

Antibody-mediated neuronal cell signaling in behavior and movement disorders

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Abstract

Behavioral and movement disorders may have antibody responses where mimicry and signal transduction may lead to neuropsychiatric abnormalities. In our study, antibodies in pediatric autoimmune neuropsychiatric disorders associated with streptococci (PANDAS) reacted with the neuronal cell surface and caudate–putamen and induced calcium–calmodulin dependent protein (CaM) kinase II activity in neuronal cells. Depletion of serum IgG abrogated CaM kinase II cell signaling and reactivity of CSF was blocked by streptococcal antigen *N*-acetyl-beta-D-glucosamine (GlcNAc). Antibodies against GlcNAc in PANDAS sera were inhibited by lysoganglioside G_{M1}. Results suggest that antibodies from an infection may signal neuronal cells in some behavioral and movement disorders.

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1. Introduction

Recently, there has been a growing interest in a group of behavior and movement disorders known as Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococci (PANDAS). PANDAS is a subset of childhood obsessive–compulsive disorder (OCD) and tic disorders where onset and exacerbation of neuropsychiatric symptoms is preceded by group A streptococcal infection (Swedo et al., 1998b). PANDAS shares common neuropsychiatric symptoms as well as the same proposed infectious etiology with the more well known group A streptococcal sequelae Sydenham's chorea (SC) (Garvey et al., 1998; Swedo, 1994).

SC is the principal neurological manifestation of acute rheumatic fever (ARF) which develops in 10–30% of cases as a result of group A streptococcal pharyngitis (Taranta and Stollerman, 1956). Although SC often occurs in combination with other manifestations of ARF, such as carditis or arthritis, isolated SC has been observed up to 6 months after pharyngitis (Stollerman, 2001; Taranta, 1959). SC is localized to the central nervous system (CNS) and is predominantly characterized by involuntary movements leading to loss of coordination and speech impairment (Marques-Dias et al., 1997). Patients exhibit an array of psychiatric and psychological abnormalities that often predate the onset of the movement disorder by 2 to 4 weeks (Marques-Dias et al., 1997; Swedo et al., 1989). As many as 70% of SC patients develop obsessive–compulsive symptoms which are indistinguishable from OCD (Swedo, 1994; Swedo et al., 1998b). Choreic episodes typically resolve within weeks of onset, however, neuropsychiatric symptoms may persist after resolution of the movement disorder (Asbahr et al., 1998, 1999).

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SC is postulated to result from a cross-reactive, anti-streptococcal antibody response directed against antigens of the basal ganglia, the area of the brain responsible for motor function, suggesting that neurological dysfunction in SC is immunologically mediated (Cunningham, 2000; Swedo et al., 1993). Sera from SC patients reacted with neurons of the basal ganglia and anti-neuronal antibodies correlated with both severity and duration of choreic episodes (Husby et al., 1976). Anti-brain antibodies were absorbed with antigens from rheumatogenic streptococcal strains, indicating that bacterial cell wall components may induce cross-reactive, neuronal antibodies (Bronze and Dale, 1993; Husby et al., 1976).

Volumetric MRI studies have demonstrated enlargement of the basal ganglia in both SC and PANDAS (Giedd et al., 1995, 1996) and immunomodulatory therapies such as plasmapheresis and intravenous immunoglobulin (IVIG) have been effective at ameliorating symptoms associated with SC and PANDAS (Garvey et al., 2005; Perlmutter et al., 1999). Antibodies to basal ganglia have been demonstrated in both SC and PANDAS raising the possibility that development of clinical manifestations in SC and PANDAS are mediated through a similar antibody-mediated mechanism of pathogenesis. However, little is known about the role

of streptococcal infection or antibodies in PANDAS (Kurlan and Kaplan, 2004).

Our previous work demonstrated that antibodies from SC recognized brain-derived lysoganglioside G_{M1} and GlcNAc, an epitope of group A streptococcal carbohydrate. SC antibodies reacted with the neuronal cell surface and induced high levels of CaM kinase II activity in human neuroblastoma cells (Kirvan et al., 2003). We tested the hypothesis that antibodies in PANDAS may be associated with a similar antibody-mediated neuronal cell signaling mechanism. The new work reported herein supports the hypothesis that the majority of PANDAS sera contain antibodies which react with lysoganglioside G_{M1} and induce CaM kinase II activation in neuronal cells. Our study is important because it demonstrates antibodies present in movement and behavior disorders which may induce antibody-mediated cell signaling mechanisms in disease.

2. Materials and methods

2.1. Patients samples

PANDAS was identified by clinical criteria of Swedo, et al., including elevated anti-streptolysin O or DNase B titers

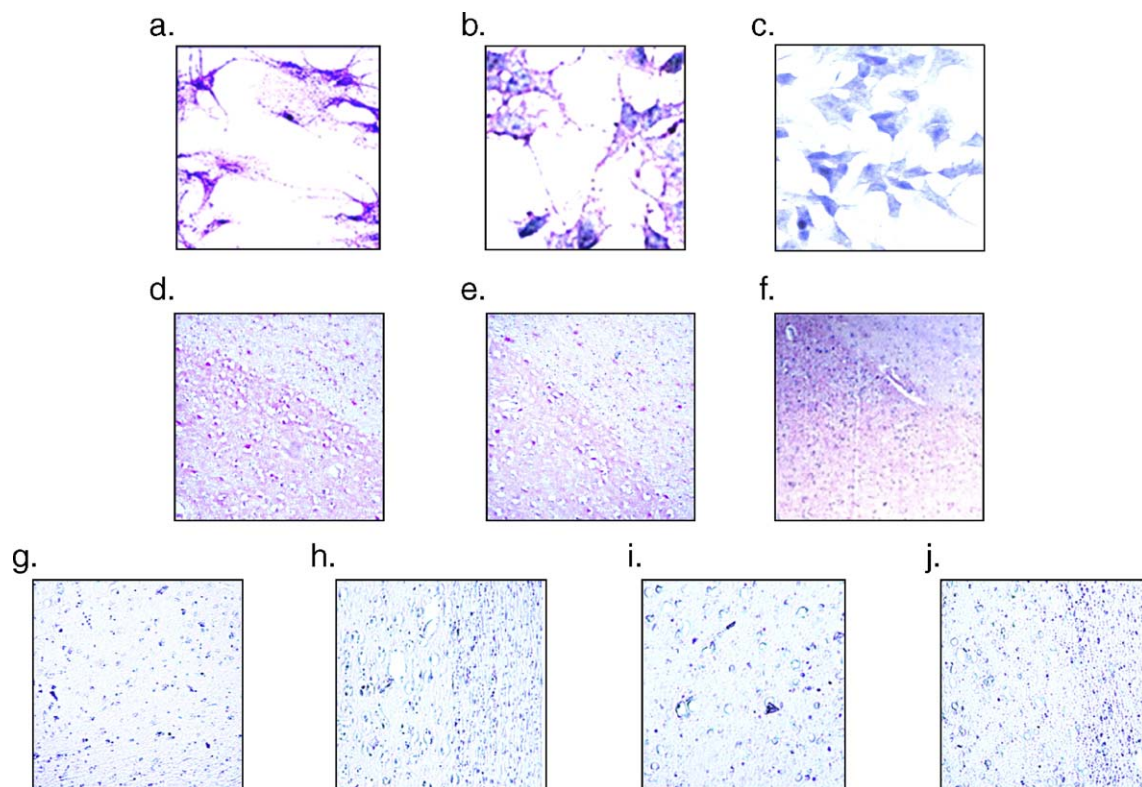


Fig. 1. Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal infection (PANDAS) antibody recognized neuronal cell surface and human caudate–putamen. (a) Acute PANDAS serum labeled human neuronal cell surface similar to acute Sydenham's chorea (SC) serum (b) Pooled normal human sera showed no reactivity (c). For cell surface immunohistochemistry, a single Sydenham's chorea serum and PANDAS serum samples, and 6 pooled age-matched controls are shown. However, all seven Sydenham's chorea and 16 PANDAS sera demonstrated neuroblastoma cell surface staining (data not shown). PANDAS (d–e) and chorea (f) cerebrospinal fluid (CSF) IgG reacted with human caudate–putamen tissue in a differential staining pattern. Four non-PANDAS control CSF (g–j) showed no binding of caudate–putamen tissue. For CSF staining of human basal ganglia tissue, one Sydenham's chorea, two PANDAS, and four non-PANDAS CSF samples were tested separately.

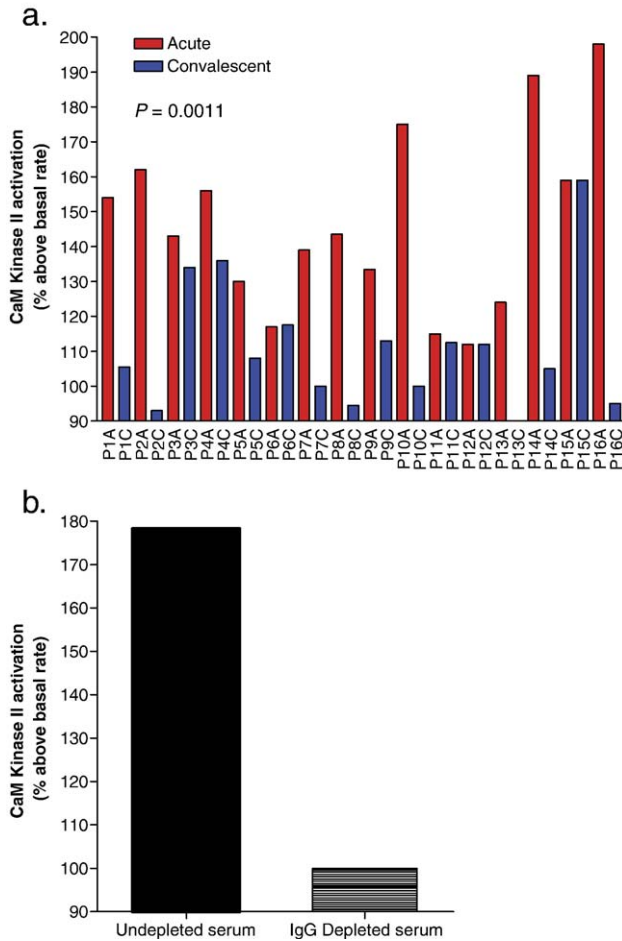


Fig. 2. Induction of calcium/calmodulin-dependent protein (CaM) kinase II in human neuroblastoma cells by acute and convalescent Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal infection (PANDAS) sera. (a) All acute PANDAS sera (P1–16A) as a group significantly ($P=0.0011$) activated CaM kinase II in comparison to all matched convalescent sera (P1–16C). (b) Depletion of IgG from PANDAS acute sera (P14A) reduced CaM kinase II activation to basal levels in comparison to undepleted serum. P values determined by Mann–Whitney two-tailed t -test.

(Swedo et al., 1998a). PANDAS subjects with clinically significant symptoms of obsessive–compulsive disorder were rated with the Children’s Yale-Brown Obsessive Compulsive Scale (CY-BOCS). Mean CY-BOCS severity score was 25.4 (± 9.3 S.D.) in the acute sample and 4.2 (± 5.5 S.D.) in the convalescent sample. PANDAS subjects with clinically significant symptoms of tics were rated with the Yale Global Tics Severity Scale (YGTSS). Mean YGTSS severity score was 20 (± 7.0 S.D.) in the acute sample and 4.0 (± 4.4 S.D.) in the convalescent sample. Non-PANDAS included OCD, tic disorders, or attention deficit/hyperactivity disorder (ADHD) patients not meeting PANDAS criteria. All subjects and their parents gave written assent or consent to participate in a study approved by the National Institute of Mental Health Institutional Review Board and conducted at the Clinical Center at the National Institutes of Health in

Bethesda, Maryland or in accordance with approved research protocols by the University of Oklahoma Institutional Review Board. Human experimentation guidelines of the U.S. Department of Health and Human Services and those of the University of Oklahoma Health Sciences Center were followed in the conduct of clinical research.

2.2. Cell lines

Human neuroblastoma line SK-N-SH (ATCC HTB-11) was routinely cultured in F12-DMEM (Gibco-BRL) media

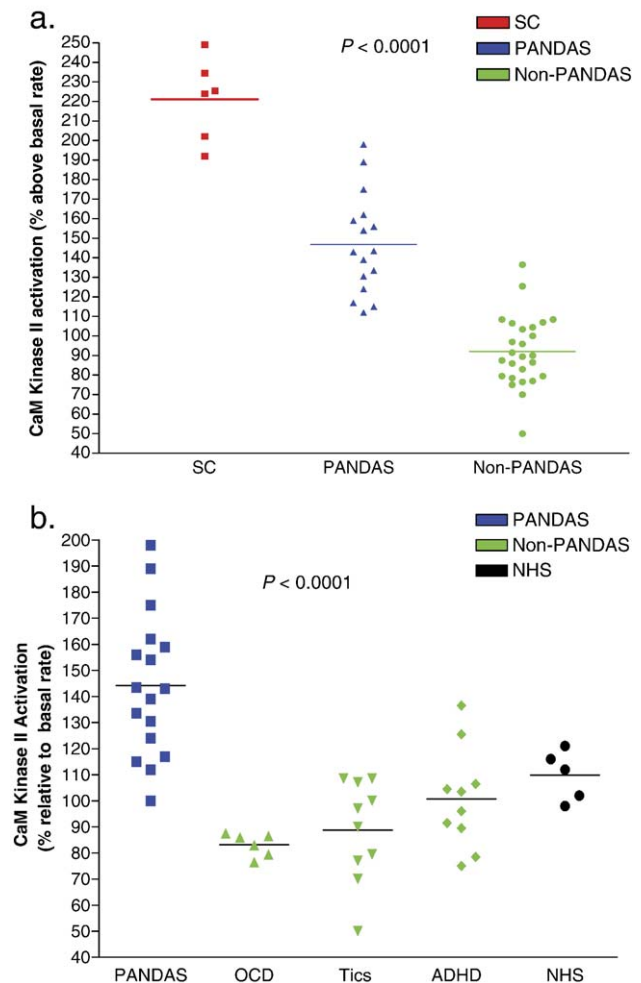


Fig. 3. Antibody-mediated calcium/calmodulin-dependent protein (CaM) kinase II activation by Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal infection (PANDAS) sera. (a) CaM kinase II activation by PANDAS acute sera was significantly ($P<0.0001$, one-way analysis of variance ANOVA) higher than seen for non-PANDAS obsessive–compulsive disorder (OCD), tic, and attention-deficit hyperactivity disorder (ADHD) sera as well as for age-matched normal human sera (NHS). Individual comparisons of groups made by Mann–Whitney two tailed t -test: SC vs PANDAS, $P=0.0006$; PANDAS vs non-PANDAS, $P<0.0001$; PANDAS vs OCD, $P=0.0004$; PANDAS vs tics, $P<0.0001$; PANDAS vs ADHD, $P=0.0005$; PANDAS vs NHS, $P<0.01$. (b) Acute Sydenham’s chorea (SC) sera produced a significant ($P<0.0001$) increase in CaM kinase II activity in comparison to other test sera.

containing 10% fetal calf serum, 1% penicillin and streptomycin, and 0.1% gentamicin at 37 °C, 5% CO₂.

2.3. Antigens

GlcNAc-BSA was prepared as previously described (Shikhman et al., 1993). Lysoganglioside G_{M1} was purchased from Sigma Chemical Co. (St. Louis, MO).

2.4. Competitive-inhibition ELISA

Competitive-inhibition ELISA was performed in triplicate as described (Galvin et al., 2000). GlcNAc conjugated to bovine serum albumin (BSA) (GlcNAc-BSA) was immobilized on the ELISA plate and lysoganglioside G_{M1} antigen was used to inhibit the serum IgG reactivity with GlcNAc. Lysoganglioside G_{M1} inhibitor solution was prepared in PBS and mixed with an equal volume of patient serum then incubated at 37 °C for 1 h and overnight at 4 °C. Fifty microliters of serum-inhibitor mixture was added to wells coated with GlcNAc-BSA (10 µg/mL). Plates were developed with alkaline phosphatase-labeled goat anti-human IgG (1:500; Sigma Chemical Co.) and *p*-nitrophenyl phosphate (Sigma 104 phosphatase substrate), prepared in diethanolamine buffer at 1 mg/mL. Optical density was measured at 405 nm using an Opsys MR microplate reader (Dynex Technologies, Chantilly, VA). Percentage of inhibition was calculated as follows: $100 \times (1 - [A_{405} \text{ inhibitor} + \text{serum} / A_{405} \text{ PBS} + \text{serum}])$. Maximal (100%) reactivity was determined by incubating serum with PBS without inhibitor.

2.5. Immunohistochemistry

Four-well chamber tissue culture slides were plated with 5×10^4 SK-N-SH cells/chamber overnight (Galvin et al., 2000; Kirvan et al., 2003). Cells were incubated with patient sera and formalin fixed. Neuronal cell surface-bound antibody was detected with biotin-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA), alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch), and Fast Red substrate (Biogenex, San Ramon, CA). Cells were counterstained with Mayer's hematoxylin (Biogenex). For caudate-putamen tissue, CSF was incubated with tissue for 1 h at room temperature and antibody binding detected as described above (Galvin et al., 2000).

2.6. Protein kinase assays

Neuronal cell lysates were prepared as previously described (Kirvan et al., 2003). Serum IgG depletion was performed using Protein G resin. Protein kinase activity was measured using CaM kinase II assay system, cAMP-dependent protein kinase assay system, and protein kinase C assay system (Promega, Madison, WI) according to manufacturer's instructions. Radioactivity retained on the membrane was determined by scintillation counting. The

specific activity of the enzyme in pmol/min/µg was determined for each sample and results presented as percentage of the basal rate.

2.7. Statistical analyses

Statistical analyses were performed for competitive inhibition of sera and signal transduction assays. *P* values were calculated by the Mann–Whitney two-tailed *t*-test for comparison of individual groups and one-way analysis of variance (ANOVA) for comparison of more than two groups.

3. Results

3.1. PANDAS IgG reacted with GlcNAc, an epitope of group A streptococcal carbohydrate and brain antigen lysoganglioside G_{M1}

To determine if PANDAS serum IgG reacted with lysoganglioside G_{M1} and GlcNAc, a competitive-inhibition

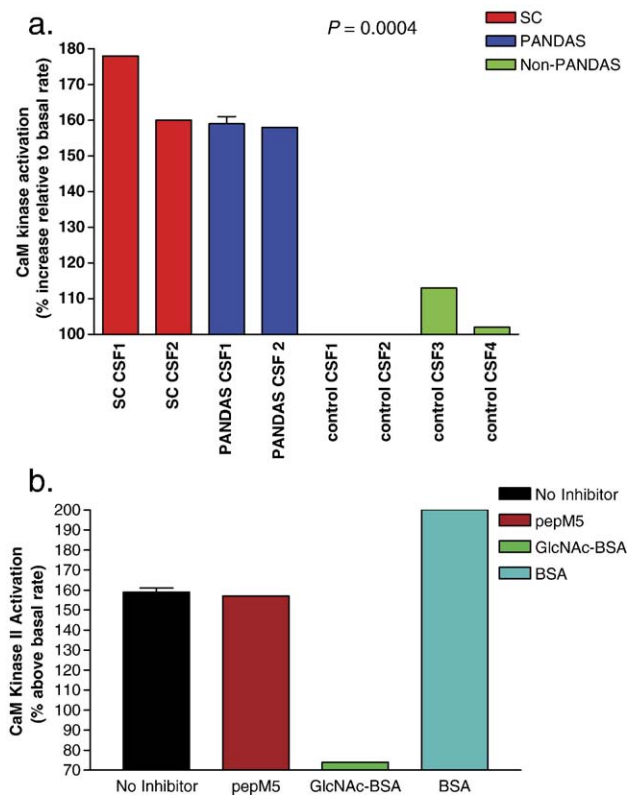


Fig. 4. Activation of calcium/calmodulin-dependent protein (CaM) kinase II by PANDAS cerebrospinal fluid (CSF). (a) Both chorea and Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal infection (PANDAS) CSF significantly ($P=0.0004$) induced CaM kinase II as compared to non-PANDAS attention deficit/hyperactivity disorder (ADHD) control CSF. *P* value determined by Mann–Whitney two-tailed *t*-test. (b) CaM kinase activity induced by PANDAS CSF was blocked by *N*-acetyl- β -D-glucosamine (GlcNAc), but not by the extracellular digestion fragment of type 5 M protein (pepM5) or bovine serum albumin (BSA). PANDAS CSF 1 and 2 were from patients P1 and P5, respectively. For CSF, CaM kinase II inhibitions (b), PANDAS CSF 1 was tested.

ELISA was utilized. Seventy-three percent (11/15) of PANDAS acute sera binding to GlcNAc-BSA was inhibited by soluble lysoganglioside G_{M1} (Table 1). In contrast, only 23% (6/26) of non-PANDAS sera were similarly inhibited. Lysoganglioside G_{M1} concentrations required to inhibit binding of PANDAS sera to GlcNAc-BSA were significantly lower ($P < 0.05$) than for non-PANDAS sera. In Table 1, the data show that lysoganglioside G_{M1} is a specific inhibitor of PANDAS IgG binding to streptococcal carbohydrate GlcNAc in vitro. The small percentage of non-PANDAS sera that were inhibited could be patients with undiagnosed overlapping syndromes. Serum anti-GlcNAc reactivity of positive control SC sera was also strongly inhibited by the lysoganglioside as expected.

Table 1
Anti-GlcNAc reactivity in PANDAS serum IgG is inhibited by lysoganglioside G_{M1} ($\mu\text{g/mL}$)

| SC | $\mu\text{g/mL}^a$ | PANDAS | $\mu\text{g/mL}$ | Non-PANDAS | $\mu\text{g/mL}$ |
|-----|--------------------|--------|------------------|------------|------------------|
| SC1 | 7.3 | P1 | 221 | T1 | 108.4 |
| SC2 | 6.9 | P2 | 177 | T2 | >500 |
| SC3 | 9.4 | P3 | >500 | T3 | 195.9 |
| SC4 | 11.4 | P5 | >500 | T4 | >500 |
| SC5 | 108.3 | P6 | 35 | T5 | >500 |
| SC6 | 20.3 | P7 | 337.1 | T6 | >500 |
| | | P8 | 500 | T7 | 105.4 |
| | | P9 | 125 | T8 | >500 |
| | | P10 | >500 | T9 | >500 |
| | | P11 | 125 | T10 | >500 |
| | | P12 | 500 | OCD1 | 40.8 |
| | | P13 | 269.3 | OCD2 | >500 |
| | | P14 | 129.4 | OCD3 | >500 |
| | | P15 | 500 | OCD4 | >500 |
| | | P16 | >500 | OCD5 | >500 |
| | | | | ADHD1 | >500 |
| | | | | ADHD2 | 374 |
| | | | | ADHD3 | >500 |
| | | | | ADHD4 | >500 |
| | | | | ADHD5 | >500 |
| | | | | ADHD6 | >500 |
| | | | | ADHD7 | 164.9 |
| | | | | ADHD8 | >500 |
| | | | | ADHD9 | >500 |
| | | | | ADHD10 | >500 |

Competitive-inhibition ELISA of serum antibody reactivity to bound *N*-acetyl- β -D-glucosamine conjugated to bovine serum albumin (GlcNAc-BSA). The concentration of lysoganglioside G_{M1} required to inhibit anti-GlcNAc antibodies in Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal infection PANDAS sera was significantly different from non-PANDAS sera ($P = 0.026$). Chorea sera binding to GlcNAc was inhibited by lysoganglioside G_{M1} to a greater degree than PANDAS ($P = 0.0007$) and non-PANDAS ($P < 0.0001$) sera. For statistical analysis, concentrations >500 $\mu\text{g/mL}$ were assumed as 501 $\mu\text{g/mL}$. P values were determined by Mann–Whitney two-tailed t -test. One-way analysis of variance (ANOVA) was also highly significant ($P < 0.0001$).

^a Concentration of lysoganglioside G_{M1} required to produce 50% inhibition of anti-GlcNAc IgG reactivity in the competitive-inhibition ELISA. Values >500 $\mu\text{g/mL}$ were considered negative for lysoganglioside G_{M1} inhibition. Positive values are in bold type.

3.2. PANDAS CSF and sera reacted with human caudate–putamen tissue and neuronal cells

PANDAS and SC acute serum IgG labeled SK-N-SH human neuronal cells (Fig. 1a,b), while no surface staining was observed for age-matched normal human sera (Fig. 1c). PANDAS CSF showed a differential IgG staining pattern of caudate–putamen tissue similar to that seen for chorea CSF (Fig. 1d–f). Non-PANDAS control CSF showed no reactivity for human caudate–putamen tissue (Fig. 1g–j). Taken together, the data indicate that PANDAS CSF and sera contain IgG specific for human basal ganglia and neuronal cells.

3.3. Sera and CSF signal transduction

Sera or CSF were added to the neuronal cell line in culture and tested for signal transduction through CaM kinase II. CaM kinase II is a multifunctional protein kinase with a broad spectrum of neuronal targets where it is thought to play a role in the CNS including behavior and neurotransmitter synthesis and release (Soderling et al., 2001). Seventy-five percent of acute PANDAS sera induced antibody-mediated activation of CaM kinase II to significantly ($P = 0.001$) higher levels than matched convalescent sera (Fig. 2a). Highest levels of CaM kinase II activation were acute sera (red bars) from patients with isolated tics (P10, P14, and P16) (Fig. 2a). To determine if antibody was responsible for CaM kinase II induction, acute PANDAS serum (P14) was depleted of IgG by protein G affinity column adsorption. IgG depleted serum did not activate CaM kinase II above the basal rate in comparison to matched undepleted serum (Fig. 2b). The data suggest that antibody is responsible for cell signaling in PANDAS serum. PANDAS sera was capable of inducing significantly ($P < 0.001$) greater CaM kinase activity than non-PANDAS sera (Fig. 3a). Chorea sera as a positive control produced significantly ($P < 0.0001$) higher levels of CaM kinase II induction than PANDAS and non-PANDAS sera as expected. No significant increase in CaM kinase II activation was found in 10 sera from non-PANDAS tics, six sera from non-PANDAS OCD, 10 sera from ADHD, or five age-matched normal control sera when compared to PANDAS (Fig. 3b).

Most importantly, PANDAS CSF showed highly elevated levels of CaM kinase II induction similar to that of positive control SC CSF (Fig. 4a). Non-PANDAS CSF showed no activation of CaM kinase II. PANDAS CSF-induced CaM kinase II activity was blocked by the streptococcal associated GlcNAc epitope, but not by group A streptococcal serotype 5 M protein, suggesting that antibodies directed against an epitope of the group A carbohydrate of *Streptococcus pyogenes* may be important in mediating cell signaling (Fig. 4b). Therefore, signaling activity found in behavior and movement disorders during the symptomatic phase of disease suggests that antibodies present in the sera and CSF were associated with and may contribute to the clinical symptoms in acute phases of disease.

4. Discussion

Our hypothesis is that antibody-mediated autoimmune mechanisms as well as infection may contribute to the pathogenesis of some movement and behavioral disorders. In SC, group A streptococcal infections induce cross-reactive antibodies that deposit in the basal ganglia and lead to CaM kinase II activation in neuronal cells (Kirvan et al., 2003). PANDAS sera were found to induce significantly higher levels of CaM kinase II activation than sera from non-PANDAS OCD, tic, and ADHD groups not associated with streptococcal infection. In fact, PANDAS patients diagnosed with isolated tics produced the highest level of CaM kinase II activity similar to chorea. Although we do not know exactly how disease producing IgG antibodies cross the blood–brain barrier, we can suggest that infection or the antibody itself may affect the blood–brain barrier. Recent work on behavior and immunity demonstrated that LPS or epinephrine affected the blood–brain barrier to allow IgG to penetrate the brain (Huerta et al., 2006).

Increased signal transduction is proposed to alter neuronal cell physiology leading to neurological dysfunction that characterizes the disorder. Reported here, antibodies from acute PANDAS sera taken in the symptomatic phase of disease activated CaM kinase II in human neuroblastoma cells. Matched convalescent sera, obtained in the absence of symptoms, did not activate CaM kinase II in comparison to acute sera. Our data support the hypothesis that antibody-induced signal transduction may be associated with clinical symptoms in subsets of movement and behavior disorders. In addition, PANDAS sera depleted of IgG did not activate CaM kinase II. Our data are consistent with plasma exchange studies, which show rapid reversal in clinical course of SC or PANDAS patients upon removal of antibody (Garvey et al., 2005; Perlmutter et al., 1999).

PANDAS CSF induced CaM kinase II activity indicating that the ability to activate CaM kinase II is present in the CNS. The level of CaM kinase II activation obtained by PANDAS CSF was comparable to cell signaling produced by SC CSF and was completely inhibited by the streptococcal associated GlcNAc epitope of the streptococcal group A carbohydrate. Although PANDAS sera induced significant levels of CaM kinase II activation, the range was lower than chorea but equivalent to values for ARF without chorea (Kirvan et al., 2003). A threshold level of CaM kinase II activity may be required to trigger choreic movement, while lower levels of CaM kinase II induction may lead to behavioral changes. CaM kinase II mediates many different learning, memory, and developmental neuronal cell pathways and has broad substrate specificity dependent on concentration, intracellular localization, and intracellular calcium levels (Bejar et al., 2002; De Koninck and Schulman, 1998; Menegon et al., 2002; Tsui et al., 2005).

CaM kinase II is a multifunctional enzyme highly concentrated in the brain with functions in neurotransmission and neuronal excitability (Greengard et al., 1993), regulation

of catecholamine release in vitro (Griffith and Schulman, 1988) and in vivo (Zhu et al., 2004), and co-localization with glutamate receptors, which are implicated in the pathology of neuropsychiatric disorders, including OCD (Chakrabarty et al., 2005). In our study, PANDAS patient sera induced CaM kinase II activity in neuronal cells compared to higher levels induced by chorea sera. Lower levels of CaM kinase II activity may contribute solely to neuropsychiatric symptoms. Support for this hypothesis comes from reports of the persistence of neuropsychiatric symptoms after the abatement of adventitious movements in chorea (Asbahr et al., 1998, 1999) and ARF patients without chorea have been shown to be at higher risk for the development of obsessive–compulsive symptoms and tics than control populations (Mercadante et al., 2000). Low level CaM kinase II activation may represent a risk factor for development of neurological disease as one of seven sera tested from uncomplicated pharyngitis was positive for CaM kinase II activation at 47% above the basal rate while the other six sera were negative (Kirvan et al., 2003). How these antibodies might cross the blood–brain barrier is not yet certain.

In PANDAS, GlcNAc binding by antibodies was blocked by lysoganglioside G_{M1} . However, PANDAS sera with the greatest GlcNAc inhibition did not necessarily show the highest induction of CaM kinase II activity or lysoganglioside inhibition. Lysoganglioside G_{M1} has been described as a putative autoantigen in chorea (Kirvan et al., 2003). Although signaling antibodies in the movement and behavioral disorders reacted with GlcNAc and lysoganglioside G_{M1} in vitro, the identity of cross-reactive antigens in vivo requires further investigation. Previously we have identified GlcNAc and lysoganglioside G_{M1} as cross-reactive antigens in Sydenham's chorea (Kirvan et al., 2003). The data in Table 1 specifically shows IgG cross-reactivity between GlcNAc and lysoganglioside G_{M1} . While the reactivities of non-inhibited IgG are unknown, the importance of Table 1 is the comparison of patient sera that shows cross-reactivity between GlcNAc and lysoganglioside G_{M1} . All SC patient sera and 73% of PANDAS sera possessed GlcNAc-specific IgG that were cross-reactive with lysoganglioside G_{M1} , in comparison to only 23% of non-PANDAS sera. Non-inhibited antibodies are presumed to be GlcNAc-specific antibodies that have no cross-reactivity to lysoganglioside G_{M1} . We believe that SC and a majority of PANDAS patients possess subsets of GlcNAc-specific IgG that cross-reacts with lysoganglioside that are likely to be involved in the pathogenesis of these disorders which are not thought to be characteristic of other streptococcal infections.

In summary, our study has revealed antibody-mediated cell signaling in movement and behavioral disorders as a potential mechanism which may produce neurological effects. Our data provides insights into to how antibodies against the streptococcal associated epitope GlcNAc may disrupt neuronal cell function and lead to disease. Finally, antibody-mediated cell signaling may be a novel mechanism

important in neuropsychiatric disorders that affect children worldwide.

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