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Short Communication

Antibodies to neuronal surface proteins in Tourette Syndrome: Lack of evidence in a European paediatric cohort

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ABSTRACT

In Tourette Syndrome (TS) a role for autoantibodies directed against neuronal proteins has long been suspected, but so far results are still inconsistent. The aim of this study was to look for antibodies to specific or undefined neuronal proteins that could be involved in the aetiology of the disease.

Sera from children with Tourette Syndrome or another chronic tic disorder (TS/TD), collected as part of the longitudinal European Multicenter Tics in Children Study, were investigated. Participants included 30 siblings of patients with TS/TD prior to developing tics (preclinical stage) and the same children after the first tic onset (onset), and 158 patients in the chronic phase undergoing an acute relapse (exacerbation). Presence of antibodies binding to rodent brain tissue was assessed by immunohistology on rat brain sections and by immunofluorescent staining of live hippocampal neurons. Live cell-based assays were used to screen for antibodies to NMDAR, CASPR2, LGI1, AMPAR and $GABA_AR$.

Immunohistology indicated evidence of antibodies reactive with brain tissue, binding mainly to the hippocampus, the basal ganglia or the cerebellum in 26/218 (12%), with 8% of the preclinical or onset sera binding to the dentate gyrus/CA3 region or cerebellum. Only two individuals (one pre-clinical, one chronic) had antibodies binding the NMDAR and the binding was only weakly positive. No other specific antibodies were detected.

Despite some immunoreactivity towards neuronal antigens on brain tissue, this was not mirrored by antibodies binding to live neurons, suggesting the presence of non-specific antibodies or those that bind non-pathogenic intracellular epitopes. NMDAR or the other neuronal surface antibodies tested were very infrequent in these patients. The evidence for pathogenic antibodies that could be causative of TS is weak.

1. Introduction

Tourette Syndrome (TS) is a chronic tic disorder (TD) with an estimated paediatric prevalence of 3–8/1000 (6–18 age range) (Leckman, 2002). Tics usually fluctuate in severity, peaking around puberty and persist into adulthood in 30–40% of cases (Martino et al., 2015). A multifactorial model was proposed for the aetiology of TDs in which genetic and environmental factors could play a synergistic role (Hoekstra et al., 2012). In particular, a dysfunctional neural-immune cross-talk in TS/TD has been proposed in line with the concept of an alteration in the immune response linked with an inflammatory status (Frick and Pittenger, 2016; Martino et al., 2015). This hypothesis is supported by findings of increased levels of the proinflammatory cytokines Tumor Necrosis Factor-alpha and Interleukin-2 (Leckman et al., 2005), decreased numbers of regulatory T cells, and raised intrathecal immunoglobulin synthesis, all suggesting an ongoing inflammatory response or predisposition to autoimmunity in these patients (Wenzel et al., 2011; Kawikova et al., 2007).

The association of specific autoantibodies with TS has frequently been suggested (Yeh et al., 2012; Morris-Berry et al., 2013), mainly based on the similarity between chronic tic disorders and other post-streptococcal conditions such as Sydenham's Chorea, Paediatric Autoimmune Neuropsychiatric Disorders Associated With Streptococcal Infections (PANDAS), or the broader PANS (Paediatric Acute-onset Neuropsychiatric Syndrome), which have been linked to molecular mimicry between β-haemolytic streptococcus (GABHS) and host neural antigens (Leckman, 2002). Since dysregulation of dopamine transmission could underlie these conditions, antibodies to the dopamine D2 receptor (DRD2) have been investigated and reported to be present in some patients (Singer et al., 2015; Dale et al., 2012). However, none of the

Abbreviations: NMDAR, N-methyl D-aspartate receptors; VGKCs, Voltage-gated potassium channels; CASPR2, contactin-associated protein-2; LGI1, leucine-rich, glioma inactivated 1; GABA A, gamma-aminobutyric acid receptor A; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; CBAs, cell-based assays; DG, dentate gyrus; CA3, Cornu Ammonis region 3; RT, room temperature; PFA, paraformaldehyde; TBS, tris-buffered saline; PBS, Phosphate-buffered saline; BSA, Bovine serum albumin; MRI, Magnetic resonance imaging; TD, Tic Disorders; TS, Tourette Syndrome; TNF-alpha, Tumor necrosis factor-alpha; IL-2, Interleukin 2

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antibodies reported have been found in a substantial proportion of patients with TD, or shown to relate clearly to treatment-responses (Singer et al., 2015). This may be partly because some positivity has been found using antibody-detection techniques (i.e. ELISA, western blotting) that are not always the most appropriate for demonstrating antibodies to neuronal surface proteins, denaturing the natural epitopes conformation (Singer et al., 2015; Yeh et al., 2012).

There have been recent reports of neuronal-surface antibodies such as NMDAR, and the voltage-gated potassium channels (VGKC), LGI1 and CASPR2-antibodies (Poot, 2015; Sühs et al., 2015). Generally, those antibodies lead to neurological disorders including idiopathic limbic encephalitis and Morvan's syndrome (Irani and Vincent, 2016), but they may also have a role in several neurodevelopmental conditions. Prenatal exposure to CASPR2 antibodies, in particular, has been associated with autism and other developmental disorders (Brimberg et al., 2016; Coutinho et al., 2017) and exposure during childhood with ADHD and Obsessive-compulsive disorder (OCD) (Poot, 2015). Interestingly, a complex chromosomal insertion/translocation with an interruption of the CNTNAP2 gene, encoding for CASPR2 was described in the TS members of a family (Verkerk et al., 2003). It was suggested that the involuntary movements were related to potassium channel dysfunction. Potassium channels are known to associate with CASPR2 in the nervous system.

Here we used procedures (cell based assay CBA; Immunohistochemistry, IHC; neuronal cultures) employed to discover new neuronal surface antibodies (e.g. Lancaster and Dalmau, 2012) hoping to identify evidence for a specific antibody that might be useful in the diagnosis and management of these challenging disorders.

2. Methods

2.1. Paediatric cohort

The samples were obtained as part of the EMTICS study, a longitudinal observational European multicenter project, with 16 participating clinical centers, enrolling paediatric participants aged 3–16 years (Schrag et al., 2018). A total of 218 samples from 188 participants were available for analysis, divided into three subgroups on the basis of disease stage. The samples were obtained from three subgroups: pre-clinical siblings of patients with TS/TD (Preclinic n = 30), taken before the presentation of clinical tic manifestations (mean time 1 year); samples from the same children during the acute phase when they had developed tics for the first time (Onset n = 30); and samples taken at the time of an exacerbation of tics in patients with TS/TD (Exacerbation n = 158) who were followed over the course of 20 months (Table 1). This study was designed to detect potential antibodies at times when they might be most evident (i.e. at Onset and Exacerbation) and to compare with the time at which they were less likely (Preclinic).

The clinical variables described were age (mean age 9 years; range 3–16 years), gender (M: F = 4:1), the presence of GABHS infections (positive swab throat in the 9% of the cohort), and tic severity using the Yale Global Tic Severity Scale (YGTSS) in the two symptomatic groups (mean Total YGTSS score 30; observed range 6–40).

2.2. Antibody screening

We looked for those antibodies that had already been described in autoimmune neurological diseases (NMDAR; CASPR2; LGI1, AMPAR, $GABA_AR$) and aimed to search for evidence of possible unknown antibodies in TS/TD. The antibody testing was conducted in the Nuffield Department of Clinical Neurosciences of the Oxford University.

2.3. Immunohistology

All samples were screened for binding to unknown antigenic targets using immunohistochemistry (IHC) staining on rat brain sections, as described (Gastaldi et al., 2017). Sprague Dawley rat brains were immersed for 1 h in 4% paraformaldehyde (PFA), cryoprotected in 40% sucrose and snap-frozen in dry-ice cooled isopentane. Twelve-µm sagittal brain sections were incubated with a solution of tris-buffered saline (TBS)-0.3% H_2O_2 for 15 min at room temperature (RT), washed and blocked in 10% normal goat serum (NGS) for 1 h before incubation with patients' serum (1:100 in 5% NGS) overnight at 4 °C. The next day slides were washed and incubated with secondary antibodies (biotinylated goat anti-human IgG antibody, Vector lab, 1:1000) for 2 h, washed, and further incubated with the avidin-biotin complex (ABC, Vectastain Elite ABC Kit Standard, 1:100, 1 h). The reaction was developed using brown 3,3′-diaminobenzidine (ImmPACT DAB peroxidase (HRP) Substrate, Vector lab) as per manufacturer's instructions. Slides were dehydrated by immersion in progressive concentration of ethanol solutions followed by p-Xylene and mounted with DPX mountant for histology (Sigma-Aldrich®). Sections were viewed using a light Microscope (Nikon Eclipse E400) and images taken using the Aperio ScanScope (Leica Biosystem).

Table 1
Summary of results.

Immunohistochemistry on sagittal rat brain sections (n = 218)	Preclinical Group (n = 30)	Tic Onset Group (n = 30)	Tic Exacerbation Group (n = 158)
Cell-based assays for neuronal surface antibodies			
Total positive sera (12%)	3 (10%)	5 (17%)	18 (11%)
Basal ganglia/striatum (7.8%)	2 (7%)	4 (14%)	11 (7%)
Hippocampus (10%)	3 (10%)	5 (17%)	14 (9%)
• DG/CA3 (5%)	2 (7%)	3 (10%)	5 (3%)
Cerebellum/ molecular layer (10.5%)	2 (7%)	4 (14%)	17 (10,7%)
Live cell-based assays			
NMDAR (n = 218) (1%)	1	0	1
CASPR2 (n = 218)	0	0	0
LGI1 (n = 218)	0	0	0
AMPAR (n = 26)	0	0	0
$GABA_AR$ (n = 26)	0	0	0
Binding to hippocampal neuronal cultures	Not done	0	0 (n = 60)

2.4. Primary neuronal cultures

Because of the higher possibility of finding specific antibodies in the sera during the acute clinical phases of the disorder, only the 30 Onset and 60 Exacerbation samples were tested for binding to rat dissociated hippocampal neuronal cultures. These were prepared following an established protocol (Kaech and Banker, 2006) and antibody testing was performed as described in previous reports (eg. Irani et al., 2010). After 12 days in culture, the live neurons were incubated with the sera (1:100) in neurobasal (NBS) medium. Coverslips were washed, fixed with PFA 3% in PBS (10 min, RT) and incubated with Alexa Fluor® Goat anti-human IgG H&L 488 secondary antibody (1:1000 in complete NBS-1% BSA) before washing and mounting with DAPI. Coverslips were visualized using a fluorescence microscope (Leica DM 2500™).

2.5. Cell based assays

Antibodies to specific antigens were investigated on human embryonic kidney (HEK) 293 T cells after transfection with the plasmid of interest, as performed in the Oxford laboratory protocol for clinical diagnoses. The specific extracellular antibodies measured in all 218 samples were NMDAR, CASPR2 and LGI1. $GABA_A$ and AMPA receptors antibodies were investigated only in those samples showing binding to brain tissue by immunohistochemistry. Live cells were incubated with subjects' serum (1:20 or 1:100) in DMEM supplemented with HEPES

and 1% Bovine serum albumin (BSA) for 1 h at RT. Coverslips were then washed and fixed in 4% PFA. After further washes, they were incubated with secondary antibodies (1:1000; Alexa Fluor™ 568 anti-human IgG H &L chain rises in goat; 1 h, RT) washed and mounted onto glass microscope slides with DAPI. Antibody binding to the expressed antigen was observed using a fluorescence microscope (Leica DM 2500™).

2.6. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 21 and GraphPad Prism 6.0. Relationships between evidence of immunoreactivity and clinical features were analysed, using the chi-square test, the Fisher's exact test and linear regression ($p = 0.05$), depending on number of groups and data distribution.

3. Results

Using immunohistology on sagittal rat brain sections, 26/218 (12%) sera showed mild to moderate IgG binding (see Fig. 1a for examples). Binding was scored as moderate in 6/26 of these samples and as weak in 20/26 (Table 1). There were no differences in binding percentages between the three groups ($p = 0.753$). In 16 of the 26 sera, the binding was not clearly regionally focussed, with similar intensity binding to the hippocampus, basal ganglia and cerebellum. In particular, there were no samples binding more strongly to the basal ganglia than to the hippocampus and cerebellum. Ten of the 26 sera, however, bound to the dentate gyrus and CA3 subfields of the hippocampus, which have been implicated previously in TS/TD, but neither these nor any other sera bound to the live primary hippocampal neurons. Only two sera bound to the NMDAR; none bound to CASPR2, LGI1, AMPAR or $GABA_A$ R. The results are summarised in Table 1 and the heatmap in Fig. 1b illustrates the results of all 28 sera that showed some evidence of immunoreactivity.

There were no relationships between these results and age ($p = 0.690$), gender ($p = 0.631$), tic severity as measured by the YGTSS ($p = 0.955$) or throat swab status ($p = 0.343$). The DG and CA3 immunoreactivity was more frequent (8%) in Preclinical or Onset patients than in those in the Exacerbation group (3%), but this difference did not reach significance ($p = 0.1$).

4. Discussion

Taking advantage of samples collected on a large longitudinal European observational study, 218 sera from 188 TS/TD paediatric participants were investigated for the possible existence of antibodies to neuronal receptors or other proteins that might be involved in the pathogenesis of the disease. Of particular importance, the samples analysed were from different stages of the disease (preclinic, acute onset, and exacerbation during the chronic phases) designed to identify putative antibodies in TS/TD. Although there was some evidence of neuronal antibody reactivity to hippocampus, basal ganglia and cerebellum, there was no apparent association between the presence of this reactivity and the symptom stage or the reported clinical variables. Only two sera presented a weak positivity for NMDAR-antibodies and this prevalence is well in line with the general population rating (about 1–3%) (Ando et al, 2016; Steiner et al, 2013). Thus, the findings of this study did not support the frequent presence of neuronal antibodies in TS/TD children.

A potentially interesting result was the binding of a proportion of serum IgG antibodies to the dentate gyrus (DG) and CA3 subfields of the hippocampus, which have been suggested to be important in the pathogenesis of TS. The hypothesis is that these areas, receiving projections from the prefrontal cortex, then modulate dopaminergic input to the ventral striatum (Albin, 2018; Peterson et al., 2007). Indeed, a brain Magnetic Resonance Imaging-MRI cross-sectional study reported an enlargement of the DG and CA3 subfields in children with TS, and reduced volumes in the same hippocampal areas in adult patients (Peterson et al., 2007). However, the DG/CA3 subfield antibody binding was only found in 10/218 (5%) of patients we examined. Although it was a little more frequent in the 5/60 (8%) preclinical and acute onset patients compared to those with a chronic disease 5/158 (3%), this was not statistically significant.

Our results are in line with those of a recent study on a group of adult participants with TS (n = 51); an anti-nuclear pattern of immunoreactivity was observed in 14% (7 out 51) of samples by immunofluorescence (IF) on hippocampus and cerebellum sections (Sühs

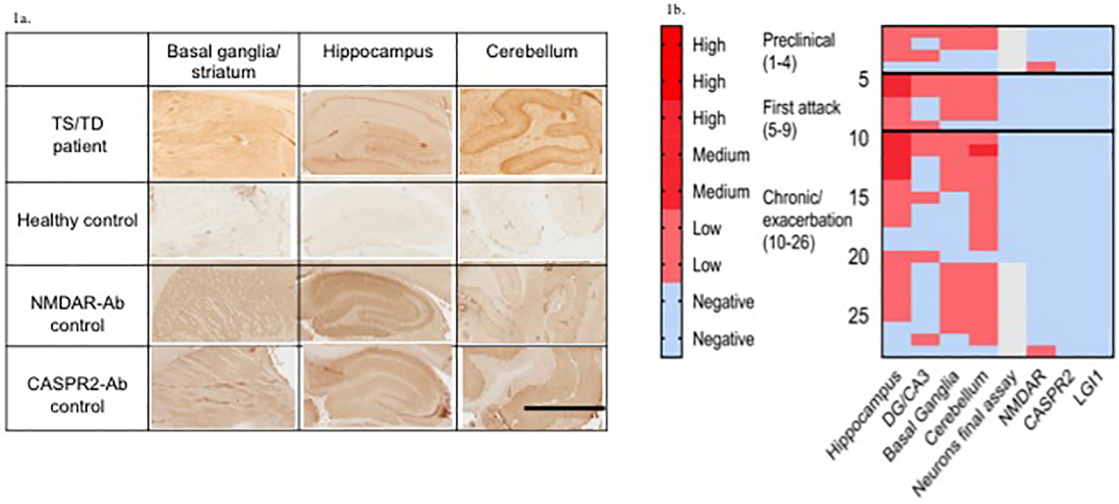

Fig. 1. Examples of immunohistology on rodent brain tissue sections. 1a. Binding of antibodies by immunohistology. An example of a TS patient's serum showing binding to basal ganglia, to the dentate gyrus DG and CA3 area of hippocampus and to the molecular layer of the cerebellum. None showed regionally specific and intense binding. IHC scalebar = 1 mm 1b. A heatmap representing results of the 28/218 (12.8%) sera that showed any neuronal immunoreactivity (on brain sections, live hippocampal cultures or cell-based assay). The results of each individual serum are represented horizontally. In each patient category (Preclinical, Onset, Exacerbation) there were sera that bound to all regions of the brain. Note that binding to the basal ganglia was less common than to other regions, binding was mainly moderate rather than strong, none of these were positive for specific antibodies but two additional patients had low titre NMDAR-Abs. Scoring scale: Blue indicates no binding detected. Pink to red colours denote the strength of the immunohistological or immunofluorescent signal from weakly positive to strongly positive. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

et al., 2015), but no antibodies against specific neuronal surface antigens were detected by CBAs, or against well-defined intracellular target antigens. Thus collectively, there is a lack of evidence of antibody reactivity in either adult or paediatric TS/TD patients.

Previous studies have been inconclusive, perhaps biased by testing sera sampled at different time points of a disease which is characterised by a waxing and waning course of tic disorders (Brimberg et al., 2012), and the use of less suitable detection techniques (i.e. western blotting; ELISA) where both intracellular and extracellular epitopes are exposed and the antigens are not in their native membrane conformation (see Martino et al., 2015 for a review). In addition, the clinical characteristics of the enrolled participants have not necessarily been restricted to TS, with some also presenting with other neurodevelopmental disorders (i.e. OCD/ADHD/Autism) (Cavanna, 2018).

Nevertheless, there has been support for immune dysregulation in patients with TS/TD. A study on cerebrospinal fluid (CSF) of adult patients with TS identified IgG oligoclonal bands in 38%, consistent with an autoimmune process in at least a subgroup of TS, and clearly different from the 3% found in the healthy population (Wenzel et al., 2011). The same group, using the CBAs and SY5Y neuronal stem-cells and astrocytes cultures, did not detect antibodies to any of the antigens in the CSF, even in those samples where there were oligoclonal bands. Other potential reactivity with brain tissue was not examined but these results do suggest immune activation within the cerebrospinal compartment which could be either primary (i.e. causative) or secondary (Baumgärtel et al., 2016).

A main limitation of our study was the absence of a matched control group of healthy children for comparison with the TS sera results. Also, only a limited number of potential specific antigens were screened and there could be others involved. Nevertheless, the strengths of this study are in the large paediatric cohort, the comparison of samples from both before and at tic onset in 30 participants, and the approaches used to look for evidence of cell-surface autoantibodies.

In conclusion, specific neuronal surface antibodies were not detected in our cohort of children with TS/TD, either by CBAs for NMDAR and other specific antibodies. There was neuronal reactivity towards rodent brain tissue sections in a limited number of samples, but this was not supported by binding to live neurons in culture. Thus, our results fail to support the involvement of a specific antibody in the pathogenesis of childhood TD/TS.

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Appendix

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Appendix A. Supplementary data

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