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Anti-Brain Autoantibodies and Altered Excitatory Neurotransmitters in Obsessive–Compulsive Disorder

Sagnik Bhattacharyya[1,*], Sumant Khanna[2], Koushik Chakrabarty[3], Anita Mahadevan[4], Rita Christopher[5] and SK Shankar[4]

[1]Division of Psychological Medicie and Psychiatry, Section of Neuroimaging, Institute of Psychiatry, King's College London, London, UK; [2]The Psychiatric Clinic, Vasant Vihar, New Delhi, India; [3]Department of Molecular Neurobiochemistry, International Graduate School of Neuroscience, Ruhr University Bochum, Universitatsstrasse 150, Bochum, Germany; [4]Department of Neuropathology, National Institute of Mental Health and Neurosciences, Bangalore, India; [5]Department of Neurochemistry, National Institute of Mental Health and Neurosciences, Bangalore, India

Although serum autoantibodies directed against basal ganglia (BG) implicate autoimmunity in the pathogenesis of obsessive–compulsive disorder (OCD), it is unclear whether these antibodies can cross the blood–brain barrier to bind against BG or other components of the OCD circuit. It is also unclear how they might lead to hyperactivity in the OCD circuit. We examined this by investigating the presence of autoantibodies directed against the BG or thalamus in the serum as well as CSF of 23 OCD patients compared with 23 matched psychiatrically normal controls using western blot. We further investigated CSF amino acid (glutamate, GABA, taurine, and glycine) levels and also examined the extent to which these levels were related to the presence of autoantibodies. There was evidence of significantly more binding of CSF autoantibodies to homogenate of BG as well as to homogenate of thalamus among OCD patients compared with controls. There was no significant difference in binding between patient and control sera except for a trend toward more bands to BG and thalamic protein corresponding to 43 kD among OCD patients compared with controls. CSF glutamate and glycine levels were also significantly higher in OCD patients compared with controls, and further multivariate analysis of variance showed that CSF glycine levels were higher in those OCD patients who had autoantibodies compared with those without. The results of our study implicate autoimmune mechanisms in the pathogenesis of OCD and also provide preliminary evidence that autoantibodies against BG and thalamus may cause OCD by modulating excitatory neurotransmission.
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INTRODUCTION

Obsessive–compulsive disorder (OCD) is a chronic disorder with a lifetime prevalence of 1.9–2.5% (Weissman *et al*, 1994) worldwide. Neuroimaging studies suggest that OCD involves hyperactivity of the ventral cognitive circuit specifically involving the basal ganglia (BG) and thalamus (Saxena *et al*, 1998; Friedlander and Desrocher, 2006). Recent evidence has also linked glutamatergic abnormalities with OCD (Rosenberg *et al*, 2000, 2004; Chakrabarty *et al*, 2005; Arnold *et al*, 2006; Dickel *et al*, 2006; Whiteside *et al*, 2006; MacMaster *et al*, 2008; Yucel *et al*, 2008), glutamate being one of the predominant excitatory neurotransmitters in the OCD circuit, with beneficial effects of glutamate-modulating agents noted in OCD (Coric *et al* 2005; Grant *et al*, 2007).

*Correspondence: Dr S Bhattacharyya, Section of Neuroimaging, Box PO67, Institute of Psychiatry, King's College London, De Crespigny Park, London SE5 8AF, UK, Tel: (+44) 20 7848 0955, Fax: (+44) 20 7848 0976, E-mail: s.bhattacharyya@iop.kcl.ac.uk
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However, the cause of OCD is still unclear with both genetic and environmental factors implicated in the causation (Hoekstra and Minderaa, 2005). Although accumulating evidence implicates autoimmunity in the causation of OCD (Pavone *et al*, 2004; Dale *et al*, 2005), it is unclear whether autoantibodies shown in the sera of OCD patients can actually cross the blood–brain barrier to bind to epitopes in the brain and whether they are causally related to OCD, particularly in light of conflicting reports from studies investigating serum antibodies in OCD and related disorders (Singer *et al*, 2005; Morer *et al*, 2008). It is also unclear whether they can cause hyperactivity in the brain pathways implicated in OCD and how that might be mediated at the neurotransmitter level.

As both BG and thalamus have a central position as per current understanding regarding the neurobiological substrate for OCD, we hypothesized that any autoantibodies would need to target either the BG or thalamus in order to cause OCD and should be detectable in the CSF of OCD patients. We also hypothesized that, independent of the presence of autoantibodies, there would be evidence of abnormal excitatory neurotransmission in OCD. We further

wanted to investigate whether the autoantibodies detected in the OCD patients would be related to abnormal excitatory neurotransmission.

We tested our hypotheses in a sample of psychotropic drug-naïve OCD patients and matched healthy controls by examining the presence of autoantibodies directed against BG and thalamus in their CSF and sera and also measuring CSF levels of excitatory neurotransmitters and modulators of excitatory neurotransmission such as glutamate and glycine and inhibitory neurotransmitters such as GABA and taurine.

MATERIALS AND METHODS

Recruitment and Assessment of Participants

This study was conducted at the Department of Psychiatry, NIMHANS, Bangalore, following a protocol approved by the departmental protocol review board (see Supplementary methods and materials for details). All psychotropic drug-naïve patients above the age of 15 years diagnosed with OCD using the Structured Clinical Interview for DSM IV-clinician version (SCID-CV) (First *et al*, 1996), who gave consent and did not have any lifetime history of psychotic disorder or mental retardation were included and rated on the Yale-Brown Obsessive Compulsive Symptom Checklist (Y-BOCS) and Severity Rating Scale (Goodman *et al*, 1989a, b). Twenty-three consenting psychotropic drug-naïve OCD patients were included in the study.

Sex-matched controls were identified from patients scheduled for various operative procedures under spinal anesthesia in a general hospital located in the same geographical area and also from staff working in the hospital. They were included into the study after obtaining informed consent and administration of SCID-CV (First *et al*, 1996) to rule out lifetime history of any psychiatric illness, head injury, or any other neurological illness. Twenty-three consenting psychiatrically normal controls were included into the study.

None of the patients and controls had a history suggestive of rheumatic fever, chorea and any other neurological or major systemic illnesses other than 16 of the controls, who were undergoing operative procedures for various non-neurological physical conditions.

Sample Collection and Storage

Lumbar CSF and serum collected aseptically from all OCD patients and control participants after overnight fasting was stored at -70°C and used subsequently for the estimation of autoantibodies directed against BG and thalamic homogenates using western blot technique. CSF samples were also used for the estimation of amino acids.

The human brain regions used for the western blot study were obtained from the Department of Neuropathology, NIMHANS, Bangalore and included a thin slice of BG containing caudate, putamen and globus pallidus, and a thalamus specimen obtained from two individuals after death from road traffic accidents. They did not suffer from any known neurological disease before death and their bodies were shifted to freezer within 1 h after death.

Western Blot Analysis

Western blotting was carried out to detect the presence of specific antibodies against BG (including caudate, putamen and globus pallidus) and thalamic homogenate using the procedure similar to Singer *et al* (1998). About 85 μg of protein from BG and thalamic homogenate were individually subjected to electrophoresis in 7.5% acrylamide gels and then transferred to polyvinylidene fluoride (PVDF) membranes. After blocking for nonspecific binding, the PVDF membranes were exposed to the primary antibody (CSF or sera from patients and controls). Following an initial standardization phase using different dilutions of sera and CSF, the final optimal dilutions of primary antibody chosen to maximize sensitivity and minimize background staining were 1 : 250 for sera and 1 : 50 for CSF. Paired sera or CSF samples (ie, one OCD patient and one control sample) were used as primary antibody to run on the same gel. Subsequently, the PVDF membranes were washed and exposed to secondary antibody (goat antihuman horseradish peroxidise-linked IgG; 1 : 3000) and the reactions were visualized using enhanced chemiluminescence reagents and protocol according to Amersham. Molecular weights of the protein bands highlighted were determined by matching with the bands of molecular weight marker run simultaneously on the PVDF membrane. The western blotting steps were carried out by SB who was blind to the diagnostic status of the individual samples that constituted each set of paired sample. Two investigators (SKS, AM) blind to subject diagnosis read the presence of bands on western blot and their relative position with reference to molecular weight markers. Another investigator not involved in reading the gels interpreted the results.

Amino Acid Estimation

CSF amino acid (glutamate, GABA, glycine, taurine) estimation was carried out by KC, who was blind to subject status, using an isocratic high-performance liquid chromatography (HPLC)–electrochemical detection system involving the use of pre-column o-pthaladehyde (OPA)–sulfite derivatization method (Rowley *et al*, 1995) in a subsample of patients for whom adequate CSF was available (22 OCD patients and 21 controls). CSF glutamate measured using an enzymatic technique from some of these patients has been reported earlier (Chakrabarty *et al*, 2005). Quantification of the amino acids and processing of the data obtained were carried out using the Shimadzu class LC-10 software package.

Statistical Analysis

Statistical analyses were carried out using the Statistical Package for Social Sciences version 12. χ^2-test was used for categorical variables and multivariate analysis of variance (MANOVA) or *t*-test for continuous variables.

RESULTS

Sociodemographic and Clinical Profile

Sociodemographic characteristics of patient and control probands were identical, except for the fact that patients (mean age ± SD: 24.65 ± 8.95 years; range: 16–46 years) were significantly younger ($p < 0.05$) than control (mean age ± SD: 32 ± 12.95 years; range: 20–65 years) probands (Table 1). The OCD patients had few comorbid psychiatric illnesses as shown in Table 1.

Table 1 Sociodemographic and Clinical Profile of Probands

	Patient probands	Control probands	*P*-value
Age in years (SD)	24.65 (8.95)	32.00 (12.95)	***<0.05*** (*t*-test)
Sex (M/F)	18:5	18:5	
Number of years of education (SD)	10.30 (4.86)	9.22 (3.52)	NS (*t*-test)
Mean duration of illness (years)	4.7 ± 4.2	NA	
Mean Y-BOCS score	26 ± 5.6	NA	
Psychiatric comorbidity in OCD patients			
Dysthymia	1	NA	
Social phobia	4	NA	
Tic disorder	3	NA	

Statistically significant '*p*' values have been depicted with bold, italicized fonts.

Autoantibodies

Molecular weight distribution of bands and thus the antigenic determinants in BG and thalamus, observed on western blot using the CSF of patients and controls (Figure 1) is shown in Table 2. There were significantly more bands, suggesting binding of CSF autoantibodies to homogenate of BG corresponding to molecular weight of 97, 43, and between 6.5 and 3 kD, among OCD patients in contrast to controls. Similarly, there were significantly more bands, suggesting binding of CSF autoantibodies to homogenate of thalamus corresponding to molecular weight of 97, 43, and between 6.5 and 3 kD, among OCD patients compared with controls.

Although there was evidence of binding of serum to BG and thalamic homogenate, this was not significantly different between the patients and controls, except for binding of serum to BG and thalamic homogenate corresponding to molecular weight of 43 kD, which was increased in OCD patients compared with controls at a trend level of significance (Table 3).

CSF Neurotransmitter Levels

On MANOVA, CSF glutamate and glycine levels were significantly higher in OCD patients compared with controls, while there was no significant difference in GABA and taurine levels (Table 4; Figure 2). There was no effect of age or gender on CSF levels of the amino acids. Furthermore, MANOVA showed evidence of a significant effect of presence of antibody against BG and thalamic antigen (OCD patients with autoantibody, $n = 20$; controls with autoantibody, $n = 14$) on CSF glycine levels in the entire group ($F(1,39) = 8.186$, $p = 0.007$) and a significant interaction effect between the presence of autoantibody and diagnosis of OCD on CSF glycine levels ($F(1,39) = 5.073$, $p = 0.030$) (Figure 3). There was no significant effect of presence of autoantibody on CSF glutamate levels in OCD patients, which was in fact slightly higher in OCD patients without autoantibody compared with those patients who had positive autoantibody status. Finally, there was no correlation between the severity of obsessive–compulsive symptoms as indexed by YBOCS score and levels of glutamate and glycine in the CSF in the OCD patients.

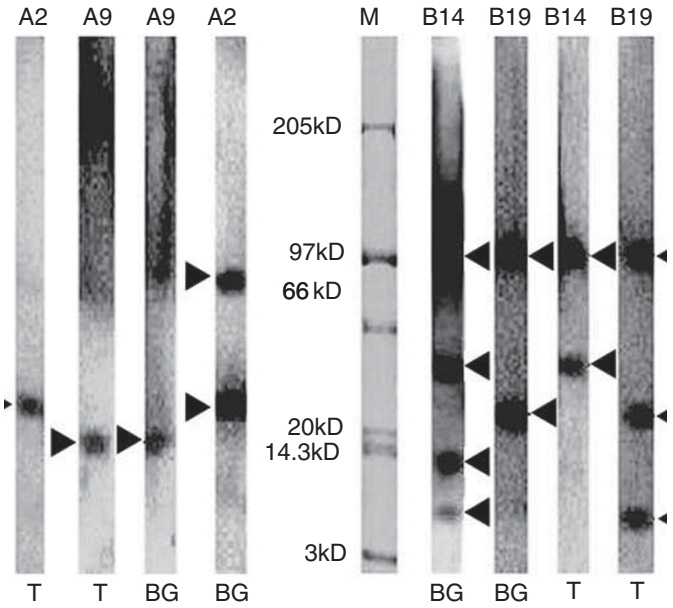

Figure 1 Representative immunoblots reacted with CSF of OCD patients and controls. Immunoblots of two patients and two controls were developed by chemiluminescence. A quantity of 85 μg of BG and thalamic homogenate in PBS was loaded in the lanes. The blots were reacted with control (A2 and A9) and patient (B14 and B19) CSF. The lane in between represents molecular weight markers (M). Lanes A2 and B14 were from the same gel, whereas lanes A9 and B19 were also from the same gel.

DISCUSSION

This study examined the presence of autoantibodies directed against the BG and thalamus in the sera and CSF and measured CSF amino acid (glutamate, GABA, taurine, and glycine) levels in a sample of psychotropic drug-naïve OCD patients compared with matched controls. Furthermore, the study examined the extent to which the CSF amino acid levels were related to the presence of the autoantibodies.

Autoantibodies

First, this study found significantly increased CSF autoantibody binding to one or more BG and thalamic antigenic proteins in the psychotropic drug-naïve OCD patients compared with psychiatrically healthy controls. To our knowledge, this is the first report implicating CSF autoantibodies in a sample of psychotropic drug-naïve OCD patients compared with psychiatrically healthy controls. The results of this study are consistent with the only previous study that has examined CSF autoantibodies in a smaller sample ($n = 6$) (Kirvan *et al*, 2006), as well as with other studies that implicate serum autoantibodies in the causation of OCD and related disorders (Pavone *et al*, 2004; Dale *et al*, 2005). The presence of autoantibodies in the CSF but not in the serum in this study might indicate intrathecal synthesis or might be a function of dilution in the larger volume of sera. Similar binding profiles against both brain

Table 2 CSF Antibody Binding to Bg and Thalamus on Western Blot

Molecular weights (kD) of brain region antigens	Number of patients with bands to BG (%) ($n=23$)	Number of controls with bands to BG (%) ($n=23$)	*P*-value	Number of patients with bands to thalamus (%) ($n=23$)	Number of controls with bands to thalamus (%) ($n=23$)	*P*-value
205–97	1 (4.3)	0	NS	0	0	
97	20 (87.0)	13 (56.5)	***< 0.05***	19 (82.6)	12 (52.2)	***< 0.05***
66	2 (8.7)	2 (8.7)	NS	1 (4.3)	0	NS
43	21 (91.3)	15 (65.2)	***< 0.05***	21 (91.3)	12 (52.2)	***< 0.005***
35	0	1 (4.3)	NS	0	1 (4.3)	NS
29	5 (21.7)	2 (8.7)	NS	4 (17.4)	2 (8.7)	NS
10	3 (13)	0	NS	3 (13)	0	NS
6.5–3	15 (65.2)	3 (13.0)	***< 0.001***	15 (65.2)	3 (13)	***< 0.001***

Statistically significant '*p*' values have been depicted with bold, italicized fonts.

Table 3 Serum Antibody Binding to Bg and Thalamus On Western Blot

Molecular weights (kD) of brain region antigens	Number of patients with bands to BG ($n=23$)	Number of controls with bands to BG ($n=23$)	*P*-value	Number of patients with bands to thalamus ($n=23$)	Number of controls with bands to thalamus ($n=23$)	*P*-value
205–97	0	0		0	2	NS
97	1	0	NS	0	1	NS
66	2	0	NS	3	1	NS
43	5	1	***0.08***	5	1	***0.08***
10	8	7	NS	8	4	NS

Statistically significant '*p*' values have been depicted with bold, italicized fonts.

Table 4 CSF Amino Acid Levels

	Glutamate	GABA	Glycine	Taurine
OCD probands ($n=22$) (nmol/ml)	3.15 ± 0.53	0.24 ± 0.07	13.41 ± 1.67	7.53 ± 0.67
Control probands ($n=21$) (nmol/ml)	2.42 ± 0.29	0.24 ± 0.10	10.83 ± 1.70	7.62 ± 0.81
Multivariate analysis of covariance on CSF amino acid levels				
Factors/covariates				
	df = 1/38	df = 1/38	Df = 1/38	df = 1/38
Diagnosis	F = 12.819	F = 0.075	F = 17.429	F = 0.069
	p = 0.001	p = NS	***p = 0.000***	p = NS
Age	df = 1/38	df = 1/38	Df = 1/38	df = 1/38
	F = 0.117	F = 0.117	F = 0.872	F = 0.668
	p = NS	p = NS	p = NS	p = NS
Gender	df = 1/38	df = 1/38	Df = 1/38	df = 1/38
	F = 0.721	F = 0.001	F = 2.507	F = 0.271
	p = NS	p = NS	p = NS	p = NS

Statistically significant '*p*' values have been depicted with bold, italicized fonts.

regions possibly indicate that the antigenic elements may be neuronal proteins present in both the BG and thalamus.

CSF Neurotransmitter Levels

This study also adds to the growing evidence for abnormal levels of excitatory neurotransmitters in OCD patients. CSF glutamate and glycine levels were significantly increased in OCD patients compared with controls. Increase in both CSF glutamate and glycine levels as noted in our study are consistent with evidence from another recent study where both CSF glutamate and glycine changed in the same direction in patients with refractory affective disorder (Frye *et al*, 2007). Overall, these results tend to suggest that abnormalities in excitatory neurotransmission may be associated with OCD symptoms, possibly by causing

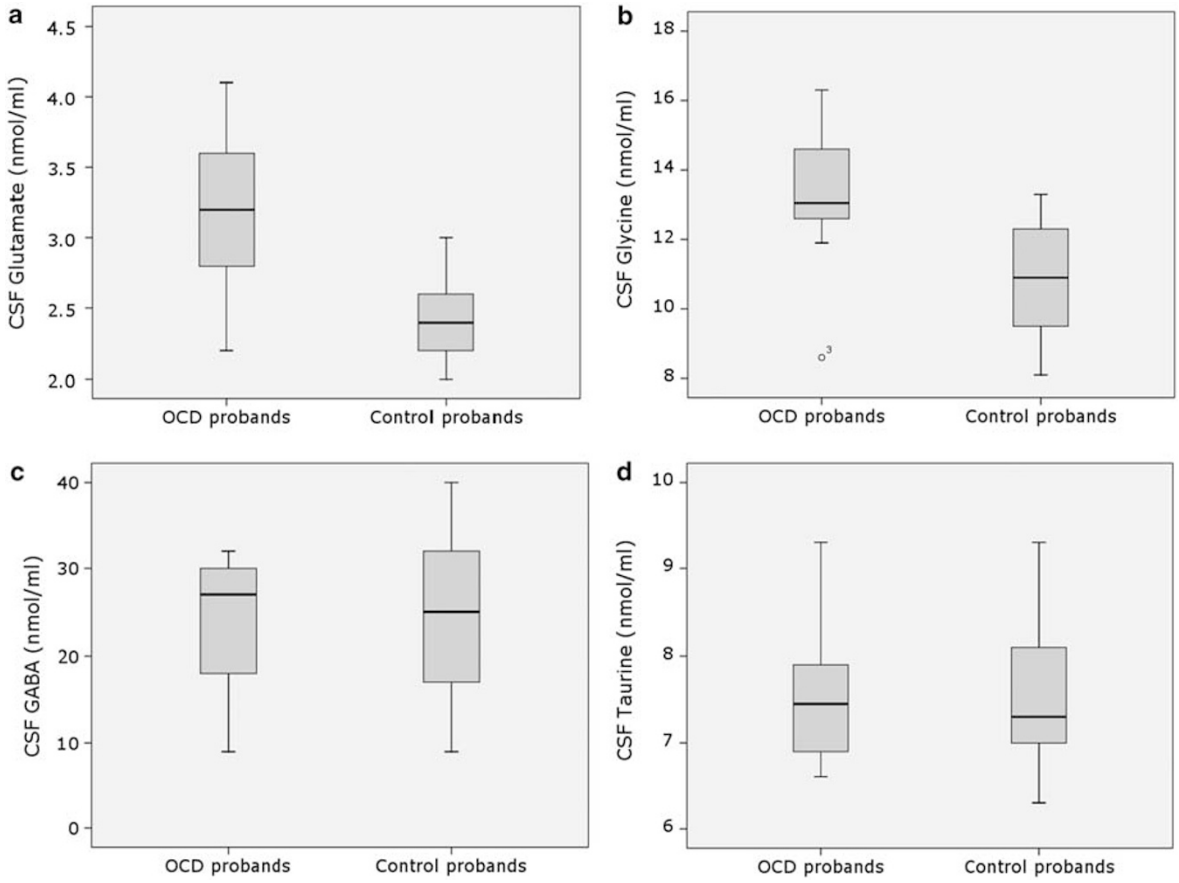

Figure 2 CSF amino acid levels. Box-plots showing the levels (*y*-axis) of glutamate (a), glycine (b), GABA (c), and taurine (d) in CSF of OCD patients and controls (*x*-axis).

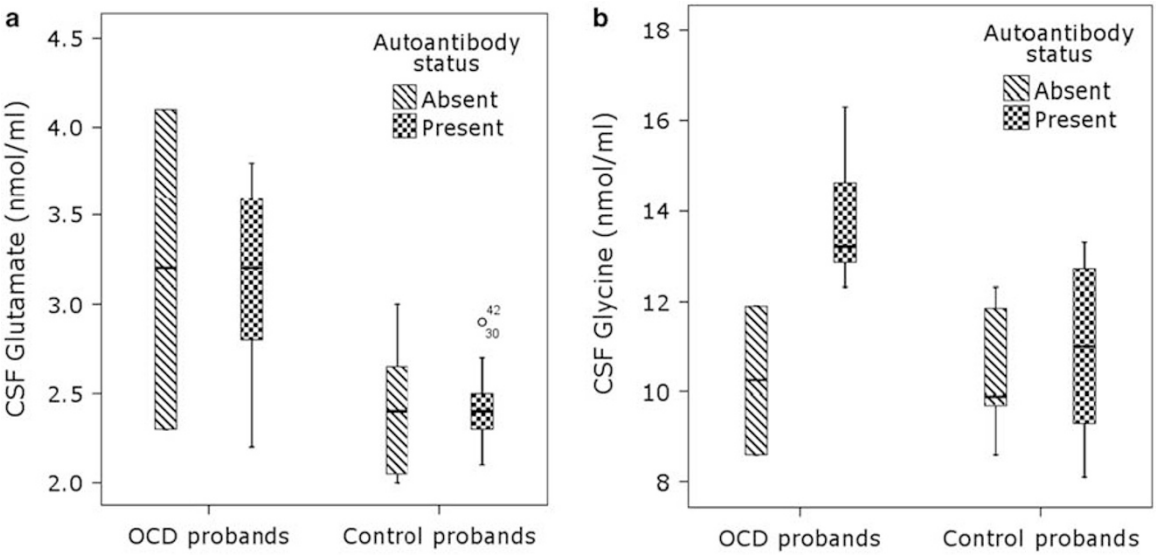

Figure 3 Effect of autoantibody status on amino acid levels. Box-plots showing the effect of autoantibody status (*x*-axis) in OCD patients and controls on levels of glutamate (a) and glycine (b) in CSF (*y*-axis).

hyperactivity in the ventral cognitive circuit. This is consistent with accumulating neuroimaging evidence of significantly altered levels of glutamate and glutamine in different parts of the OCD circuit in the brain of OCD patients compared with controls (Rosenberg *et al*, 2000, 2004; Whiteside *et al*, 2006; Yucel *et al*, 2008). However,

earlier neuroimaging studies suggest that the direction of changes in brain glutamate and glutamine may be region and gender-specific. While some studies report greater levels in those with more severe obsessive–compulsive symptoms (Whiteside *et al*, 2006), which decrease in parallel with decrease in symptoms after treatment with anti-obsessional medications (Rosenberg *et al*, 2000), others report an association with lower levels (Rosenberg *et al*, 2004; Yucel *et al*, 2008) that may be gender specific and correlate with symptom severity (Yucel *et al*, 2008). However, there was no effect of gender in the CSF levels of neurotransmitters in this study, nor was any correlation observed with symptom scores.

Association Between CSF Autoantibodies and Excitatory Neurotransmitter Levels

This study also provides preliminary evidence that abnormal levels of excitatory neurotransmitters might be related to anti-BG or thalamic autoantibodies. CSF glutamate levels were increased in all patients with OCD compared with the control group, irrespective of whether they had autoantibodies or not, but were slightly higher in those OCD patients who did not have autoantibodies compared with those who did. On the other hand, CSF glycine levels were significantly increased compared with the control group, only in those OCD patients who had autoantibodies in their CSF. But this needs to be interpreted with caution, as most of the OCD patients in this study were also positive for autoantibody in their CSF. However, the contrasting effects of the presence of autoantibody on CSF glutamate and glycine levels in the OCD patients suggest that this is unlikely to be a spurious association. The lack of overlap between the ranges of CSF glycine levels in OCD patients with and those without autoantibody, whose CSF glycine levels were clearly in the range of the control population, also support this contention.

In light of these results, one may speculate that the association between abnormal glutamate levels and OCD may result through different paths, either by antibodies cross-reacting against antigenic proteins in critical regions of the OCD circuit or through non-immunological mechanisms, consistent with current understanding regarding the heterogeneity of OCD. On the basis of these results, one may also speculate that autoantibodies directed against BG and thalamic antigens do not cause glutamatergic abnormalities directly. But they possibly cause glutamatergic abnormalities by modulating the central levels of glycine (Figure 4). This is consistent with evidence that glutamatergic neurotransmission is tightly controlled by synaptic concentration of glycine, which is a co-agonist of glutamate at NMDA receptors (Johnson and Ascher, 1987) and evidence of potentiation of glutamatergic neurotransmission by increase in glycine levels (Depoortere *et al*, 2005; Leonetti *et al*, 2006).

However, these results need to be considered in the context of some of the limitations of this study. First, in light of the multiple statistical comparisons that we carried out, inclusion of a larger sample of patients than was possible in this study would have further strengthened the significance of the findings, especially those relating neurotransmitter levels to the presence or absence of antibodies. Another potential limitation of this study is that the control sample was older compared with the patients. As exposure to certain infections are more likely in the younger age group, it is possible that the significantly increased autoantibodies detected in the OCD patients in this study was a result of the increased likelihood of the younger OCD patients to have been exposed to such infections resulting in higher titers of antibodies cross-reactive against brain tissue compared with the older control participants. But this does not influence the results of this study that relate to the levels of neurotransmitters in the CSF, where we did not find any effect of age on their levels. Overall, these two limitations reflect the logistical difficulties in carrying out a study involving an invasive procedure in drug-naïve patients. One could argue that they may also limit the generalizability of these findings especially as the OCD patients in this study were atypical in that they had less comorbidity than is usual in epidemiologically derived samples (Ruscio *et al*, 2008). However, the aim of this study was not to identify autoantibodies as the single definitive cause responsible for all cases of OCD, which is likely to be multifactorial in origin. Hence, the results of this study are generalizable to the extent that they provide complementary evidence that may implicate autoimmune mechanisms in certain types of OCD. Other limitations include not complementing the initial western blots with isolation and identification of the specific brain proteins or techniques such as ELISA, which would have helped in identifying lower titer antibodies. Potential differences in protein loading, incubation, or blocking between gels may have also influenced our results. We attempted to overcome these by running the patient and control samples (either CSF or sera) in pairs on the same

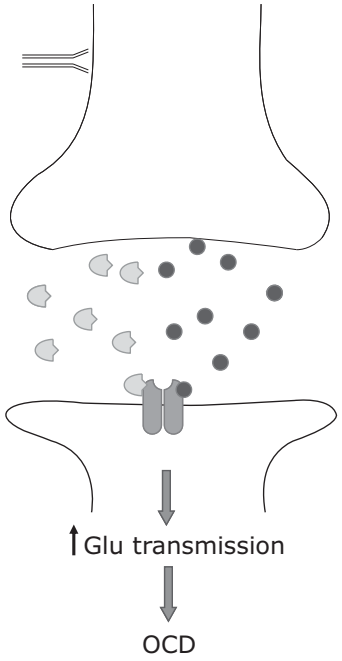

Figure 4 Schematic of autoimmune causation of OCD. Schematic showing anti-brain autoantibodies may cause OCD by indirectly augmenting glutamatergic neurotransmission through a direct increase of local glycine levels. Autoantibody: ═, glutamate: ●, glycine: ●, NMDA receptor: ▮.

gels, with the researcher (SB) blind to the status of the individual samples. Inclusion of a group of non-OCD psychiatric patients similar to those with other anxiety disorders or depression would have helped to determine whether the presence of autoantibodies and dysfunction of excitatory neurotransmission were specific to OCD. Similarly, inclusion of tissue outside brain (eg, muscle) and from other brain regions that are not part of the OCD circuit as antigen would have helped to determine whether these autoantibodies were specifically directed against components of the OCD circuit. Furthermore, although the increased levels of CSF glutamate and glycine as observed in OCD patients in this study may be considered as an indirect measure of increased glutamatergic tone (Frye *et al*, 2007), they are not the same as increase in these neurotransmitters in the cortical and subcortical components of the OCD circuit, as various processes such as neuronal release and transport, glial uptake, diffusion barriers, sequestration, and degradation may modify CSF levels of these neurotransmitters.

This study also provides preliminary evidence of how autoantibodies may cause OCD through altering the levels of excitatory neurotransmitters. In addition, this study provides evidence for the occurrence of autoantibodies even in non-tic related and adult OCD patients. Although, association does not necessarily indicate causation, the highly significant association of CSF autoantibodies and excitatory amino acid levels in OCD patients and the interaction effect of the presence of autoantibody and diagnosis on CSF glycine level, not only implicates autoimmune mechanisms in certain types of OCD, but also suggests that excitatory amino acid abnormalities may be the final common pathway in both immunologically and non-immunologically mediated OCD. This is particularly significant in light of converging evidence implicating glutamate transporter genes in OCD (Arnold *et al*, 2006; Dickel *et al*, 2006) as well evidence of autoantibody-mediated alteration of neuronal signal transduction through the induction of CaM kinase II, which is modulated by glutamate in disorders that are either characterized by obsessive–compulsive symptoms or have them as a comorbidity (Kirvan *et al*, 2003, 2006). However, our findings are to be considered with caution and would need to be complemented with further characterization of the antigenic element in OCD as well as experimental evidence regarding the mechanism by which the autoantibodies might mediate neurotransmitter alterations combined with *in vivo* evidence of neurotransmitter alterations in different parts of the OCD circuit.

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DISCLOSURE

The authors report no biomedical financial interests or potential conflict of interest.

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