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## Differential binding of antibodies in PANDAS patients to cholinergic interneurons in the striatum

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### Abstract

Pediatric Autoimmune Neuropsychiatric Disorder Associated with Streptococcus, or PANDAS, is a syndrome of acute childhood onset of obsessive-compulsive disorder and other neuropsychiatric symptoms in the aftermath of an infection with Group A beta-hemolytic *Streptococcus* (GABHS). Its pathophysiology remains unclear. PANDAS has been proposed to result from cross-reactivity of antibodies raised against GABHS with brain antigens, but the targets of these antibodies are unclear and may be heterogeneous. We developed an *in vivo* assay in mice to characterize the cellular targets of antibodies in serum from individuals with PANDAS. We focus on striatal interneurons, which have been implicated in the pathogenesis of tic disorders. Sera from children with well-characterized PANDAS (n = 5) from a previously described clinical trial (NCT01281969), and matched controls, were infused into the striatum of mice; antibody binding to interneurons was characterized using immunofluorescence and confocal microscopy. Antibodies from children with PANDAS bound to ~80% of cholinergic interneurons, significantly higher than the <50% binding seen with matched healthy controls. There was no elevated binding to two different populations of GABAergic interneurons (PV and nNOS-positive), confirming the specificity of this phenomenon. Elevated binding to cholinergic interneurons resolved in parallel with symptom improvement after treatment with intravenous immunoglobulin. Antibody-mediated dysregulation of striatal cholinergic interneurons may be a locus of pathology in PANDAS. Future clarification of the functional consequences of this specific binding may identify new opportunities for intervention in children with this condition.

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## INTRODUCTION

Obsessive-compulsive disorder (OCD) and tic disorders often first appear in childhood [1–3]. In a minority of pediatric OCD cases, onset is unusually abrupt and is accompanied by a range of comparably severe associated neuropsychiatric symptoms. This syndrome has been named Pediatric Acute-onset Neuropsychiatric Syndrome (PANS) [4, 5]. In some instances, this abrupt onset is seen with or after resolution of an infectious illness, suggesting an immune-mediated pathogenesis [6]. Temporal association with infection by group A beta-hemolytic *Streptococcus* infection (GABHS, or *Streptococcus pyogenes*) has been noted with particular frequency; this association has been termed Pediatric Autoimmune Neuropsychiatric Disorder Associated with Streptococcus, or PANDAS [7–9].

By analogy with the pathophysiology of Sydenham’s chorea, a neuropsychiatric disorder that also occurs following GABHS infection, it was proposed that infection in susceptible children triggers an autoimmune reaction through molecular mimicry, a process in which host antibodies directed against *Streptococcus pyogenes* cross-react with human proteins [5, 8, 10–12]. In Sydenham’s chorea (SC), for example, antibodies from patients have been found to cross-react both against neuronal lysoganglioside and streptococcal N-acetyl-beta-D-glucosamine [12]. Other antibody targets have been described in SC, including the dopamine D2 receptor [13, 14]. Numerous studies have sought to better characterize the PANDAS clinical subgroup, clarify the associated pathophysiology, and identify the brain targets of the autoantibodies [8]. Studies in animals have confirmed the ability of anti-Streptococcal antibodies to produce neural and behavioral abnormalities, further justifying the pursuit of antibody targets that may explain pathogenesis [15–19]. Despite this progress, the PANDAS diagnosis remains somewhat controversial, and its pathophysiology remains to be clearly elucidated [9].

Based on the hypothesized autoimmune etiology, a variety of immunomodulatory therapies have been investigated in children with PANDAS [8]. An early controlled study indicated efficacy of both plasmapheresis and intravenous immunoglobulin (IVIG), compared to placebo [20]. Subsequent clinical experience has continued to suggest benefit from these approaches in some cases [21]. A recent two-site study, performed at Yale and the National Institute of Mental Health, identified children with PANDAS by particularly stringent criteria and treated them with IVIG or placebo (NCT01281969). While IVIG did not separate from placebo during the blinded phase, response rates were robust after the administration of open-label IVIG, which all participants were offered, if their symptoms remained severe after completion of the double-blind phase [22].

Functional and structural abnormalities of the cortico-basal ganglia circuitry have been described in both OCD and tic disorders and are central to most current thinking about their pathophysiology [23–26]. Pathological abnormalities in the striatum have been also reported in PANDAS. Giedd and colleagues [27, 28] found enlarged striatal volume in patients with PANDAS, similar to that seen in those with acute Sydenham’s chorea. Striatal abnormalities have been reported to resolve in conjunction with symptoms, either after plasmapheresis [28] or spontaneously [29]. More recently, inflammation of the striatum has been reported in

PANDAS and Tourette syndrome patients, as measured by positron emission tomography using a marker of microglial activation [30].

The hypothesis that PANDAS derives from molecular mimicry implies the presence of antibodies in PANDAS patients that cross-react with brain antigens, as has been documented in Sydenham's chorea [12]. Indeed, such reactivity has been documented *ex vivo* [31]. Reactivity of antibodies from individuals with PANDAS against several neuronal proteins, including tubulin, lysoganglioside, and dopamine receptors, has been reported, as has antibody-mediated activation of CaM kinase II; these findings have not been consistent across all studies, perhaps due to etiological heterogeneity [11, 32–35].

The current investigation draws upon recent evidence implicating striatal interneuronal abnormalities in the pathophysiology of tic disorders. *Post mortem* analyses have identified reduced density of specific populations of striatal interneurons in patients with Tourette syndrome; these include cholinergic interneurons (CINs) and GABAergic interneurons expressing the markers parvalbumin and nNOS [36–38]. Our laboratory has shown that recapitulation of these post-mortem findings in mice, using experimental depletion of cholinergic or parvalbumin-expressing interneurons in the dorsal striatum, produces tic-like phenomenology [39–41]. This suggests that striatal interneuronal dysfunction may play a causal role in tic pathophysiology. The contribution of interneuronal pathology to OCD is less clear.

To date, no studies have investigated reactivity of antibodies from individuals with PANDAS with epitopes present on interneurons. We investigated interneuron targets of PANDAS antibody reactivity using an *in vivo* model. We infused serum from children with PANDAS into the striatum of mice and characterized the cellular targets of serum antibodies using double immunofluorescence with a panel of cell-specific markers. Samples were drawn from the treatment trial described above, NCT01281969; cases matched particularly stringent clinical criteria for PANDAS (not just PANS), including acute onset of OCD symptoms, presence of characteristic associated symptoms, and documented GABHS infection [22]. Individuals who responded clinically to IVIG treatment were selected for analysis, as responders may be most likely to have antibody-mediated pathophysiology. We report the first evidence for interneuron-reactive antibodies in the pathogenesis of PANDAS.

## METHODS AND MATERIALS

### Samples from patients diagnosed with PANDAS

Sera from children with PANDAS were obtained from a recent IVIG trial, NCT01281969 [22]. This randomized, double-blind trial investigated the efficacy of IVIG on PANDAS symptoms. Subjects in the clinical trial were required to meet all diagnostic criteria for PANDAS, including a positive test for beta hemolytic *Streptococcus*, and to have moderate to severe OCD symptoms, as assessed by the Children's Yale-Brown Obsessive Compulsive Scale (CY-BOCS [42]). Ratings were performed by trained clinicians, blinded to the treatment status [12].

For the pilot study (Supplementary Figure 2), three children with PANDAS were selected from this cohort, without applying further selection criteria. To enhance the probability of identifying subjects with antibody-mediated pathophysiology in the main experiment (Figures 1–3), we selected subjects who had a positive clinical response to IVIG, defined as a >35% decrease in the Y-BOCS score (see Figure 4A). One subject from the pilot experiment met these additional criteria and was included in the main experiment (subject B in the pilot, #3 in the main experiment). Two subjects in the main experiment received IVIG during the blinded treatment phase; two received placebo during the blinded phase and IVIG during the subsequent open-label phase, 6 weeks later. The fifth subject received open-label IVIG infusion and follow-up on the same schedule as the others after being excluded from the trial due to anxiety associated with a lumbar puncture. Serum samples used in this analysis were obtained at baseline and at 12 weeks (time points 1 and 3 in the clinical trial; see Figure 4A). The second time point was thus either 6 (2 subjects) or 12 weeks (3 subjects) after IVIG infusion. Of the five subjects in the main experiment, two had mild tics at baseline; three had no tics.

Control subjects for the pilot experiment were drawn from healthy control samples collected at Yale. For the main experiment, well-screened healthy subjects matched to the PANDAS sample for age and gender, also evaluated at the NIMH and stored under equivalent conditions and for equivalent time, were used as controls (see Table 1). None of these healthy controls had clinically significant OCD or tic symptoms.

Serum samples were stored at  $-80^{\circ}\text{C}$  until use. These investigations were approved by the Human Investigations Committees of Yale University and the National Institute of Mental Health. All subjects provided assent and parental informed consent. Samples were fully anonymized at the clinical site at NIMH before being sent to the laboratory at Yale for analysis.

## Mice

All experiments were conducted under the auspices of the Yale Institutional Animal Care and Use Committee, in accordance with NIH guidelines. Adult female and male C57Bl/6 wild-type or bitransgenic BAC transgenic mice [43], aged 2–4 months, were used for all experiments; there were no significant differences between results from male and female mice. (These BAC transgenic mice were used to identify binding to D1- and D2-expressing medium spiny neurons; this staining was uninformative and is not reported here.) 16 mice were infused in the pilot experiment (2–3 mice/serum sample); 20 mice were infused in the first experiment (2 mice with each of 10 sera), and 20 were used in the follow-up treatment experiment (5 patients; 2 mice with serum from before IVIG treatment and 2 mice with serum from after IVIG treatment).

## Surgery and serum infusions

Custom bilateral cannulae were manufactured to target the striatum (3.25 mm long; 4.6 mm space between guides; Plastics One, Roanoke, VA). Cannulae were chronically implanted using standard stereotaxic technique, targeting +0.50 mm anteroposterior,  $\pm 2.3$  mm mediolateral, relative to bregma [44]. Animals were anesthetized during surgery with

ketamine:xylazine 100:10 mg/kg. Guide cannulae were affixed to the skull using C&B-Metabond® Quick! Cement System (Parkell Inc.). Guide cannula patency was ensured with customized dummy cannulae that projected 0.5 mm beyond the end of the guide. Animals recovered for at least 5 days prior to first serum infusion.

Undiluted serum was infused into the striatum simultaneously in the two hemispheres at a fixed rate of 0.1µl/min through customized injectors projecting 0.5 mm beyond the end of the guide cannulae, using a PHD Ultra Pump (Harvard Apparatus).

Pilot experiments determined optimal infusion parameters. Piloting was performed with pooled human serum (Sigma: H4522). Serum was infused unilaterally, varying the volume and number of infusions and the time between the last infusion and animal sacrifice; saline was infused on the contralateral side (Supplementary Figure 1). IgG deposition was identified using immunohistochemistry with rabbit anti-human IgG (1:200 Abcam, Cambridge, MA) and visualized using diaminobenzidine (ABC Kit; Vector Laboratories, Burlingame, CA, USA). Optical density was quantified using ImageJ (NIH, Bethesda, MD, USA), as previously described [45]. Optimal IgG deposition was achieved when animals were infused for 5 consecutive days with 0.5 µl serum and then sacrificed 5 days after the last infusion; these conditions were used for all subsequent experiments.

### Immunofluorescence and confocal microscopy

Mice were euthanized without perfusion. Brains were quickly dissected, fixed in 4% paraformaldehyde for 48h at 4°C, and then transferred into a 30% sucrose solution and allowed to equilibrate at 4°. Brains were sectioned at 30 µm using a cryostat; sections were stored in cryoprotectant solution (30% glycerin + 30% ethylene glycol in PBS) at -20°C until use.

Free-floating sections were washed with PBS, treated with Bloxall Solution (Vector Laboratories), washed again in PBS, incubated in 5% normal donkey serum solution in PBS + 0.3% Triton X100 for 1 hour at room temperature, and then overnight at 4° C in the same solution with rabbit anti-human IgG 1:200 (Abcam), together with goat anti-choline acetyltransferase (ChAT; 1:1000, Sigma, St. Louis, MO, USA), mouse anti-neuronal nitric oxide synthase (nNOS; 1:100, Santa Cruz Biotechnology, Dallas, TX), or mouse anti-parvalbumin (PV; 1:500; Sigma). For the anti-nNOS and anti-PV antibodies, an additional blockade with Mouse-on-Mouse (MOM) reagent (Vector Laboratories) was included to prevent reactivity of the secondary antibody with endogenous mouse immunoglobulin, following the manufacturer's instructions. Slices were washed 3X with PBS and then incubated with fluorescent secondary antibodies (1:400, Invitrogen, Carlsbad, CA, USA) for one hour at room temperature. Secondary antibodies were: for ChAT immunostaining, Alexa 555-conjugated donkey anti-rabbit and Alexa 633-conjugated donkey anti-goat; for nNOS and PV immunostaining, Alexa 555-conjugated donkey anti-rabbit and Alexa 488-conjugated donkey anti-mouse. Samples were washed again and mounted in Vectashield HardSet Mounting Medium (Vector Labs).

Confocal imaging was performed by sequential scanning of slices on an Olympus Fluoview FV-1000 confocal microscope, using a Kalman filter with an acquisition speed of 4 pixel/sec. 15  $\mu\text{m}$  z-stacks were generated by using a fixed step size of 0.5  $\mu\text{m}$ .

### **Quantification of serum antibody binding to interneurons**

Two images were obtained from each slice (one per side); several slices per mouse were used to provide a representative sample of the entire mouse striatum, for a total of 8–12 images per mouse. Single- and double-positive cells were counted throughout all images, blind to experimental condition. Cell counts were averaged to produce a single value for each animal. Two mice were infused with each serum; technical variation in infusion and cell counting was very low.

### **Statistical analysis**

Data were analyzed using Prism 6.0 (GraphPad), except for linear mixed model analysis, which was performed using SPSS 24 (IBM). The unit of analysis was the patient/serum sample, not the slice or the animal. Each data point in the final analysis averages cell counts from ~20 slices (8–12 slices from each of 2 animals).

We have examined a small number of rigorously characterized samples, rather than a larger but potentially more heterogeneous clinical group. With an  $N$  of five samples per group in the main experiment, we therefore have 80% power to detect only very large effects ( $d > 1.7$ ). We reasoned that examination of a small, homogeneous sample would lead to smaller variance and thus to greater power than examination of a larger but more heterogeneous group.

## **RESULTS**

### **Pilot study**

In a small pilot study, we infused serum from three individuals with PANDAS and three controls into the striatum of adult mice. Binding to ChAT-positive interneurons was elevated after infusion of PANDAS serum, relative to controls; binding to PV-positive interneurons was unchanged (Supplementary Figure 2). These pilot data motivated a larger experiment with more carefully selected PANDAS subjects and optimally matched controls.

Serum infusion produced microgliosis and astrogliosis in the affected striatum. However, this glial activation was qualitatively equivalent after infusion of control and PANDAS serum (Supplementary Figure 3).

### **Main experiment: Subjects**

Five subjects with PANDAS were selected from the participants in the clinical trial [22] on the basis of their robust clinical response to IVIG; one of these overlapped with the smaller group used in the pilot experiment. These subjects had no history of acute rheumatic fever or Sydenham's chorea, autism, or schizophrenia, or a history of previous immunomodulatory therapy. They were matched for age and gender with 5 healthy controls (see Table 1).

PANDAS subjects had slightly but significantly higher IgG levels at baseline than controls ( $p = 0.02$ ).

### **Elevated binding of IgG from PANDAS serum to cholinergic but not GABAergic interneurons**

IgG deposition onto ChAT-positive cholinergic interneurons (CINs) was seen after infusion of both PANDAS and control serum. A larger number of ChAT-positive interneurons, expressed as a fraction of total ChAT<sup>+</sup> cells, was bound by antibodies in serum collected at baseline from PANDAS patients, compared to controls (Figure 1). This increased binding had a very large effect size of  $d = 8.1$ . The total number of ChAT-positive interneurons was not altered by PANDAS serum infusion, indicating that this difference does not derive from a non-specific toxic effect ( $t[8] = 0.89$ ,  $p > 0.4$ ).

We pooled these CIN binding data with data from the pilot experiment (Supplementary Figure 2) using a linear mixed model, coding serum number as a random variable to account for the fact that one sample was included in both experiments. This confirmed a highly significant effect of diagnosis ( $F[1,23] = 75.8$ ;  $p < 0.001$ ).

In contrast, binding of IgG to PV<sup>+</sup> and nNOS<sup>+</sup> interneurons did not differ between PANDAS and control samples (Figure 2, 3). The nominal trend was towards decreased binding in both cases.

### **Reversal of CIN antibody deposition after IVIG treatment**

These five patients were selected in part because they all improved substantially following IVIG treatment (Figure 4A). In an independent experiment, we infused sera from the same patients, collected at baseline and 6–12 weeks after IVIG treatment. Infusion was again into 2 mice per serum sample. Binding of IgG to CINs was significantly reduced in convalescent serum (Figure 4B). Improvement in CY-BOCS after treatment correlated significantly with reduction of IgG binding to cholinergic interneurons (Figure 4C).

## **DISCUSSION**

We have identified elevated binding of serum antibodies to cholinergic interneurons (CINs) in the striatum in children with stringently defined PANDAS that responded to IVIG treatment. This represents the first time that immunoreactivity against interneurons in the striatum has been examined in PANDAS and identifies CINs as a potentially important locus of pathology in the disorder.

Several lines of evidence implicate CINs in tic disorders. In postmortem brain from patients with Tourette syndrome, ChAT-positive CINs are reduced in number by approximately 50% in the caudate and the putamen [37, 38]. Moreover, selective virus-mediated depletion of CINs in the dorsolateral striatum of mice, which reproduces the ~50% loss seen post-mortem, leads to tic-like stereotypies after either acute stress or psychostimulant challenge [39]. Mutations in the Slit and Trk-like family member 1 (SLITRK1) gene have been associated with Tourette syndrome in rare cases [46, 47]; in the adult striatum, expression of this gene is restricted to CINs [48]. Finally, expression of a number of acetylcholine-related

genes in blood has been reported to correlate with tic severity in Tourette syndrome, and these genes appear to be differentially spliced in patients [49].

No similar analyses have been reported in OCD. However, the comorbidity between TS and OCD in youth has been reported to be as high as 50% [50], and the two conditions appear to have overlapping pathophysiology [51]. Tic and OCD comorbidity is 60% in PANDAS [7].

In conjunction with our findings, this literature suggests that antibodies that recognize striatal cholinergic interneurons may have a pathogenic role in PANDAS. It is noteworthy that tics were not prominent in the subjects we characterized: two had mild tics at baseline, and three had none. Therefore, the elevated binding of IgG to CINs documented here is not specific to tic symptoms, but rather is associated with PANDAS more generally.

Elevated binding of IgG in PANDAS serum to CINs is specific: there is no differential binding to other interneuron types examined. This specificity is in contrast to post mortem evidence that both PV<sup>+</sup> fast spiking interneurons and the nNOS-expressing GABAergic interneurons have also been shown to be affected in adults with severe tics [36–38]. IgG levels were slightly higher in controls, which represents a weakness in our study; however, the specificity of binding increases confidence that differential binding to cholinergic interneurons is not a consequence of this or of other nonspecific abnormalities in the serum, but rather a reflection of specific populations of antibody.

Cholinergic interneurons are well positioned to regulate information processing in the basal ganglia. They differentially regulate D1- and D2-expressing medium spiny neurons through activation of metabotropic ACh receptors [52, 53] and modulate activity of GABAergic interneurons [54]. Cholinergic dysregulation in the dorsal striatum may thus produce an imbalance between the direct and indirect pathways through the basal ganglia, leading to disinhibition of off-target behaviors [55, 56]. Our previous studies of the consequences of cholinergic interneuron depletion *in vivo* show that functional perturbation of these cells can produce relevant phenomenology [39]. We hypothesize that antibody deposition on cholinergic interneurons leads to functional abnormalities, and thereby to PANDAS symptomatology. How antibody binding to these cells might perturb their function, however, remains unclear. Cholinergic interneurons express D2 dopamine receptors, and antibodies in PANDAS have been reported to bind to these receptors in some studies [32]{Morris-Berry, 2013 #39;Cox, 2013 #37}; this represents one possible molecular target that might explain the preferential binding to these interneurons seen here. *In vitro*, antibodies from PANDAS patients can activate calcium-calmodulin kinase-II in immortalized human neuron-like cells [11, 33, 35]. However, this has not been demonstrated in cholinergic cells.

Our model rests on the assumption that antibodies that recognize cholinergic interneurons in mice will have similar binding in the human brain; this is a limitation. An additional limitation is that binding to tissue does not permit ready identification of the molecular targets to which PANDAS antibodies are binding; this is an important focus of future work. However, the approach employed here has unique advantages. While inflammation and microglial activation can be visualized *in vivo* in humans [30], IgG deposition cannot, and cell-type specificity of binding is difficult to characterize. Analysis of post-mortem tissue



can be used to characterize abnormalities in chronic illness, and post-mortem analysis of individuals with severe TS has revealed microglial activation [38]; but tissue for post-mortem analysis is scarce and often heterogeneous, and repeated characterization after treatment, such as we present here, is obviously not possible. Furthermore, the use of human post-mortem tissue to evaluate autoantibodies in human serum samples is limited by numerous technical challenges, including interference from formaldehyde tissue preservatives and non-specific brain IgG deposition. Thus, the use of murine brain tissue for the characterization of human anti-brain antibodies may represent an important “first-pass” tool for antibody identification. A similar approach has been used, with success, in the identification of antibodies in autoimmune encephalitis, particularly anti-NMDA receptor encephalitis [57]. An additional advantage is that we can investigate binding by cell type, rather than molecular target, which may be a more sensitive approach if antibody targets are heterogeneous.

IVIG treatment has been reported to ameliorate symptoms in PANDAS in some cases [20–22]. In the recent clinical trial from which our clinical samples are drawn, patients who received blinded IVIG treatment did not separate from controls in the primary analysis of the blinded phase, but a majority of patients responded after receiving IVIG during either the blinded or open-label phase [22]. Because our goal here was to identify potential pathophysiological mechanisms, and not to establish characteristics of the full clinical cohort, we selected subjects who demonstrated a robust clinical response to IVIG. This clinical response was paralleled by reduced binding of antibodies to striatal cholinergic interneurons. This correlation supports the conclusion that IVIG may produce clinical response by reducing pathogenic antibodies. Similar declines in antibody reactivity have been reported in convalescent serum from PANDAS [15] and Sydenham’s chorea [12], though not previously to our knowledge after IVIG treatment.

These results do not exclude the possibility of other pathogenic cellular targets in PANDAS serum. PANDAS is likely to be etiologically heterogeneous. D1- and D2-expressing medium spiny neurons represent the majority of striatal neurons; and several studies have suggested that they may be targeted by autoantibodies in PANDAS, in at least some cases [32–35].

In addition, our results do not establish that this abnormality is unique to PANDAS. We have examined a small number of extensively characterized patients, selected so as to maximize the *a priori* likelihood of identifying antibody-mediated pathophysiology. Future examination of larger cohorts and a more diverse set of patients, including individuals with Tourette syndrome, non-PANDAS pediatric-onset OCD, and Sydenham’s chorea would better demarcate the clinical correlates of antibody reactivity against CINs and might reveal different patterns of antibody deposition in distinct populations of interneurons in other groups of patients. The approach described here is extremely laborious; now that the CINs have been identified as a cellular target of interest, it may be possible to do more focused analyses in these broader clinical cohorts using *ex vivo* methods.

In summary, our *in vivo* approach to characterizing antibody reactivity in patients with PANDAS has identified a novel candidate pathophysiology: specific autoantibody binding to striatal cholinergic interneurons. This focus on cholinergic interneurons fits well with the

developing appreciation of the role of these cells in tic disorders. Identification of the specific antigens on these cells and the functional consequences of antibody binding may open new avenues for the understanding and treatment of PANDAS and related conditions.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

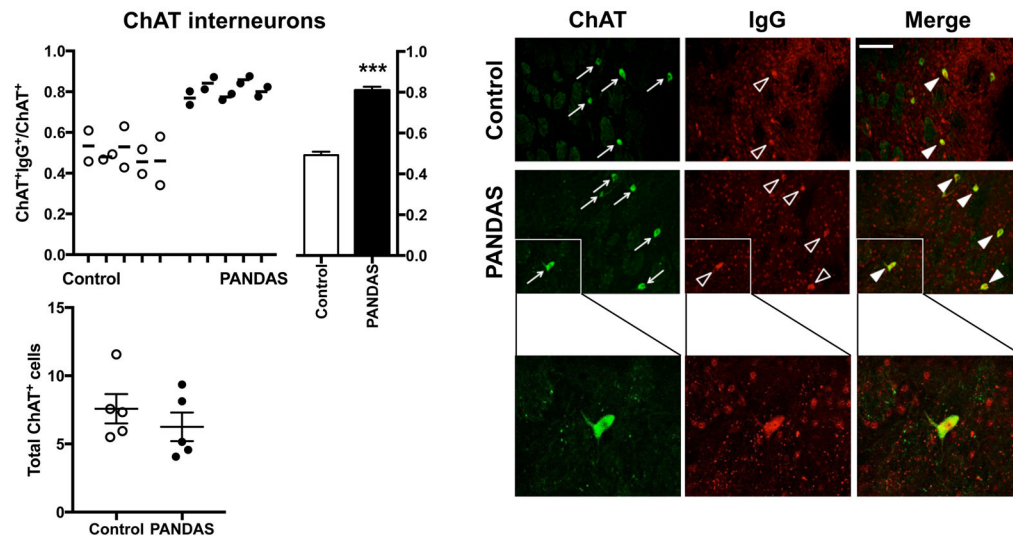
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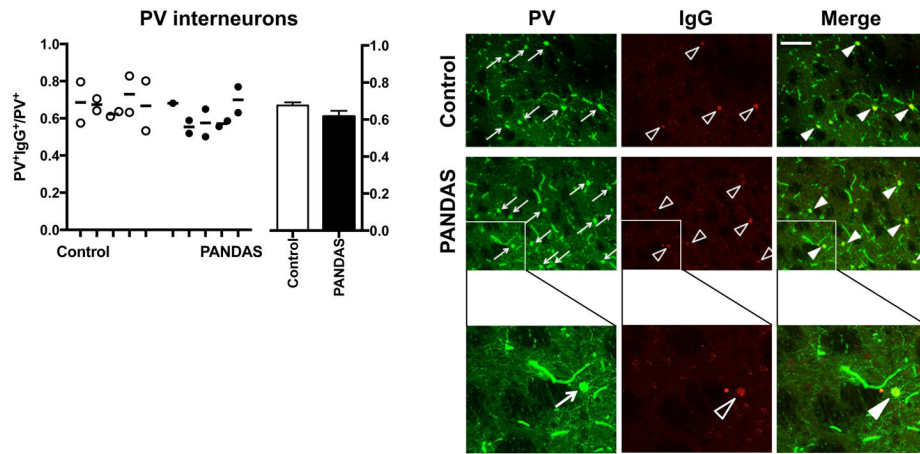
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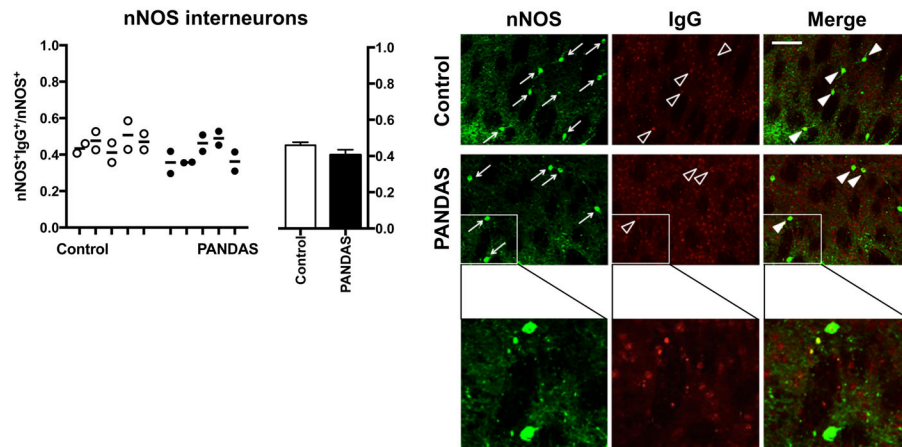


**Figure 1. PANDAS autoantibodies differentially bind to ChAT interneurons in the basal ganglia**  
 Deposition of antibodies from control and PANDAS sera (collected at baseline from highly symptomatic patients) onto ChAT-positive interneurons (CINs) *in vivo* was evaluated by double immunofluorescent staining and confocal microscopy, using anti-human IgG (red) and anti-ChAT antibodies (green). Antibodies from both control and PANDAS serum were deposited onto CINs, but a significantly larger number of ChAT<sup>+</sup>IgG<sup>+</sup> cells (arrowheads), as a fraction of total ChAT<sup>+</sup> cells, was observed in PANDAS samples, compared to healthy controls (N=5 samples per group, 2 mice per sample; two-tailed t-test:  $t[8] = 12.90$ ;  $p < 0.0001$ ). Insets illustrate binding to a single ChAT<sup>+</sup> cell at higher magnification. Scale bar = 100  $\mu\text{m}$ .



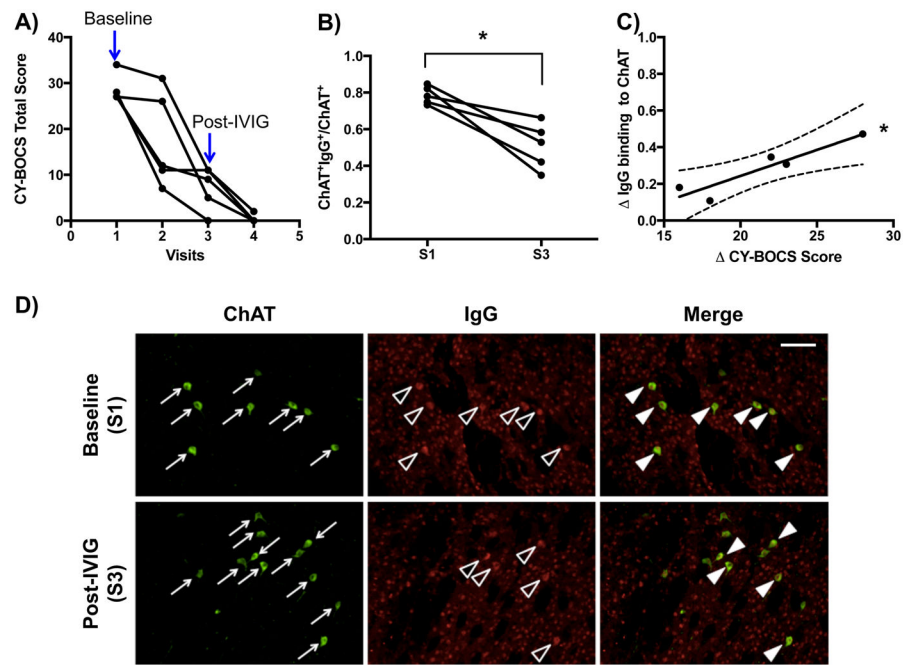
**Figure 2. PANDAS autoantibodies do not show elevated binding to parvalbumin-positive GABAergic interneurons**

No differences between PANDAS and control serum in antibody deposition onto PV-positive interneurons were observed (two-tailed t-test:  $t[8] = 1.7$ ,  $p = 0.13$ ). Scale bar = 100  $\mu$ m.



**Figure 3. PANDAS autoantibodies do not show elevated binding to nNOS-positive GABAergic interneurons**

No differences between PANDAS and control serum in antibody deposition onto nNOS interneurons were observed (two-tailed t-test:  $t[8] = 1.6$ ,  $p = 0.15$ ). Scale bar = 100  $\mu$ m.



**Figure 4. Resolution of elevated ChAT binding in post-IVIG serum**

**A.** These patients all showed a significant clinical response to IVIG treatment. Individual patients' changes in the Children's Yale-Brown Obsessive Compulsive Scale (CY-BOCS) score across four evaluation sessions are shown (see also Table 1). Antibody binding to interneurons was assayed at baseline (Visit 1; Figures 1–3) and 12 weeks later (Visit 3). **B.** IgG binding to ChAT<sup>+</sup> interneurons was markedly reduced in serum collected from all five PANDAS patients after IVIG treatment (evaluation 3), relative to baseline (paired t-test:  $t[4] = 4.4$ ;  $p = 0.012$ ). **C.** Change in IgG binding to CINs correlated significantly with improvement in CY-BOCS after IVIG treatment ( $r^2 = 0.86$ ,  $p = 0.023$ ). Scale bar = 100  $\mu\text{m}$ .



Table 1

## Subjects

Patients were selected from the larger group who participated in a clinical trial of IVIG for the treatment of PANDAS [11], on the basis of clinical response to IVIG treatment and positive ANA at baseline. Healthy controls were previously screened by the same clinical site, at the NIMH.

Subjects	SEX	AGE (at trial initiation)	RACE/ETHNICITY	TIME OF IVIG (weeks)	Baseline CY-BOCS	Week 12 CY-BOCS	Baseline IgG (mg/dL)	ANA prior to IVIG
<b>PANDAS</b>								
1	F	9.5 y	M/NH	6	27	5	1250	+
2	F	8.9 y	W/NH	0	28	0	898	+
3	F	6.7 y	W/NH	0	27	11	1160	+
4	M	8.2 y	W/NH	6	34	11	1120	+
5	M	7.5 y	W/NH	0*	27	9	1200	+
<b>CONTROL</b>								
1	F	5.1 y	W/NH	-	-	-	896	+
2	F	8.0 y	W/NH	-	-	-	963	+
3	M	8.1 y	M/NH	-	-	-	768	-
4	M	7.8 y	M/H	-	-	-	877	-
5	F	7.3 y	W/NH	-	-	-	1040	-
<b>P-value</b>							0.02	NS

Race/ethnicity (parental report): M - mixed; W - white; H - Hispanic or Latino; NH - not Hispanic or Latino. Patients received a single IVIG treatment, either during the blinded clinical trial (at 0 weeks) or during a subsequent open-label phase (at 6 weeks).

\* This patient was not randomized due to an unrelated adverse event (anxiety during a lumbar puncture) but was treated open-label with IVIG and followed up on the same schedule as those who were randomized.