, which could contribute to the development of ASD.

Immune system-related irregularities associated with ASD involve the innate as well as the adaptive arms of the immune system. Immune findings in ASD are varied and include increased numbers of circulating monocytes, decreased natural killer cell lytic activity, abnormal cytokine and immunoglobulin levels and decreased peripheral lymphocyte numbers and responsiveness (Ashwood et al., 2006; Croonenberghs et al., 2002; Molloy et al., 2006; Stubbs and Crawford, 1977; Sweeten et al., 2003; Trajkovski et al., 2004; Warren et al., 1987; Warren et al., 1990; Yonk et al., 1990). The presence of several putative autoantibodies to various elements of the nervous system has also been reported in ASD, including antibodies directed against myelin basic protein (MBP), brain serotonin receptor, neurofilament proteins, brain endothelial cell proteins, heat shock protein as well as autoantibodies directed against epitopes within the cerebellum (Cabanlit et al., 2007; Connolly et al., 1999; Evers et al., 2002; Plioplys et al., 1994; Silva et al., 2004; Singer et al., 2006; Singh et al., 1998; Singh et al., 1997a; Singh et al., 1997b; Singh et al., 1993; Todd et al., 1988; Vojdani et al., 2002). It is important to note that many of these autoantibodies are not unique to individuals with ASD, nor are they found in all subjects with ASD. MBP self-reactive antibodies, for example, are often identified in individuals with multiple sclerosis (Egg et al., 2001).

The neuropathology of ASD is still in its infancy (Amaral et al., 2008). Yet, cerebellar irregularity is one of the most consistent findings in the brains of subjects with ASD. For example, in a review by Brambilla et al. of the 24 post-mortem cases of autism reported in which the cerebellum was studied, 19 demonstrated a lower number of Purkinje cells (Brambilla et al., 2003). The lack of cells is most commonly observed in the cerebellar hemispheres. Other abnormalities observed in the cerebellum or cerebellum-related brain regions, such as changes over time in the inferior olive, deep cerebellar nuclei, and increases in white matter have also been described in postmortem tissue (Bailey et al., 1998; Kemper and Bauman, 1998). Interestingly, very little information is available with respect to the other neuronal cell types found in the cerebellum, although Yip *et al.* recently described reduced GAD 67 mRNA in the basket and stellate cells of the molecular layer in postmortem autism tissue (Yip et al., 2007).

Previous studies describing the presence of antibodies to various neural proteins as well as neuropathological indications for a reduced number of Purkinje cells led us to examine plasma from a cohort of extremely well-characterized children with ASD as well as age-matched typically developing and developmentally delayed controls using a two-pronged approach. First, to look for the presence of specific autoantibodies to brain tissue, we examined plasma from children with ASD and controls for reactivity to human brain protein extracts using western blot analysis. Second, to identify specific autoantibodies that were directed to neural

structures using immunohistochemistry, plasma of subjects with ASD were examined for their ability to bind to sections from the *Macaca fascicularis* monkey cerebellum. These approaches enabled us to determine both the apparent molecular weight and the cellular location of the target molecule(s).

Methods/Materials

Subjects/ Sample Collection

The study protocol followed the ethical guidelines of the most recent Declaration of Helsinki (Edinburgh, 2000), and was approved by the Institutional Review Boards of the UC Davis School of Medicine and the State of California, and all subjects enrolled in the study had written informed consent provided by their parents and assented to participate if developmentally able. Subjects for this study were enrolled through the M.I.N.D. (Medical Investigations of Neurodevelopmental Disorders) Institute clinic. The M.I.N.D. clinic sample population consisted of children diagnosed on the autism spectrum (ASD) (n=63) and their siblings (n=25). There were two separate control populations: one consisted of age-matched typically-developing children (n=63); the other contained children who are developmentally delayed but do not have ASD (n=21) (Table 1). A diagnosis of ASD was confirmed in all subjects using the Autism Diagnostic Interview–Revised (ADI-R) and the Autism Diagnostic Observation Schedule (ADOS) (DiLavore et al., 1995; Joseph et al., 2002; Lord et al., 2001; Lord et al., 1997). Final autism case status is defined as meeting criteria on the communication, social interaction and repetitive behavior domains of the ADI-R with onset prior to 36 months and scoring at or above the social plus communication cutoff for autism on the ADOS module 1 or 2.

The ADI-R was used to define onset of ASD. The ADI-R provides a standardized, semi-structured interview and a diagnostic algorithm for the DSM-IV and the ICD-10 definitions of autism (Association, 1994) (World Health Organization (WHO), 1992) (Steinhausen and Erdin, 1992). The Social Communication Questionnaire was used to screen for characteristics of ASD among the subjects with developmental disabilities, the typically developing controls, and the siblings of subjects with ASD. Children who scored above the screening cut-off were fully assessed using the ADI-R and ADOS.

Blood samples for western blot were obtained from the 172 individual subjects at the time of assessment. A number of these subjects were also analyzed by immunohistochemical staining, including 34 children diagnosed with ASD, 23 age-matched typically-developing controls, 14 siblings of children with ASD, and 11 children diagnosed with developmental delay but not ASD. (Table 2).

Antibodies and reagents

Human adult cerebellum protein medley (BD Bioscience Clontech, Palo Alto, CA) was used to screen for potential autoantibodies in the plasma by western blot. Human kidney protein was used as negative tissue control (BD Bioscience Clontech, Palo Alto, CA). Horseradish peroxidase-conjugated goat anti-human IgG was used as a secondary antibody (Zymed, San Francisco, CA), and SuperSignal Chemiluminescent Substrate was used to develop the blot. (Pierce, Rockford, IL)

Immunoblotting

To determine the incidence of plasma containing antibodies that were reactivity to human brain extracts, SDS-PAGE was performed using 12% Tris-HCL Mini Ready gels (Bio-Rad, Hercules, CA). A final concentration of 300 µg/ml of adult human brain extracts from the cerebellum, extracts from human kidney, and 5 µl of magic mark protein standard were loaded into the gel

and electrophoresed for 1hr and 30mins at a constant current of 30mAmps. After gel electrophoresis, proteins were transferred at 30 volts overnight to a nitrocellulose membrane, dried, and stored at 4°C. For immunoassay, the membranes were blocked with 5% milk in PBS buffer containing 0.05% Tween 20 (PBST) for 1 hr at room temperature. Blots were incubated in plasma from subjects with ASD (n=63), TD controls (n=63), typically developing siblings (n=25), and DD subjects (n=21) diluted 1:500 in 5% milk and PBS-Tween for 1 hr at room temperature. They were washed 5 times for 5 min duration with PBST followed by 1hr incubation with HRP-conjugated goat anti-human IgG diluted 1:10,000. To visualize the signal, blots were developed using a SuperSignal Chemiluminescent Substrate according to the manufacturer's instructions. Strips were imaged and analyzed using a FluorChem 8900 imager and AlphaEaseFC imaging software. (Alpha Innotech Corporation, San Leandro, CA). Since multiple blots were used in this project, a reference subject, who demonstrated reactivity to all bands noted in this study, was run as a standard control on each blot.

Cerebellum from a healthy adult male macaque monkey was prepared by suspending 1.0 g of fresh tissue in 10mL of 20mM HEPES-OH, pH 7.5, containing 320mM sucrose, 1mM EDTA, 5mM DTE, and 1 mg/mL of a mixture of protease inhibitors (1mM PMSF and 1 tablet Complete™ (Roche Diagnostics) per 50mL of suspension buffer) and phosphatase inhibitors (0.2mM Na₂VO₃ and 1mM NaF). The suspension was homogenized using a Teflon/potter homogenizer and centrifuged at 800g for 10 min to remove nuclei and undissolved material and diluted ten-fold with 50mM Tris-HCl, pH 6.8, containing 25% glycerol and 1% lithium dodecyl sulfate (LDS), and concentrated again to reach a protein concentration of about 12.5 mg/mL. A total of 15 µg/lane was loaded on a 12%Tris-HCL Mini Ready gel (Bio-Rad, Hercules, CA) in alternate lanes with 15 µg/lane of human cerebellum and run as described above. The resulting blots were probed with plasma at a dilution of 1:400.

Rf and Molecular Weight

Measurement of band R_f, the ratio of the distance migrated by the protein to that of the marker dye front, and molecular weights (MW) was normalized to the IgG band for each sample. Bands were manually selected and the R_f and MW were automatically calculated using AlphaEase FC Imaging Software (AlphaInnotech). AlphaEase FC assumes the origin (R_f value=0.00) is located at the top of the screen and the dye front (R_f value= 1.00) is at the bottom of the screen. Using these points as a frame of reference, AlphaEase calculates R_f values for the intermediate bands. The MW of the band was calculated based on the graph of the known marker bands. In addition to the MW, an R_f difference of +/- 0.005 was used as a distinguishing factor between bands.

Immunohistochemistry (IHC)- Animals and Fixation

We choose nonhuman primate tissue as our antigen source due to the highly conserved nature of autoantigens as well as their similar, but compressed neurodevelopment when compared with humans (Webb et al., 2001). All procedures were carried out under an approved University of California-Davis Institutional Animal Care and Use Protocol and strictly adhered to National Institutes of Health policies on primate animal subjects. The brain sections were obtained from two different male adult macaque monkeys (*Macaca fascicularis*), aged six years, nine months and nine years, five months at the time of sacrifice. The brains from these animals had been used for neural tract tracing studies and library sections were used for the present study. No animals were sacrificed expressly for these studies. The sections were obtained from animals for which no health problems were reported in either case.

Tissue fixation and histological processing were performed according to procedures described by Pitkänen and Amaral (Pitkanen and Amaral, 1998). Briefly, the animals were deeply anesthetized and perfused intracardially with the fixative 1% paraformaldehyde in 0.1 M

phosphate buffer (pH 7.4) at 4° C at a rate of 250 ml/ min for 2 minutes, followed by 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) at 4°C at a rate of 250 mls/minute for 10 minutes. The flow rate was then reduced to 100 mls/minute for 50 minutes.

The brain was then blocked stereotactically, extracted from the skull and post-fixed in 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) solution for 6 hours. Finally, the brain was cryoprotected in a solution containing 10% glycerol and 2% dimethylsulfoxide (DMSO) for 1 day followed by 20% glycerol and 2% DMSO for 3 days. The fixed brain was then frozen using the isopentane method described by Rosene et al. and stored at -70°C until sectioning (Rosene et al., 1986). Frozen sections were cut in the coronal plane at a thickness of 30 µm with a sliding microtome and placed into a cryoprotectant tissue collecting solution (30% ethylene glycol, 25% glycerol in 0.05 M sodium-phosphate buffer; TCS). The sections were stored at -80°C until they were processed immunohistochemically.

Immunohistochemistry – Tissue processing procedures

Free-floating sections were processed using the method described by Gerfen and Sawchenko with modifications (Gerfen and Sawchenko, 1984). Briefly, 30 µm-thick coronal sections from the brain were taken from storage and were placed into nets and washed (3×10 min with 0.1M PBS). The following steps were done with rotation at room temperature, except for the primary incubation, which was at 4°C with rotation. Prior to primary incubation, the sections were pretreated with hydrogen peroxide (1.6% hydrogen peroxide in 0.1M PBS-Fisher) for fifteen minutes in order to remove endogenous peroxidases and reduce background staining. During incubations, the tissue was maintained in solution at all times. The sections were blocked for four hours using 5% normal mouse serum (Pierce) in 0.1M PBS and 0.5% Triton X-100 (Fisher). The nets were incubated with the primary antibody (subject plasma) at a dilution of 1:100 in 0.1M PBS, 2% normal mouse serum and 0.3% Triton X-100 for 40–48 hrs. The sections were then washed with 2% normal goat serum (Biogenesis) in 0.1M PBS (3×10min) and incubated with biotinylated mouse anti-human monoclonal IgG (Zymed) in 0.1M PBS, 2% normal mouse serum and 0.3% Triton X-100 for one hour. Sections were then washed with 2% normal goat serum in 0.1M PBS (3×10min) followed by incubation for 45 min with ABC peroxidase (Biostain Super ABC/Peroxidase Basic Kit; Biomedex) incubation in 0.1M PBS. Following the first ABC peroxidase incubation, the sections were washed in 2% normal goat serum in 0.1M PBS. The incubation with the secondary was repeated for 45 min, followed by washes with 0.1M PBS only. The second ABC peroxidase incubation was 30 min, followed by washes with 0.05M Tris buffer. Next, the sections were incubated with 3,3'-diaminobenzidine (DAB) (Fisher) in 0.05M Tris with 0.04% hydrogen peroxide for 30 min. The sections were then washed twice with 0.05M Tris, once with 0.02M KPBS, and stored covered at 4°C in 0.02M KPBS. Sections were then mounted from phosphate buffer onto gelatin coated slides and dried with a fan, then placed in a 37°C incubator for 24 hrs to dry.

Silver Nitrate/Gold Chloride Intensification

In order to increase the signal to noise ratio of the immunohistochemical reaction, a silver nitrate/gold chloride intensification procedure was followed. Prior to intensification, the slides were defatted in a mixture of equal parts chloroform and 100% ethanol for a total of 4 hrs. The sections were then hydrated through graded ethanols, and placed in a 37°C incubator overnight. Following the overnight incubation, the slides were rinsed with running, deionized water for 10 min, followed by incubation in a 1% silver nitrate solution maintained at 56°C with the use of a water bath, for 40 min. The slides were then rinsed with running, deionized water for 10 min, and incubated for 10 min in a 0.2% gold chloride solution with rotation at room temperature. Following a second rinse, the slides were stabilized in a 5% sodium thiosulfate solution at room temperature for 15 min with rotation followed by an additional rinse in running, deionized water for 10 min. Sections were then dehydrated through graded ethanols,

followed by xylene and coverslipped using DPX mounting medium (Sigma Aldrich). Slides were observed by brightfield microscopy on a Nikon Eclipse E600 or a Leica Leitz DMRB microscope with the use of Spot Diagnostic Instruments Digital Camera System and software. Preliminary identification of the various cell types was accomplished using morphological features as well as their location within the cerebellum. The slides were each evaluated by two different observers (SW and DA). Both observers were blind to the classification of the subject plasma used for staining the slides. Disputes in ranking were resolved by joint reanalysis and ranking.

Nissl Staining

An adjacent series of sections was stained with thionin to establish the cytoarchitectural boundaries in the cerebellum. Individual sections were taken from storage, washed in PBS then further fixed in 10% formalin for two weeks. Prior to staining, the slides were defatted in 1:1 chloroform and 100% ethanol, re-hydrated, and placed in 0.25% thionin for 20 sec. Immediately following thionin incubation, the slides were rinsed in deionized water then dehydrated through graded ethanol and xylene. The slides were then coverslipped and dried in the hood for at least 1 week before viewing.

Statistics

The differences between each study group with respect to the presence of Golgi cell staining were determined using a non-parametric, two-tailed Mann-Whitney test. The Fisher's exact test was used to compare for the presence of antibodies by western blot in subjects with ASD to each of the control groups (TD controls, siblings and DD group). A 95% confidence interval was determined using the approximation of Katz (InStat GraphPad Software, version 3.0a). To determine the association between the presence of antibodies that were reactive to brain proteins at approximately 52 kDa by western blot with the presence antibodies that were reactive by IHC to cells in the cerebellum, we ran the Fisher's Exact Test, where the presence or absence of band reactivity was correlated with the intensity of staining, separated into two groups: moderate to intense (++ , +) and absent to weak ($\pm$ or -). *P*-values < 0.05 were considered statistically significant (InStat GraphPad Software, version 3.0a).

Results

Western blot analysis

Western blot analysis revealed that the presence of antibodies directed against brain proteins varied among subjects (Figure 1a). Interestingly, 13/63 (21%) of the subjects with ASD demonstrated specific reactivity at approximately 52kDa against human cerebellar proteins compared to only 1/63 (2%) of the typically developing controls ($p=0.0010$). None of the sibling controls ($p=0.0162$) had reactivity to this apparently 52kDa band. This approximately 52kDa band was present in only one of the subjects in the developmental delay group ($p>0.05$) (Table 3). When a western blot was performed with adult macaque (*Macaca mulatta*) cerebellum as the source of protein and compared to a blot that contained human cerebellum, we noted that the 52kDa band was present in the monkey preparation and was reactive with antibodies from the subjects with ASD but not controls (Figure 1b).

Immunohistochemical analysis

The cerebellar cortex consists of 3 layers that are, from superficial (the pial surface) to deep, the molecular layer, the Purkinje cell layer, and the granular layer. The molecular layer contains relatively few cells and is populated by basket and stellate cells. The Purkinje cell layer contains a monolayer of fairly large Purkinje cells. The densely cellular granular layer contains tightly packed granule cells, unipolar brush cells, Lugaro cells and Golgi cells.

Background staining noted in the sections not exposed to primary antibody typically consisted of intermittent, varying levels of stained basket/stellate cells, frequently restricted to the edges of the cerebellar folia. Basket/stellate cells were observed to have some degree of staining in all samples, including those processed in the absence of primary antibody, and were therefore considered to be an artifact and not included in the analysis. Infrequently, staining of the white matter in the cerebellum was also observed, more often in patients with ASD. Further identification of this staining pattern is in progress.

A striking pattern of immunoreactivity, most frequently found in the plasma from subjects with ASD, was noted at the interface of the molecular and granule cell layers (Figure 2A). At higher magnification, it was apparent that the staining of these cells was prominent in the somal cytoplasm (Figure 2C) and continued into their proximal dendrites that primarily entered the molecular layer, but also extended deeply into the granular layer. The intracellular staining typically appeared punctuate, extending into the dendrites of the labeled cells. Based on their size, morphology, and dendritic orientation, it appeared unlikely that the antibodies were reactive to Purkinje cells. Moreover, in immunohistochemically stained sections counterstained with a Nissl stain, it was apparent that the Purkinje cells were completely negative for autoantibody reactivity (Figure 3). Based on the size of the labeled neurons and the distribution of their dendrites, we concluded that Golgi cells, which are large interneurons located in the granular layer, were the likely target cell. Other large cells in this region, such as the Lugaro cells have most of their dendritic trees located within the granule cell layer (Melik-Musyan and Fanardzhyan, 2004). There was substantial variability of the staining across sections from the ASD group. To summarize the findings, staining of the Golgi cells was scored using a four parameter system; (++), (+), (-/+), and (-). The (++) designation refers to the staining being continuous and intense in the majority of the referenced cell type (Figure 4A). The notation of (+) refers to the staining being present in a large number of the referenced cell type (Figure 4B). The notation of (-/+) refers to positive staining that is intermittent throughout the section (Figure 4C). The description of (-) refers to the absence of specific staining in the referenced cell type (Figure 4D).

Overall, when reactivity to Golgi cells was compared between subject groups, there were significantly more subjects in the ASD study population who had Golgi-reactive autoantibodies when compared to the TD control subjects ($p=0.0003$), the subjects with DD ($p=0.0349$), and the siblings of the AU group ($p=0.004$). Intense (++) Golgi cell staining was observed in the plasma from 21% (7/34) of subjects with ASD, and was not seen for any of the typically developing controls (0/23) or subjects with developmental delay (0/11) (Table 4). Intense (+) staining was observed with the plasma from one sibling of a subject with ASD. There was some reactivity (+) for 2/23 of the controls subjects (9%) and slight reactivity (-/+) to Golgi cells observed in plasma from 36% (4/11) of subjects with developmental delay and in 14% (1/14) of the siblings of subjects with ASD.

Of further interest was the relationship between immunoreactivity to the Golgi cells in the cerebellum and western blot evidence for the presence of autoantibodies to the approximately 52kDa human cerebellar protein in subjects with ASD (Table 5). There was a significant correlation between the presence of reactivity to the 52 kDa band by immunoblot and immunohistochemical staining of the Golgi cell of the cerebellum in the ASD group ($r=0.76$; $p=0.0001$) but not the controls. Thus, when plasma from a subject with ASD was reactive to the 52 kDa cerebellar protein by western blot analysis, correspondingly the same subject was typically found to have intense to moderate (+++, +) staining of Golgi cells by immunohistochemistry.

Finally, we did not find a relationship between behavioral outcome and incidence of autoantibody production either to the 52 kDa band or by IHC. This included a breakdown of subjects based on a diagnosis of regressive versus early onset autism.

Discussion

We have carried out a detailed examination for the presence antibodies that are directed against cerebellar antigen(s) in subjects with autism spectrum disorders (ASD), by western blot and immunohistochemistry. A substantial number (21%) of individuals with ASD were found to have specific plasma antibodies that were directed against proteins of approximately 52kDa molecular weight from human cerebellum. In contrast, such reactivity was absent in the plasma of all but two control subjects: one typically developing control and one with developmental delay without ASD. To determine which neural or glial component of the cerebellum these antibodies identified, we carried out immunohistochemical analysis of sections from the cerebellum of *Macaca fascicularis* monkey brain. We demonstrated a consistent, cell-specific staining in 21% of subjects with ASD, compared to 0% of either control group. Of particular note was the association found between plasma reactivity to a distinct band at approximately 52 kDa as determined by western blot of both human and monkey cerebellar proteins and the presence of reactivity to Golgi cells. This finding raises the possibility that the autoantibodies specific for the Golgi cells in the cerebellum may be recognizing a protein that is approximately 52 kDa. However, it is possible that the approximately 52 kDa protein is not isomorphic with the Golgi cell antigen(s) that are recognized by plasma from children with ASD. The identity of the 52 kDa band is currently unknown. Previous studies have cited autoantibodies to neural proteins in the blood of some children with ASD, including antibodies to glial fibrillary acid protein (GFAP) that has an apparent molecular weight of approximately 50 kDa (Singh, et al., 1997b). Through absorption studies and western blot analysis, we have determined that the 52 kDa band does not appear to be GFAP (data not shown). Therefore, due to the apparent specificity of autoantibody reactivity to an apparently 52 kDa band in the subjects with ASD, further identification of this protein is currently in progress.

Previous studies published by our group have described the presence of autoreactivity to bands, predominantly in the hypothalamus and thalamus, which appear to be specific for a subpopulation of children with ASD (Cabanlit, et al., 2007). While such reactivity appears to a lesser extent in the cerebellum and are certainly of interest to us, only the 52 kDa band correlated with the presence of Golgi cell staining.

Our finding of selective Golgi cell immunoreactivity adds to a growing literature on the presence of autoantibodies to neuronal tissue in subjects with ASD. In a recent study by Singer et al, a variety of brain-specific autoantibodies were identified in the serum of subjects with autism spectrum disorders by both ELISA and western blot analysis (Singer et al., 2006). Interestingly, the authors found that subjects with autism disorder as well as their non-autistic siblings had denser bands of antibody reactivity at 73 kD in the cerebellum and cingulate gyrus when compared with controls (Singer et al., 2006). More recently, using fetal rat brain, Zimmerman and colleagues reported reactivity by immunoblotting in subjects with autism (Zimmerman et al., 2007). Siblings of ASD-affected individuals could be differentiated based on their antibody pattern of reactivity. However, the pattern of antibody reactivity observed in subjects with ASD when compared to subjects with other neurodevelopmental disorders, such as developmental delay without autism, did not distinguish between groups.

The potential role of autoantibodies in nervous system disorders has been widely investigated. Elevated levels of circulating autoantibodies to nervous system components have been reported in a number of psychiatric disorders including schizophrenia, obsessive-compulsive disorder, neuropsychiatric symptoms associated with systemic lupus erythematosus (SLE), pediatric

autoimmune neuropsychiatric disorders associated with streptococcal infection (PANDAS), and Gilles de la Tourette's Syndrome (TS) (Huerta et al., 2006; Jones et al., 2005; Kiessling et al., 1994; Pandey et al., 1981; Rothermundt et al., 2001; Snider and Swedo, 2004; Yeh et al., 2005). While the findings of some of these studies remain controversial, they all point to the potential for autoantibodies to play a role in neural dysfunction.

Autoantibodies, such as those described herein, have the potential to exert their effects through several different mechanisms. For example, some autoantibodies act as ligands and are able to bind to a receptor and induce hyperactivity, or even excitotoxic death through excessive signaling. An example of this mechanism was demonstrated in a recent study of patients with cognitive and neuropsychiatric symptoms associated with systemic lupus erythematosus (SLE) (Kowal et al., 2004). The authors found that antibodies to dsDNA from the serum of patients with SLE cross-react with the NR2a polypeptide of the NMDA receptor (NMDAR). In a subsequent study by this group, following lipopolysaccharide (LPS) exposure to compromise blood brain barrier integrity, passive intravenous transfer of serum with reactivity to DNA and NMDAR extracted from lupus patients elicited cognitive impairments in the recipient mice. Brain histopathology revealed that the passively transferred human antibodies were capable of causing apoptotic neuronal death in the hippocampus (Huerta et al., 2006). Alternatively, autoantibodies to neural antigens can act as an antagonist by blocking an essential pathway that may lead to abnormal nervous system development and/or function. Finally, autoantibodies may also mediate tissue destruction through either complement-mediated or cell-mediated cytotoxicity. Any of these scenarios could potentially result in alterations in neuronal function, receptor density and/or distribution, or an increase or reduction in the release of neurotransmitters and/or cytokines. It is currently premature to speculate whether the Golgi cell-reactive autoantibodies are of pathogenic significance or merely an epiphenomenon. Thus, we are pursuing additional studies to determine whether the antibodies that we have identified here may have deleterious effects on brain structure and function in animal models.

The autoantibodies described herein demonstrate intense, defined reactivity for the Golgi cell, which is a large interneuron that participates in the glomeruli of the granule cell layer and acts as a down-regulatory neuron. As discussed above, such antibodies could have an impact, through various potential mechanisms, on down-stream function. For example, Golgi cells modulate the activity of the mossy fiber to granule cell excitatory synapses, thus influencing the input that leaves the cerebellum via the Purkinje cells (De Schutter et al., 2000; Hirano et al., 2002). Interference in this neural pathway could have profound effects on cerebellar and ultimately cerebral function.

In order for the Golgi-cell specific autoantibodies to be of pathological significance, they must be able to access their target antigens in the cerebellum. While the brain has historically been described as an immune-privileged site, a more accurate depiction is an immunologically customized site (Cohen and Schwartz, 1999; Schwartz and Cohen, 2000). Immune responses are tightly regulated, and as in other parts of the body, immune surveillance also occurs routinely in the central nervous system, even in the presence of an intact blood-brain barrier (Hickey, 2001). Antibodies in the circulation are capable of reaching the brain when there is a disturbance in the blood brain barrier, such as was noted above in the Kowal LPS model (Kowal et al., 2004). Further, autoantibodies have been shown to reach intracellular targets, even those in the nucleus, in living cells (Alarcon-Segovia et al., 1978; Yanase et al., 1997). This penetration has been shown to result in cell cycle changes, alterations in gene expression, as well as apoptotic cell death.

At present, the pathological significance of elevated levels of autoantibodies to cerebellar protein(s) in ASD is unclear. These autoantibodies may be pathologically relevant, or merely an epiphenomenon of abnormal central nervous system development or brain injury in children

with ASD. We are currently investigating the distribution of the target antigen in other regions of the brain. Moreover, further work is under way to both replicate these findings in a larger study cohort, and to determine the protein targets of these antibodies, the identification of which is key to deciphering their potential role in ASD. Once these issues are addressed, we will better understand the pathological significance, for at least some forms of ASD, and of the cerebellum-specific autoantibodies described herein.

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Figure 1.

a. Western blot of human cerebellum proteins. Representative blots, shown from left to right, include plasma reactivity of two subjects with ASD, a typically developing control (TD), and a subject with developmental delay (DD), along with the molecular weight markers. The arrow denotes a protein with an apparent molecular weight of approximately 52 kDa that is specifically recognized by the autoantibodies from subjects with ASD.

b. Western blot comparing reactivity to human and monkey cerebellum proteins. Cerebellum protein extracts from human and Rhesus macaque were run together in alternate lanes to determine the presence of the 52 kDa band in monkey cerebellum. The left lane of each duplex contains the human cerebellum preparation (H), while the right lane contains adult monkey cerebellum (M). The left blot was reacted with plasma from a child with autism (AU) while the right was reacted with plasma from a typically developing control (TD).

Figure 2.

Immunohistochemical staining of macaque monkey cerebellum with plasma from a subject with intense (++) reactivity. A) Low magnification (4X objective) photomicrograph of one folia in a coronal section of the cerebellum. This image illustrates the consistent staining of Golgi cells lying just subjacent to the molecular layer. B) Higher magnification (40X objective) phase-contrast photomicrograph of a subset of the cells from panel A. Note that the labeled neurons have both apical dendrites that extend into the molecular layer and basal dendrites that remain in the granular layer. C) High magnification (100X objective) photomicrograph of immunostained Golgi cells. Panel C illustrates that the immunoreactivity has a particulate appearance and is distributed within the somal cytoplasm and extends into the proximal

dendrites. Calibration bars: A) 250 μm , B) 50 μm , C) 20 μm . Abbreviations: GL: granular layer. ML: molecular layer.

Figure 3.

Nissl counterstained (blue) immunohistochemically processed coronal sections through the macaque monkey cerebellum illustrating an additional (++) case not illustrated in preceding figures. A: low power (10X objective) photomicrograph illustrates the Golgi cells (dark brown) at the interface between the molecular and granule cell layers. Panels B and C are higher magnification (20 X) photomicrographs and illustrate Golgi cells with dark brown reaction product and lightly Nissl-stained Purkinje cells (arrows) which are devoid of immunohistochemical reaction product. Calibration bars equal 100µm. Bar in panel C also applies to panel B.

Figure 4.

This figure illustrates the range of immunohistochemical staining intensities and patterns observed in this study. Panel A illustrates strong and selective staining of Golgi cells (++). Panel B illustrates less intense and less consistent staining of Golgi cells (+). Panel C depicts intermittent positive staining (-/+) and panel D illustrates a lack of specific staining in the referenced cell type (-). Calibration bar equals 100 μ m and applies to all panels.

Figure 5.

Immunohistochemical staining of macaque monkey cerebellum with plasma from six individual subjects. Despite slight differences in the nature of the staining, each of these cases was rated as (++) for Golgi cell staining. Panels A and B both represent strong and selective staining of Golgi cells similar to the subject presented in Figure 2. Of particular note in both panels A and B is the particulate dendritic staining in the molecular layer. In panel B, there is also some light staining of basket cell somata in the deep portion of the molecular layer. Panels C and D illustrates staining resulting from plasma from subjects whose antibodies produced both positive staining of the Golgi cells as well as more intense staining of the deep molecular layer basket cells. As illustrated in Figure 4, there is occasionally light staining of these basket

cells in negative control sections. E illustrates a case in which the staining was lighter using the same concentration of plasma and procedures as in cases shown in panels A and B, nonetheless, the staining is selective for Golgi neurons. In F, the staining is also light but selective for Golgi neurons. More prominent dendritic labeling is observed in the superficial portion of the molecular layer. Calibration bar equals 100 μ m and applies to all panels.

Table 1

Demographics of study subjects for western blot analysis.

Study Population	Median Age (mos.)	Range (mos.)	Gender
ASD (N=63)	53	30 to 184	Female=3 Male=60
Typically Developing Controls (N=63)	68	25 to 168	Female=15 Male=48
Developmental Delay (N=21)	37.5	30 to 57	Female=4 Male=17
Siblings of ASD Subjects (N=25)	61	21 to 160	Female=12 Male=13

Table 2

Demographics of study subjects used for immunohistochemistry.

Subject Population	Median Age (mos.)	Age Range (mos.)	Male	Female
ASD (N=34)	65.5	31–164	28	6
Typically Developing Controls (N=23)	43	30–108	14	9
Developmental Delay (N=11)	48	25–65	9	2
Siblings of ASD Subjects (N=14)	77.5	30–120	9	5

Table 3

Western blot results reactivity to the human cerebellum extract.

Study Population	~52 kDa Band Positive N (%)
ASD N=63	13 (21) ^{a, b}
Typically Developing Controls N=63	1 (2)
Developmental Delay Controls N=21	1 (5)
Siblings of ASD Subjects N=25	0 (0)

^a ASD vs. Typically Developing Controls; $p = 0.0010$

^b ASD vs. Sibling Controls; $p = 0.0162$

Immunohistochemical staining of cynomolgus monkey cerebellum with plasma from children with ASD and controls.

Table 4

Subject Population	Golgi Cell Reactivity						
	Staining intensity				No staining		
	++ (N)	(%)	+	(N)	%	± (N)	%
ASD ^a (N=34)	7	21%	3		9%	12	35%
Typically Developing Controls (N=23)	0	0%	2		9%	1	4%
Developmental Delay (N=11)	0	0%	0		0%	4	36%
Siblings of ASD Subjects (N=14)	1	7%	0		0%	1	7%
						12	86%

^a ASD vs. TD; *p* = 0.0003
ASD vs. DD; *p* = 0.0349
ASD vs. Sibs; *p* = 0.0040

Table 5

The association between reactivity of autoantibodies to an ~52 kDa cerebellar protein by immunoblot analysis and positive Golgi cell reactivity in the cerebellum.

Presence of ~52 kDa band	Golgi Cell Reactivity			
	Staining intensity			No staining
Study population	++ (N)	+ (N)	± (N)	– (N)
ASD (N=19)				
~52 kDa band (+)	6 [*]	1	1	0
~52 kDa band (–)	0	1	7	3
Typically developing controls (N=15)				
~52 kDa band (+)	0	0	1	0
~52 kDa band (–)	0	0	0	14
Siblings of ASD Subjects (N=8)				
~52 kDa band (+)	0	0	0	0
~52 kDa band (–)	0	0	0	8
Developmental Delay (N=4)				
~52 kDa band (+)	0	0	0	1
~52 kDa band (–)	0	0	1	2

* $r = 0.76$; $p = 0.0001$ within the ASD study population.