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Antibodies to surface dopamine-2 receptor in autoimmune movement and psychiatric disorders

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Recent reports of autoantibodies that bind to neuronal surface receptors or synaptic proteins have defined treatable forms of autoimmune encephalitis. Despite these developments, many cases of encephalitis remain unexplained. We have previously described a basal ganglia encephalitis with dominant movement and psychiatric disease, and proposed an autoimmune aetiology. Given the role of dopamine and dopamine receptors in the control of movement and behaviour, we hypothesized that patients with basal ganglia encephalitis and other putative autoimmune basal ganglia disorders harboured serum autoantibodies against important dopamine surface proteins. Basal ganglia encephalitis sera immunolabelled live surface cultured neurons that have high expression of dopamine surface proteins. To detect autoantibodies, we performed flow cytometry cell-based assays using human embryonic kidney cells to express surface antigens. Twelve of 17 children (aged 0.4–15 years, nine males) with basal ganglia encephalitis had elevated immunoglobulin G to extracellular dopamine-2 receptor, compared with 0/67 controls. Immunofluorescence on wild-type mouse brain showed that basal ganglia encephalitis sera immunolabelled microtubule-associated protein 2-positive neurons in striatum and also in cultured striatal neurons, whereas the immunolabelling was significantly decreased in dopamine-2 receptor knock-out brains. Immunocytochemistry confirmed that immunoreactivity localized to the surface of dopamine-2 receptor-transfected cells. Immunoabsorption of basal ganglia encephalitis sera on dopamine-2 receptor-transfected human embryonic kidney cells decreased immunolabelling of dopamine-2 receptor-transfected human embryonic kidney cells, neurons and wild-type mouse brain. Using a similar flow cytometry cell-based assay, we found no elevated immunoglobulin G binding to dopamine 1, 3 or 5 receptor, dopamine transporter or *N*-methyl-D-aspartate receptor. The 12 dopamine-2 receptor antibody-positive patients with encephalitis had movement disorders characterized by parkinsonism, dystonia and chorea. In addition, the patients had psychiatric disturbance with emotional lability, attention deficit and

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psychosis. Brain magnetic resonance imaging showed lesions localized to the basal ganglia in 50% of the patients. Elevated dopamine-2 receptor immunoglobulin G was also found in 10/30 patients with Sydenham's chorea, 0/22 patients with paediatric autoimmune neuropsychiatric disorders associated with streptococcal infection and 4/44 patients with Tourette's syndrome. No dopamine-1 receptor immunoglobulin G was detected in any disease or control groups. We conclude that assessment of dopamine-2 receptor antibodies can help define autoimmune movement and psychiatric disorders.

Keywords: encephalitis; autoantibody; dopamine receptor; movement disorders; Sydenham's chorea

Abbreviations: DAT = dopamine transporter; GFP = enhanced green fluorescent protein; HEK = human embryonic kidney; IgG = immunoglobulin G; MAP2 = microtubule-associated protein 2; NMDAR = *N*-methyl D-aspartate receptor; PANDAS = paediatric autoimmune neuropsychiatric disorders associated with streptococcal infection

Introduction

There is increasing evidence that autoimmune encephalitis represents a significant subgroup of encephalitis in children and adults, which are defined by the presence of antibodies against important proteins involved in neurotransmission (Dalmau *et al*., 2008; Lai *et al*., 2009; Irani *et al*., 2010*a, b*). These antibodies have subsequently been used to define the clinical spectrum of autoimmune CNS diseases. Despite this, many cases of encephalitis present a diagnostic challenge and the cause is unexplained (Granerod *et al*., 2010). We have previously described a basal ganglia encephalitis associated with movement disorders, and have proposed this syndrome to be autoimmune and treatable with immune therapy (Dale *et al*., 2001, 2002, 2004).

Basal ganglia encephalitis is part of the spectrum of autoimmune basal ganglia disorders with the classic autoimmune basal ganglia disorder being Sydenham's chorea, in which there is good evidence from clinical and therapeutic studies to support the autoimmune hypothesis and the presence of autoantibodies (Church *et al*., 2002; Kirvan *et al*., 2003; Garvey *et al*., 2005; Brilot *et al*., 2011; Brimberg *et al*., 2012; Walker *et al*., 2012). The other putative autoimmune basal ganglia disorder is paediatric autoimmune neuropsychiatric disorders associated with streptococcal infection (PANDAS), although the evidence for an autoimmune disorder is less clear and the entity remains controversial (Singer *et al*., 2005; Kirvan *et al*., 2006; Brilot *et al*., 2011). Finally, it has been proposed that an autoimmune subgroup may exist in Tourette's syndrome, although results remain controversial (Martino *et al*., 2009; Murphy *et al*., 2010; Felling and Singer, 2011).

Dopamine is a crucial neurotransmitter in the brain and dopaminergic dysfunction is thought to underlie common human diseases, such as Parkinson's disease, Tourette's syndrome and schizophrenia (Beaulieu and Gainetdinov, 2011). Dopamine signalling is mediated through dopamine receptors, which are G-protein-coupled seven-transmembrane domain receptors. Five receptors have been identified in humans and are divided into two groups: D1- (D1R and D5R) and D2-class receptors (D2R, D3R and D4R) (Kebabian and Calne, 1979). D1- and D2-class receptors have high expression in basal ganglia, for example striatum (caudate–putamen), but also in cortex, hippocampus and substantia nigra (Beaulieu and Gainetdinov, 2011). Modulation of D2R expression in the basal ganglia has been associated with schizophrenia, depression and movement disorders (Nikolaus *et al*., 2009*a*, *b*; Beaulieu and Gainetdinov, 2011). Movement and psychiatric disorders associated with D2R antibody are biologically plausible as D2R is intimately linked to the control of movement and behaviour. For example, myoclonus dystonia is associated with *D2R* gene mutations (Klein *et al*., 1999); the best treatment of psychosis, chorea and Tourette's syndrome are D2R antagonists; and dystonia–parkinsonism is a recognized side-effect of D2R antagonists.

Herein, we report the presence of antibodies to extracellular epitopes of D2R antibodies in autoimmune movement and psychiatric disorders.

Materials and methods

Patients and controls

We investigated 17 patients seen between 2000 and 2011 with clinical or radiological features compatible with a basal ganglia encephalitis (Table 1), previously termed encephalitis lethargica (Dale *et al*., 2004). The 17 patients had clinical features suggestive of basal ganglia involvement (movement disorder sometimes with a psychiatric or sleep disorder). Previous serological testing had been negative for autoantibodies to *N*-methyl-D-aspartate receptor (NMDAR) ($n = 17$), voltage-gated potassium channel complexes ($n = 17$), glycine receptor ($n = 10$), glutamic acid decarboxylase ($n = 10$), leucine-rich, glioma-inactivated 1 protein ($n = 10$) and contactin-associated protein 2 ($n = 10$) (Dale *et al*., 2009; Suleiman *et al*., 2011). All 17 patients fulfilled criteria of encephalitis as previously defined, including encephalopathy ($n = 17$), focal neurological signs ($n = 17$), fever ($n = 9$), MRI inflammatory lesions ($n = 8$), EEG compatible with encephalitis ($n = 7$) and cerebrospinal fluid (CSF) pleocytosis ($n = 6$) (Granerod *et al*., 2010). Patients were investigated empirically with a total of 349 negative aetiological investigations (mean 20, range 6–31; Supplementary Table 1). All patients were treated briefly with intravenous antibiotics and acyclovir until bacterial infection or herpes simplex virus infection was excluded. Follow-up outcomes were assessed by child neurologists including functional differences compared with age-matched peers. Formal neuropsychology was only performed in four patients. Serum used in antibody studies was from the first week of the acute admission and before immune therapy (except in Supplementary Fig. 3 where convalescent samples were also used).

In order to define antibody specificity, we used serum from 67 paediatric controls as outlined in Supplementary Table 2. The controls

Table 1 Summary of clinical details in the patient cohorts

Disorder	Basal ganglia encephalitis (*n* = 17)	Sydenham's chorea (*n* = 30)	PANDAS (*n* = 22)	Tourette's syndrome (*n* = 44)
Male: female	9:8	6:24	14:8	39:5
Age at onset, mean (range) (years)	6.7 (0.4–15)	10.7 (2–17)	5.6 (2–9)	6.4 (2–13)
Age at serum sampling mean (range)	6.7 (0.4–15)	10.8 (2–17)	9 (4–14)	10.5 (5–15)
Country of origin (ethnicity), *n*	Australian (White) 6; UK (White) 3; Australia (Asian) 2; UK (Black) 4; UK (Indian) 1; New Caledonia (Melanesian) 1	Israel (White) 12[a]; UK (White) 5; Australian (White) 2; Israel (Arab) 1; UK (Indian) 1; Australia (Aboriginal) 5; Samoa (Polynesian) 2; Tonga (Polynesia) 1; Fiji (Melanesian) 1	US (White) 14; Australia (White) 6; US (Hispanic) 2	Australian (White) 30; Australian (Asian) 8; Australian (Arab) 4; Australian (Aboriginal) 1; New Zealand (Maori) 1
Movement disorder, *n*	Dystonia 10; parkinsonism 7; chorea 4; ocular flutter; motor tics 1 each	Chorea 30	Motor and vocal tics 17; motor or vocal tics only 3	Motor and vocal tics 44
Course of disease, *n*	Monophasic 14; relapse after improvement 3	Monophasic 22; relapse 7; persistence 1	Abrupt onset and relapsing remitting course 18; abrupt onset then static 4	Wax and wane with no complete remission 44
Psychiatric features, *n*	Any psychiatric 12; agitation 5; emotional lability 4; psychosis 3; depression 2	Any psychiatric 23; emotional lability 21; abrupt personality change 20; ADHD 6; GAD 5; depression 5; psychosis 1	Any psychiatric 22; abrupt personality change 22; OCD or OCB 16; other emotional lability 11; depression 10; ADHD 8	Any psychiatric 33; ADHD 22; OCD or OCB 17; ODD 9; depression 8; GAD 8; separation anxiety 8; psychosis 1
Associated symptoms, *n*	Encephalopathy 12; sleep disorder 7; reduced consciousness 6; mutism 4	Dysarthria 19; carditis 19 Arthritis or arthralgia 12; fever 5	Motoric hyperactivity 12; frequent urination 11; learning difficulties 2	Learning difficulties 6; autism 5
Positive anti-streptolysin-O titre (>200 IU/ml)	3/12 tested	28/30 tested[b]	14/22 tested[b]	16/37 tested

[a]Ashkenazi Jew *n* = 9, Sephardi Jew *n* = 3.
[b]Anti-Streptolysin-O titre-negative patients had positive anti-DNAse B (>300 IU/ml) or anti-group A carbohydrate (ACHO) titres (>2.76 U/ml).
ADHD = attention deficit hyperactivity disorder; GAD = generalized anxiety disorder; OCD/OCB = obsessive compulsive disorder/behaviour; ODD = oppositional defiant disorder.

included viral encephalitis, NMDAR encephalitis, inflammatory demyelination and basal ganglia disease of defined metabolic, genetic or degenerative origin.

For Sydenham's chorea, all patients ($n = 30$) fulfilled criteria, namely modified Jones criteria, clinical characteristics and evidence of streptococcal infection (Cardoso *et al*., 1997). There was a preceding throat infection in 19, preceding vaccine in one and no clinical precedent in 10. Serum was from the acute phase (<3 months) in 24, subacute phase (3–6 months) in three or during relapse of Sydenham's chorea in two or persistence of Sydenham's chorea in one (Table 1).

All patients with PANDAS ($n = 22$) fulfilled criteria proposed for PANDAS (Swedo *et al*., 1998) at illness onset. The main differentiating feature of PANDAS from other neuropsychiatric disorders was the infection-associated abrupt onset and rapid deterioration of symptoms that often occurred 'overnight', and the evidence of recent streptococcal infection. In total, 18 of the 22 patients had further ongoing infection-associated relapses, followed by remissions, which were often associated with the use of antibiotics, referred to as a 'sawtooth' course. Four of the patients had abrupt onset, but then a static course without remissions. All patients had serological evidence of streptococcal infection associated with infectious triggers. All patients had active tics and psychiatric disorders at the time of serum sampling (Table 1).

All patients with Tourette's syndrome ($n = 44$) fulfilled the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition criteria for Tourette's syndrome. All patients had active tic disorders at the time of serum sampling. No patients had the PANDAS phenotype (Table 1).

Patient sera had immunoglobulin G (IgG) concentrations measured by nephelometry (BN ProSpec, Siemens), and IgG values were within the normal range (6.2–14.4 g/l).

Ethics approval for this study was granted by the Children's Hospital at Westmead ethics Committees (HREC 2007/035, SSA 07/CHW/58, HREC/09/CHW/56 and SSA/09/CHW/143), and written informed consent from all patients was obtained.

Cloning and expression of human D1R, D2R, D3R, D5R, DAT and NMDAR

Extracellular N-terminus haemagglutinin-tagged human D1R, D2R and D5R complementary DNAs were obtained from the Missouri S&T cDNA Resource Centre (www.cdna.org). Intracellular N-terminus FLAG-tagged human dopamine transporter (DAT), human D3R and extracellular C-terminus haemagglutinin-tagged syntaxin-4 complementary DNAs was kindly given by Prof J.A. Javitch (Colombia University, NY, USA), and Prof D.E. James (Garvan Institute, Sydney, Australia), respectively. Sequence-verified D1R, D2R and D5R complementary DNAs were subcloned into pIRES2-GFP vector (Clontech), a vector suitable for expression of N-terminal haemagglutinin-tagged transmembrane antigens with enhanced green fluorescent protein (GFP) reporter under control of an internal ribosome entry site enabling both dopamine receptor and GFP to be co-expressed in cells separately. The human subunit 1 of NMDAR (kind gift from Prof A. Vincent, Oxford, UK) was also subcloned into the pIRES2-GFP vector, but was untagged. Human DAT and human D3R were subcloned into a modified pcDNA4 vector to express intracellular GFP in frame/fusion with DAT/D3R. We used syntaxin-4 as a control to exclude immunoreactivity of sera against the haemagglutinin tag. Syntaxin-4 is a single-pass type IV membrane protein of 298 amino acids with only three extracellular amino acids (C terminus amino acids 296–298) followed by the haemagglutinin tag. Syntaxin-4 therefore 'anchors' the haemagglutinin tag at the cell surface, and is a useful control for haemagglutinin immunoreactivity.

Lipofectamine™ was used to transfect human embryonic kidney 293 (HEK293) cells to obtain surface antigen-expressing cells ($HEK293^{NR1+, D1R+, D2R+, D3R+, D5R+ \text{ and } DAT+}$ cells) according to the manufacturer's instructions (Invitrogen). Control cells ($HEK293^{NR1-, D1R-, D2R-, D3R-, D5R- \text{ and } DAT-}$ cells) were obtained by transfection of HEK293 cells with empty vectors, either pcDNA-GFP or pIRES2-GFP. Additionally, GFP-positive $HEK293^{D2R+}$ cells were sorted by flow cytometry, and cultured under 250 μg/ml of Geneticin® (Invitrogen) in order to obtain a polyclonal stable transfectant.

Cell-based assay for detection of antibodies to cell surface D1R, D2R, D3R, D5R, DAT and NMDAR

We used fluorescence-activated cell sorting analysis to detect antibody binding of patient serum IgG to surface brain antigens transfected in HEK293 cells as previously described (Brilot *et al*., 2009, 2011). Two days after transfection, the cells were harvested using Versene™ (Invitrogen) and washed in phosphate-buffered saline (PBS) supplemented with 2% foetal bovine serum (FBS) (PBS/FBS). Then, 50 000 cells were incubated with serum at a 1:100 dilution in a V-bottom plate (Corning) for 30 min at room temperature. Cells were then washed three times with 200 μl PBS/FBS and incubated with Alexa Fluor® 647-conjugated goat anti-human IgG secondary antibody (Invitrogen) for 30 min at room temperature. Cells were washed three times with PBS/FBS and then resuspended in 50 μl PBS/FBS before analysis. Before acquisition, viability dye 7-AAD (BD Biosciences) was added to the cells to exclude dead cells. A total of 10 000 events/well were recorded on a BD LSRII instrument equipped with a high-throughput sampler (BD Biosciences). Data analysis was performed using FlowJo software (TreeStar) and Excel (Microsoft). Binding was expressed as mean fluorescence intensity as previously described (Brilot *et al*., 2009, 2011). Levels of antibody binding in GFP-positive-transfected cells were expressed as ΔMFI. ΔMFI was determined by the subtraction of mean fluorescence intensity obtained with $HEK293^{NR1-, D1R-, D2R-, D3R-, D5R- \text{ and } DAT-}$ cells from the mean fluorescence intensity obtained with $HEK293^{NR1+, D1R+, D2R+, D3R+, D5R+ \text{ and } DAT+}$ cells. A ΔMFI value greater than mean + 3 SDs of values of the healthy control samples was considered positive. Each experiment was performed at least three times. Cell-based assays were performed by blinded investigator and data were unblinded in order to calculate the threshold of positivity. All cell-based assays were optimized on prior assessment of antigen surface expression. Surface expression on transfected cells was analysed by flow cytometry after staining with a rabbit polyclonal anti-haemagglutinin antibody (Clontech), mouse monoclonal anti-human extracellular NMDAR subunit 1 antibody (BD Biosciences), rat monoclonal anti-human extracellular DAT (Santa Cruz Biotechnology Inc.) or goat polyclonal anti-human extracellular D3R (Merck) in combination with an Alexa Fluor® 647-conjugated appropriate secondary antibody (Invitrogen). Dot plots shown in Figs 1, 2, 4 and 6 were generated using Excel or Prism software version 4.0b (GraphPad Software, Inc.).

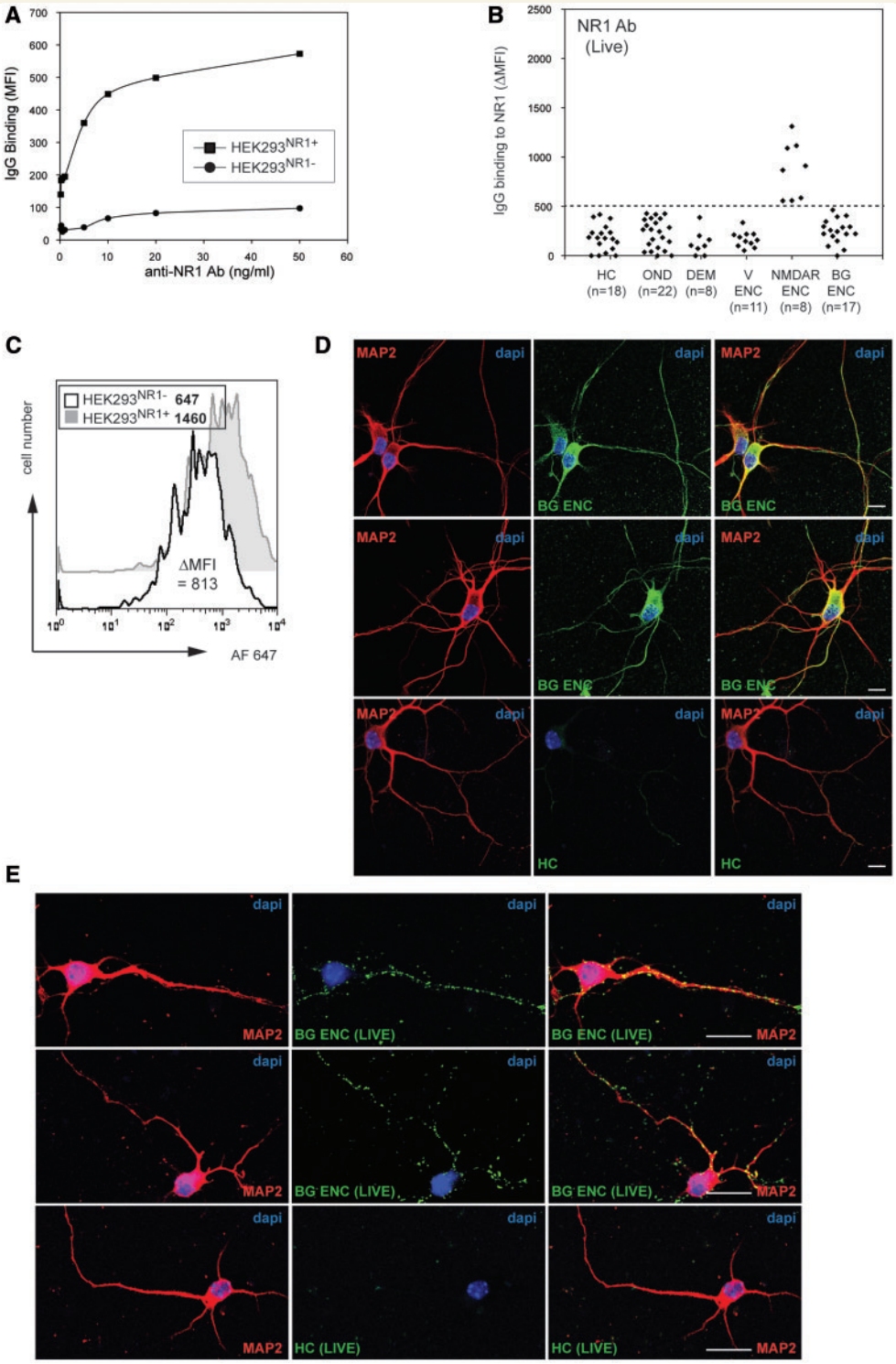

Figure 1 Basal ganglia encephalitis serum is negative for NMDAR antibody but is immunoreactive to hippocampal and striatal primary neurons. (**A**) Antibody reactivity to the subunit 1 of NMDAR (NR1) was determined by flow cytometry cell-based assay using HEK293^{NR1+} cells expressing surface NR1 subunit of NMDAR, and HEK293^{NR1-} cells. (**B**) Sera from healthy control (HC), other neurological disease (OND), first episode of demyelination syndromes (DEM), viral encephalitis (V ENC), NMDAR encephalitis (NMDAR ENC) and basal ganglia encephalitis (BG ENC) were incubated with live HEK293^{NR1+} and HEK293^{NR1-} cells at 1:50 dilution, followed by AF647-conjugated anti-human IgG secondary antibody and flow cytometry analysis. ΔMFI was calculated using mean fluorescence intensity obtained with live HEK293^{NR1+} and HEK293^{NR1-}. Positivity threshold was determined by ΔMFI of three standard deviations above the mean of healthy controls (dotted line on graph). NR1 antibody was detected in 0/17 patients with basal ganglia encephalitis and 0/59 controls, but in all eight patients with NMDAR encephalitis. Representative dot plot out of three experiments is shown. (**C**) Representative example of flow cytometry histograms for one NMDAR antibody-positive patient. Mean fluorescence intensity values are noted in legend. (**D**) Fixed and live

(continued)

Immunocytochemistry on primary culture of murine hippocampal and striatal neurons and HEK293 cells

Embryonic Day 16.5 mouse hippocampal and striatal neurons were cultured as previously described (Fath *et al*., 2009). Primary neurons and stably transfected HEK293 were fixed with 4% paraformaldehyde and incubated with patient or control sera (diluted at 1:50), primary antibodies, purified human IgG and/or immunoabsorbed sera. Cells were then washed and incubated with a secondary Alexa Fluor® 647-conjugated anti-human IgG antibody, or appropriate secondary antibodies (Invitrogen). Alternatively, live neurons were washed and incubated with patient and control sera (diluted 1:50) followed by incubation with appropriate secondary antibody before fixation/permeabilization and incubation with anti-rabbit polyclonal anti-microtubule-associated protein 2 antibody (MAP2, Millipore). To confirm that D2R was the main target antigen, two sera from control and D2R antibody-positive patients (selected for positivity for D2R antibody by flow cell-based assay and adequate volume of serum available) were serially incubated with six wells of live unpermeabilized $HEK293^{D2R+}$ or $HEK293^{D2R-}$ cells (Irani *et al*., 2010*a*; Lai *et al*., 2010). Successful immunoabsorption of D2R IgG was assessed by flow cytometry cell-based assay. We purified IgG from sera using protein G-agarose and Microcon (Millipore).

To visualize neurons and HEK293 cells, we used a confocal SP5 Leica microscope with ×100 1.4 NA oil immersion lenses (with a digital zoom of ×1.5 for Fig. 1D and ×1.43 for Fig. 1E). Pictures were overlaid using Metamorph software version 7.1 (Molecular Devices) and ImageJ software version 1.44o (National Institute of Health). Additionally, images from Alexa Fluor® 647-conjugated anti-human IgG staining shown in Figs 1 and 4 were pseudo-coloured in green for clarity.

Immunofluorescence on mouse brain from wild-type and D2R knock-out mice

Adult genotyped D2R knock-out mice on C57B6 background and wild-type littermates were deeply anaesthetized with an intraperitoneal injection of sodium pentobarbitone (100 mg/kg) and then transcardially perfused with heparinized 0.1 M PBS at 37°C followed by fresh 4% paraformaldehyde in 0.1 M PBS. Brains were embedded in optimum cutting temperature medium and immediately frozen in a dry ice–acetone bath. Sixteen-micrometre thick cryostat sections were thaw mounted on SuperFrost Plus® glass slides (Merzel) and stored at −20°C. Sections were washed in 0.1 M PBS, then blocked and permeabilized for 30 min at room temperature with 0.3% Triton® X-100 (Sigma) and 15% normal goat serum (Sigma) in 0.1 M PBS, and incubated overnight at 4°C with sera from healthy controls, and patients with basal ganglia encephalitis, NMDAR encephalitis, Sydenham's chorea and Tourette's syndrome (diluted 1:100 in 0.1 M PBS supplemented with 0.3% Triton® X-100 and 5% normal goat serum). Slides were washed in 0.1 M PBS and incubated with the appropriate secondary antibodies (Invitrogen) for 2 h at room temperature. Then, slides were incubated with anti-MAP2 antibody for 2 h at room temperature, washed and incubated with the secondary appropriate antibody for 2 h at room temperature. Slides were washed, mounted and images were acquired by confocal microscopy using a ×20 0.7 NA objective with or without a digital zoom of ×3.05. Analysis of serum binding to wild-type and D2R knock-out brain was performed in a blinded manner. All procedures on animals are approved by the Florey Neuroscience Institutes animal ethics committee and conform to the National Health and Medical Research Council of Australia's published code of practice.

Statistical analysis

Chi-square with Yates correction test was used to compare patient and control groups. A $P < 0.05$ was considered significant.

Results

Surface D2R IgG antibody in patients with basal ganglia encephalitis

We first optimized flow cytometry for detection of autoantibodies using NMDAR antibody-positive sera and HEK cells expressing the NMDAR subunit 1. The mean intensity of the fluorescence correlated with antibody concentration (Fig. 1A). Using the mean + 3 SDs to establish the threshold for positivity, NMDAR antibodies were found only in the NMDAR encephalitis group ($n = 8$) compared with all other groups ($n = 76$), including basal ganglia encephalitis, healthy controls, other neurological diseases and viral encephalitis (Fig. 1B and C; chi-square test with Yates correction, $P < 0.0001$), validating flow cytometry as an appropriate method to detect autoantibodies in patient sera.

Although negative for surface NMDAR antibody, basal ganglia encephalitis sera immunolabelled cell bodies and MAP2-positive dendrites of fixed non-permeabilized striatal neurons (Fig. 1D) and dendrites and cell surface of live primary neurons (Fig. 1E), suggesting that these sera had IgG that bound antigens expressed at the surface of neurons. In order to investigate the dopamine signalling pathway specifically, we expressed disease-relevant conformational surface D2R at the surface of HEK293 cells, and used these cells to detect antibody against D2R (Fig. 2). Importantly, due to the lack of a commercial antibody that binds to an extracellular epitope of D2R, we labelled the extracellular N-terminus of D2R with a haemagglutinin tag, which was readily detected on

Figure 1 Continued
(**E**) neurons (15 days *in vitro*) were co-labelled with sera from two patients with basal ganglia encephalitis or one healthy control sera followed by AF647 anti-human IgG secondary antibody (pseudocoloured in green) and stained with anti-neuron-specific microtubule-associated protein 2 (MAP2) antibody (red) followed by appropriate secondary antibody. Compared with healthy control sera, sera from patients with basal ganglia encephalitis immunolabelled non-permeabilized dendrites and cell bodies of fixed MAP2-positive neurons, whereas surface dendrites were immunolabelled in live MAP2-positive neurons. Scale bars: **D** = 6.6 μm; **E** = 20 μm. Representative images are shown. Nuclei are stained with DAPI (4′,6-diamidino-2-phenylindole). MFI = mean fluorescence intensity.

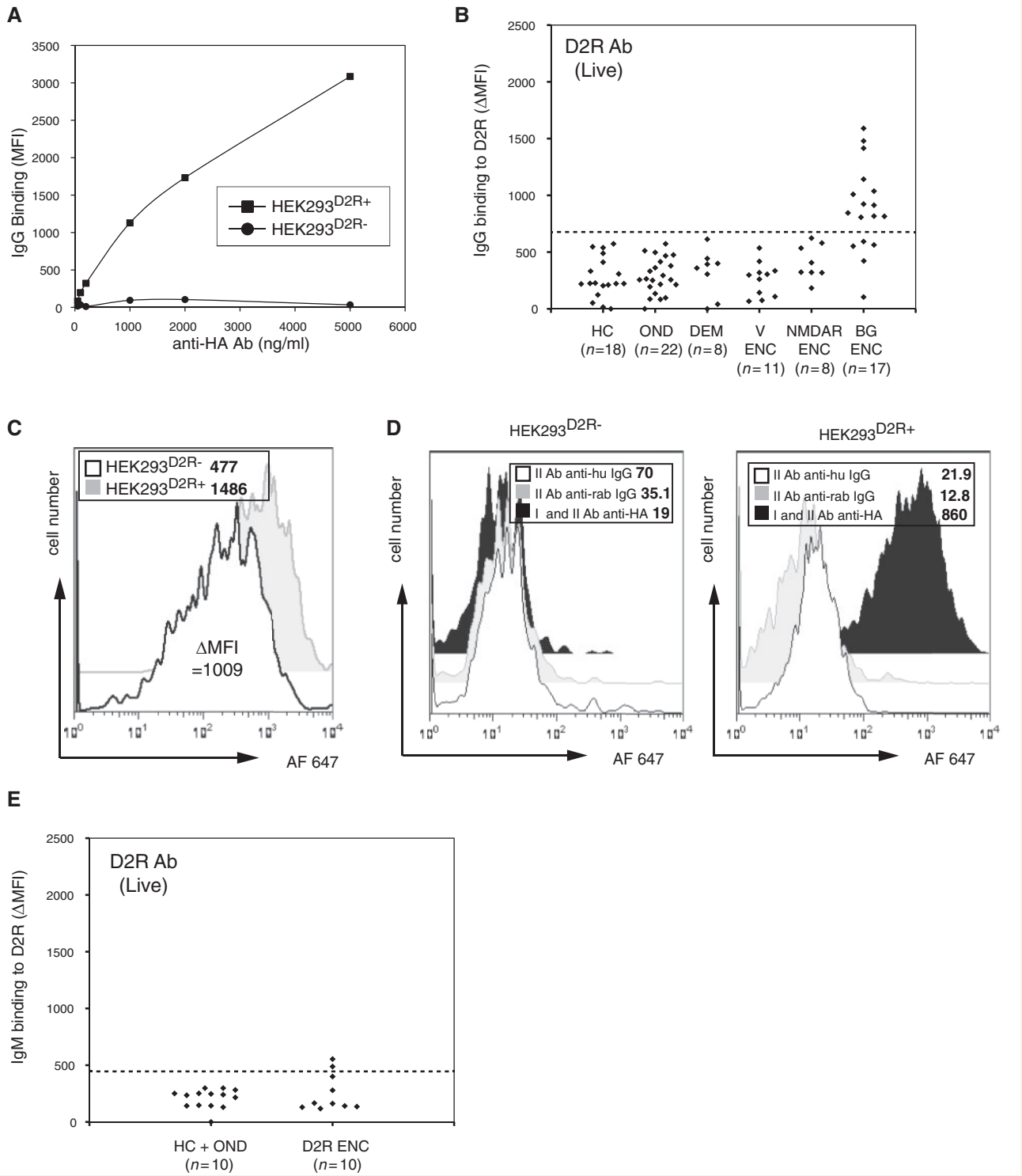

Figure 2 Basal ganglia encephalitis is associated with D2R antibody. (**A**) Antibody reactivity to D2R was determined by flow cytometry cell-based assay. (**B**) Human surface D2R IgG antibody was detected in 12/17 basal ganglia encephalitis samples and 0/67 controls. Dotted line on graph represents the positivity threshold. Representative dot plot out of five experiments is shown. Samples were considered positive if they were above threshold at least four times out of five repeated experiments. (**C**) Representative example of flow cytometry histograms for one D2R antibody-positive patient and (**D**) for staining controls on live HEK293^{D2R-} (*left*) and HEK293^{D2R+} cells (*right*). Controls shown are cells stained with secondary AF647-conjugated anti-rabbit IgG antibody only [II Ab anti rab IgG; secondary antibody for the anti-haemagglutinin (HA) Ab], secondary AF647-conjugated anti-human IgG antibody only (II Ab anti hum IgG; secondary antibody for human serum). For comparison, staining of cells with anti-haemagglutinin antibody followed by the appropriate secondary antibody is also shown (I and II Ab anti-HA). Only the latter staining gives high mean fluorescence intensity on HEK293^{D2R+} cells. Mean fluorescence intensity values are noted in legend. (**E**) Low levels of immunoglobulin M (IgM) were detected in 2/10 patients positive for D2R IgG antibody. Sera from 10/12 D2R-positive patients with encephalitis were available for testing due to inadequate sera. Representative dot plot out of three experiments is shown.

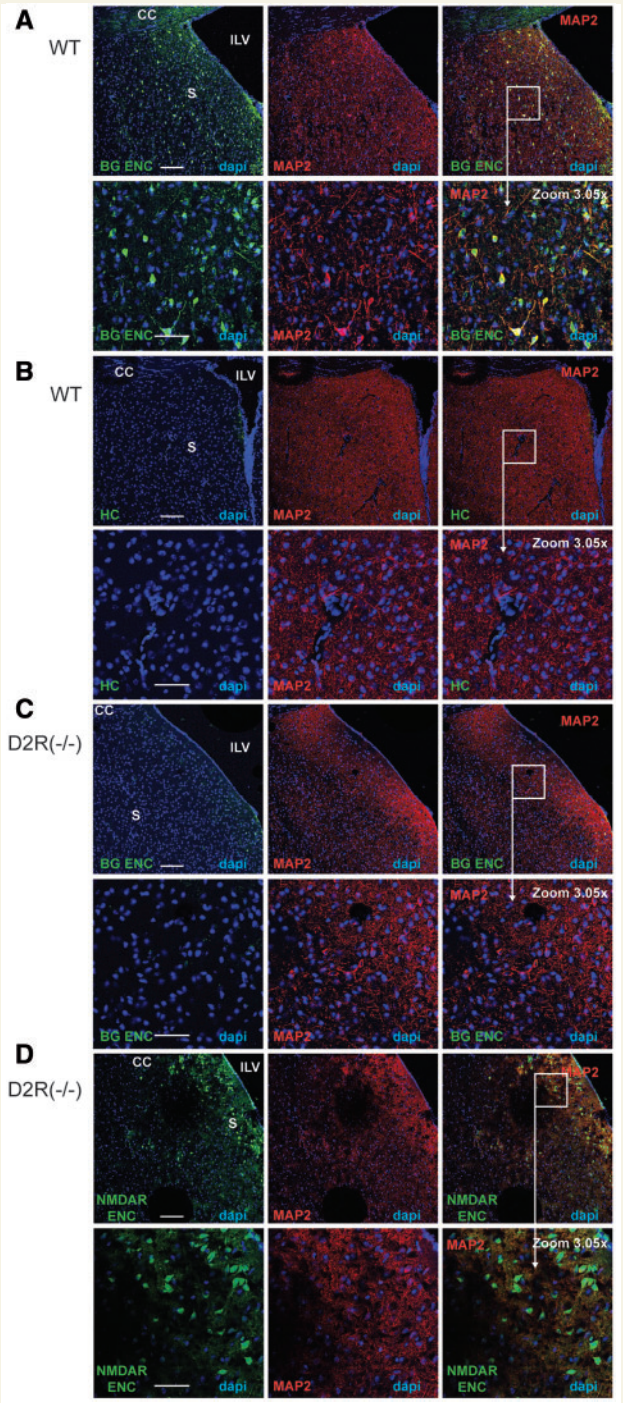

Figure 3 Immunoreactivity of basal ganglia encephalitis, NMDAR encephalitis and healthy control sera in the striatum of wild-type and D2R knock-out mice. Basal ganglia encephalitis (BG ENC, green) immunolabelled dendrites and cell bodies of MAP2$^+$ neurons (red) in striatum of wild-type (WT) mouse (**A**). In contrast, healthy control (HC) serum showed no significant immunolabelling to wild-type brain (**B**). The immunolabelling of basal ganglia encephalitis serum was significantly decreased in D2R knock-out D2R(−/−) striatum (**C**), whereas NMDAR encephalitis immunolabelling was still observed (NMDAR ENC; **D**). The same basal ganglia encephalitis serum is showed in panels **A** and **C**. *Lower* rows in **A–C** are 3.05-fold digital zoom from *top* images (white square), and show co-localization between MAP2 and serum staining. CC = corpus callosum; S = striatum; ILV = left lateral ventricle. Nuclei stained with DAPI. Scale bar = 100 μm and 50 μm in zoomed images. Representative images are shown.

unpermeabilized cells by immunocytochemistry and flow cytometry (Fig. 2A and D). An empty vector was negative for surface D2R staining, as were cells incubated with primary and secondary antibody controls (Fig. 2A and D). As shown in Fig. 1A, mean fluorescence intensity was directly dependent on antibody titre (Fig. 2A). Twelve of the 17 patients with basal ganglia encephalitis had serum antibodies to surface D2R (71%), compared with 0/67 controls (Fig. 2B and C; chi-square test with Yates correction, $P < 0.0001$). In contrast, only two patients showed any IgM reactivity to the cells (Fig. 2E). We were unable to demonstrate D2R antibodies in the four available CSFs by flow cytometry or by immunocytochemistry (data not shown).

To further determine whether basal ganglia encephalitis sera bound to neurons, six sera (from three patients with basal ganglia encephalitis and three from healthy control subjects) were applied to wild-type mouse brains, and co-labelled with the neuronal marker MAP2. Basal ganglia encephalitis samples brightly co-immunolabelled MAP2-positive neurons in the striatum (Fig. 3A), whereas healthy control sera did not (Fig. 3B). Importantly, the basal ganglia encephalitis immunolabelling was significantly decreased in D2R knock-out striatum (Fig. 3C), whereas NMDAR encephalitis immunolabelling was still observed (Fig. 3D). Immunoreactivity was clearly visible on the cell surface of unpermeabilized HEK293^{D2R+} cells, and co-localized with surface haemagglutinin-D2R staining, whereas there was no staining with IgGs purified from healthy control sera (Fig. 4A). To confirm the specificity for D2R, we successfully immunoabsorbed sera from two patients with basal ganglia encephalitis using HEK293^{D2R+} cells (Fig. 4B), and found that absorption decreased immunolabelling of HEK293^{D2R+} cells, primary neurons and wild-type mouse brains (Fig. 4C–E).

In addition, we show that the D2R antibody-positive sera did not bind to an extracellular haemagglutinin-tagged irrelevant antigen (syntaxin-4) or extracellular N-terminal haemagglutinin-tagged D5R (Supplementary Fig. 2), further confirming that sera are immunoreactive against D2R and not haemagglutinin.

Absence of surface D3R, D1R, D5R and DAT IgG antibody in patients with basal ganglia encephalitis

To determine whether the sera might also contain antibodies to other proteins in the dopamine signalling pathway, we performed similar cell-based assays with cells expressing D1R, D3R and DAT. We could not detect IgG against human surface D3R, D1R or DAT in any patient or control sera (Supplementary Fig. 1).

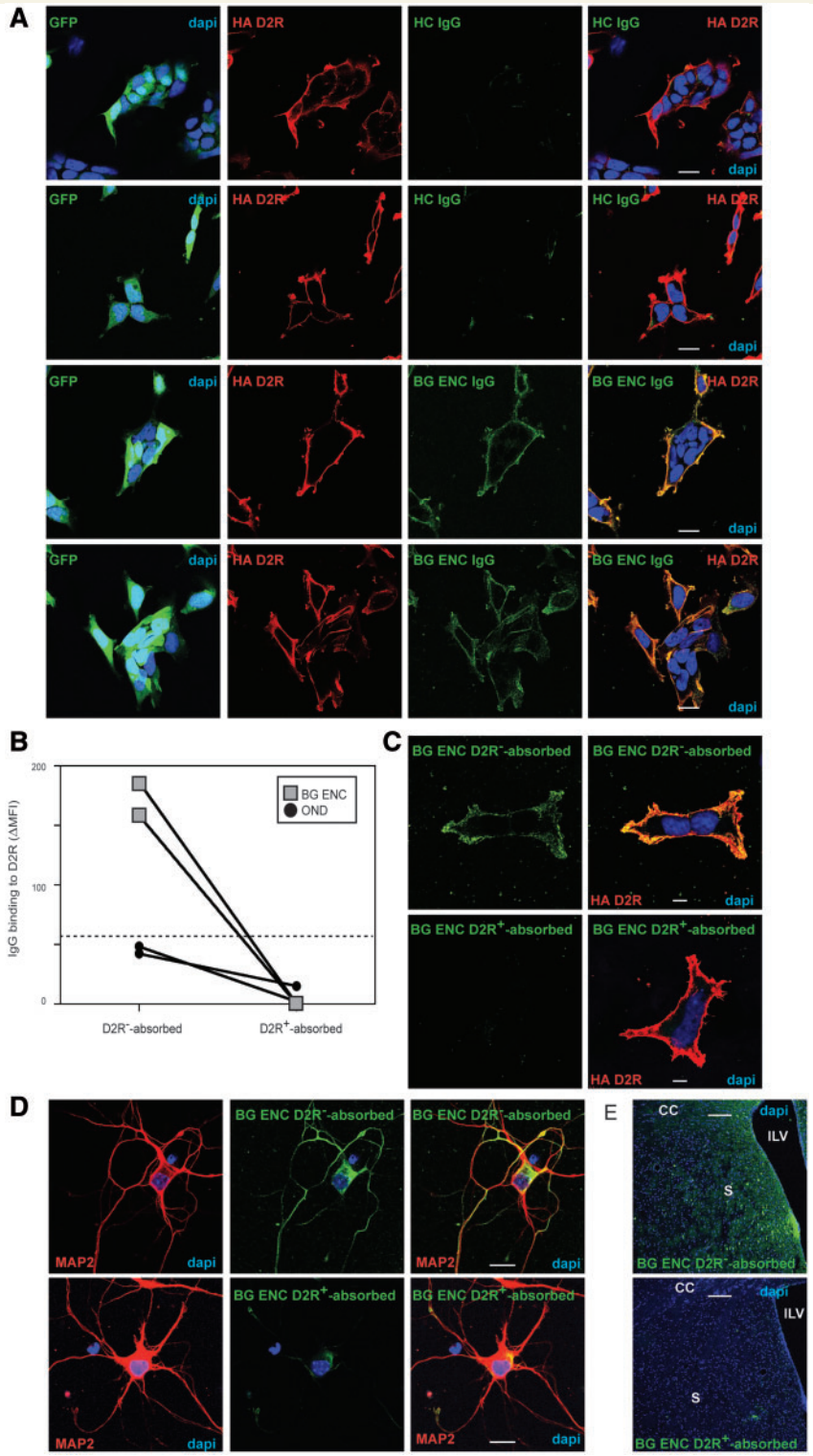

Figure 4 (**A**) Immunocytochemistry on fixed unpermeabilized HEK293^{D2R+} cells confirmed basal ganglia encephalitis IgG co-labelled GFP-positive and haemagglutinin-D2R-positive (red) cells (GFP shown in green, *left* panel). Both haemagglutinin-D2R and basal ganglia encephalitis (BG ENC) IgG immunoreactivities are localized on the cell surface, whereas GFP is cytoplasmic. Healthy control (HC) IgG showed no significant immunoreactivity. Nuclei stained with DAPI. Scale bar = 20 μm. (**B**) Immunoreactivity to D2R in HEK293^{D2R-}- and HEK293^{D2R+}-immunoabsorbed basal ganglia encephalitis and other neurological diseases (ONDs) sera was assessed by flow cell-based assay. HEK293^{D2R-}-immunoabsorbed basal ganglia encephalitis sera were above the threshold of positivity (dotted line), whereas the immunoabsorption of basal ganglia encephalitis with HEK293^{D2R+} cells decreased the immunoreactivity to D2R. Immunoabsorption of basal ganglia encephalitis sera with HEK293^{D2R+} cells also significantly reduced the surface labelling of HEK293^{D2R+} cells (**C**), primary MAP2^{+} neurons (**D**) and brain wild-type sections (**E**) compared with immunoabsorption with HEK293^{D2R-} cells. CC = corpus callosum; S = striatum; lLV = left lateral ventricle. Nuclei stained with DAPI. Scale bars: **C** = 6.70 μm; **D** = 10 μm; **E** = 100 μm. Representative images are shown.

Clinical features of D2R antibody-positive basal ganglia encephalitis

Table 2 summarizes the clinical characteristics and features of the 12 patients with D2R antibody-positive basal ganglia encephalitis. There was an even sex distribution, and children of all ages were affected. The children were of mixed ethnic background (Caucasian 5/12, Afro-Caribbean 3/12, East Asian 2/12, South Asian 1/12 and Polynesian 1/12). Symptom onset frequently occurred in the post-infectious or post-vaccine setting. Five out of 12 had clinical history and serology suggestive of preceding infection with β-haemolytic *Streptococcus* ($n = 3$), mycoplasma pneumonia ($n = 1$) and enterovirus ($n = 1$). The symptoms at onset were variable, but lethargy, psychiatric symptoms, abnormal movements or gait disturbance were typical. The established clinical syndrome was dominated by a spectrum of movement disorders, including dystonia, parkinsonism and chorea. Many of the patients with dystonia had coarse tremor compatible with dystonic tremor. Oculogyric crises occurred in three patients with dystonia or parkinsonism, and ocular flutter occurred in one child with chorea and ataxia. Psychiatric disturbance occurred in 9/12, particularly agitation, emotional lability, anxiety and psychotic symptoms. Sleep disturbance, lethargy, drowsiness, brainstem dysfunction, seizures and ataxia occurred less commonly. Investigation for a tumour was not routinely performed, although examination for neuroblastoma in four patients was negative. Brain MRI was normal in 6/12 (Supplementary Table 3), but when abnormal, showed inflammatory changes localizing to basal ganglia and brainstem structures (Fig. 5). The CSF was abnormal in 9/12 patients, although pleocytosis occurred in only three patients, and the median CSF cell count was 1 monocyte/mm^3 (Supplementary Table 3). The EEG was either normal or showed non-specific slowing compatible with encephalopathy. No patient had epileptic features on EEG.

Due to the diagnostic uncertainty, the patients were treated empirically and some patients did not receive immune therapy (Supplementary Table 3). The outcome was variable with a full recovery occurring in only 5/12 (Supplementary Tables 3 and 4). Motor, cognitive and psychiatric morbidity commonly occurred. Dystonia, dysexecutive cognitive problems, attention deficit disorder and psychosis were characteristic outcomes. Five out of 12 patients had problems with learning, with rigid thinking and dysexecutive features, although formal neuropsychology was only performed in four patients. Two patients with abnormal acute scans have shown basal ganglia atrophy and gliosis on follow-up; these patients have persistent cognitive and psychiatric morbidity.

Although the cohort was treated empirically, the most recent patients have been treated aggressively and early with high dose methylprednisolone, oral prednisolone taper and concomitant intravenous immunoglobulin; these patients have made a complete recovery (Supplementary Tables 3 and 4). Acute and convalescent sera were available for three D2R antibody-positive patients (Supplementary Fig. 3). The two patients diagnosed retrospectively only received steroid treatment, have been left with permanent disability and have elevated D2R antibody on

Table 2 Clinical comparison of D2R antibody-positive ($n = 12$) and negative ($n = 5$) basal ganglia encephalitis patients

Characteristic	D2R antibody-positive ($n = 12$)	D2R antibody-negative ($n = 5$)
Demographics		
Sex: male	6/12	2/5
Age, mean, median (range)	7.4, 5.5 (1.6–15)	6.5 (0.4–10)
Non-White	7/12	1/5
Prodromal symptoms		
Infection	6/12	4/5
Vaccination	2/12	0/5
Drug	1/12	0/5
Nil	3/12	1/5
Symptom presentation		
Lethargy, drowsy	5/12	2/5
Movement disorder	4/12	0/5
Paranoia, psychosis, hallucinations	3/12	0/5
Agitation, anxiety	2/12	2/5
Gait disturbance, ataxia	2/12	0/5
Seizure	2/12	1/5
Movement disorder		
Any movement disorder	12/12	5/5
Dystonia, including dystonic tremor	5/12	4/5
Parkinsonism	5/12	2/5
Oculogyric crisis	3/12	0/5
Chorea	3/12	0/5
Hemidystonia and hemichorea	1/10	0/5
Ocular flutter	1/12	0/5
Psychiatric symptoms		
Any psychiatric change or personality change	9/12	3/5
Agitation	5/12	0/5
Psychosis or hallucinations	3/12	0/5
Emotional lability, anxiety	2/12	3/5
Compulsive touching	2/12	0/5
Aggression	1/12	0/5
Sleep disorder		
Any sleep disorder	6/12	3/5
Somnolence, lethargy	4/12	2/5
Insomnia	2/12	1/5
Other		
Fever at any stage	6/12	3/5
Encephalopathy[a]	12/12	5/5
Reduced consciousness or confusion	5/12	3/5
Autonomic or hiccough	3/12	0/5
Ophthalmoplegia	2/12	0/5
Pyramidal weakness	2/12	1/5
Mutism	2/12	2/5
Seizures	2/12	2/5
Ataxia	2/12	0/5
MRI abnormal[b]	6/12	2/5

[a]Encephalopathy definition as per Granerod *et al*. (2010).
[b]Lesions localized to basal ganglia only.

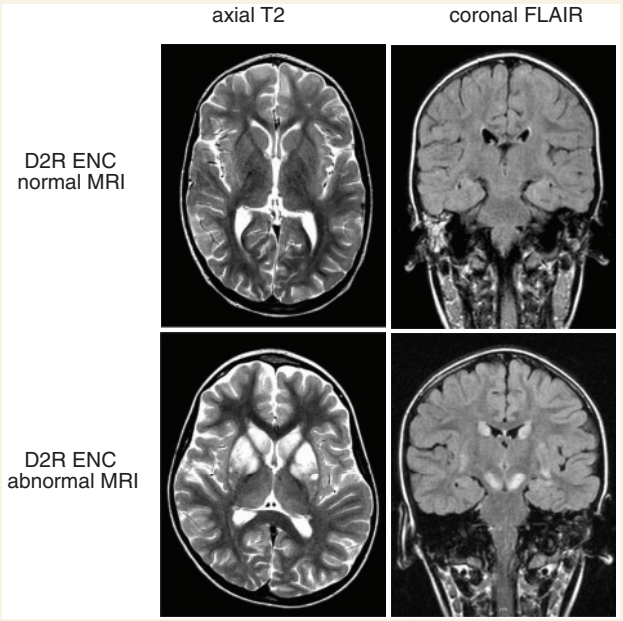

Figure 5 D2R antibody-positive encephalitis (ENC) is associated with normal and abnormal brain MRI. MRI were normal in 6/12 D2R antibody-positive patients with acute encephalitis (*upper* panel), and abnormal in 6/12 D2R antibody-positive patients with acute encephalitis (*lower* panel). In MRI abnormal patients, T_2 axial image (*left* panel) shows enhancement in caudate, putamen and globus pallidus, and T_2 FLAIR coronal image (*right* panel) shows enhancement in substantia nigra.

convalescent testing. The prospectively diagnosed patient was treated with both steroid and intravenous immunoglobulin, has made a complete clinical recovery and D2R antibody has normalized on convalescent testing (Supplementary Fig. 3).

Spectrum of D2R antibody-associated movement and psychiatric disorders

We measured serum IgG reactivity to D2R in other putative autoimmune basal ganglia disorders, including Sydenham's chorea, PANDAS and Tourette's syndrome. We detected surface D2R IgG antibody in the sera of 10/30 patients with Sydenham's chorea (30%), 0/22 with PANDAS (0%) and 4/44 with Tourette's syndrome (9%) compared with 0/40 of control subjects (Fig. 6A, chi-square test with Yates correction, Sydenham's chorea versus controls, $P < 0.0001$, Tourette's syndrome versus controls, $P < 0.11$). Samples were considered positive if they were above threshold at least three times out of four repeated experiments. We could not detect any surface D1R IgG antibody in any sera (Fig. 6B). Immunolabelled live neurons from D2R antibody-positive patients with Sydenham's chorea and Tourette's syndrome (Fig. 6C), co-labelled with MAP2 in the striatum of wild-type mouse (Fig. 7A and B) and the immunolabelling was significantly decreased in D2R knock-out striatum, suggesting that D2R is the main antibody target in these patients (Fig. 7C and D).

Clinical features of D2R antibody-positive Sydenham's chorea and Tourette's syndrome

No obvious differences were observed between Sydenham's chorea patients positive or negative for D2R antibody (Supplementary Table 5). Clinical histories of patients with D2R antibody-positive Tourette's syndrome are presented in the Supplementary material. Interestingly, 2/5 of the females with Tourette's syndrome were D2R antibody positive, whereas only 2/39 males with Tourette's syndrome were D2R antibody positive.

Clinical and demographic findings of D2R antibody positivity in autoimmune basal ganglia disorders

We identified a total of 26 individuals with D2R antibody in basal ganglia disorders. D2R antibodies were more common in females (17/45) compared with males (9/68, chi-square test with Yates correction, $P < 0.005$), and non-White (13/35) compared with White patients (13/78, chi-square test with Yates correction, $P < 0.05$). Streptococcal-O titres were positive (>200 IU/ml) in all of the patients with Sydenham's chorea, but in only 3/11 of the patients with basal ganglia encephalitis. The movement disorder phenotype varied according to gender. In females positive for D2R antibodies, the movement disorders in descending order of frequency were chorea ($n = 13$), dystonia–parkinsonism ($n = 3$) and tics ($n = 2$); in D2R antibody-positive males, the movement disorders were dystonia–parkinsonism ($n = 7$), tics ($n = 2$) and chorea ($n = 1$).

Discussion

We have previously shown that 10 out of 20 patients with encephalitis and dyskinesia, encephalopathy and seizures, previously considered to be a dyskinetic form of encephalitis lethargica (Dale *et al*., 2001, 2002), had NMDAR antibodies, indicating that they have NMDAR encephalitis (Dale *et al*., 2009). Herein, we show that some patients with basal ganglia encephalitis without NMDAR antibodies, and with a syndrome dominated by movement disorders, have serum IgG antibodies against surface D2R, which we call D2R antibody-positive encephalitis. Whereas we did not find these antibodies in a large number of relevant healthy and disease controls, they were also present in a proportion of patients with Sydenham's chorea and Tourette's syndrome.

D2R is an essential receptor that regulates dopaminergic neurotransmission. Alternative splicing of D2R generates two major variants, D2-short and -long, which differ by the presence of an additional 29 amino acids in the third intracellular loop (Giros *et al*., 1989). D2-long has been shown to be predominantly expressed postsynaptically, whereas D2-short is expressed presynaptically and functions as an autoreceptor (Usiello *et al*., 2000). Although both isoforms have an identical extracellular sequence, we used D2-long to detect the antibodies, and further studies are needed to determine whether the autoantibodies can

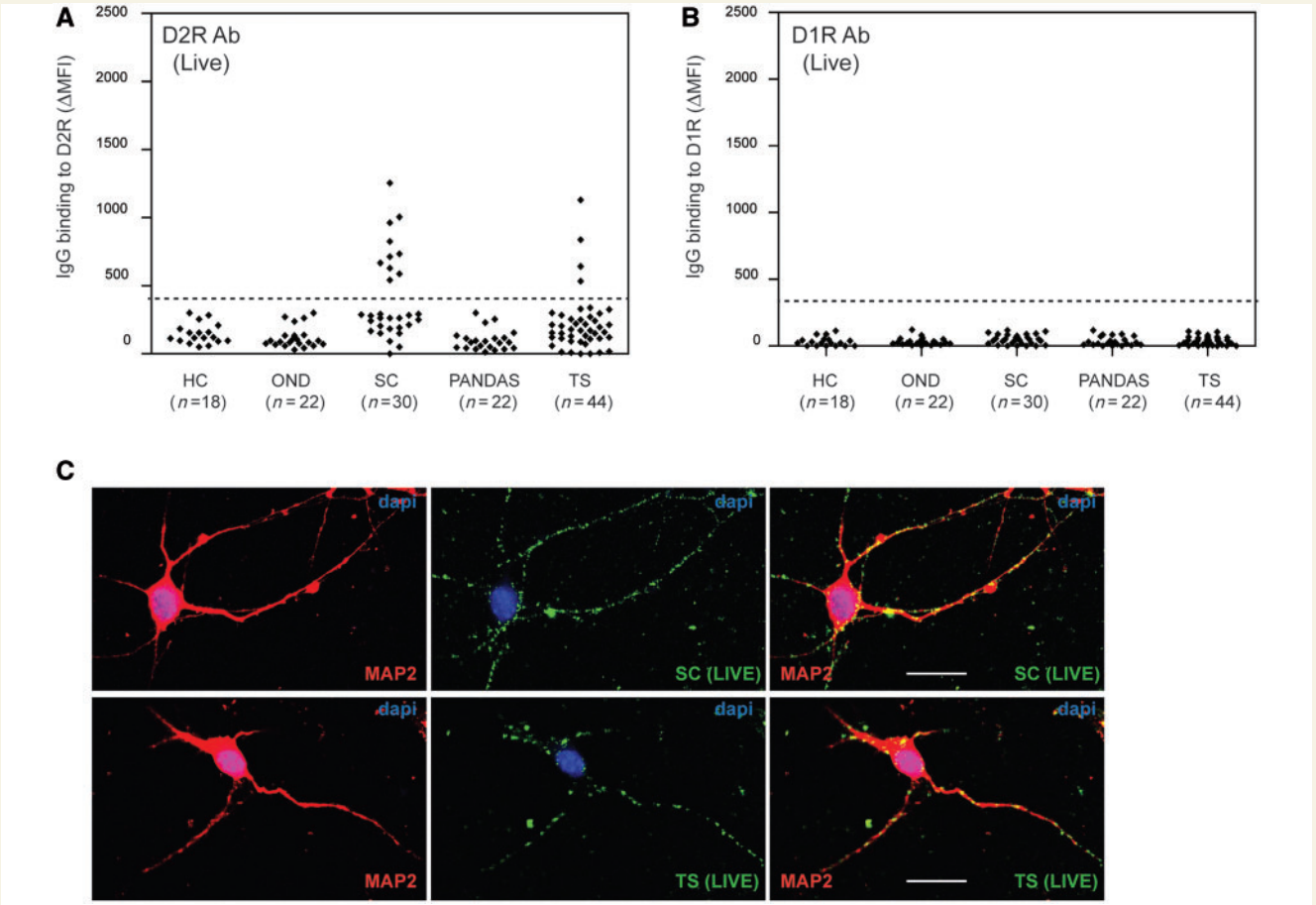

Figure 6 Sydenham's chorea and Tourette's syndrome, but not PANDAS, are associated with D2R antibody. (**A**) Human surface D2R IgG antibody was detected in 10/30 patients with Sydenham's chorea (SC), 0/22 with PANDAS, 4/44 with Tourette's syndrome (TS) and 0/40 healthy control (HC) and other neurological diseases (OND) control subjects. (**B**) Patients with Sydenham's chorea, PANDAS and Tourette's syndrome do not have D1R antibody. Dotted lines on graphs (**A** and **B**) represent the positivity threshold. Representative dot plots out of four (D2R) and three (D1R) experiments are shown. (**C**) Live neurons (15 days *in vitro*) were co-labelled with sera from one D2R IgG-positive patient with Sydenham's chorea or one D2R IgG-positive patient with Tourette's syndrome, followed by AF647 anti-human IgG secondary antibody (pseudocoloured in green), and stained with anti-MAP2 antibody (AF555, red). Both sera from patients with Sydenham's chorea and from Tourette's syndrome immunolabelled surface dendrites of $MAP2^+$ neurons. Scale bar = 20 μm. Representative images are shown. Nuclei stained with DAPI.

also bind to the D2-short receptor, and to define the epitope(s) involved in autoantibody binding. In contrast, although basal ganglia-expressed D1R, D2R and D3R are closely related (up to 72% amino acid homology), we did not identify antibodies to surface D1R and D3R, or to the DAT, in any of the sera tested, but that does not exclude the possibility that they exist in other disease entities.

The techniques used to detect antibodies against surface and conformational epitopes are critical. The use of cell-based assays, such as ours, either using flow cytometry or via immunofluorescence, has been highly successful in identifying novel antibody-associated diseases (Dalmau *et al*., 2007; Hutchinson *et al*., 2008; Brilot *et al*., 2009; Dale *et al*., 2009; Lai *et al*., 2009, 2010; Irani *et al*., 2010*a, b*; Lancaster *et al*., 2010) and also in patients with demyelinating disease (O'Connor *et al*., 2007; Brilot *et al*., 2009; McLaughlin *et al*., 2009; Probstel *et al*., 2011; Waters *et al*., 2012). Importantly, antibodies that bind to the extracellular domain of important proteins involved in neurotransmission are highly likely to be pathogenic (Dalmau *et al*., 2008; Lai *et al*., 2009; Hughes *et al*., 2010; Vincent *et al*., 2011; Bien *et al*., 2012), although pathogenic mechanisms have not yet been demonstrated in most instances. In contrast, previous studies of patients with basal ganglia encephalitis used western blotting, a technique that does not readily recognize extracellular epitopes of membrane proteins, and have not proved to be helpful in identifying highly relevant cell surface antigenic targets (Dale *et al*., 2004, 2006). Our findings contrast with a recent report that found D1R and D2R antibodies in both patients with Sydenham's chorea and those with PANDAS, but this study used ELISA and western blotting with D2R- and D1R-enriched membrane fractions, which would reveal intracellular epitopes (Brimberg *et al*., 2012).

In our cohort, the D2R antibody-positive patients with encephalitis were children, but it is probable that adults will also be affected. Although a search for tumours was not systematic, no

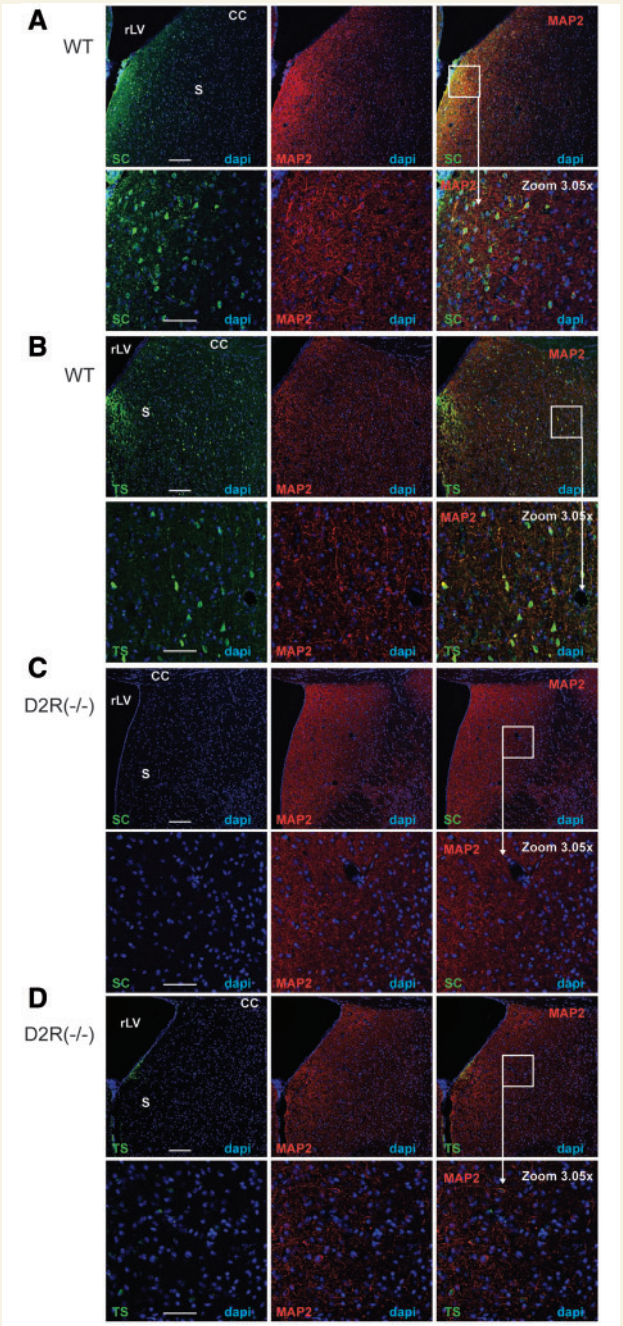

Figure 7 Immunoreactivity of Sydenham's chorea and Tourette's sydrome sera in the striatum of wild-type and D2R knock-out D2R(−/−) mice. D2R IgG antibody-positive Sydenham's chorea (SC, **A**) and Tourette's syndrome (TS, **B**) sera (green) immunolabelled dendrites and cell bodies of MAP2-positive neurons (red) in striatum of wild-type mouse. The immunolabelling of sera from patients with Sydenham's chorea (**C**) and from Tourette's syndrome (**D**) was significantly decreased in D2R knock-out striatum. The same Sydenham's chorea and Tourette's syndrome sera are showed in **A** and **C**, and **B** and **D**, respectively. *Lower* rows in **A–D** are 3.05-fold digital zoom from *top* images (white square), and show co-localization between MAP2 and serum staining. CC = corpus callosum; S = striatum; rLV = right lateral ventricle. Nuclei stained with DAPI. Scale bar = 100 and 50 μm in zoomed images. Representative images are shown.

tumours were found during the follow-up and a paraneoplastic syndrome seems unlikely, but cannot be excluded. There were no discernable clinical or radiological differences between the 12 D2R antibody-positive and the five D2R antibody-negative patients, and the five patients with basal ganglia encephalitis negative for D2R antibody may have antibodies below the detection limit of our assays, an alternative antigenic target or an unidentified viral infection.

Movement disorders dominated the clinical syndrome of D2R antibody-positive encephalitis, and included dystonia, dystonic tremor, oculogyric crises, parkinsonism and chorea, sometimes co-existing in the same patient. Psychiatric features were also common but not universal, particularly agitation, anxiety and psychosis. Sleep disturbance was also common, as was other brainstem dysfunction. Seizures occurred only in 20% of the D2R antibody-positive encephalitis, significantly less than paediatric NMDAR encephalitis (77%) (Florance *et al*., 2009). A relapsing course affected 3/12 patients, which is a similar rate to NMDAR encephalitis (Dalmau *et al*., 2011).

MRI was normal in 50% of the D2R antibody-positive encephalitis cases, but when abnormal, the lesions were localized to the basal ganglia. Interestingly, MRI is also often normal in NMDAR encephalitis (Dale *et al*., 2009; Florance *et al*., 2009), which emphasizes the importance of autoantibodies in the identification of these disorders. EEG was rarely contributory and often normal, further suggesting D2R antibody-positive encephalitis predominantly affects subcortical rather than cortical regions, in contrast to NMDAR encephalitis, which affects both (Irani *et al*., 2010*b*; Dalmau *et al*., 2011).

We were unable to detect CSF D2R antibodies in the few samples available, which could be due to a lack of sensitivity of these assays or represent a genuine absence of intrathecal immunoglobulin synthesis in these patients. The detection and role of intrathecal versus serum antibody may vary in different antibody-associated brain diseases, as may pathogenic mechanisms and pathological findings (Martinez-Hernandez *et al*., 2011; Bien *et al*., 2012), as discussed elsewhere (Dalmau *et al*., 2011; Lancaster *et al*., 2011; Vincent *et al*., 2011). One proposed but not proven mechanism of antibody access into the brain parenchyma is penetration directly from the bloodstream, which could be influenced by the presence of systemic inflammation (Dalmau *et al*., 2008; Vincent *et al*., 2011). Indeed fever or a preceding infection was common in D2R antibody-positive patients with encephalitis, although no consistent infectious precipitant was identified.

The best therapeutic approach is difficult to assess since recovery was variable with frequent residual motor, cognitive and psychiatric morbidity. However, taking lessons from the treatment of NMDAR encephalitis, recently admitted patients with D2R antibody-positive encephalitis have been treated promptly and aggressively with high dose steroids and intravenous immunoglobulin, and have made good recoveries with normalization of D2R antibody titre in one patient. Further study to determine the optimal therapeutic approach is required. Additionally, although immune therapy is recommended in autoimmune encephalitis, a spontaneous and complete recovery occurred without immune

therapy in two of the patients suggesting that the autoimmune process can be spontaneously reversible.

There are a number of similarities between basal ganglia encephalitis and Sydenham's chorea; both syndromes are typically post-infectious movement disorders evolving over days or weeks, both syndromes can respond to immune therapies and both syndromes have a small but significant risk of relapse (Garvey *et al*., 2005; Walker *et al*., 2012). Although recent streptococcal infection was evident in all D2R antibody-positive patients with Sydenham's chorea, only a minority of patients with basal ganglia encephalitis had positive streptococcal serology, suggesting that D2R antibody is not dependent upon streptococcal immunoreactivity. The D2R antibody-positive patients with encephalitis also tended to have a more severe clinical syndrome than those with Sydenham's chorea, and were more likely to have basal ganglia radiological abnormalities and residual impairments. Intriguingly, Greenfield and Wolfsohn (1922) reported that the pathology of basal ganglia encephalitis (so called encephalitis lethargica) and Sydenham's chorea showed significant similarities, with perivenous inflammation predominantly affecting the striatum.

In contrast to Sydenham's chorea, we did not detect D2R antibody in patients with PANDAS, highlighting a further immunological difference between Sydenham's chorea and PANDAS, already suggested in our previous work (Brilot *et al*., 2011). The hallmark of PANDAS is the rapid infection-associated deteriorations, which typically occur overnight. Likewise, improvements in PANDAS can occur very rapidly in association with the use of antibiotics. This rapid remission with antibiotic therapy would be considered atypical of accepted autoantibody-associated CNS disorders, such as NMDAR encephalitis, which are characterized by a slower evolution and remission of disease over weeks and months (Dalmau *et al*., 2008). We therefore suggest that other immune mechanisms may be operating in PANDAS. One caveat is that serum sampling in basal ganglia encephalitis and Sydenham's chorea was performed during the acute phase, whereas serum sampling in PANDAS and Tourette's syndrome was performed during the chronic active phase. Longitudinal studies are needed to understand temporal fluctuations of D2R antibodies in these patient subgroups. An interesting finding in our report is the presence of D2R antibodies in a small subgroup of children with Tourette's syndrome, a common movement and neuropsychiatric disorder. Dopaminergic dysfunction is largely accepted as being a prime abnormality in Tourette's syndrome (Felling and Singer, 2011). Furthermore, although still controversial, there is a body of literature describing immune abnormalities in patients with Tourette's syndrome, and the presence of an autoimmune subgroup in Tourette's syndrome has been proposed for some time (Martino *et al*., 2009; Felling and Singer, 2011; Landau *et al*., 2012). The role of autoantibodies in chronic neurological and psychiatric disease are an important and growing area of interest, including voltage-gated potassium channel complex antibody in epilepsy (McKnight *et al*., 2005), NMDAR antibody in schizophrenia (Zandi *et al*., 2011) and NMDAR antibody of the immunoglobulin A isotype in atypical dementia (Pruss *et al*., 2012). The role of D2R antibodies in large cohorts of Tourette's syndrome and associated disorders merits further investigation.

Finally, we found that females and patients of non-White ethnicity were positively associated with D2R antibodies, which are described risk factors for NMDAR encephalitis and autoimmunity in general (Florance *et al*., 2009; Dalmau *et al*., 2011; Vincent *et al*., 2011).

We propose that D2R antibody can define movement and psychiatric disorders of autoimmune origin. D2R antibody may prove to be an essential diagnostic biomarker to help early intervention and improve outcome in patients with movement and psychiatric disorders.

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Conflict of interest: A patent has been filed by the F.B. and R.C.D. (University of Sydney) claiming D2R as target for autoantibodies.

Supplementary material

Supplementary material is available at *Brain* online.

References

Beaulieu JM, Gainetdinov RR. The physiology, signaling, and pharmacology of dopamine receptors. Pharmacol Rev 2011; 63: 182–217.

Bien CG, Vincent A, Barnett MH, Becker AJ, Blumcke I, Graus F, et al. Immunopathology of autoantibody-associated encephalitides: clues for pathogenesis. Brain 2012; 135: 1622–38.

Brilot F, Dale RC, Selter RC, Grummel V, Kalluri SR, Aslam M, et al. Antibodies to native myelin oligodendrocyte glycoprotein in children with inflammatory demyelinating central nervous system disease. Ann Neurol 2009; 66: 833–42.

Brilot F, Merheb V, Ding A, Murphy T, Dale RC. Antibody binding to neuronal surface in Sydenham chorea, but not in PANDAS or Tourette syndrome. Neurology 2011; 76: 1508–13.

Brimberg L, Benhar I, Mascaro-Blanco A, Alvarez K, Lotan D, Winter C, et al. Behavioral, pharmacological, and immunological abnormalities after streptococcal exposure: a novel rat model of sydenham chorea and related neuropsychiatric disorders. Neuropsychopharmacology 2012. Advance Access published on April 25, 2012, doi: 10.1038/npp.2012.56.

Cardoso F, Eduardo C, Silva AP, Mota CC. Chorea in fifty consecutive patients with rheumatic fever. Mov Disord 1997; 12: 701–3.

Church AJ, Cardoso F, Dale RC, Lees AJ, Thompson EJ, Giovannoni G. Anti-basal ganglia antibodies in acute and persistent Sydenham's chorea. Neurology 2002; 59: 227–31.

Dale RC, Candler PM, Church AJ, Wait R, Pocock JM, Giovannoni G. Neuronal surface glycolytic enzymes are autoantigen targets in post-streptococcal autoimmune CNS disease. J Neuroimmunol 2006; 172: 187–97.

Dale RC, Church AJ, Benton S, Surtees RA, Lees A, Thompson EJ, et al. Post-streptococcal autoimmune dystonia with isolated bilateral striatal necrosis. Dev Med Child Neurol 2002; 44: 485–9.

Dale RC, Church AJ, Cardoso F, Goddard E, Cox TC, Chong WK, et al. Poststreptococcal acute disseminated encephalomyelitis with basal ganglia involvement and auto-reactive antibasal ganglia antibodies. Ann Neurol 2001; 50: 588–95.

Dale RC, Church AJ, Surtees RA, Lees AJ, Adcock JE, Harding B, et al. Encephalitis lethargica syndrome: 20 new cases and evidence of basal ganglia autoimmunity. Brain 2004; 127: 21–33.

Dale RC, Irani SR, Brilot F, Pillai S, Webster R, Gill D, et al. N-methyl-D-aspartate receptor antibodies in pediatric dyskinetic encephalitis lethargica. Ann Neurol 2009; 66: 704–9.

Dalmau J, Gleichman AJ, Hughes EG, Rossi JE, Peng X, Lai M, et al. Anti-NMDA-receptor encephalitis: case series and analysis of the effects of antibodies. Lancet Neurol 2008; 7: 1091–8.

Dalmau J, Lancaster E, Martinez-Hernandez E, Rosenfeld MR, Balice-Gordon R. Clinical experience and laboratory investigations in patients with anti-NMDAR encephalitis. Lancet Neurol 2011; 10: 63–74.

Dalmau J, Tuzun E, Wu HY, Masjuan J, Rossi JE, Voloschin A, et al. Paraneoplastic anti-N-methyl-D-aspartate receptor encephalitis associated with ovarian teratoma. Ann Neurol 2007; 61: 25–36.

Fath T, Ke YD, Gunning P, Gotz J, Ittner LM. Primary support cultures of hippocampal and substantia nigra neurons. Nat Protoc 2009; 4: 78–85.

Felling RJ, Singer HS. Neurobiology of Tourette syndrome: current status and need for further investigation. J Neurosci 2011; 31: 12387–95.

Florance NR, Davis RL, Lam C, Szperka C, Zhou L, Ahmad S, et al. Anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis in children and adolescents. Ann Neurol 2009; 66: 11–8.

Garvey MA, Snider LA, Leitman SF, Werden R, Swedo SE. Treatment of Sydenham's chorea with intravenous immunoglobulin, plasma exchange, or prednisone. J Child Neurol 2005; 20: 424–9.

Giros B, Sokoloff P, Martres MP, Riou JF, Emorine LJ, Schwartz JC. Alternative splicing directs the expression of two D2 dopamine receptor isoforms. Nature 1989; 342: 923–6.

Granerod J, Ambrose HE, Davies NW, Clewley JP, Walsh AL, Morgan D, et al. Causes of encephalitis and differences in their clinical presentations in England: a multicentre, population-based prospective study. Lancet Infect Dis 2010; 10: 835–44.

Greenfield JG, Wolfsohn JM. The pathology of Sydenham's chorea. Lancet 1922; 2: 603–6.

Hughes EG, Peng X, Gleichman AJ, Lai M, Zhou L, Tsou R, et al. Cellular and synaptic mechanisms of anti-NMDA receptor encephalitis. J Neurosci 2010; 30: 5866–75.

Hutchinson M, Waters P, McHugh J, Gorman G, O'Riordan S, Connolly S, et al. Progressive encephalomyelitis, rigidity, and myoclonus: a novel glycine receptor antibody. Neurology 2008; 71: 1291–2.

Irani SR, Alexander S, Waters P, Kleopa KA, Pettingill P, Zuliani L, et al. Antibodies to Kv1 potassium channel-complex proteins leucine-rich, glioma inactivated 1 protein and contactin-associated protein-2 in limbic encephalitis, Morvan's syndrome and acquired neuromyotonia. Brain 2010a; 133: 2734–48.

Irani SR, Bera K, Waters P, Zuliani L, Maxwell S, Zandi MS, et al. N-methyl-D-aspartate antibody encephalitis: temporal progression of clinical and paraclinical observations in a predominantly non-paraneoplastic disorder of both sexes. Brain 2010b; 133: 1655–67.

Kebabian JW, Calne DB. Multiple receptors for dopamine. Nature 1979; 277: 93–6.

Kirvan CA, Swedo SE, Heuser JS, Cunningham MW. Mimicry and autoantibody-mediated neuronal cell signaling in Sydenham chorea. Nat Med 2003; 9: 914–20.

Kirvan CA, Swedo SE, Snider LA, Cunningham MW. Antibody-mediated neuronal cell signaling in behavior and movement disorders. J Neuroimmunol 2006; 179: 173–9.

Klein C, Brin MF, Kramer P, Sena-Esteves M, de Leon D, Doheny D, et al. Association of a missense change in the D2 dopamine receptor with myoclonus dystonia. Proc Natl Acad Sci USA 1999; 96: 5173–6.

Lai M, Hughes EG, Peng X, Zhou L, Gleichman AJ, Shu H, et al. AMPA receptor antibodies in limbic encephalitis alter synaptic receptor location. Ann Neurol 2009; 65: 424–34.

Lai M, Huijbers MG, Lancaster E, Graus F, Bataller L, Balice-Gordon R, et al. Investigation of LGI1 as the antigen in limbic encephalitis previously attributed to potassium channels: a case series. Lancet Neurol 2010; 9: 776–85.

Lancaster E, Lai M, Peng X, Hughes E, Constantinescu R, Raizer J, et al. Antibodies to the GABA(B) receptor in limbic encephalitis with seizures: case series and characterisation of the antigen. Lancet Neurol 2010; 9: 67–76.

Lancaster E, Martinez-Hernandez E, Dalmau J. Encephalitis and antibodies to synaptic and neuronal cell surface proteins. Neurology 2011; 77: 179–89.

Landau YE, Steinberg T, Richmand B, Leckman JF, Apter A. Involvement of immunologic and biochemical mechanisms in the pathogenesis of Tourette's syndrome. J Neural Transm 2012; 119: 621–6.

Martinez-Hernandez E, Horvath J, Shiloh-Malawsky Y, Sangha N, Martinez-Lage M, Dalmau J. Analysis of complement and plasma cells in the brain of patients with anti-NMDAR encephalitis. Neurology 2011; 77: 589–93.

Martino D, Dale RC, Gilbert DL, Giovannoni G, Leckman JF. Immunopathogenic mechanisms in tourette syndrome: a critical review. Mov Disord 2009; 24: 1267–79.

McKnight K, Jiang Y, Hart Y, Cavey A, Wroe S, Blank M, et al. Serum antibodies in epilepsy and seizure-associated disorders. Neurology 2005; 65: 1730–6.

McLaughlin KA, Chitnis T, Newcombe J, Franz B, Kennedy J, McArdel S, et al. Age-dependent B cell autoimmunity to a myelin surface antigen in pediatric multiple sclerosis. J Immunol 2009; 183: 4067–76.

Murphy TK, Kurlan R, Leckman J. The immunobiology of Tourette's disorder, pediatric autoimmune neuropsychiatric disorders associated with Streptococcus, and related disorders: a way forward. J Child Adolesc Psychopharmacol 2010; 20: 317–31.

Nikolaus S, Antke C, Muller HW. In vivo imaging of synaptic function in the central nervous system: I. Movement disorders and dementia. Behav Brain Res 2009a; 204: 1–31.

Nikolaus S, Antke C, Muller HW. In vivo imaging of synaptic function in the central nervous system: II. Mental and affective disorders. Behav Brain Res 2009b; 204: 32–66.

O'Connor KC, McLaughlin KA, De Jager PL, Chitnis T, Bettelli E, Xu C, et al. Self-antigen tetramers discriminate between myelin autoantibodies to native or denatured protein. Nat Med 2007; 13: 211–7.

Probstel AK, Dornmair K, Bittner R, Sperl P, Jenne D, Magalhaes S, et al. Antibodies to MOG are transient in childhood acute disseminated encephalomyelitis. Neurology 2011; 77: 580–8.

Pruss H, Holtje M, Maier N, Gomez A, Buchert R, Harms L, et al. IgA NMDA receptor antibodies are markers of synaptic immunity in slow cognitive impairment. Neurology 2012; 78: 1743–53.

Singer HS, Hong JJ, Yoon DY, Williams PN. Serum autoantibodies do not differentiate PANDAS and Tourette syndrome from controls. Neurology 2005; 65: 1701–7.

Suleiman J, Brenner T, Gill D, Brilot F, Antony J, Vincent A, et al. VGKC antibodies in pediatric encephalitis presenting with status epilepticus. Neurology 2011; 76: 1252–5.

Swedo SE, Leonard HL, Garvey M, Mittleman B, Allen AJ, Perlmutter S, et al. Pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections: clinical description of the first 50 cases. Am J Psychiatry 1998; 155: 264–71.

Usiello A, Baik JH, Rouge-Pont F, Picetti R, Dierich A, LeMeur M, et al. Distinct functions of the two isoforms of dopamine D2 receptors. Nature 2000; 408: 199–203.

Vincent A, Bien CG, Irani SR, Waters P. Autoantibodies associated with diseases of the CNS: new developments and future challenges. Lancet Neurol 2011; 10: 759–72.

Walker K, Brink A, Lawrenson J, Mathiassen W, Wilmshurst JM. Treatment of sydenham chorea with intravenous immunoglobulin. J Child Neurol 2012; 27: 147–55.

Waters PJ, McKeon A, Leite MI, Rajasekharan S, Lennon VA, Villalobos A, et al. Serologic diagnosis of NMO: a multicenter comparison of aquaporin-4-IgG assays. Neurology 2012; 78: 665–71.

Zandi MS, Irani SR, Lang B, Waters P, Jones PB, McKenna P, et al. Disease-relevant autoantibodies in first episode schizophrenia. J Neurol 2011; 258: 686–8.