Stroke Patients Develop Antibodies That React With Components of N-Methyl-d-Aspartate Receptor Subunit 1 in Proportion to Lesion Size

Maggie L. Kalev-Zylinska, MBChB, PhD, FRCPA; Wymond Symes, MSc; Kevin C.E. Little, PhD; Peng Sun, MSc; Daying Wen, MSc; Linzi Qiao, MSc; Deborah Young, PhD; Matthew J. During, MBChB, DSc, FRACP, FACCP; P. Alan Barber, MBChB, PhD, FRACP

Background and Purpose—Antibodies against neuronal antigens develop in patients after stroke and some may serve as biomarkers of neuronal injury. We aimed to determine whether antibodies against subunit 1 (GluN1) of the N-methyl-d-aspartate receptor also develop after stroke and if so, whether they correlate with stroke characteristics.

Methods—Forty-eight patients with ischemic stroke and 96 healthy controls were tested for the presence of serum antibodies targeting GluN1. Testing was conducted using 20-kDa recombinant GluN1-S2 peptide (by ELISA and Western blotting) and on rat brain tissue (by Western blotting and immunohistochemistry). Clinical examinations and computed tomographic brain scans were performed to assess clinical state and infarct size and location.

Results—Of the 48 patients with ischemic stroke, 21 (44%) had antibodies that reacted with the recombinant GluN1-S2. There was no evidence of antibody binding to intact GluN1 in brain tissue. Western blot appearances suggested reactivity with GluN1 degradation products. Patients with anti–GluN1-S2 antibodies were more likely to have higher National Institutes of Health Stroke Scale scores, larger infarcts, and more frequent cortical involvement. Of the 96 controls, only 3 (3%), all aged >50 years, had antibodies that reacted with GluN1-S2 at low levels.

Conclusions—Antibodies that bind recombinant GluN1-S2 peptides (but not the intact GluN1 protein) develop transiently in patients after stroke in proportion to infarct size, suggesting that these antibodies are raised secondarily to neuronal damage. The anti–GluN1-S2 antibodies may provide useful information about the presence and severity of cerebral infarction. This will require confirmation in larger studies. (Stroke. 2013;44:2212-2219.)

Key Words: antibody ■ GluN1 ■ imaging ■ ischemic ■ NMDAR ■ NR1

In ischemic stroke, the breakdown of the blood–brain barrier facilitates exposure of brain antigens to the peripheral immune system and as a result, patients develop antibodies against various neuronal proteins.1–3 Immunogenic antigens include subunit 2 (GluN2) of the N-methyl-d-aspartate receptor (NMDAR).4–6 NMDARs are highly expressed in the cerebral cortex, and their normal activity is critical for neuronal connectivity and survival.7 During brain ischemia, high glutamate levels cause NMDAR overactivation that initiates neuronal cell death.8,9 Along with GluN2 subunits, NMDARs also contain obligate GluN1 components.10 Feedback regulation of NMDAR overactivity during ischemia involves proteolytic cleavage of both GluN2 and GluN1, including by enzymes involved in coagulation and fibrinolysis (thrombin, plasmin, tissue-type plasminogen activator [tPA]).11–16 Different types of cleavage events increase or reduce NMDAR activity.11,12,16,17 Cleaved GluN2 peptides are found circulating in peripheral blood of patients with stroke.18 When released to the peripheral blood or regional lymph nodes,19 that is outside of the immunologically privileged brain environment, GluN2 peptides trigger autoantibody development.4–6 Anti-GluN2 antibodies are detected soon after stroke and their levels correlate with infarct size.4,6 High levels of anti-GluN2 antibodies may be predictive of stroke and other adverse neurological outcomes in high-risk situations.20 Antibodies targeting GluN1 epitopes have not been previously reported in patients with stroke. GluN1 binds glycine (NMDAR coligand), regulates receptor activity, and contributes toward intracellular signaling pathways, including those involved in neuroprotection.21–24 Similar to

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From the Departments of Molecular Medicine and Pathology (M.L.K.-Z., W.S., K.C.E.L., P.S., D.W., M.J.D.), Pharmacology and Clinical Pharmacology (L.Q., D.Y.), and Centre for Brain Research (L.Q., D.Y., P.A.B.), The University of Auckland; LabPlus Haematology (M.L.K.-Z.) and Department of Neurology (P.A.B.), Auckland District Health Board, Auckland, New Zealand; and Departments of Molecular Virology, Immunology and Medical Genetics, Neuroscience and Neurological Surgery, Ohio State University, Columbus, OH (M.J.D.).

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Correspondence to Maggie L. Kalev-Zylinska, MBChB, PhD, FRCPA, Molecular Medicine and Pathology, The University of Auckland, Private Bag 92019, Auckland, ACM 1142, New Zealand. E-mail m.kalev@auckland.ac.nz

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2212
GluN2, GluN1 is also cleaved during brain ischemia.11-14,17 We hypothesized that the proteolytic cleavage of GluN1 associated with stroke would lead to the development of anti-GluN1 antibodies.

Methods

Patients and Controls

Forty-eight patients with acute ischemic stroke admitted to our stroke unit were enrolled into this study. Written informed consent was obtained from all participants, and all study procedures were approved by the regional Ethics Committee. Neurological dysfunction was assessed using the National Institutes of Health Stroke Scale (NIHSS) score at hospital admission and discharge. Patients were classified according to the Trial of Org 10172 in Acute Stroke Treatment and the Oxfordshire Community Stroke Project criteria. All 48 patients had computed tomographic brain scans performed and 11 also had MRI brain scans. Infarct size and location were classified using the Alberta Stroke Program Early computed tomography (ASPECT) score, which has a range of 0 to 10, with lower scores indicating larger infarcts.25 Patients with ASPECT scores ≤7 were considered to have medium to large infarcts. All 48 patients had the anti–GluN1-S2 antibody screen performed within 48 hours of stroke onset. Where possible, anti-GluN1-S2 antibody testing was also performed at day 7 after stroke onset. Patients with a positive antibody result during hospitalization were restested 28 months after the stroke.

Two control groups were used: 46 healthy laboratory workers aged ≤30 years and 50 healthy blood donors aged ≥50 years. Control individuals with a history of stroke and systemic lupus erythematosus were excluded, as anti-NMDAR antibodies may be seen in systemic lupus erythematosus.26,27 Blood donors were matched to patients with stroke for sex but imperfectly matched for age, as older people tend not to donate blood. All participants had 10 mL of blood collected at each time-point into BD Vacutainer SST II tubes (Becton Dickinson, Franklin Lakes, NJ). Within hours from collection, blood was spun at 4000 g for 10 minutes at 4°C. Serum was separated and stored in small aliquots at −80°C, until testing.

Generation of the Recombinant GluN1-S2 Peptides

Sequences encoding GluN1-S2 were amplified by polymerase chain reaction from mouse GluN1 cDNA28 using forward 5'-aaggtccttcctggtgctggatcg-3' and reverse 5'-ccgaagttgctttcaaaagtagg-3' primers. This region carries 100% amino acid homology with human GluN1-S2. Polymerase chain reaction products were cloned into PCR-TOPO2.1 (Life Technologies, Carlsbad, CA), sequence verified and subcloned into pET3 bacterial expression plasmid (Novagen), according to standard protocols (see Methods in the online-only Data Supplement). Recombinant protein was sedimented in 100 mM NaH2PO4, 10 mM/L Tris-HCl, pH 8.0, and washed 3× with repeated sonication. Size and purity of the recombinant protein was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (15%) under denaturing conditions.

Anti–GluN1-S2 ELISA

Human serum immunoglobulin G (IgG) binding recombinant GluN1-S2 was measured by ELISA according to a method previously described for anti-GluN2 antibodies29 with minor modifications. Flat-bottom 96-well ELISA plates (Nunc MaxiSorp, ThermoFisher Scientific, Rockford, IL) were coated for 24 to 48 hours using 1 μg/well of recombinant GluN1-S2 peptides diluted in an alkaline buffer (0.05 mol/L Na2CO3 and 0.05 mol/L NaHCO3; pH 9.6). Wells were washed with phosphate buffered saline containing 0.03% Tween-20 and blocked with 3% fetal calf serum in phosphate buffered saline containing 0.05% Tween-20 (200 μL/well) for 3 hours at room temperature. Human sera (diluted 1:200 in 1% fetal calf serum–phosphate buffered saline containing 0.05% Tween-20, 100 μL/well) were incubated overnight at 4°C. Plates were washed with phosphate buffered saline containing 0.05% Tween-20 and incubated with goat antihuman IgG linked to horseradish-peroxidase (1:5000; Jackson Immunoresearch, West Grove, PA) for 3 hours. After further washes, antibodies that bound were detected using Turbo 3,3',5,5'-tetramethylbenzidine peroxidase substrate (ThermoFisher Scientific). Optical density was read at 450 nm in Synergy 2 Multi-Detection Microplate Reader (BioTek Instruments Inc, Winooski, VT). Each serum sample was tested in triplicate and each run was repeated ≥3x. To minimize variations between plates, optical density readings were converted to a Z score: (optical density sample–mean optical density controls)/SD of controls.29 Three negative and 1 positive control were run on each plate. Threshold for positivity was defined as higher than 2×SD above the mean of measurements obtained in 46 young healthy people, aged ≤30 years. Sera from GluN1-S2–vaccinated rats were included as positive controls, diluted 1:3000. The coefficient of variation was <10% between ELISA plates and <3% between replicates on the same plate.

Purification of IgG and Western Blotting

Human serum IgG was purified on 2 mL Immobilized Protein G Columns (ThermoFisher Scientific) using ImmunoPure (G) IgG Purification Kit (ThermoFisher Scientific). Purified IgG was dialyzed against phosphate buffered saline using Slide-A-Lyzer dialysis cassettes (ThermoFisher Scientific). Anti–GluN1-S2 antibodies were preabsorbed from samples using recombinant GluN1-S2 added in excess (see Methods in the online-only Data Supplement). Protein extracts from rat hippocampus were prepared and quantified as previously described.30 Protein samples (0.5 μg of recombinant GluN1-S2 or 30 μg of brain proteins) were loaded per lane, separated by SDS-PAGE (15% and 10%, respectively), transferred to a nylon cell membrane (Hybond-ECL, Amersham, Piscataway, NJ), and processed as previously reported.30 The following primary antibodies were used: human serum (1:500), purified human IgG (1:750), or anti-GluN1 antibody control (MAB363, Millipore, Billerica, MA; 1:3000). After washes, horseradish-peroxidase–linked secondary antibodies were applied: antihuman (1:20000; Jackson) or antimouse (1:10000; Santa Cruz Biotechnology, Santa Cruz, CA). Signal was developed using ECLPlus (ThermoFisher Scientific) in a FujiFilm LAS-3000 phosphorimager (Life Science, Stamford, CT).

Immunohistochemistry

Rat brain sections, 40 μm thick, were processed as previously reported30 with minor modifications (see Methods in the online-only Data Supplement). Human sera or purified human IgG were diluted 1:200 and incubated on sections overnight. Antibodies that bound were detected with 3,3′-diaminobenzidine (Sigma-Aldrich, St Louis, MO). Images were taken using a DS-Qi1 digital camera and supporting Nikon Instruments-Elements software connected to an Eclipse Ti-U inverted research microscope.

Statistical Analysis

Data are presented as the mean±SD or median (range), as indicated. The SPSS version 16 (Chicago, IL) software package for Mac was used to perform statistical analysis. To compare between groups, cross-tabulations with significance tests were performed for data in categories (Fisher exact test if n<5). Analysis for mean differences between groups was conducted using independent samples t test (2-sided). Paired samples t test was used to assess changes in the ELISA Z-scores over time. General linear model and linear regression were applied to test the relationship between antibody levels (ELISA Z scores) and patient age, sex, NIHSS, and ASPECT scores. P values <0.05 were considered statistically significant.

Results

Forty-eight patients with ischemic stroke were tested for the presence of antibodies targeting recombinant GluN1.
sequences (Figure 1A). Patient characteristics at inclusion into the study are shown in Table 1. The median NIHSS score for patients on admission was 5 (0–23). Slightly more than half of the strokes were total or partial anterior circulation infarcts and just under one third were lacunar infarcts. The median ASPECT score was 9 (3–10), determined from computed tomographic scans performed at a median of 3 hours and 20 minutes from hospital admission. Cardiac embolism was the most frequent cause of the stroke (38%) and 29% of infarcts were because of small vessel disease. Cortical involvement was seen in 35% of patients. Four (8%) patients were treated with recombinant tPA. The control group included 50 healthy blood donors (mean age, 55±6 years; 25 women) and 46 healthy laboratory workers (mean age, 25±5 years; 25 women), without a history of prior stroke or systemic lupus erythematosus.

**Stroke Patients Develop Antibodies That Bind Recombinant GluN1-S2**

All 48 patients had initial ELISA testing for the presence of anti-recombinant GluN1-S2 antibodies performed at 2±1 days after stroke. Thirty (63%) patients were retested at hospital discharge, 7±2 days after stroke onset. Sixteen of the 18 patients not retested had been discharged before day 7, 1 had died, and 1 declined further testing. Eighteen of the 21 (86%) patients with the evidence of antibodies at the initial test were retested at 18±6 months after stroke. Of the 3 patients who had the anti–GluN1-S2 antibodies detected on admission, but were not retested long-term, 1 died, 1 declined, and 1 could not be located.

Twenty-one of 48 (44%) patients had antibodies that bound recombinant GluN1-S2 at initial testing (Figure 1B). The mean antibody level for all patients with stroke was 2.12±1.89, as

![Figure 1. Anti–GluN1-S2 antibodies by ELISA. A, Schematic of the full-length human GluN1 protein (adapted from http://www.bristol.ac.uk/synaptic/receptors/) and the recombinant GluN1-S2 peptide equivalent to 658 to 809 aa region of GluN1. Black arrow points to the recombinant GluN1-S2 on SDS-PAGE at ≈20 kDa. B, Levels of anti–GluN1-S2 antibodies shown as ELISA Z scores in 3 experimental groups: young healthy people (n=46), patients with stroke (n=48), and healthy blood donors (n=50). In each group, mean values of ELISA Z scores are indicated as black horizontal lines. The threshold for positivity is marked. C, Anti–GluN1-S2 antibody levels in individual patient at 3 time-points: day 2±1 (admission), day 7±2 (discharge), and 18±6 months after stroke. Side legend lists samples in the order of ELISA Z scores (from highest to lowest).](http://stroke.ahajournals.org/)

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expressed by the ELISA $Z$ score. In 8 patients, $Z$ scores were >4.5, max 5.9. Anti–GluN1-S2 antibodies were not detected in 46 healthy controls, aged ≤30 years. Three (6%) of control blood donors, aged ≥50 years, had anti–GluN1-S2 antibody levels that were mildly elevated (mean $Z$ score, 0.21±1.36; Figure 1B).

Thirty patients with stroke had repeat antibody testing performed on day 7 (Figure 1C). Sixteen of these 30 patients were anti–GluN1-S2 negative on the first test and remained negative at day 7. Fourteen of 30 patients were antibody positive on the first test, and 11 of these 14 had small increases in levels at day 7 (paired samples $t$ test, $P=0.022$). Anti–GluN1-S2 antibody levels declined in all 18 of the antibody-positive patients who were retested at 18±6 months ($P<0.001$), but in 8 patients antibody levels remained above the threshold for positivity (Figure 1C). Western blotting was used to confirm that antibodies bound recombinant GluN1-S2 and those who did not. All 4 patients treated with recombinant tPA (STR 011, 013, 032, 039) had anti–GluN1-S2 antibodies detected (Fisher exact test, $P=0.031$). Antibody-positive patients tended to have greater clinical deficit on admission, compared with those without antibodies, as defined by the NIHSS score ($P=0.091$; Table 1). Higher antibody levels correlated with smaller reductions in the NIHSS scores between admission and discharge ($P=0.033$; Table 2). The reason for this is unknown, but may be related to the fact that these patients had more severe strokes from the outset. Patients with anti–GluN1-S2 antibodies had greater infarct size, as determined by the ASPECT score and Oxfordshire Community Stroke Project criteria (Table 1). There was an inverse correlation between the ASPECT score and the antibody levels (linear regression, $R^2=0.121$; $P=0.015$). Cortical involvement was also more common in patients with the antibodies (57%) compared with those without (19%), which underscores antibody association with larger infarcts (Table 1).

Patients With Large Strokes Are More Likely to Have Anti–GluN1-S2 Antibodies

There was no difference in the age, sex, risk factor profile (Table 1), or Trial of Org 10172 in Acute Stroke Treatment criteria ($\chi^2$ test; $P=0.320$) between patients who had antibodies binding recombinant GluN1-S2 and those who did not. All 4 patients treated with recombinant tPA (STR 011, 013, 032, 039) had anti–GluN1-S2 antibodies detected (Fisher exact test, $P=0.031$). Antibody-positive patients tended to have greater clinical deficit on admission, compared with those without antibodies, as defined by the NIHSS score ($P=0.091$; Table 1). Higher antibody levels correlated with smaller reductions in the NIHSS scores between admission and discharge ($P=0.033$; Table 2). The reason for this is unknown, but may be related to the fact that these patients had more severe strokes from the outset. Patients with anti–GluN1-S2 antibodies had greater infarct size, as determined by the ASPECT score and Oxfordshire Community Stroke Project criteria (Table 1). There was an inverse correlation between the ASPECT score and the antibody levels (linear regression, $R^2=0.121$; $P=0.015$). Cortical involvement was also more common in patients with the antibodies (57%) compared with those without (19%), which underscores antibody association with larger infarcts (Table 1).

Table 1. Clinical Characteristics and Features of Stroke in all Patients With Stroke, and According to the Presence or Absence of Anti–GluN1-S2 Antibodies

<table>
<thead>
<tr>
<th></th>
<th>All Stroke Patients (n=48)</th>
<th>Anti–GluN1-S2 Antibodies Present (n=21)</th>
<th>Anti–GluN1-S2 Antibodies Absent (n=27)</th>
<th>PValue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean±SD), y</td>
<td>70±17</td>
<td>72±17</td>
<td>68±17</td>
<td>0.416*</td>
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<tr>
<td>Women, n (%)</td>
<td>25 (52)</td>
<td>14 (67)</td>
<td>11 (41)</td>
<td>0.074†</td>
</tr>
<tr>
<td>Previous stroke, n (%)</td>
<td>7 (15)</td>
<td>2 (10)</td>
<td>5 (19)</td>
<td>0.445‡</td>
</tr>
<tr>
<td>Previous TIA, n (%)</td>
<td>5 (10)</td>
<td>3 (14)</td>
<td>2 (7)</td>
<td>0.641‡</td>
</tr>
<tr>
<td>Risk factors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>33 (69)</td>
<td>12 (57)</td>
<td>21 (78)</td>
<td>0.126†</td>
</tr>
<tr>
<td>Atrial fibrillation, n (%)</td>
<td>14 (29)</td>
<td>6 (29)</td>
<td>8 (30)</td>
<td>0.936†</td>
</tr>
<tr>
<td>Ischemic heart disease, n (%)</td>
<td>13 (27)</td>
<td>7 (33)</td>
<td>6 (22)</td>
<td>0.390†</td>
</tr>
<tr>
<td>Hyperlipidemia, n (%)</td>
<td>12 (25)</td>
<td>6 (29)</td>
<td>6 (22)</td>
<td>0.614†</td>
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<tr>
<td>Current smoker, n (%)</td>
<td>11 (23)</td>
<td>2 (10)</td>
<td>9 (33)</td>
<td>0.083‡</td>
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<tr>
<td>Peripheral vascular disease, n (%)</td>
<td>6 (13)</td>
<td>3 (14)</td>
<td>3 (11)</td>
<td>1.0‡</td>
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<td>Diabetes mellitus, n (%)</td>
<td>3 (6)</td>
<td>1 (5)</td>
<td>2 (7)</td>
<td>1.0‡</td>
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<td>NIHSS§ on admission</td>
<td>5 (0–23)</td>
<td>5 (1–23)</td>
<td>4 (0–14)</td>
<td>0.091*</td>
</tr>
<tr>
<td>NIHSS§ on discharge</td>
<td>4 (0–23)</td>
<td>6 (0–23)</td>
<td>4 (2–10)</td>
<td>0.216*</td>
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<td>OCSP</td>
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<td>TACI, n (%)</td>
<td>5 (10)</td>
<td>4 (19)</td>
<td>1 (4)</td>
<td>0.04†</td>
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<tr>
<td>PACI, n (%)</td>
<td>21 (44)</td>
<td>12 (57)</td>
<td>9 (33)</td>
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<tr>
<td>LACI, n (%)</td>
<td>15 (31)</td>
<td>3 (14)</td>
<td>12 (44)</td>
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<td>POCI, n (%)</td>
<td>7 (15)</td>
<td>2 (10)</td>
<td>5 (19)</td>
<td></td>
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<tr>
<td>ASPECT score§</td>
<td>9 (3–10)</td>
<td>9 (3–10)</td>
<td>10 (8–10)</td>
<td>0.003*</td>
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<tr>
<td>Cortical involvement, n (%)</td>
<td>17 (35)</td>
<td>12 (57)</td>
<td>5 (19)</td>
<td>0.006†</td>
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ASPECT indicates Alberta Stroke Program Early computed tomography; LACI, lacunar infarct; NIHSS, National Institutes of Health Stroke Scale; OCSP, Oxfordshire Community Stroke Project; PACI, partial anterior circulation infarct; POCI, posterior circulation infarct; TACI, total anterior circulation infarct; and TIA, transient ischemic attack.

*Independent samples $t$ test; †$\chi^2$ test; ‡Fisher exact test (2-sided); and §median (range).
brain proteins were conducted for all 48 patients with stroke and 50 blood donors. There was no clear evidence of antibody binding to intact 120 kDa GluN1 protein for any of stroke or control samples tested (Figure 3; Figure I in the online-only Data Supplement). However, anti–GluN1-S2-positive samples reacted with more brain proteins than anti–GluN1-S2-negative samples. STR032, taken from tPA-treated patient, was the most reactive with brain proteins. Among different Western blot reactivities, anti–GluN1-S2-positive samples frequently bound to proteins at ≈66 kDa, known to contain GluN1 degradation products (marked by the commercial anti-GluN1 antibody control; Figure 3A–3C, arrowheads). Preabsorption of stroke samples with recombinant GluN1-S2 attenuated binding to proteins at ≈66 kDa, implying contribution from anti–GluN1-S2 antibodies to this binding pattern (Figure 3B). Western blot reactivities weakened over time, demonstrating relationship with stroke event (Figure 3C).

Immunohistochemistry on rat brain sections was performed using 4 anti–GluN1-S2-negative and 8 anti–GluN1-S2-positive samples, including 4 from patients treated with tPA (Figure 4). None of these samples reproduced binding of the commercial anti-GluN1 antibody control, supporting the Western blot findings that stroke antibodies do not bind full-length GluN1. Samples from tPA-treated patients produced the most staining on sections, but patterns appeared nuclear and staining was not removed by the preabsorption with recombinant GluN1-S2. This suggests that most of the staining on sections was because of antibodies other than anti–GluN1-S2. The immunohistochemistry pattern is not unexpected and may

<table>
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<th>Explanatory Variable</th>
<th>β-Coefficient Estimate</th>
<th>95% Confidence Interval</th>
<th>P Value</th>
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<tr>
<td>Age</td>
<td>0.004</td>
<td>−0.037 to 0.045</td>
<td>0.853</td>
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<tr>
<td>Sex</td>
<td>1.068</td>
<td>−0.321 to 2.457</td>
<td>0.125</td>
</tr>
<tr>
<td>Delta NIHSS*</td>
<td>0.354</td>
<td>0.031 to 0.677</td>
<td>0.033</td>
</tr>
<tr>
<td>ASPECT score ≤7</td>
<td>−1.662</td>
<td>−3.277 to −0.047</td>
<td>0.044</td>
</tr>
</tbody>
</table>

ASPECT indicates Alberta Stroke Program Early computed tomography; and NIHSS, National Institutes of Health Stroke Scale.

*Change in the NIHSS score between admission and discharge.
We hypothesize that the proteolytic cleavage of GluN1 may provide a trigger for the development of anti–GluN1-S2 antibodies. The state of immune privilege would normally prevent antibody responses in the brain. However, the blood–brain barrier is disrupted in stroke, facilitating dissemination of neuronal antigens into blood and lymph nodes. The presence of anti–GluN1-S2 IgG early after stroke suggests memory of the GluN1-S2 antigen. The most obvious situations under which the immune system may have encountered GluN1-S2 would have been during previous episodes of cerebral ischemia. Our results do not confirm an association with prior stroke but patient numbers are small, and such correlations will need to be pursued in larger cohorts. Antibodies that develop in patients with stroke may be an epiphenomenon, but their functional consequences cannot be excluded. Because anti–GluN1-S2 antibodies do not bind endogenous NMDARs, they are unlikely to target intact cells. However, anti–GluN1-S2 antibodies may recognize damaged cells that bear fragmented NMDARs and target these cells for immune clearance. Future studies are required to determine whether anti–GluN1-S2 antibodies contribute to tissue damage or repair after stroke.

The anti–GluN1-S2 antibodies found in patients with stroke are different from those that define the syndrome of the anti–NMDAR antibody-induced limbic encephalitis. This is an autoimmune, typically paraneoplastic condition where anti–NMDAR antibodies are triggered by ectopic NMDAR expression usually in patients with ovarian teratoma. In this syndrome, anti–NMDAR antibodies display characteristic binding to GluN1 and GluN1–GluN2 heteromers, as well as robust hippocampal staining on rat brain sections. The epitope for anti–NMDAR antibodies has been recently located within a small region of the GluN1 amino-terminal domain, which is different from the GluN1-S2 domain used in this study.

A number of limitations apply to this work. This was an exploratory study, patient numbers were small, and outcome was not assessed at 18±6 months. We did not have access to the medical history of the apparently healthy blood donors, and it is possible that some had conditions that led to the production of anti–GluN1-S2 antibodies. There are also technical limitations related to the production of recombinant GluN1-S2. It was generated in E coli and contained not only full-length peptides, but also their degradation products and multimeric complexes. ELISA results against recombinant GluN1-S2 were dichotomized arbitrarily, although with well established criteria. It is possible that some patients may have been misclassified as antibody negative if their antibody levels or the affinity of antibody binding fell below the threshold for positivity.

Future studies will need to test larger cohorts of patients with stroke and controls to confirm clinical and radiological correlations, and determine any association with long-term outcome and vascular risk factors. The use of smaller, synthetic GluN1 peptides would help define S2 sequences that are most immunogenic. IgM antibodies should be measured in tPA treatment may reveal additional binding of anti–GluN1-S2 antibodies in tissue. Imaging studies could help elucidate whether the presence of anti–GluN1-S2 antibodies relates to a higher level of blood–brain barrier breakdown or whether these are unrelated events.
In conclusion, antibodies that bind recombinant GluN1-S2 peptides (but not intact GluN1) develop transiently in patients after stroke in proportion to infarct size, which suggests a response induced by neuronal damage. The most direct clinical implication of this work is that anti–GluN1-S2 antibodies may provide information about the presence and severity of cerebral infarction. This will require confirmation in larger studies.

Acknowledgments
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Disclosures
None.

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Stroke Patients Develop Antibodies that React with Components of NMDA Receptor Subunit 1 in Proportion to Lesion Size

Maggie L. Kalev-Zylinska, MBChB, PhD, FRCPA; Wymond Symes, MSc; Kevin C. E. Little, PhD; Peng Sun, BSc; Daying Wen, MSc; Linzi Qiao, BSc; Deborah Young, PhD; Matthew J. During MBChB, DSc, FRACP, FACP; P. Alan Barber, MBChB, PhD, FRACP.

Corresponding author: M. L. Kalev-Zylinska, Molecular Medicine and Pathology, The University of Auckland, Private, Bag 92019, Auckland, ACM 1142, New Zealand. Tel.: +64 9 923 4481; fax: +64 9 367 7121; e-mail: m.kalev@auckland.ac.nz

Supplemental Methods

Generation of Recombinant GluN1-S2 Peptides
Sequences encoding GluN1-S2 region (689-840 aminoacids [aa] of mouse GluN1) were amplified by PCR from the mouse GluN1 cDNA (accession number D10028) using forward: 5'-aaggtcttcctggtgctggatcg-3' and reverse: 5'-ccgaattcgttctcaaaatgag-3' primers. At the aa level, this region shares 100% homology with human GluN1 (658-809 aa). PCR products were cloned into PCR-TOPO2.1 (Life Technologies, Carlsbad, CA), sequence verified and subcloned into pET3 bacterial expression plasmid (Novagen, Madison, WI), modified to contain BamHI/EcoRI restriction sites. Recombinant GluN1-S2 peptides were expressed in the Escherichia coli BL21 strain (Novagen) according to standard protocols. Briefly, 500 ml ampicillin-containing Luria Broth media was inoculated with bacteria and grown at 37°C until optical density (OD) reached 0.6-1.0 at 600 nm. Protein expression was induced with 0.4 mM isopropyl β-D-thiogalactoside (IPTG; Sigma-Aldrich, St Louis, MO). After a further 3 h, bacteria were pelleted by centrifugation at 7,000 g and re-suspended in 50 mM Tris-HCl, 2 mM EDTA, pH 8.0. Inclusion bodies containing recombinant protein were isolated from cell lysates using lysozyme (0.1 mg/ml; Boehringer Ingelheim GmbH, Germany) and sonicated on ice. Protein was sedimented in 100 mM NaH2PO4, 10 mM Tris-HCl, pH 8.0 and washed three times with repeated sonication and centrifugation at 12,000 g. Size and purity of the recombinant protein was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (15%) under denaturing conditions.

Purification of IgG and Pre-absorption with Recombinant GluN1-S2
Human serum IgG was purified on 2 ml Immobilized Protein G Columns (Thermo Fisher Scientific) using ImmunoPure (G) IgG Purification Kit (Thermo Fisher Scientific), according to manufacturer's instructions. Briefly, serum samples were equilibrated in
ImmunoPure Binding buffer pH 8.0, mixed well and spun at 10,000 g for 20 min at 4°C. Protein G columns were equilibrated in Binding buffer by letting the buffer drain through columns by gravity. Equilibrated serum was applied to columns and allowed to flow through. Columns were washed with 10 ml Binding buffer and IgG eluted with 6 ml ImmunoPure Elution buffer, pH 2.8 (Thermo Fisher Scientific). Elution fractions of 1 ml were collected into microfuge tubes containing 50 µl of neutralisation buffer (1 M Tris, pH 9.5). IgG concentrations were determined spectrophotometrically at 280 nm. Protein G columns were regenerated in 5 ml elution buffer, equilibrated in 5 ml binding buffer and re-used. Purified IgG was dialysed against PBS (with three changes) over 18 h at 4°C using Slide-A-Lyzer dialysis cassettes (Thermo Fisher Scientific) (10,000 molecular weight cut-off).

Human IgG was pre-absorbed with the recombinant GluN1-S2 peptides by adding excess antigen according to the equation: n(antigen) = n(antibody) x 2[MW(antigen)/MW(antibody)] (where MW [molecular weight] of GluN1-S2 = 20 kDa and MW of human IgG = 150 kDa). Mixtures were incubated overnight at 4°C with constant rotation and spun at 10,000 rpm for 5 min prior to further use.

Immunohistochemistry
Rat brain sections, 40 µm thick, were processed as previously reported with minor modifications. Endogenous peroxidases in brain tissue were inhibited by floating sections in the solution containing 1% H$_2$O$_2$ in 50% methanol for 20 min. After washes, sections were blocked in 5% goat serum, 5% bovine serum albumin (BSA) in 0.3% PBS-TritonX for 1 h. Human sera or purified human IgG (before and after pre-absorption with the recombinant GluN1-S2 peptides) were diluted 1:200 in the buffer containing 5% goat serum, 2% BSA and 0.2% TritonX, and incubated overnight. The following morning, sections were washed in PBS-0.2% TritonX and anti-human peroxidase-labeled secondary antibody (1:500; Jackson) applied for 3 h. Further washes followed with the subsequent incubation in ExtrAvidin-Peroxidase (Sigma-Aldrich) diluted 1:500 for 2 h. Binding of antibodies was detected with 3,3’-diaminobenzidine (Sigma-Aldrich) at 0.5 mg/ml.

Supplemental Figure

Supplemental Figure S1.
**Supplemental Figure Legend**

**Supplemental Figure S1.** Western Blots on Rat Brain Proteins Using Anti-GluN1-S2-Negative Stroke and Donor Samples. Sera from anti-GluN1-S2-negative patients (A) and blood donors (B) were tested for binding with rat brain proteins. Patient identification numbers are shown. Arrows point to full-length GluN1 highlighted by the commercial anti-GluN1 positive antibody control (MAB363; Millipore), and arrowheads...
to GluN1 degradation products at ~66 kDa. A, Anti-GluN1-S2-negative samples do not demonstrate any binding at 120 or 66 kDa positions. B, Anti-GluN1-S2-positive but not anti-GluN1-S2-negative donor samples react with proteins at ~66 kDa.

**Supplemental References:**