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SHORT COMMUNICATION

No evidence for the presence of neuronal surface autoantibodies in plasma of patients with schizophrenia

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Abstract
The immune system has been implicated in the etiology of schizophrenia. Autoimmunity by antibodies against neuronal cell surface antigens has been proposed as one of the pathological mechanisms. We examined plasma samples of 104 patients diagnosed with schizophrenia for the presence of autoantibodies against neuronal cell surface antigens using cultured hippocampal neurons and transfected HeLa cells. None of the samples tested positive for the presence of these autoantibodies. Based on our results it seems unlikely that autoantibodies against neuronal cell surface antigens play a role in the pathogenesis of schizophrenia, although further studies using cerebrospinal fluid are needed.

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1. Introduction

Different lines of evidence from the genetic (Ripke et al., 2014), epidemiological (Benros et al., 2014) and the immunological field (Tomasik et al., 2014) suggest that the immune system is involved in the pathogenesis of schizophrenia. However, the exact pathological mechanism explaining how the immune system contributes to schizophrenia is still unknown. In the 1960s autoimmunity was already proposed as a potential pathological mechanism (Heath and Krupp, 1967). More recent support for this theory is provided by studies showing a higher prevalence of autoimmune disorders in patients with schizophrenia (Benros et al., 2014) and the association of schizophrenia with genetic loci that are involved in adaptive immune responses and autoimmunity, including the MHC region (Ripke et al., 2014; The Network and Pathway Analysis Subgroup of the Psychiatric Genomics Consortium, 2015).

One of the key mechanisms involved in autoimmune disorders is the production of self-reactive antibodies, the so-called autoantibodies. Some neurological autoimmune disorders, such as anti-NMDA receptor encephalitis, are caused by autoantibodies targeting neuronal cell surface antigens. These pathogenic autoantibodies can impair the function of their target protein in various ways, for example by clustering and internalization of the receptor leading to diminished cell surface expression (for review see van Coevorden-Hameete et al. (2014)). This leads to changes in synaptic transmission and neuronal excitability, resulting in neurologic and/or psychiatric symptoms. Isolated episodes with psychotic symptoms can occur in patients with anti-NMDA receptor encephalitis (Kayser et al., 2013). It has therefore been postulated that neuronal surface autoantibodies could cause a clinical syndrome similar to schizophrenia (Coutinho et al., 2014).

Several groups have investigated the presence of neuronal autoantibodies in patients with schizophrenia. The presence of antibodies against brain antigens in general has been examined by incubating rat brain tissue slices with patients' sera. Anti-brain antibodies were found to be increased in some studies but not in others, thoroughly reviewed elsewhere (Jones et al., 2005). The last decade, multiple studies have focused on the seroprevalence of autoantibodies targeting specific neuronal surface antibodies, such as NMDA (Pearlman and Najjar, 2014), dopamine, AMPA and GABA receptor antibodies (Ezeoke et al., 2013; Müller et al., 2014), with inconclusive evidence.

We hypothesized that known and/or yet unknown autoantibodies against neuronal cell surface antigens are involved in the pathogenesis of schizophrenia in a subgroup of patients. In this study we therefore set out to examine the prevalence of neuronal autoantibodies by screening plasma of 104 patients with schizophrenia using live rat hippocampal neurons. In addition, cell-based assays (CBA) were used to test these samples for autoantibodies against a selection of 24 neuronal cell surface antigens that have been associated with schizophrenia in GWAS studies (Ayalew et al., 2012; Greenwood et al., 2011; Levinson et al., 2011; Sullivan et al., 2008).

2. Experimental procedures

2.1. Participants

Plasma samples from a subgroup of 104 patients diagnosed with schizophrenia and schizophreniform disorder that participated in the Genetic Risk and Outcome of Psychosis (GROUP) study in the Netherlands were used for this study. Further details of this study are described elsewhere (Korver et al., 2012). In brief, inclusion criteria for patients participating in the original study were: (1) Age range of 16 to 50 years, (2) a diagnosis of non-affective psychotic disorder according to DSM-IV criteria, (3) good command of the Dutch language, (4) and able and willing to give written informed consent. A plasma sample of a patient with autoimmune encephalitis caused by anti-GluR1 antibodies was included as positive control.

2.2. Commercial antibodies

The following antibodies were used in this study: mouse anti-myc (Santa Cruz Biotechnology, 9E10), mouse anti-v5 (Life Technologies, R960-25) and Alexa 488- and Alexa 568- conjugated anti-mouse and -human IgG secondary antibodies (Life Technologies).

2.3. DNA constructs

DNA constructs for the 24 candidate antigens, including a tag to identify transfected cells, were either present in our lab, or a gift from other laboratories. Table 1 depicts the specifications of all DNA constructs used in this study.

2.4. Immunocytochemistry of live primary hippocampal neurons

Cultures of primary hippocampal neurons were prepared from embryonic day 18 rat brains. Neurons were grown in Neurobasal medium (Life Technologies) supplemented with B27, 0.5 μM glutamine, 12.5 μM glutamate and penicillin/streptomycin. Neurons were plated on coverslips coated with poly-L-lysine (30μg/ml) and laminin (2 μg/ml) at a density of 75,000/well. Neurons were then incubated with plasma (1:50) in unconditioned medium for 1 hour at 37 °C, washed in medium and fixed for 10 min with 4% paraformaldehyde (PFA)/4% sucrose at room temperature. Cells were incubated with the secondary-antibody in GDB buffer (0.1% bovine serum albumin (BSA), 0.4 M NaCl, 15 mM phosphate buffer, pH 7.4) for 1 h at room temperature. Neurons were then washed in PBS and mounted on slides in Vectashield mounting medium containing DAPI (Vector Laboratories).

2.5. Cell-based assay

HeLa cells were cultured in Ham's F10/DMEM (50%/50%) containing 10% fetal calf serum and 1% penicillin/streptomycin. HeLa cells were detached using trypsin/EDTA and were plated on 16 well Tissuetek chamber slides (Thermoscientific) and were transfected using Polyethylenimine (1 mg/ml, PEI max, Polysciences, 24765-2). Cells were fixed for 10 min using 4% PFA and incubated overnight at 4 °C with patient plasma (1:400) in PBS+ (PBS with 1% BSA and 0,1% triton). Cells transfected with Grik3 or KCNIP constructs were also incubated with mouse anti-myc tag or mouse anti-v5 tag primary antibodies. After washing, cells were incubated with secondary antibodies in PBS+ for 1 hour at room temperature. Cells were washed and mounted in Vectashield mounting medium containing DAPI (Vector Laboratories).

Table 1 DNA constructs used in the cell-based assay.

Protein	Gene	Species	Vector	Tag	Source
Glutamate receptor 1	Gria1	rat	pGW2	GFP	Casper Hoogenraad, Universiteit Utrecht, Netherlands
Glutamate receptor 2	Gria2	rat	pGW2	GFP	Casper Hoogenraad, Universiteit Utrecht, Netherlands
Metabotropic glutamate receptor 1	GRM1	human	pGW2	GFP	Casper Hoogenraad, Universiteit Utrecht, Netherlands
Metabotropic glutamate receptor 3	GRM3	human	pGW2	GFP	Casper Hoogenraad, Universiteit Utrecht, Netherlands
Metabotropic glutamate receptor 5	Grm5	mouse	pGW2	GFP	Casper Hoogenraad, Universiteit Utrecht, Netherlands
Glutamate receptor ionotropic, Kainate 3	Grik3	rat	pcDNA3.1	Myc	Christophe Mulle, Université Bordeaux, France
Glutamate receptor ionotropic, NMDA 1	Grin1	rat	pEGFP	YFP	Casper Hoogenraad, Universiteit Utrecht, Netherlands
Glutamate receptor ionotropic, NMDA 2a	Grin2a	rat	pGW2	GFP	Casper Hoogenraad, Universiteit Utrecht, Netherlands
Glutamate receptor ionotropic, NMDA 2b	Grin2b	rat	pGW2	GFP	Casper Hoogenraad, Universiteit Utrecht, Netherlands
Glutamate receptor ionotropic, NMDA 3	Grin3a	rat	pEGFP	GFP	Casper Hoogenraad, Universiteit Utrecht, Netherlands
Neuronal membrane protein M6a	Gpm6a	rat	pEGFP	GFP	Camila Scorticati, Universidad nacional de San Martín, Argentina
Glutamate receptor ionotropic, Kainate 4	GRIK4	human	pGW2	GFP	Casper Hoogenraad, Universiteit Utrecht, Netherlands
Brain-derived neurotrophic factor	Bdnf	rat	pEGFP	GFP	Casper Hoogenraad, Universiteit Utrecht, Netherlands
Receptor tyrosine-protein kinase erbB4	ERBB4	human	pEGFP	GFP	Frank Jones, Tulane University Health Sciences Center, New Orleans, USA
Kv channel-interacting protein 1	KCNIP1	human	pcDNA3.1	v5	Priit Pruunsild, University of Heidelberg, Germany
Kv channel-interacting protein 2	KCNIP2	human	pcDNA3.1	v5	Priit Pruunsild, University of Heidelberg, Germany
Kv channel-interacting protein 3	KCNIP3	human	pcDNA3.1	v5	Priit Pruunsild, University of Heidelberg, Germany
Kv channel-interacting protein 4	KCNIP4	human	pcDNA3.1	v5	Priit Pruunsild, University of Heidelberg, Germany
5-Hydroxytryptamine receptor 4 (isoform d)	HTR4	human	pcDNA3.1	YFP	Frank Lezoualc'h, Inserm, France
5-Hydroxytryptamine receptor 4 (isoform e)	HTR4	human	pcDNA3.1	YFP	Frank Lezoualc'h, Inserm, France
Neurexin-1	Nrxn1	rat	unknown	GFP	Edwin Chapman, Howard Hughes Medical Institute, USA
D (1A) Dopamine receptor	DRD1	human	pIRES2-GFP	GFP	Fabienne Brilot, University of Sydney, Australia
D (2) Dopamine receptor	DRD2	human	pIRES2-GFP	GFP	Fabienne Brilot, University of Sydney, Australia
D (1B) Dopamine receptor	DRD5	human	pIRES2-GFP	GFP	Fabienne Brilot, University of Sydney, Australia

2.6. Imaging

Both the HeLa cells and the neurons were scored using a Nikon eclipse 80i. Confocal images were acquired with the Zeiss LSM 700 using the 40 × (oil) objective.

3. Results

We included plasma samples of 104 patients, 102 patients were diagnosed with schizophrenia and 2 patients were diagnosed with schizophreniform disorder. The cohort consisted of 81 males and 23 females. The average age of the included patients was 30.7 years (SD ±7.6, range 20–55). The average duration of illness was 7.1 years (SD±3.9, range 2.1–23.4).

Plasma samples were examined for surface staining by live incubation on primary hippocampal neurons. The positive control showed the typical surface staining seen in patients with neuronal autoantibodies (Figure 1A). All samples in the cohort showed an aspecific background staining of which one example is shown in Figure 1B. None of the 104 plasma samples were positive using this approach. The plasma samples were next tested for autoantibodies against 24 neuronal antigens using CBA. The different tags identified the transfected cells. The positive control showed an increased staining of HeLa cells cotransfected with GluR1 and GluR2 as shown in Figure 2A. None of the plasma samples were positive for autoantibodies against any of the 24 proteins, of which one example is shown in Figure 2B.

4. Discussion

In this study we examined the prevalence of autoantibodies against neuronal cell surface antigens in patients diagnosed with schizophrenia or schizophreniform disorder. Plasma samples of 104 patients were screened using cultured hippocampal neurons and a CBA using 24 candidate neuronal membrane proteins. None of the samples were positive in either of these assays.

One other study used a similar approach by screening sera of patients with schizophrenia for neuronal autoantibodies using cultured hippocampal neurons and rat brain slices. In this study 4/80 patients and 4/40 controls showed reactivity with neuronal surface antigens, unfortunately the targets of these autoantibodies have not been identified and therefore their clinical relevance remains unknown (Masdeu et al., 2012). However, the fact that these antibodies occurred more frequent in controls does not support a role for antibodies in the pathogenesis of schizophrenia.

Using the CBA we examined the presence of several known and unknown autoantibodies. In line with some prior studies we did not find autoantibodies against subunits of the NMDA-receptor (Masdeu et al., 2012; Rhoads et al., 2011) or subunits of the dopamine, AMPA and GABA receptor (Müller et al., 2014). However, there is still an ongoing debate about the presence of anti-NMDA receptor antibodies in patients with schizophrenia as some studies reported a seroprevalence of up to 10% (Hammer et al., 2014; Steiner et al., 2013). It is important to stress that the majority of the seropositive patients had antibodies of the IgA and IgM subclass, whereas only antibodies of the IgG class seem to be clinically relevant (Gresa-Arribas et al., 2014; Kayser and Dalmau, 2014; Titulaer and Dalmau, 2014). Therefore, in our study only antibodies of the IgG subclass were assessed.

Strengths of our study include the use of two different screening methods, offering the opportunity to explore the presence of yet unknown autoantibodies and to further validate findings on known autoantibodies. In addition, positive findings on surface autoantibodies found using a CBA can be validated using hippocampal neurons. A possible shortcoming of our study is the use of plasma samples instead of cerebrospinal fluid (CSF). For anti-NMDA receptor antibodies it has been shown that testing CSF samples has a higher sensitivity (Gresa-Arribas et al., 2014) and this may also apply for other neuronal autoantibody mediated disorders. Moreover, most included patients were diagnosed years ago. It seems worthwhile to perform a similar

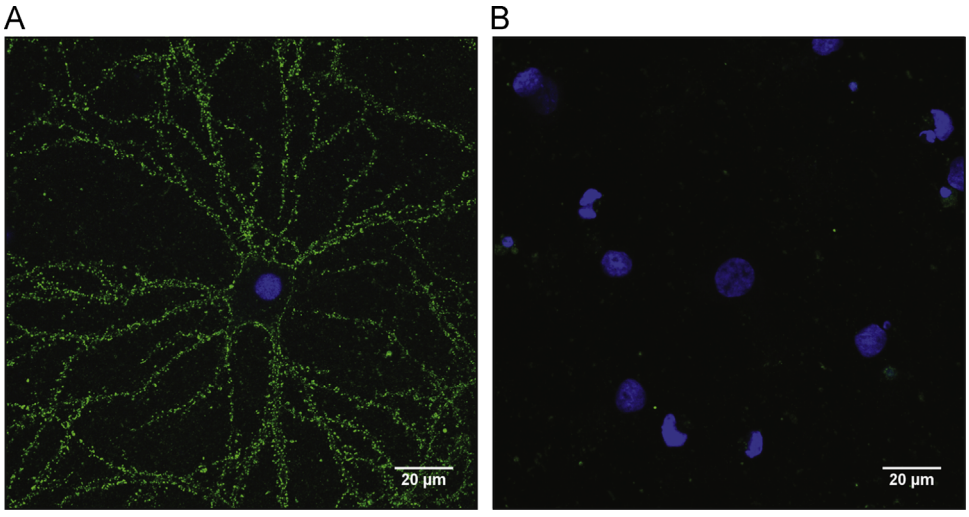

Figure 1 (A) Representative picture of surface labeling of rat hippocampal neurons by plasma from the positive control. (B) Typical example of background staining by a plasma sample from schizophrenia cohort. Human antibodies labeled green, nuclei blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

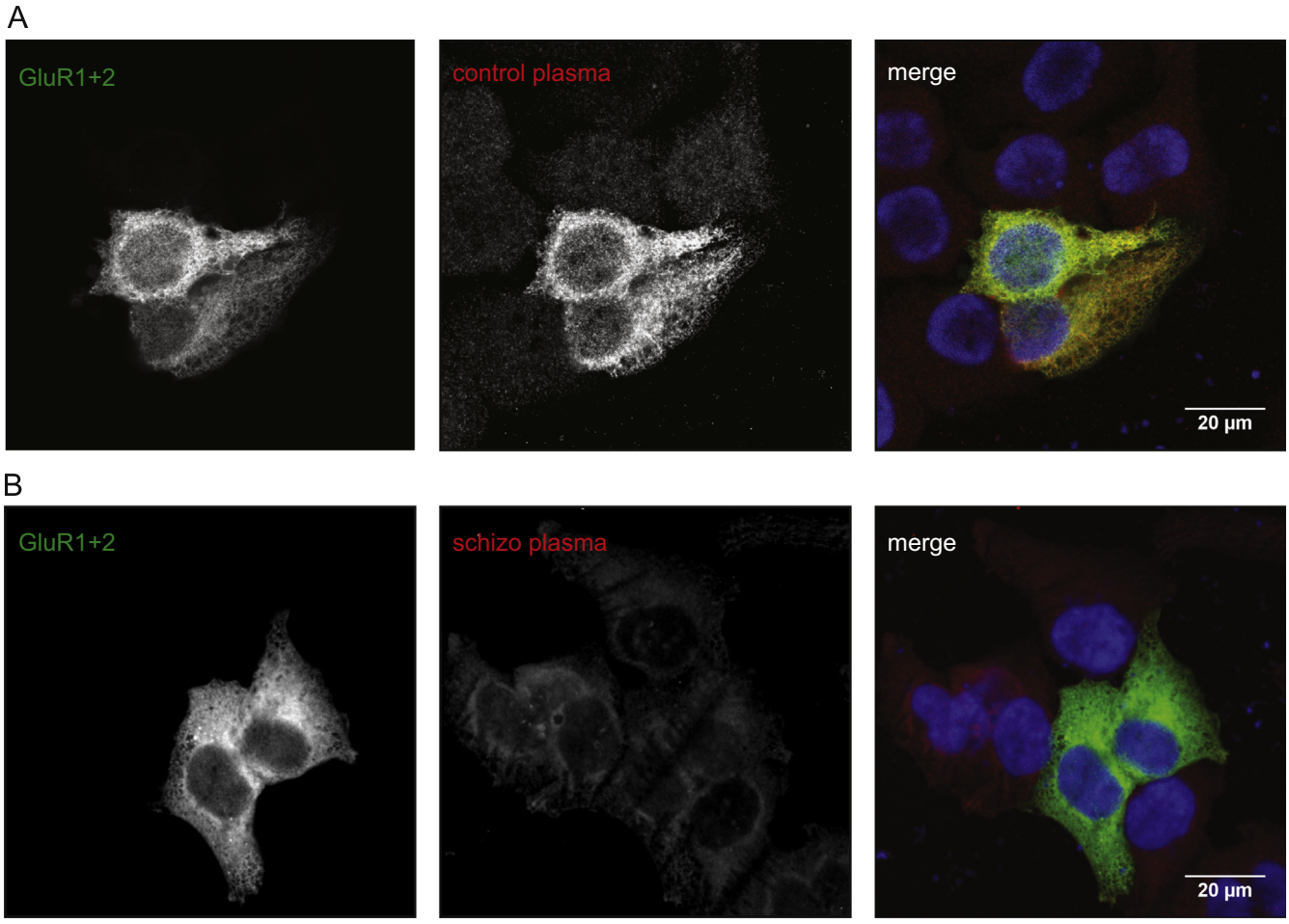

Figure 2 (A) Representative picture of increased staining of HeLa cells cotransfected with AMPA receptor subunits GluR1 and GluR2 by plasma from the positive control with anti-GluR1 antibodies. (B) Typical example of background staining by a plasma sample from the schizophrenia cohort. Transfected cells labelled green, human antibodies red, nuclei blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

screening in acutely ill patients with a first episode psychosis, preferably using both blood and CSF samples, as some autoantibodies might only be detectable in CSF in an early phase of the disease. In addition, some types of neuronal autoantibodies have a higher prevalence in specific subgroups, for example anti-NMDA receptor antibodies in females with a teratoma (Dalmau et al., 2007). Future studies could therefore focus on specific subgroups of patients with schizophrenia or psychosis, such as patients with a post-partum psychosis (Bergink et al., 2015), with neurologic or other characteristic signs of autoimmune encephalitis (van Mierlo et al., 2015) or with a history of a malignancy (Dalmau et al., 2007). Lastly, performing a similar screening in patients with bipolar disorder could prove to be of value (León-Caballero et al., 2015).

In conclusion this study does not support a role for autoantibodies against neuronal cell surface antigens in the pathogenesis of schizophrenia. However, studies using cerebrospinal fluid from a large group of first episode patients are needed to further evaluate the potential role of autoantibodies in schizophrenia.

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Contributors

All authors contributed to and have approved the final manuscript. MHvCH, LPM and HCvM performed the experiments and collected the data. HCvM and MHvCH wrote the first draft of the manuscript. LdW, EdG and RSK designed and supervised the study. GROUP Investigators designed the GROUP project and revised the manuscript.

Conflict of interest

All authors declare that they have no conflicts of interest.

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