

SUPPLEMENTAL MATERIAL

Inhibition of NMDA receptor function with an anti-GluN1-S2 antibody impairs human platelet function and thrombosis

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Supplemental Methods

Mapping of anti-GluN1 epitopes

The epitopes of anti-GluN1 antibodies were mapped by an antigen-capture ELISA using a library of 74 overlapping synthetic peptides spanning all extracellular regions of the GluN1 protein (Mimotopes, PepSetsTM, Melbourne, Australia; Supplemental Table S1). The 13 aa linker was included in the library although it was not a part of the native protein. Each synthetic peptide was 16 aa long, containing hexamer repeats overlapping the adjacent peptides at both ends and a biotin label at the 5'-end.

Microtitre plates (Nunc Maxisorp; ThermoFisher Scientific, Rockford, IL) were coated with streptavidin (5 µg per well) at 37°C for 4 h. Plates were washed and stored at 4°C until use (up to a month). Prior to epitope profiling, plates were blocked with 5% (weight per volume [w/v]) skim milk in 1x PBS containing 0.05% Tween20 (PBS-T). Blocking was conducted overnight at 4°C with gentle shaking. Plates were washed 3 times for 5 min in 1x PBS-T and coated with biotinylated peptides (250 pmol per well) in 1x PBS at room temperature for 3 h; triplicate wells were coated with each peptide. The antibody diluent was applied to control wells to obtain background absorbance. Plates were washed as above.

Pooled rat sera were diluted 1:200 in 1% skim milk (w/v) in 1x PBS-T. The anti-GluN1-S2 (BD556038; BD Biosciences, San Jose, CA) was diluted 1:500 and control samples (pools of human sera previously identified as positive and negative) 1:100. Samples were applied to wells in triplicates and incubated overnight at 4°C with gentle shaking. Plates were washed and secondary peroxidase-conjugated antibodies applied diluted 1:10,000 in 1x PBS-T (anti-rat, Sigma-Aldrich, Saint Louis, MO; anti-mouse, Jackson ImmunoResearch, West Grove, PA; and anti-human, Sigma-Aldrich). Plates were incubated at room temperature for 3 h and washed as above. O-phenylenediamine dihydrochloride (OPD) was added as a substrate and plates were incubated in the dark for 30 min. The reaction was stopped with 1N H₂SO₄. The absorbance was measured at 450 nm using Gen5 v1.04.5 software (BioTek Instruments Inc., Winooski, VT). Antibody levels were expressed as Z-scores calculated relative to the negative control, with a positivity threshold set greater than 2x SD from the mean of negative controls.

Supplemental Tables

Table S1. Sequences of synthetic peptides generated to span extracellular regions of GluN1.

No.	Hydro	MW	Peptide sequence	No.	Hydro	MW	Peptide sequence
1	0.64	2,402.9	STMHLLTFALLFSCSF	38	0.33	2,337.7	VIPNDRKIIWPGGETE
2	0.52	2,241.7	LFSCSFARAACDPKIV	39	0.04	2,307.6	PGGETEKPRGYQMSTR
3	0.34	2,227.7	CDPKIVNIGAVLSTRK	40	0.38	2,457.9	YQMSTRLKIVTIHQEP
4	0.18	2,458.8	VLSTRKHEQMFREAVN	41	0.41	2,405.8	TIHQEPFVYVKPTMSD
5	0.06	2,441.7	FREAVNQANKRHGSWK	42	0.18	2,300.6	KPTMSDGTCKEEFTVN
6	0.29	2,348.6	RHGSWKIQLNATSVTH	43	0.31	2,292.6	EEFTVNGDPVKKVICT
7	0.35	2,182.5	ATSVTHKPNAIQMALS	44	0.23	2,114.4	KKVICTGPNDTSPGSP
8	0.54	2,249.6	IQMALSVCEDLISSQV	45	0.37	2,203.5	TSPGSPRHTVPQCCYG
9	0.61	2,238.6	LISSQVYAILVSHPT	46	0.72	2,299.8	PQCCYGFVCDLLIKLA
10	0.40	2,256.5	VSHPTPNDFHTPTPV	47	0.51	2,462.9	LLIKLARTMNFITYEVH
11	0.55	2,330.7	FTPTVSYTAGFYRIP	48	0.38	2,325.6	FTYEVHLVADGKFGTQ
12	0.58	2,337.8	GFYRIPVLGLTTRMSI	49	-0.12	2,349.6	GKFGTQERVNNSNKKE
13	0.38	2,399.8	TTRMSIYSDKSIHLSF	50	0.20	2,351.7	NSNKKEWNGMMGELLS
14	0.50	2,395.7	SIHLSFLRTVPPYSHQ	51	0.53	2,158.6	MGELLSGQADMIVAPL
15	0.49	2,494.9	PPYSHQSSVWFEMMRV	52	0.47	2,359.8	MIVAPLTINNERAQYI
16	0.70	2,592.1	FEMMRVYNWNHIILLV	53	0.22	2,504.8	ERAQYIEFSKPFKYQG
17	0.32	2,287.6	HIILLVSDDEHGAAQ	54	0.46	2,387.9	PFKYQGLTILVKKEIP
18	0.02	2,412.7	EGRAAQKRLETLLER	55	0.28	2,389.8	VKKEIPRSTLDSFMQP
19	0.19	2,433.8	TLLEERESKAQKVLQF	56	0.29	2,317.5	DSFMQPFQSTLSTEGE
20	0.29	2,273.6	EKVLQFDPGTKNVTAL	57	0.28	2,284.5	LSTEGEVNAAEEGFLL
21	0.20	2,343.7	KNVTALLMEARDLEAR	58	0.32	2,463.8	EEGFLLVDRPEERIT
22	0.20	2,315.6	RDLEARVIILSASEDD	59	0.13	2,390.7	PEERITGINDPRLNP
23	0.16	2,142.3	SASEDDAATVYRAAAM	60	0.25	2,418.8	PRLRNPSDKFIYATVK
24	0.47	2,304.7	YRAAAMLNMTGSGYVW	61	0.34	2,459.8	IYATVKQSSVDIYFRR
25	0.34	2,236.5	GSGYVWLVGEREISGN	62	0.30	2,628.0	DIYFRRQVELSTMYRH
26	0.30	2,258.6	REISGNALRYAPDGII	63	0.13	2,468.8	STMYRHMEKHNYESAA
27	0.33	2,165.5	APDGIIGLQLINGKNE	64	0.05	2,292.5	NYESAAEAIQAVRDNK
28	0.20	2,124.3	INGKNEAHISDAVGV	65	0.37	2,355.7	AVRDNKLHAFIWDASV
29	0.38	2,150.4	SDAVGVVAQAVHELLE	66	0.37	2,354.6	IWDSAVLEFEASQKCD
30	0.19	2,360.6	VHELLEKENITDPPRG	67	0.37	2,328.7	ASQKCDLVTTGELFFR
31	0.29	2,272.6	TDPPRGCVGNTNIWKT	68	0.32	2,330.7	GELFFRSGFGIGMRKD
32	0.48	2,404.9	TNIWKTGPLFKRVLMS	69	0.25	2,359.7	IGMRKDSPWKQNVSL
33	0.15	2,281.7	KRVLMSKYADGVTGR	70	0.35	2,317.6	QNVSLSILKSHENGFM
34	-0.06	2,307.5	DGVTGRVEFNEDGDRK	71	0.25	2,553.8	HENGFMEDLDKTWVRY
35	0.16	2,414.7	EDGDRKFANYSIMNLQ	72	0.15	2,453.7	KTWVRYQECDSRSNAP
36	0.42	2,389.8	SIMNLQNRKLVQVGII	73	0.22	2,355.6	SRSNAPATLTTFE
37	0.34	2,295.6	VQVGIIYNGTHVIPNDR	74	0.33	2,328.5	APATLTTFE

Table S2. Haematological parameters in rats vaccinated with recombinant GluN1 peptides and controls in week 10.

	Experimental groups			Statistical analysis		
	Vehicle	Luciferase	GluN1	<i>P</i> value (1-way ANOVA)	<i>P</i> value <i>post-hoc</i> (Veh : GluN1)*	<i>P</i> value <i>post-hoc</i> (Luc : GluN1)*
Haemoglobin (g L ⁻¹)	154 ± 2	152 ± 1	148 ± 2	0.0735	0.0718	0.2324
Mean Cell Volume (fL)	53.5 ± 0.5	51.1 ± 0.6	50.5 ± 0.5	0.0013	0.0016	0.7511
Mean Cell Haemoglobin (pg)	18.1 ± 0.2	17.5 ± 0.2	17.3 ± 0.2	0.0093	0.0086	0.6764
Platelet count (x10 ⁹ L ⁻¹)	933 ± 25	835 ± 75	932 ± 51	0.3722	0.9998	0.4391
Mean Platelet Volume (fL)	8.0 ± 0.1	8.3 ± 0.1	8.2 ± 0.2	0.2370	0.3819	0.9393
White Cell Count (x10 ⁹ L ⁻¹)	10.36 ± 0.76	12.55 ± 1.16	15.73 ± 1.60	0.0194	0.0155	0.1803
Neutrophil count (x10 ⁹ L ⁻¹)	1.56 ± 0.14	2.92 ± 0.20	5.83 ± 0.74	<0.0001	<0.0001	0.0003
Lymphocyte count (x10 ⁹ L ⁻¹)	8.13 ± 0.70	8.56 ± 0.97	8.87 ± 0.88	0.8340	0.8195	0.9634
Reticulocyte count (%)	2.91 ± 0.05	3.09 ± 0.08	3.05 ± 0.09	0.3114	0.4872	0.9347
Reticulocyte-Haemoglobin (Ret-He; pg)	20.9 ± 0.3	20.6 ± 0.2	20.2 ± 0.2	0.1314	0.1178	0.3904
Ferritin (ng mL ⁻¹)	997.0 ± 192.5	1009.9 ± 78.9	1006.4 ± 236.3	0.9988	0.9994	>0.9999

*One-way ANOVA with Dunnett's *post-hoc*.

Abbreviations: Luc, luciferase; Veh, vehicle.

Table S3. Haematological parameters in rats vaccinated with recombinant GluN1 peptides and controls in week 20.

	Experimental groups			Statistical analysis		
	Vehicle	Luciferase	GluN1	<i>P</i> value (1-way ANOVA)	<i>P</i> value <i>post-hoc</i> (Veh : GluN1)*	<i>P</i> value <i>post-hoc</i> (Luc : GluN1)*
Haemoglobin (g L ⁻¹)	153 ± 1	149 ± 2	148 ± 2	0.1293	0.1161	0.8026
Mean Cell Volume (fL)	53.4 ± 0.7	50.7 ± 0.6	48.7 ± 0.7	0.0001	<0.0001	0.0894
Mean Cell Haemoglobin (pg)	17.9 ± 0.2	17.2 ± 0.2	16.6 ± 0.2	0.0004	0.0003	0.0927
Platelet count (x10 ⁹ L ⁻¹)	930 ± 67	883 ± 54	899 ± 47	0.8431	0.9174	0.9777
Mean Platelet Volume (fL)	8.3 ± 0.1	8.4 ± 0.1	8.1 ± 0.1	0.4204	0.7187	0.3934
White Cell Count (x10 ⁹ L ⁻¹)	12.49 ± 0.80	14.07 ± 1.07	13.98 ± 1.29	0.5981	0.6563	0.9977
Neutrophil count (x10 ⁹ L ⁻¹)	2.07 ± 0.17	2.71 ± 0.18	4.22 ± 0.49	0.0007	0.0009	0.0089
Lymphocyte count (x10 ⁹ L ⁻¹)	9.27 ± 0.78	9.76 ± 0.88	8.48 ± 0.90	0.5580	0.8163	0.5320
Reticulocyte count (%)	2.58 ± 0.08	2.58 ± 0.16	2.57 ± 0.07	0.9982	0.9981	0.9992
Reticulocyte-Haemoglobin (Ret-He; pg)	20.4 ± 0.2	20.3 ± 0.2	19.7 ± 0.2	0.0302	0.0356	0.0931
Ferritin (ng mL ⁻¹) †	1029.1 ± 199.8	685.0 ± 71.9	338.4 ± 43.8	0.0004	0.0004	0.0092

*One-way ANOVA with Dunnett's *post-hoc*. †Data was transformed.

Abbreviations: Luc, luciferase; Veh, vehicle.

Supplemental Figures and Figure Legends:

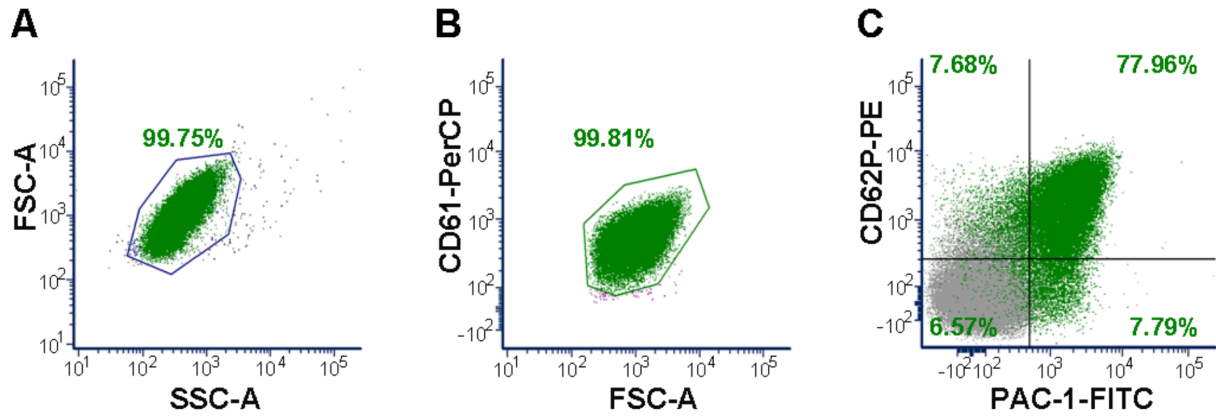


Figure S1. Gating on platelets by flow cytometry. Dot plots are shown demonstrating platelet gates based on: (A) forward and side scatter characteristics (FSC-A – SSC-A); (B) CD61 expression (CD61-PerCP) and FSC-A. Doublets were excluded based on SSC-H – SSC-A and FSC-H – FSC-A characteristics (not shown). (C) An example of CD62P expression and PAC-1 binding on platelets activated with 2.5 M μ M ADP (green) and non-activated controls (grey).

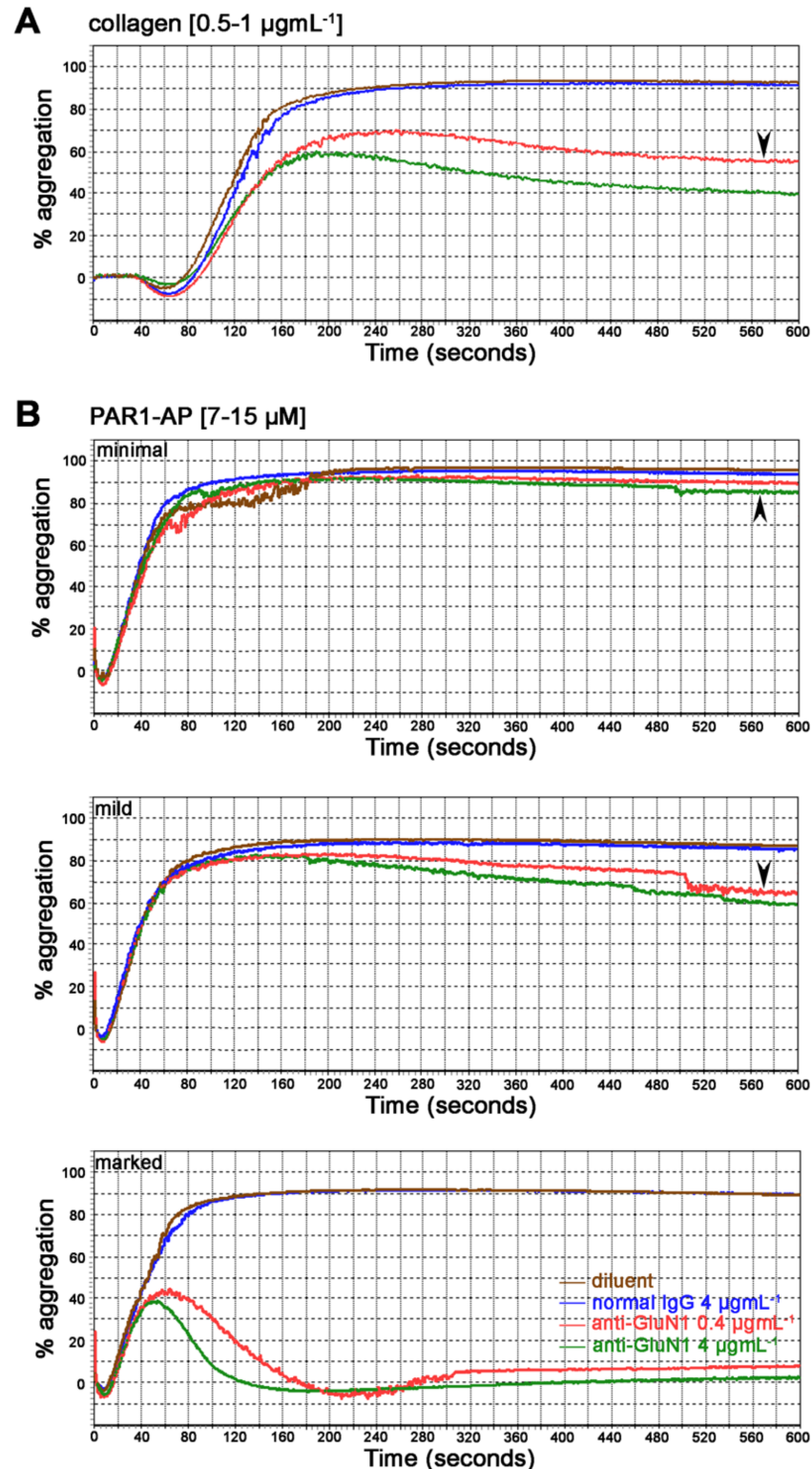


Figure S2. Inhibition of aggregation by anti-GluN1-S2 in the presence of collagen and PAR1-AP. Human platelets were pre-treated with either anti-GluN1-S2 (0.4 and $4 \mu\text{g mL}^{-1}$) or control mouse IgG ($4 \mu\text{g mL}^{-1}$) and platelet aggregation was induced using collagen ($0.5-1 \mu\text{g mL}^{-1}$) or PAR1-AP ($7-15 \mu\text{M}$). Representative examples of aggregation traces are shown. In the presence of PAR1-AP, responses varied between donors from minimal, mild to marked. Arrowheads point to signs of platelet disaggregation occurring over time.

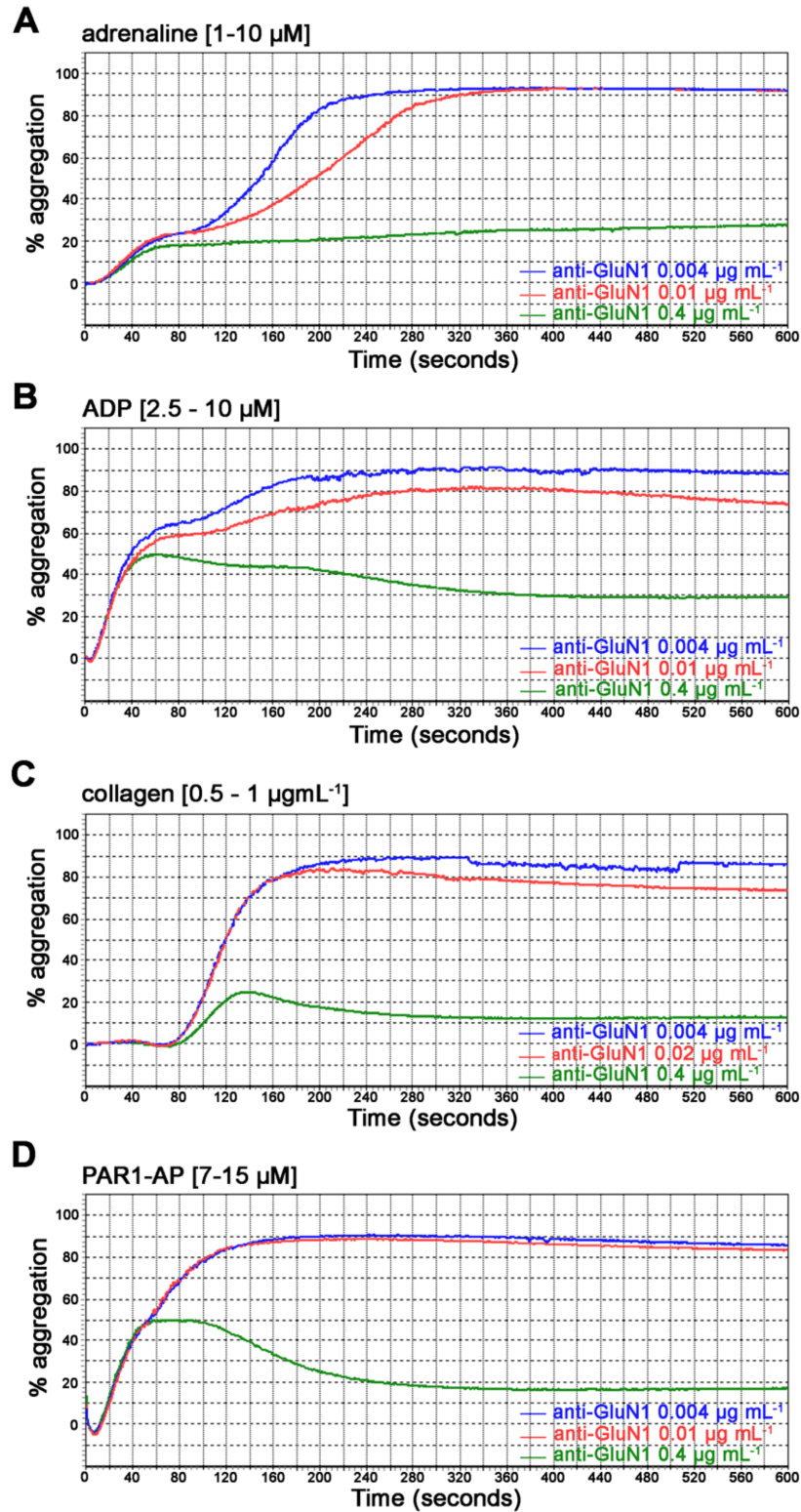


Figure S3. Titration of effects by anti-GluN1-S2 on platelet aggregation. Aggregation was induced using (A) adrenaline (5-10 μM), (B) ADP (2.5-10 μM), (C) collagen (0.5-1 $\mu\text{g mL}^{-1}$) and (D) PAR1-AP (7-15 μM) in the presence of anti-GluN1-S2 from 0.004 to 0.4 $\mu\text{g mL}^{-1}$. Representative examples of aggregation traces are shown. Inhibition of aggregation by anti-GluN1-S2 was concentration-dependent and most potent in the presence of adrenaline and ADP.

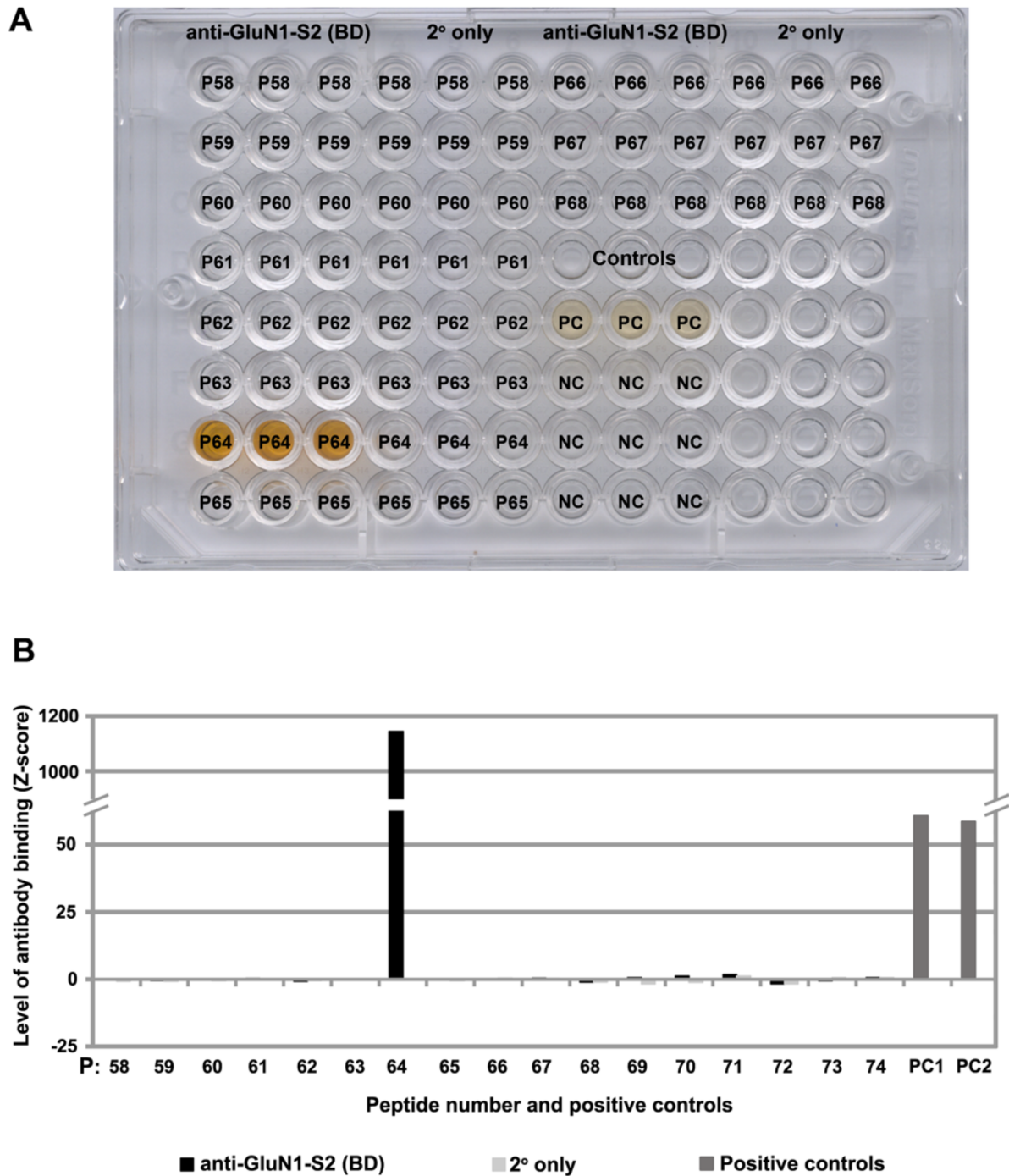


Figure S4. Identification of the anti-GluN1-S2 (BD) epitope. (A) An image of an antigen-capture ELISA plate showing strong and specific binding of anti-GluN1-S2 (BD Biosciences; diluted at 1:500) to peptide number 64 (P64). Numbers P58 to P68 refer to synthetic GluN1 peptides used to map the antibody epitope (sequences are in Table S1). Wells were coated with each peptide in triplicates. Yellow colour indicates reactive wells. Pooled reactive and non-reactive sera were used as positive and negative controls, respectively (PC and NC; diluted 1:100). (B) Levels of antibody binding with selected synthetic GluN1 peptides (as indicated) quantified relative to the negative control (Z-score).

Supplemental Video S1. Thrombus formation in flowing human blood under control conditions. Human whole blood was pre-treated with normal mouse IgG ($10\ \mu\text{g mL}^{-1}$) and perfused over microcapillary slides coated with type I collagen at a shear rate of $1800\ \text{s}^{-1}$.

Supplemental Video S2. Impact of anti-GluN1-S2 on thrombus formation in flowing human blood. Human whole blood was pre-treated with anti-GluN1-S2 ($10\ \mu\text{g mL}^{-1}$) and perfused over microcapillary slides coated with type I collagen at a shear rate of $1800\ \text{s}^{-1}$. Compared with control thrombi seen in Supplemental Video S1, thrombi forming in the presence of anti-GluN1-S2 are less dense and unstable; platelets are seen to detach from the initially formed thrombi.