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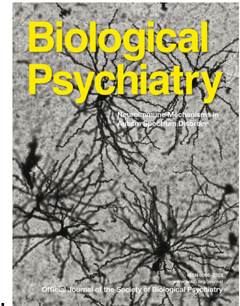

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Cell- and single molecule-based methods to detect anti-NMDA receptor autoantibodies in first episode psychosis patients from the OPTiMiSE project

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Circulating autoantibodies against glutamatergic N-methyl-D-aspartate receptor (NMDAR-Ab) have been reported in a proportion of patients with psychotic disorders, raising hopes for more appropriate treatment for these antibody-positive patients. However, the prevalence of circulating NMDAR-Ab in psychotic disorders remains controversial with detection prevalence rates, and immunoglobulin (Ig) classes, varying considerably between studies, perhaps because of different detection methods. Here, we compared the results of serum assays for a large cohort of first episode psychosis patients (FEP) using classical cell-based assays in three labs and a single molecule-based imaging method. Most assays and single molecule imaging in live hippocampal neurons revealed the presence of circulating NMDAR-Ab in approximately 5% of FEP patients. However, some heterogeneity between cell-based assays was clearly observed, highlighting the urgent need of new sensitive methods to detect the presence of low-titer NMDAR-Ab in seropositive patients that cannot be clinically identified from seronegative ones.

The detection of autoantibodies against neurotransmitter receptors in patients with neurological and psychiatric disorders has raised hopes for a better understanding of the molecular cascades underlying these pathologies and for treating patients who are antibody-positive with immunotherapy (1). The link between psychotic disorders and autoimmunity is, in fact, an old concept that has regained strong support partly thanks to the discovery of anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis (2). In this disorder, autoantibodies directed against the GluN1 subunit of NMDAR (NMDAR-Ab) are strongly associated with psychotic symptoms, sometimes catatonia, and profound reversible neurologic deterioration (2). Remarkably, approximately 80% of patients recover with immunotherapy directed to remove the antibodies and antibody-producing plasma cells (corticosteroids, intravenous immunoglobulins, plasma exchange, rituximab, or cyclophosphamide). The process of recovery is slow, spanning over months, with initial improvement of autonomic dysfunction, and gradual improvement of deficits of memory, attention, and behavior (3). In patients with schizophrenia, the prevalence and clinical significance of circulating NMDAR-Ab remains controversial with detection prevalence rates varying considerably between studies (4). Recent articles reported the presence of NMDAR-Ab in schizophrenic patients using a detection method that has provided different outcomes in schizophrenic or FEP patients in the past (5-8). The extent to which discrepancies reflect different sensitivities between labs or between NMDAR-Ab assays is unclear (9). Moreover, most assays depend on demonstrating the presence of antibodies binding to the NMDAR, but do not address whether the antibodies significantly alter the expression, localization or function of the NMDAR. In the present study, we discuss and address these key issues by using three different assays to assess independently samples from the same patients with FEP. We also employ an innovative single nanoparticle-imaging method to examine the presence of such autoantibodies.

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Methods and materials

Participants

From the OPTiMiSE cohort, 298 FEP patients, having minimal or no exposure to anti-psychotics, were examined (Table 1).

NMDAR-Ab detection using cell-based assays (CBA)

Live CBA

Serum samples were first tested for the presence of NMDAR-Ab in Lab A (Lyon) using a cell-based assay on human embryonic kidney cells (HEK293) ectopically expressing both GluN1 and GluN2B-NMDAR subunits, as previously described (10). Briefly, HEK293 were grown on glass coverslips in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum. 24h later, cells were co-transfected (Lipofectamine LTX, Invitrogen) with plasmids coding for GluN1-GFP (Green Fluorescent Protein) and GluN2B subunits of the NMDAR. The co-transfection rate was not assessed in each sample as we previously validated that most cells efficiently co-express the different cDNA constructs. To prevent cytotoxicity, cells were supplemented with 500 µM ketamine. 48h after transfection, cells were subsequently incubated in a saturation buffer (DMEM, 25mM HEPES, 1% BSA, 5% normal goat serum) for 1h and with patient serum at an initial 1:20 dilution for 2h at room temperature. Cells were then washed in DMEM HEPES and fixed (1% paraformaldehyde, 15 min) before incubation with Cy3-conjugated anti-human IgG for 1h. Finally, cells were washed in phosphate buffered saline (PBS) and DMEM-HEPES, and mounted on slides in Mowiol mounting medium. Binding of human IgG to live NMDAR-expressing HEK293 cells was visualized using an epifluorescence microscope (Axiophot, Zeiss) and was scored from 0 to 4 according to the

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percentage of transfected cells (green) colocalized with human IgG staining (red) as following: negative (0 = no binding), weak positive (binding to a low percentage of transfected cells, <2) and positive (>2). All samples were tested by two independent blinded observers and all positive and weakly positive sera were retested. The final score was the mean of the scores of the independent assays from the 2 blinded scorers.

All Lab A positive samples were tested in Lab B (Nuffield Department of Clinical Neurosciences, Oxford) using a similar live CBA, as described previously (11). In this case, HEK293 cells were transfected with plasmids containing cDNAs of the NMDAR subunits GluN1 and GluN2B at a ratio of 1:3, along with an independent plasmid expressing eGFP. Cells were incubated in human serum at an initial 1:20 dilution for 1h at room temperature before fixation with 4% formaldehyde, followed by 45 min incubation with Alexa Fluor 568 goat anti-human IgG (Invitrogen anti-IgG heavy and light chains). Binding of human IgG to the cell membrane of live HEK293 cells (*i.e.* those expressing eGFP) was assessed using an epifluorescence microscope (Leica DM2500); intensity of fluorescence of each sample was rated on a visual scoring system of 0-4. Scores of 0 and 1 were rated as negative, 1.5 as weak positive and 2-4 as positive. All positive samples were repeated at dilutions of 1:20, 1:100 and 1:500 to confirm IgG specificity using goat anti-human IgG Fc, followed by AlexaFluor 568 donkey anti-goat IgG (Invitrogen), and also checked for non-specific binding using HEK293 cells that had been transfected with another antigen. The titer of the antibody was given as the dilution of serum giving a score of 1.

Euroimmun CBA kit

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In order to assess potential sensitivity and specificity differences between live and fixed CBA, all positive sera identified in Lab A were also tested in Lab C (King's College, London) using the commercial CBA kit (Euroimmun AG, Luebeck, Germany). Schematically, this kit uses acetone-prefixed cells (to enable transport) expressing the GluN1a subunit only, which mostly form intracellular GluN1-NMDAR homomers (membrane homomers are possible). Small chips of the cells are placed onto glass cover slips and sold for detection of antibodies to the NMDAR (12) using in-house reagents. The cells were incubated for 30 min in human serum at an initial 1:10 dilution, followed by fluorescein-labelled anti-human IgG. Binding of IgG to transfected cells was assessed using fluorescence microscopy, and rated as positive or negative. All samples were scored by two independent scorers along with positive control samples supplied by the manufacturers. Slides were further reviewed (coded) by a final scorer at another laboratory (University College London, Department of Neuroimmunology) which performs the assay as part of routine clinical testing. Positive samples were tested again at dilutions of 1:100 and 1:1000 and the final titer was calculated according to manufacturer instructions.

IgG purification and live CBA

As human serum contains a myriad of molecules and generates high fluorescence background in any cultured cell systems, IgG were purified (concentrations 1.44 – 4.04 mg/ml) as previously described (13) from all positive sera of patients with a first psychotic episode. All samples were dialyzed against PBS and solutions were used at pH 7.4. To further confirm the presence of IgG directed against NMDAR, lab A (Lyon) performed an additional live CBA using purified IgG from the positive sera (Figure 1A). The concentration of the purified patient IgG

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used for the assay was, on average, 165 μg/ml. The term “IgG” relate thus to the IgG purified from patients.

NMDAR-Ab detection using single nanoparticle imaging

To ascertain the presence of NMDAR-Ab, we performed single nanoparticle detection on live cultured rat hippocampal neurons (10). Live cultured hippocampal neurons were incubated for 10 min at 37°C with either purified IgG from seropositive patients (5 μg/ml, 2/6 positive and 3/4 weak positive samples randomly selected among the 14 samples) or a commercial anti-GluN1 antibody (Alomone, 5 μg/ml). Neurons were then washed and incubated for 10 min with QD655 rabbit F(ab')2 anti-human IgG (Invitrogen, 1:5000) or QD655 goat F(ab')2 anti-rabbit IgG (Invitrogen, 1:5000). Non-specific binding was blocked by adding BSA 1% (Vector Laboratories) to the QD solution. QDs were illuminated by using a mercury lamp and appropriate excitation/emission filters, and fluorescence signals were detected using an EM-CCD camera (Evolve, Photometrics). Images were obtained with an acquisition time of 50 ms with up to 500 consecutive frames (Metamorph, Universal Imaging Corp). By fitting the fluorescence signal to a 2D Gaussian function, QD-anti-IgG/IgG complexes were localized with a 20 nm pointing accuracy and their 2D trajectories were constructed by correlation analysis between consecutive images using a Vogel algorithm. Surface diffusion parameters were extracted from each 2D trajectory: i) the Mean Square Displacement (MSD) over time, which represents the area explored by the receptor over time and illustrates the type of diffusion undergone by the receptor (*e.g.* freely-diffusing or confined), and ii) the instantaneous diffusion coefficient, D ($\mu m^2/s$), which reflects the mobility of the receptor within the plasma membrane, was calculated from linear fits of the first four points of the MSD *versus* time function such as: $MSD(t)=<r2>(t)= 4Dt$.

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Statistics

All values are expressed as mean ± SEM. Comparisons between groups were performed with non-parametric Mann-Whitney or Kolmogorov-Smirnov (distributions) tests. Significance levels were defined as *$p<0.05$, **$p<0.01$, ***$p<0.001$

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Results

Detection of NMDAR-Ab in the sera of first-episode psychotic patients using different assays

Part of the Optimise project, 298 FEP patients were recruited during their hospitalisation and included after approval by ethical committee and written informed consent for their participation (Table 1). A live CBA was first performed to determine the presence of circulating NMDAR-Ab in the whole cohort. Of the 298 sera tested in Lab A using an in-house live CBA (Figure 1A), 14 bound to NMDAR-expressing HEK293 cells (Figure 1B), with 8 samples scored as positive and 6 samples as weak positive according to the visual scoring scale (see Methods section); there was no obvious clinical differences between seropositive and seronegative patients (Table 1). In order to compare the results between antibody detection methods, we then used either live or fixed CBAs, performed in three independent laboratories, on these positive samples (Figure 1A). The 14 sera were then retested in Lab B using in-house live CBA. This second independent assay detected IgG antibodies binding to the NMDAR in 9 samples, and 5 sera that were negative or below the threshold defined for positivity in clinical testing (Figure 1C), with a very good correlation between the scores from the two laboratories (Figure 1D). Noteworthy, the negative samples of Lab B correspond to the weak positive ones of lab A, suggesting some minor appreciation difference between labs. In contrast to these outcomes, the fixed CBA performed in Lab C detected only 1 positive and 1 weak positive sera out of the 14 samples (Figure 1C). Indeed, one of the two "positive" samples titrated to 1:10 only (weak positive) and the other to 1:10 and 1:100 (positive), suggesting much lower sensitivity of the fixed cells, at least for IgG antibodies detection.

An additional live CBA using purified IgG from these 14 seropositive samples was performed in Lab A. Purified IgG from anti-NMDAR encephalitis patients were used as a positive control to

validate the assay (Figure 1B). 8 out of 14 purified IgG samples were found to be positive (3 weak binding), consistent with the previous serum scores obtained in Lab A and B (Figure 1C). Furthermore, from the 8 positive patients from live CBA (Lab A), 5 were in the purified IgG assay (the other being border lines); from the 5 positive patients from live CBA (Lab B), 4 of them correspond to the positive ones in the purified IgG assay (Lab A). To note, we did not look for IgM and IgA antibodies. Overall, the results clearly demonstrate that seropositivity is highly influenced by the detection method selected, and various in-house criteria for positivity, with fixed CBA showing low detection rates for IgG NMDAR-Abs from FEP patients.

Single nanoparticle imaging as a sensitive antibody detection test

Live CBA might not be sensitive enough to detect low titers. In order to further confirm that there were NMDAR-Ab in the samples, we took advantage of the single nanoparticle imaging technique to characterize the behavior of patients' purified IgG on NMDARs in live hippocampal neurons (Figure 2A-C). Indeed, single nanoparticle imaging gives access to the diffusion properties of individual labelled receptors over time (14) through a specific high-affinity ligand-target receptor interaction that recognizes the extracellular domain of the molecular target in live cells. In addition, labelling needs to be performed at low QD densities so that it can be optically resolved (typically <1 molecule/μm^3) and to avoid cross-linking in the case of multivalent ligands (*e.g.* antibody). Hence, the molecular behaviour of membrane target(s) detected by QD/IgG complexes is virtually independent of the IgG concentration present in the sample, contrary to classical CBA. In this assay, purified IgG from 5 out of the 14 seropositive samples (randomly selected from both positive and weak positive samples) were directly coupled to fluorescent nanoparticles called Quantum Dots (QDs). The surface dynamics of such QD-purified IgG's membrane target(s) were then compared to those of

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endogenous GluN1-NMDAR (tagged with commercial anti-GluN1 antibody). Noteworthy, very few unspecific trajectories were detected in absence of anti-NMDAR IgG (Figure 2C). The mean square displacement (MSD) curve, an indicator of the surface explored by the targeted membrane receptor, and the instantaneous diffusion coefficients from the positive and weakly positive patients were indistinguishable, and not different to those of endogenous GluN1-NMDAR (Figure 2D). These data are consistent with the binding of QD-human IgG complexes to NMDAR because the presence of IgG against other neuronal membrane neurotransmitter receptors will provide different diffusion signatures (15, 16). As an example, IgG against dopamine receptors, reported in few FEP patients (17), would have different diffusion coefficient and MSD curve (18). Together, these data indicate that single nanoparticle imaging provides a quantitative diagnosis, and limits the human bias inherent to the CBA scoring step, constituting a powerful and alternative detection tool.

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Discussion

The field of “autoimmune psychiatry” is contentious with different results from different laboratories in Europe and the USA. Here, in a coded study of 298 patient sera, we found a small proportion (around 5%) of patients with FEP that were positive for autoantibodies against the NMDAR. The NMDAR-Ab were effectively detected using live cell-based assays, and confirmed by single nanoparticle imaging. These results support earlier studies (19, 20), and further highlight the need to define the role of NMDAR-Ab in the etiology of the disease.

While the hypothesis of a subgroup of psychotic patients associated with anti-brain antibodies is not novel *per se*, recent studies have rekindled discussion about the immune hypothesis of psychiatric disorders. Over the last decade, several studies have reported the presence of circulating NMDAR-Ab in varying proportions of patients with psychosis-related disorders (21-24); however, several other studies have reported contradictory outcomes (6-8, 25). Such discrepancies could reflect the heterogeneity of autoantibody detection methods between studies (9, 26). We here confirmed that the choice of cell-based assay (*e.g.* live *versus* fixed cells) substantially influences the outcome of NMDAR-Ab seropositivity. In particular, the fixed CBA showed low sensitivity (2/14) and might be more suitable for patients with full-blown anti-NMDAR encephalitis, as virtually all seropositive samples from patients with FEP detected using live CBA were negative using this method. Live CBA appears to be more sensitive as it detected NMDAR-Ab in various clinical conditions (first-episode psychosis and encephalitis) and provided homogenous outcomes despite the human bias that falls to the visual scoring system. In addition, CBA employing live mammalian cells have the advantage of exposing autoantibodies to extracellular domains of receptors in a native conformation, consistent with the pathological condition. To note, each CBA has its own technical specificity, which call for caution when establishing strict comparisons. For instance, transfecting HEK cells with two

(e.g. lab A) or three (e.g. lab B) plasmids can generate different expression profile and detection in the cells, potentially influencing the relative detection rate. It should also be stressed out that the conformation of NMDARs in the human brain may be different from the one in HEK cells, or even rodent neurons. Testing the reactivity of NMDAR-Ab in human or primate brain tissue will surely be of great value for our understanding of these autoimmune disorders.

In search of a new approach that could identify the presence of NMDAR-Ab at low titers, we performed an additional live test using single nanoparticle imaging in hippocampal neurons to define the live "signature", *i.e.* the diffusion regime of the autoantibodies' membrane target. We could show that the signature of the CBA defined "NMDAR-Ab" was indistinguishable from that of native GluN1-NMDAR. In particular, single nanoparticle imaging relies on individual antibodies binding to their target and is therefore independent of the sample titer, contrary to other methods which are dependent on their being sufficient antibodies to provide a detectable signal. By taking advantage of this imaging approach, we were able to detect the presence of NMDAR-Ab in low titer samples (weak positives). Implementing such an additional approach in the clinical diagnostic would thus be of great help, although such a cutting-edge imaging method is technically challenging and time-consuming. Emerging assays with high-screening capacity single molecule-based approaches, such as PALM or uPAINT, and automatized fluorescence reading devices will likely provide efficient, sensitive and reproducible antibody detection tests. Since neurotransmitter receptors have distinct diffusion characteristics in the plasma membrane of neurons these approaches could even reveal the nature of the targeted receptors (15). Thus, the development of multi-approach screening methods, including CBA and cutting-edge single molecule detection methods, and clinical investigations will certainly shed new and key lights on the link between

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autoantibodies and psychotic disorders in subsets of patients. Investigating in vivo the pathogenicity of these NMDAR-Ab is surely needed for our understanding of these pathologies, potentially raising hope for appropriate therapeutic strategies.

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Author Contributions

Drs Groc and Leboyer had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Jézéquel, Lepleux, Gréa, Rogemond, and Pollak contributed equally to this work. All authors reviewed and approved the final version of the manuscript. Study concept and design: McGuire, Honnorat, Leboyer, Groc. Acquisition, analysis, or interpretation of data: Jézéquel, Gréa, Rogemond, Pollak, Lepleux, Jacobson, Groc. Drafting of the manuscript: Jézéquel, Groc. Critical revision of the manuscript for important intellectual content: Jézéquel, Pollak, Vincent, Honnorat, McGuire, Leboyer, Groc. Statistical analysis: Jézéquel, Groc. Obtained funding: McGuire, Leboyer, Groc. Study supervision: Jézéquel, Leboyer, Groc.

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A.V. and the University of Oxford hold patents for antibody assays and A.V. receives a proportion of royalties. The other authors report no biomedical financial interests or potential conflicts of interest.

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Figure legends

Figure 1. Comparison of live *versus* fixed cell-based assays for NMDAR-Ab detection in the serum of first-episode psychotic patients**. (A)** Comparative scheme illustrating the different steps of the cell-based assays (CBA) performed in the 3 independent laboratories included in the study. Note the relative homogeneity between Lab A and Lab B protocols, compared to the detection test performed in Lab C, but Lab B includes an additional step for confirmation of IgG specificity. **(B)** Representative images of the live CBA performed in Lab A. GluN1-GFP/GluN2B-expressing HEK293 cells ("GFP", green) were incubated with purified IgG from first-episode psychotic (first ep.) patients' sera ("IgG", red). Note the presence of transfected cells binding patients' purified IgG (white arrowheads), illustrating the detection of positive and weak positive sera. Samples from patients with known anti-NMDAR encephalitis ("enceph.") were used a positive control. Scale bar= 50 µm. **(C)** Table summarizing the number of samples scored as "positive" and "weak positive" relative to the type of CBA (live or fixed CBA, serum or purified IgG) and the laboratory that performed the detection test. The number of total seropositive samples dramatically decreases when sera are submitted to the fixed CBA. **(D)** Comparison and correlation of the test scores between Lab A and Lab B (x-axis, binding score from Lab A; y-axis, dilution beyond which binding was no longer positive from Lab B).

Figure 2. Single nanoparticle imaging to detect circulating NMDAR-Ab. Schematic description of a single nanoparticle imaging experiment for the detection of NMDAR-Ab at the surface of cultured hippocampal neurons. **(A)** First, purified IgG from first-episode psychotic patient are directly coupled to functionalized QD (anti-human Fab fragment) at a ratio 1:1. Live

hippocampal neurons are then incubated for 10 min with single QD-purified IgG complexes which will bind to the NMDAR. **(B)** Single QD-purified IgG complexes, attached to the NMDAR membrane target, are then detected and tracked over 500 frames with a 20Hz frequency of acquisition. By fitting the fluorescence signal to a 2D Gaussian function, QD-purified IgG complexes can be localized with a pointing accuracy of 10-30 nm. The surface density of nanofluorescent particles must be low enough to ensure good spatial resolution. **(C)** The different trajectories collected over the 500 frames are then reconnected. *Left upper panel*: Neuronal field with representative trajectories of the membrane target bound to QD-purified IgG. Scale bar= 20µm. *Right panel*: Enlarged single trajectories (25s duration) of the surface receptors targeted by endogenous surface GluN1-NMDAR (anti-GluN1 subunit antibody), purified IgG from seropositive patients ("positive", 5 µg/ml), purified IgG from weak seropositive patients ("Weak positive", 5 µg/ml) or negative control without primary IgG. Scale bar= 500 nm. **(D)** Diffusion parameters are finally extracted from QD-purified IgG trajectories for quantification. *Left panel*: comparison of the mean square displacement (MSD) curves obtained with five patients' purified IgG (2 positive and 3 weakly positive) or anti-GluN1 subunit antibody (mean ± SEM; Positive, n= 2012 trajectories; weak positive, n= 1409 trajectories; endogenous GluN1, n=113). *Right panel*: comparison of the mean instantaneous diffusion coefficients ($\mu m^2/s$) between positive, weak positive and endogenous GluN1-NMDAR surface trajectories (mean ± SEM; positive= 0.16 ± 0.015, n=175 trajectories randomly selected from 2012 trajectories; weak positive= 0.17 ± 0.016, n=180 randomly selected from 1409 trajectories; endogenous GluN1= 0.15 ± 0.016, n=113; ANOVA followed by Newman-Keuls comparison test; 3 independent experiments; no statistical difference between groups). To note, same statistical conclusion is reached when comparing initial dataset (Positive= n= 2012 trajectories; weak positive, n= 1409 trajectories;

endogenous GluN1, n=113; ANOVA followed by Newman-Keuls comparison test; no statistical difference between groups).

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Table. Demographics, Diagnostic, and comparison between seropositive and -negative patients

	Cohort	Seropositive	Seronegative
Patients, No.	298	14 (4.7%)	284 (95.3%)
Age, mean (SD)	26.06 (5.97)	26.77 (6.61)	26.02 (5.95)
Sex, No. (percent)	Male 196 (65.8%) Female 102 (34.2%)	Male 8 (57.1%) Female 6 (42.9%)	Male 188 (66.2%) Female 96 (33.8%)
Duration of psychosis (mo.), mean (SD)	5.91 (5.89)	4.23 (6.44)	5.99 (5.86)
DSM-IV diagnosis, No. (percent)			
Schizophreniform disorder	131 (44 %)	6 (42.9%)	125 (44%)
Schizophrenia undiff. type	28 (9.4%)	2 (14.3%)	26 (9.2%)
Schizophrenia disorganised type	6 (2%)	1 (7.1 %)	5 (1.8%)
Schizophrenia paranoid type	110 (36.9%)	5 (35.7 %)	105 (37%)
Schizophrenia residual type	1 (0.3%)	0 (0%)	1 (0.4 %)
Schizoaffective disorder	21 (7.0 %)	0 (0 %)	21 (7.4 %)

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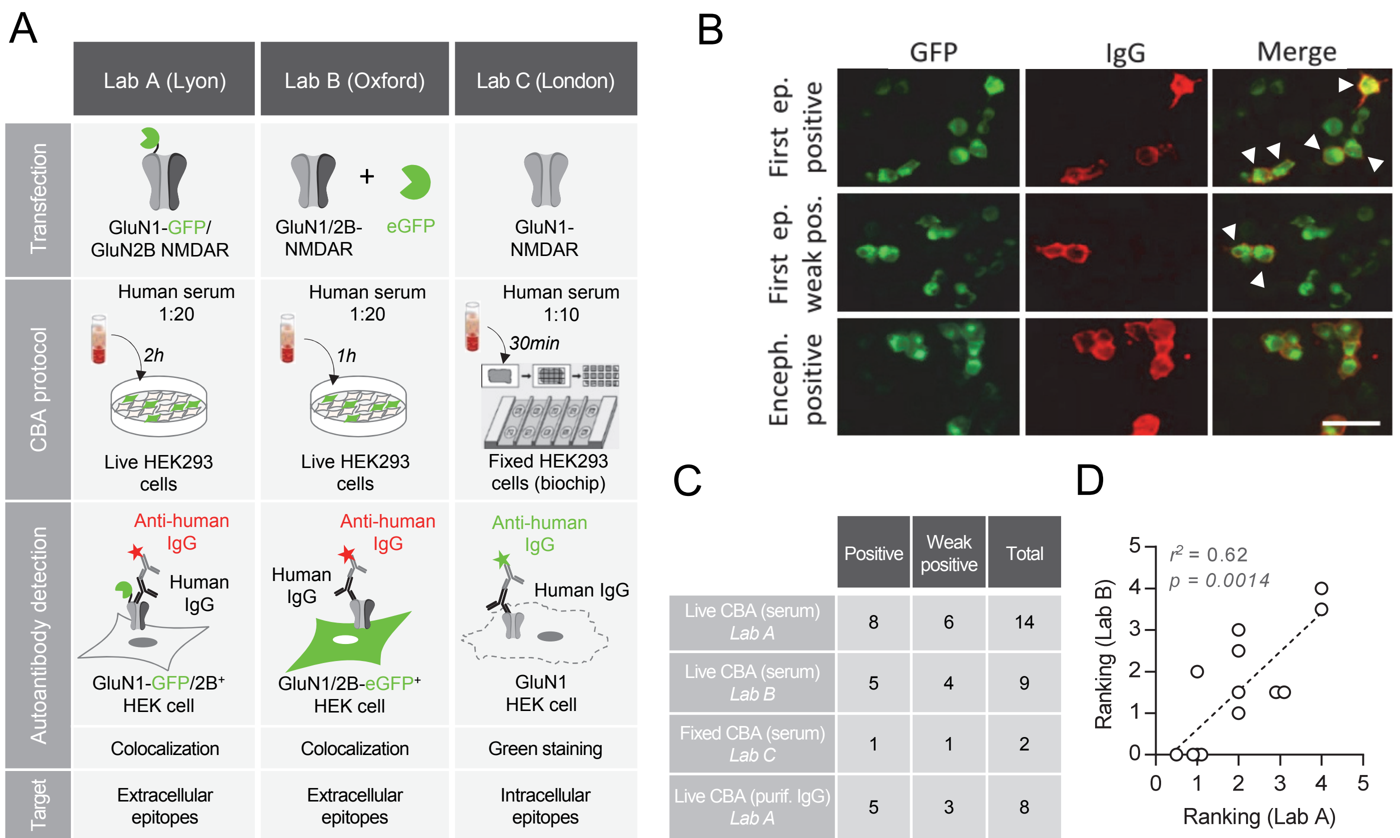
Figure 1



	Positive	Weak positive	Total
Live CBA (serum) *Lab A*	8	6	14
Live CBA (serum) *Lab B*	5	4	9
Fixed CBA (serum) *Lab C*	1	1	2
Live CBA (purif. IgG) *Lab A*	5	3	8

Figure 2