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Autoantibodies to Select Brain Regions in Autism

Wyatt Harlan Rivas
Utah State University

AUTOANTIBODIES TO SELECT BRAIN REGIONS IN AUTISM

By

Wyatt Harlan Rivas

Thesis submitted in partial fulfillment
of the requirements for the degree

of

UNIVERSITY HONORS
WITH DEPARTMENT HONORS

in

Biology

Approved:

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Thesis/Project Advisor	Department Honors Advisor

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Director of Honors Program

UTAH STATE UNIVERSITY
Logan, Utah

2003

AUTOANTIBODIES TO SELECT BRAIN REGIONS IN AUTISM

Wyatt H. Rivas

Introductory Statement:

My honor's project involved research, determining specific brain regions affected by autoantibodies in autism, under Dr. Vijendra K. Singh in the Department of Biology over a one-year period. During that time, I've been exposed to a wide variety of bench research methods and protocols. As part of the undergraduate research training, I also received training in laboratory safety and blood-borne pathogen handling and was certified by the Environmental Health and Safety Center on campus. The whole experience has been a wide eye opener. I've learned a tremendous amount, namely skills and knowledge that I'll carry with me throughout my career, under the tutelage of Dr. Singh. I presented our research on April 17th at the annual 2003 student showcase (See miniature version of the poster on next page). The following is a description of our research.

AUTOANTIBODIES TO SELECT BRAIN REGIONS IN AUTISM

Wyatt H. Rivas and Vijendra K. Singh

Department of Biology, Biotechnology & Genomics Research Center
Utah State University,Logan, Utah

ABSTRACT

Autism, a neurodevelopmental disorder, may involve abnormal immune reaction such as autoimmunity to the brain. Autoimmunity is generally characterized by the presence of organ-specific autoantibodies, for example brain-specific autoantibodies in autism. Thus we conducted a study of autoantibodies against three brain regions, namely the caudate-putamen nucleus (CP), cerebral cortex (CC), and cerebellum (CE). These brain regions were dissected out from the brain of a Sprague-Dawley rat and homogenized for protein separation by SDS-PAGE. Autoantibodies were detected by immunoblotting technique in the serum of autistic children (n=42) and normal children (n=11). We found that many autistic children had autoantibodies to neural proteins: 46% of sera were positive for CP, 20% of sera were positive for CC, and 2% of sera were positive for CE. Normal children did not harbor these autoantibodies. Because of the highest number of autistic sera positive for autoantibodies to CP, we suggest that the caudate-putamen nucleus might be affected in the brain of autistic children. Subsequently, we made a supernatant (CPS) of the CP homogenate and repeated immunoblotting assays for autoantibodies. We detected autoantibodies to three proteins of the CPS with molecular weights of approximately 160 kD, 115 kD and 49 kD. The nature of these proteins is currently under investigation.

INTRODUCTION

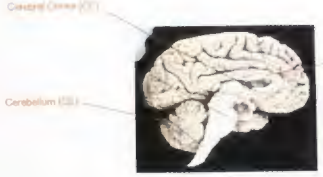
Autism is an early-onset disorder of the developing central nervous system (CNS). The disorder causes severe deficits of higher mental functions such as social interaction, language, communication, imagination and cognition. Today, autism is the fastest-growing developmental disability, according to the Autism Society of America (December 2002). Autism affects more than one-half of a million Americans and many more worldwide, but very little is known about the etiology and pathogenesis of the disorder.

Current theories include genetic factors, immune factors, environmental factors and yet other unidentified factors. We focused on immune factors such as autoimmunity in autism. Indeed, the evidence is rapidly accumulating to support an "autoimmune hypothesis" in autism, which was recently described in a review article [3]. Autoimmunity in autistic children is indicated by the presence of brain-specific autoantibodies, impaired lymphocyte functions, abnormal cytokine regulation, viral associations and indirect association of certain immunogenetic factors [1-3].

In the present study, our goal was to determine if autistic children harbor brain-specific autoantibodies particularly with regards to different brain regions. Based on the known neurobiology of autism, we selected three brain regions: Cerebellum (CE), Cerebral Cortex (CC), and Caudate-putamen (CP) nucleus. Laboratory studies described here provide initial evidence for an autoimmune reaction to caudate nucleus in children with the disorder.

MATERIALS AND METHODS

1. Subjects. The study included a total of 53 children: (i) 42 Autistic children, and (ii) 11 Normal children. Of the 42 autistic children, 5 had a diagnosis of pervasive developmental disorder (PDD) and 37 had a diagnosis of autism. The clinical diagnoses of autism was made essentially according to the standard DSM-IV criteria of the American Association of Psychiatrists, Washington, DC. The Institutional Review Board (IRB) of Utah State University reviewed and approved our research protocol that involved the use of human blood samples.
2. Brain Autoantibody Detection. This was performed essentially according to our published methods of immunoblotting technique [1-3]. A Sprague-Dawley rat was sacrificed to remove the brain. Then the brain was dissected to collect caudate-putamen (CP), cerebral cortex (CC) and cerebellum (CE). Their location in the human brain is diagrammatically shown in the Figure below. Each brain region was separately homogenized in Tween-20 containing Tris-Buffered Saline (TBST) and stored frozen. Later on in the study, the CP homogenate was centrifuged in a microcentrifuge to make CP supernatant (CPS). Proteins were separated by SDS-PAGE in 12% polyacrylamide Ready Gels (Bio-Rad Labs). Then they were transferred to nitrocellulose membranes (NCM) by the double-sandwich technique. The NCMs, after blocking with BSA-buffer, were used for antibody detection by our standard procedure of immunoblotting [1-3]. All sera were screened at 1:25 dilution. The secondary antibody was goat-antihuman polyvalent immunoglobulins-alkaline phosphatase-conjugate. A reaction was scored positive if a purplish-blue band was observed on the immunoblot.



RESULTS AND OBSERVATIONS

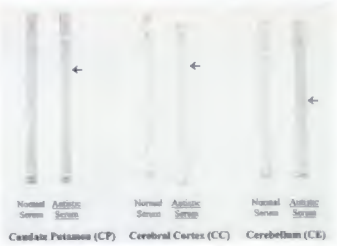


Figure 1. Typical immunoblots of CP, CC, and CE autoantibodies. Note that the black arrows are pointing to positive bands due to antibodies in autistic children but not in normal children.

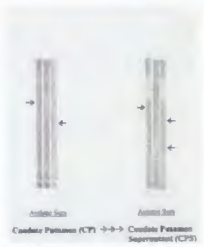
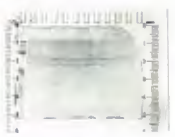
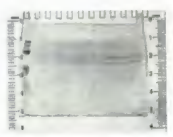


Figure 2. Representative CP&CPS immunoblots reacted with autistic sera. Note that black arrows point to positive reactions. These bands corresponded to proteins of 160 kD, 115 kD and 49 kD molecular weights (see below Figure 4 & 5).



CP gel showing protein pattern



CPS gel showing protein pattern

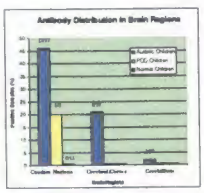
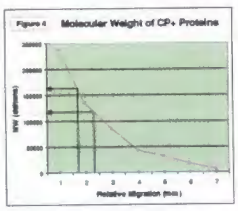
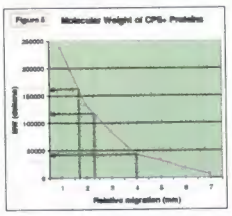


Figure 3. Distribution of brain autoantibodies in three different brain regions. Note that the highest number of autistic sera were positive for CP proteins.





Figures 4 & 5. Molecular weight determination of CP and CPS Proteins immunoreactive for autoantibodies. Note that autoantibodies reacted to two major proteins (160 kD and 115 kD) in both CP and CPS. (See 4 other proteins (49 kD) in CPS only. The reference standards were tuberculosis protein standards purchased from Bio-Rad Labs.

KEY FINDINGS

1. We found that approximately 46% of autistic children but none of the normal children harbored brain-specific autoantibodies, in particular autoantibodies to the caudate nucleus (CP) of the brain (Figure 1 & 2).
2. Approximately 20% of autistic children also had antibodies to cerebral cortex proteins.
3. Only one out of 37 autistic children (a very low number of positive sera) had antibodies to cerebellum (Figure 1 & 2).
4. In the caudate nucleus (CP), the immunoreactive proteins had approximate molecular weights of 160 kD, 115 kD and 49 kD (Figure 3 – 5).

CONCLUSION

We detected autoantibodies to caudate nucleus of the brain in autistic children but not in normal children. This is the first study of this kind ever. This immunological evidence may suggest the involvement of caudate nucleus in the neuropathology of autism.

REFERENCES

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2. Singh VK., et al. Abnormal measles-mumps-rubella antibodies and CNS autoimmunity in children with autism. *Journal of Biomedical Science* 9: 359-364 (2002).
3. Singh VK. Cytokine regulation in autism. In *Cytokines and Mental Health* (Z. Kronfol, ed.), Kluwer Academic Publishers, Boston, MA, pp. 369-383 (2003).

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Autism Research Institute, San Diego, California

Poster presented at the
2003 Utah State University Student Showcase
Sponsored by the Office of the Provost and the Vice President for Research
April 17, 2003; Logan, Utah, USA

ABSTRACT:

Autism, a neurodevelopmental disorder, may involve abnormal immune reaction such as autoimmunity to the brain. Autoimmunity is generally characterized by the presence of organ-specific autoantibodies, for example the brain-specific autoantibodies in autism. Thus, we conducted a study of autoantibodies against three brain regions, including the caudate-putamen nucleus (CP), cerebral cortex (CC), and cerebellum (CE). These brain regions were dissected out from the brain of a Sprague-Dawley rat and homogenized for protein separation by SDS-PAGE. Autoantibodies were detected by immunoblotting technique in the serum of autistic children (n=42) and normal children (n=11). We found that many autistic children had autoantibodies to neural proteins: 46% of sera were positive for CP, 20% of sera were positive for CC, and 2% of sera were positive for CE. Normal children did not harbor these autoantibodies. Because of the highest number of autistic sera positive for autoantibodies to CP, we suggest that the caudate-putamen nucleus might be affected in the brain of autistic children. Subsequently, we made a supernatant (CPS) of the CP homogenate and repeated immunoblotting assays for autoantibodies. We detected autoantibodies to three proteins of the CPS with molecular weights of approximately 160 kD, 115 kD and 49 kD. The nature of these proteins is currently under investigation.

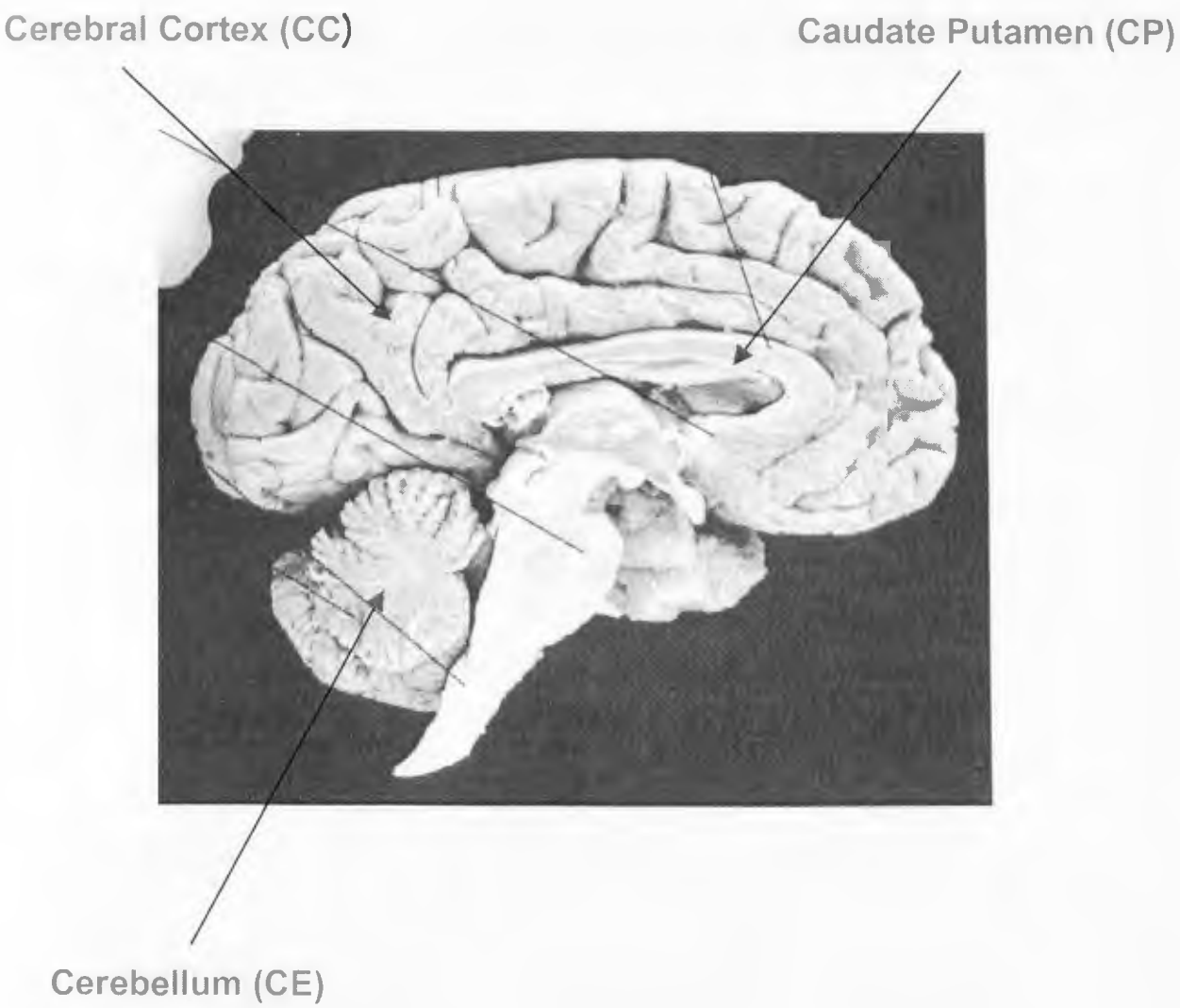
INTRODUCTION:

Autism is an early-onset disorder of the developing central nervous system (CNS). The disorder causes severe deficits of higher mental functions such as social interaction, language, communication, imagination and cognition. Today, autism is the fastest-growing developmental disability [1]. Autism affects more than one-half of a million Americans and millions more worldwide but very little is known about the etiology and pathogenesis of the disorder.

Current theories include genetic factors, immune factors, environmental factors and yet other unidentified factors [2,3]. We focused on immune factors such as autoimmunity in autism [4,5]. Indeed, the evidence is rapidly accumulating to support an "autoimmune hypothesis" in autism, as recently described in a review article [6]. Autoimmunity in autistic children is shown by several autoimmune factors: brain-specific autoantibodies [2,3,7], impaired lymphocyte functions [8-11], abnormal cytokine regulation [6], viral associations [12] and indirect association of certain immunogenetic factors [13].

In the present study, our goal was to determine if autistic children harbor brain-specific autoantibodies particularly with regards to different brain regions. This study is an extension of a pilot study previously conducted in Dr. Singh's laboratory [14]. Based on the known neurobiology of autism, we selected three brain regions: Cerebellum (CE), Cerebral Cortex (CC), and Caudate-Putamen (CP) nucleus (See Figure 1). Laboratory studies described here provide initial evidence for an autoimmune reaction to caudate nucleus in children with autistic disorder.

Figure 1: Showing Three Brain Regions Selected in the Study



MATERIALS AND METHODS:

1. Subjects: The study included a total of 53 children: (i) 42 Autistic children; and (ii) 11 Normal children. Of the 42 autistic children, 5 had a diagnosis of pervasive developmental disorder (PDD) and 37 had a diagnosis of autism. The clinical diagnosis of autism was made essentially according to the standard DSM-IV criteria of the American Association of Psychiatrists, Washington, DC. The Institutional Review Board (IRB) of Utah State University reviewed and approved our research protocol that involved the use of human blood samples.

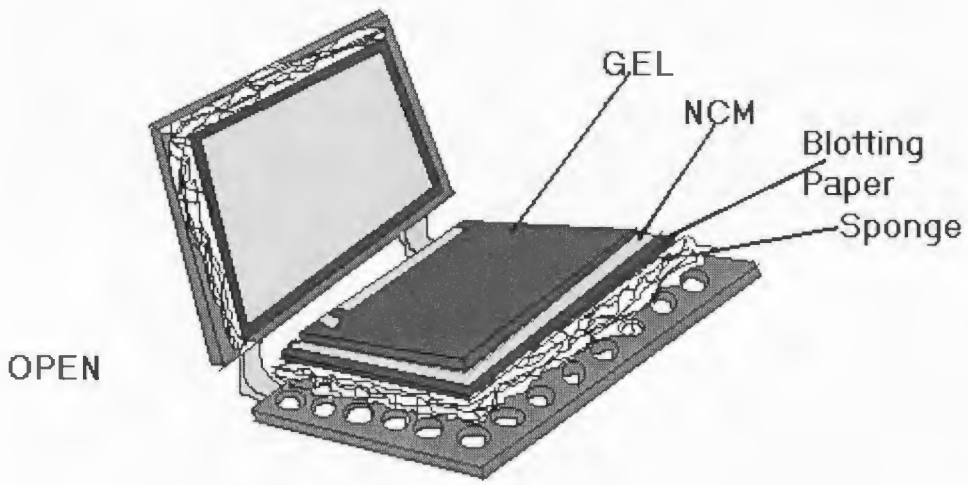
2. Brain Autoantibody Detection: This was performed essentially according to our published methods of immunoblotting technique [4,5,12]. A Sprague-Dawley rat was sacrificed to remove brain. Then the brain was dissected to collect caudate-putamen (CP), cerebral cortex (CC) and cerebellum (CE). Their location in the human brain is diagramatically shown in Figure 1. Each brain region was separately homogenized in Tween-20 containing Tris-Buffered Saline (TBST) and stored frozen. Later on in the study, the CP homogenate was centrifuged in a microcentrifuge to make CP supernatant (CPS).

3. Protein Electrophoresis & Transfer: The separate brain regions were each combined with a 4X sample buffer in a microcentrifuge tube and boiled for five minutes and then allowed to cool. Using a micropipette, 100 microliter aliquots were placed in a long horizontal well along the top of the gels. Standards were also placed in side wells. The electrophoresis apparatus was turned on and the proteins migrated down the gel with the help from a 150 mV current for about an

hour. The individual proteins were separated out by molecular weight, larger proteins move slower, during this step. The proteins were then transferred onto nitrocellulose membranes (NCMs). To do this, each gel was sandwiched on either side by a sponge, blotting paper, and NCM (See "Sandwich" diagram). After securing the sandwich they were placed in a transfer buffer and left for 20-24 hours. The NCM's were dried and then blocked for a minimum of 2 hours by soaking in 100 ml of TBST buffer (Tris-Buffered Saline Tween) containing 1% (w/v) bovine serum albumin (BSA).

4. Immunoassay: The NCMs, after blocking with BSA, were used for antibody detection by our standard immunoblotting procedure. The NCMs were cut into narrow blots (3-4 mm wide) and placed in a 1:25 pre-dilution serum / TBS. After incubation for an hour the blots were washed three times with TBST. Then they were incubated in the presence of the secondary antibody that was alkaline phosphatase-conjugated-goat-antihuman immunoglobulins (pre-diluted at 1:2,500). This is a polyvalent antibody that contains IgG, IgM and IgA. Again this was allowed to incubate for an hour and again washed three times with TBST. The blots were incubated in the color reagent (AP substrate reagent purchased from Bio-Rad Labs.) and the color was allowed to develop. A reaction was scored positive if a purplish-blue band appeared (See Figure 2). During the experiment, all serum samples were treated the same. To prevent potential bias, all samples were analyzed in a double-blind fashion and the code was broken after the completion of the laboratory analysis.

Sandwich Diagram



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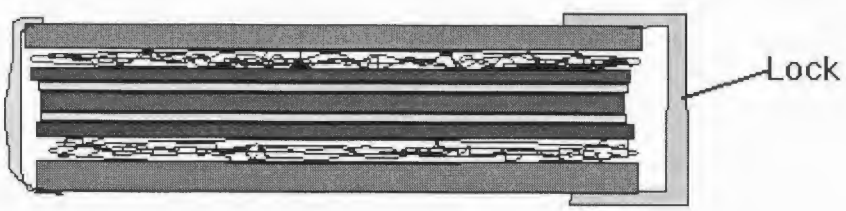

RESULTS and DISCUSSION:

At first, the brain proteins of CP, CC and CE were separated from different regions and transferred to nitrocellulose membranes. This was followed by immunoassays in the presence of patient or control serum, which served as the source of autoantibody. The representative positive reactions are shown in Figure 2. Autistic serum showed a positive reaction but normal serum did not. The autoantibody screen revealed that the highest number of autoantibody-positive autistic sera was with the CP region (46%) as compared to CC region (20%) or CE region (2%) (Figure 3). This finding suggests that the neurons in the caudate nucleus (CP) might be a target of autoimmune pathology in autism but more research is necessary to firmly establish this observation.

Because CP region was the most immunoreactive region, an attempt was made to extract proteins from this brain region followed by immunoblotting. First, the protein patterns of CP homogenate and CP supernatant (CPS) were resolved. The protein patterns of CP (Figure 4a) and CPS (Figure 4b) resembled quite closely. Then, the immunoassay with autistic sera showed that they were positive for two major proteins in CP (Figure 5); they had molecular weights of approximately 160,000 and 115,000 (Figure 6a). These two proteins also showed positive reaction in CPS but an additional protein band was positive at a molecular weight of approximately 49,000 (Figure 5 & 6b). Considering the fact that CP and CPS contain a large number of proteins, it is interesting to find only two or three proteins showing autoantibody positive reactions in autistic children.

This must imply a significant level of specificity with respect to the proteins of the CP region. Once they have been fully characterized, these proteins could potentially serve as markers of neuropathology in autism. This aspect of brain-autoimmunity and autism research is currently under investigation in Dr. Singh's laboratory. In summary, the key findings of this study are:

1. We found that approximately 46% of autistic children but none of the normal children harbored brain-specific autoantibodies, in particular autoantibodies to caudate nucleus of the brain.
2. Approximately 20% of autistic children also had antibodies to cerebral cortex. Normal children did not harbor these antibodies.
3. Only one out of 37 autistic children (a very low number of positive sera) had antibodies to cerebellum.
4. In the caudate nucleus, the immunoreactive proteins showed approximate molecular weights of 160 kD, 115 kD and 49 kD.

CONCLUSION:

We detected a significant percentage of autoantibodies to the caudate nucleus of the brain in autistic children, and none in normal children. Three unique caudate nucleus proteins were isolated and molecular weights determined. This is the first study of this kind. This new immunological evidence may suggest the involvement of caudate nucleus in the neuropathology of autism.

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FIGURE 2

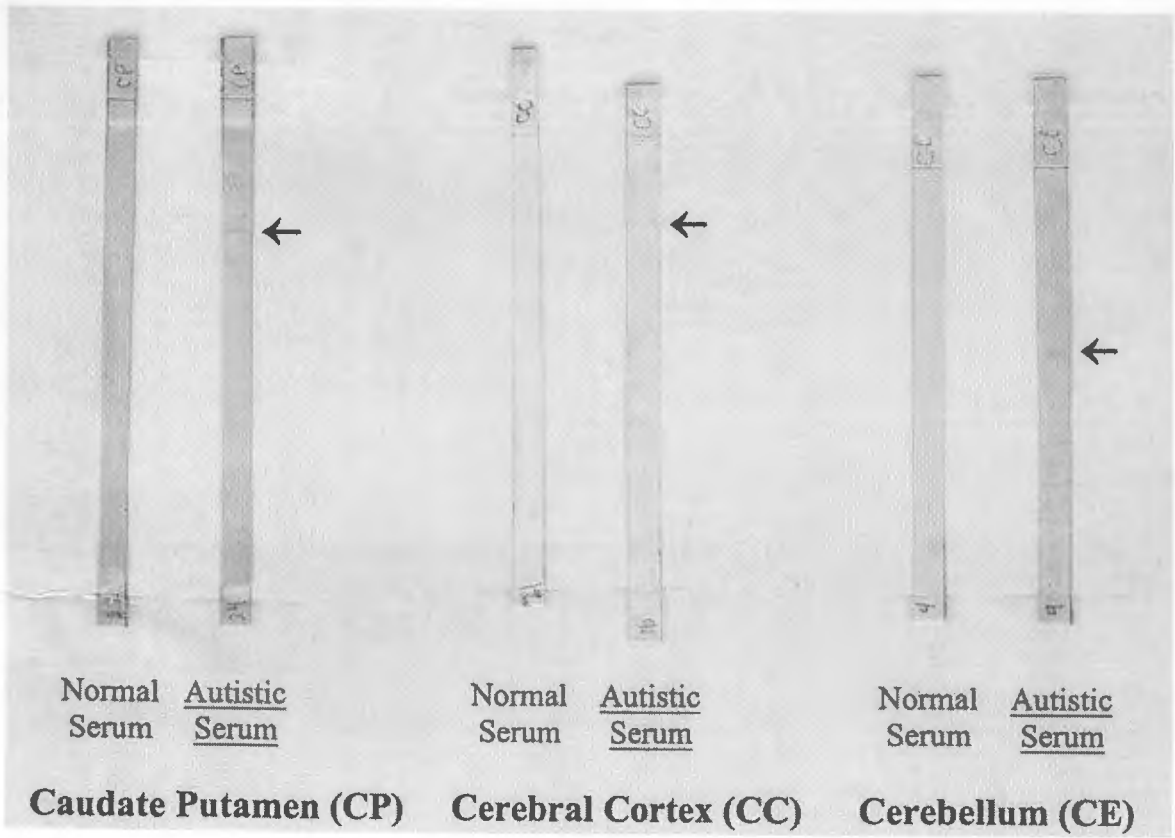


Figure 2. Typical immunoblots of CP, CC, and CE autoantibodies: Note that the black arrows are pointing to positive bands due to antibodies in autistic children but not in normal children.

FIGURE 3

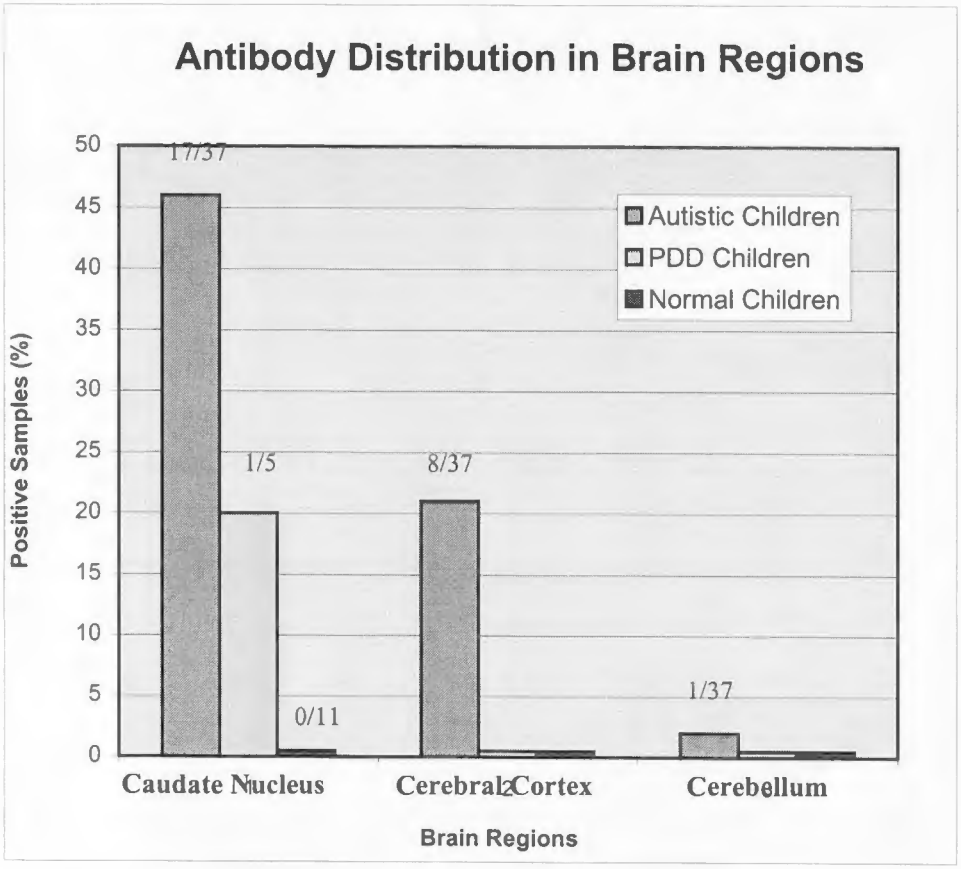


Figure 3. Distribution of brain autoantibodies in three different brain regions. Note that the highest number of autistic sera (17/37) were positive for CP proteins but not for CC or CE proteins.

FIGURE 4a & 4b

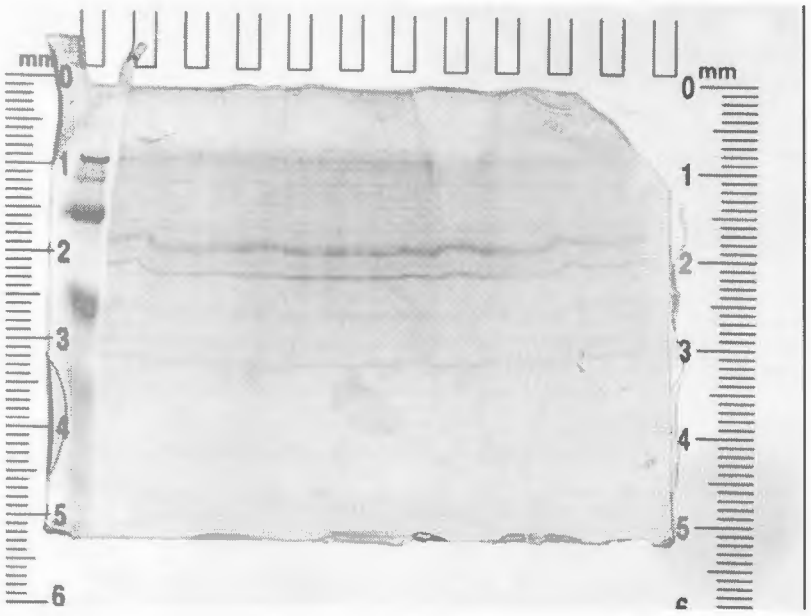


Fig. 4a. CP gel showing Protein Pattern

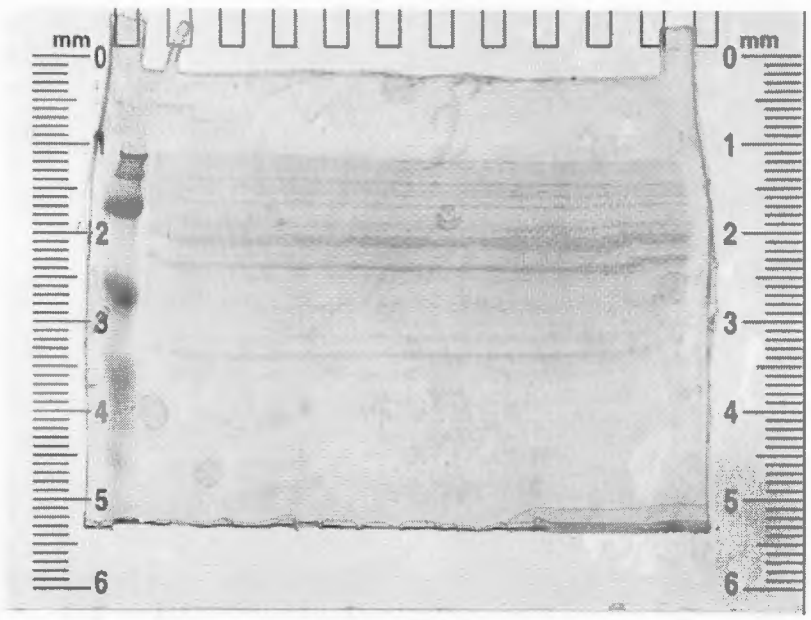


Fig. 4b. CPS gel showing Protein Pattern

FIGURE 5

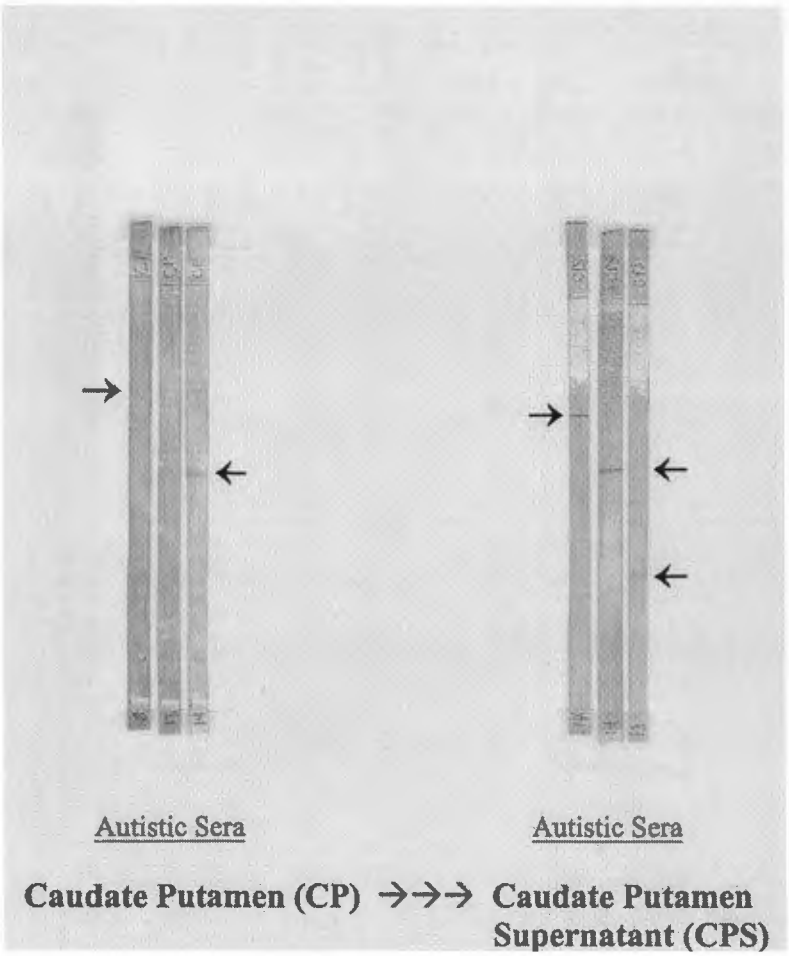
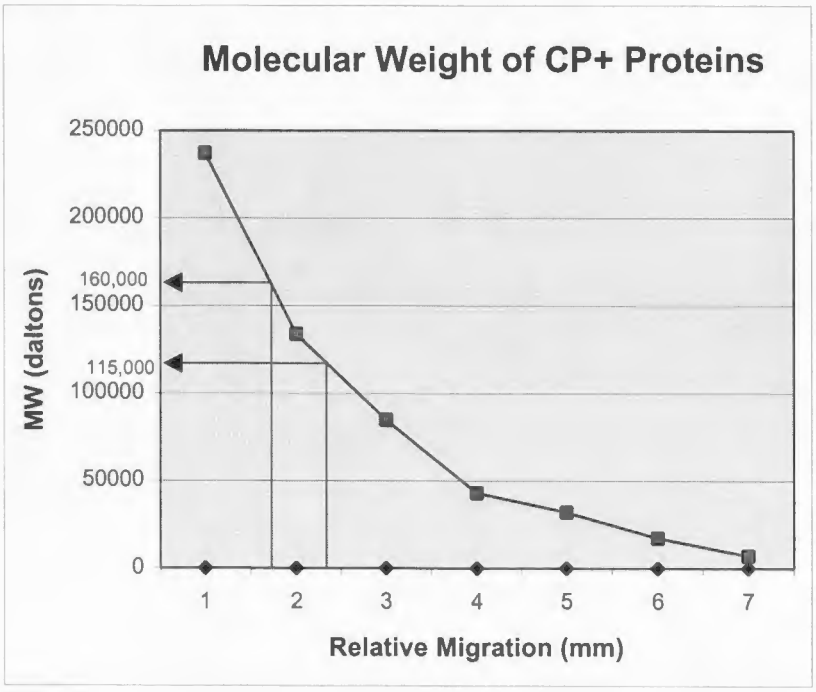


Figure 5. Representative CP& CPS immunoblots reacted with autistic sera. Note that black arrows point to positive reactions. These bands corresponded to proteins of 160 kD, 115 kD and 49 kD molecular weights as shown in Figures 6a & 6b on the next page).

FIGURE 6a & 6b



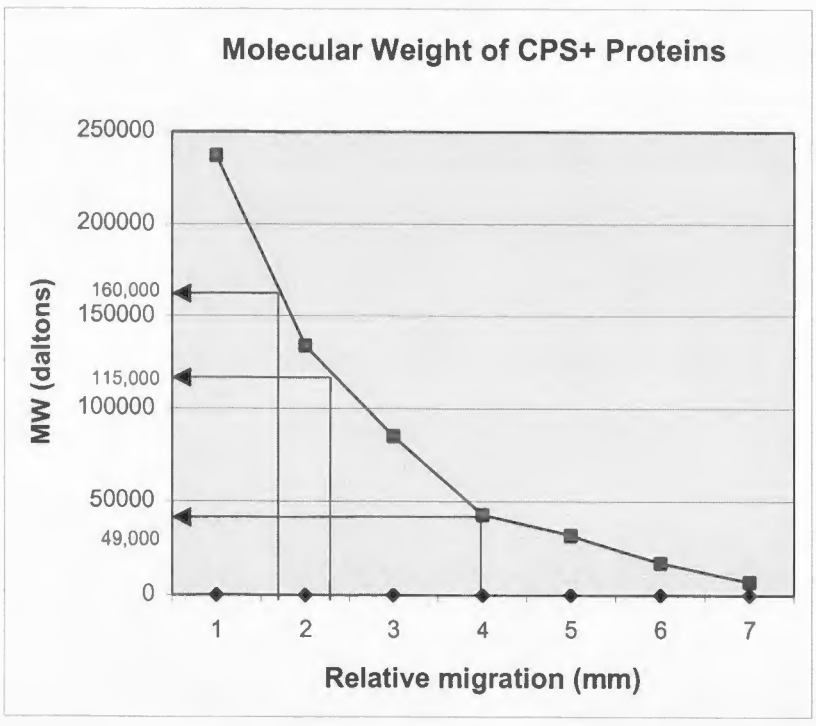


Figure 6a & 6b. Molecular weight determination of CP and CPS Proteins immuno-reactive for autoantibodies. Note that autoantibodies reacted to two major proteins (160 kD and 115 kD) in both CP (Fig.6a) and CPS (Fig. 6b), plus a third protein (49 kD) in CPS only.