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Citation for final published version:

Zelek, Wioleta M., Bevan, Ryan J. , Nimmo, Jacqui, Dewilde, Maarten, De Strooper, Bart and Morgan, B. Paul 2024. Brain-penetrant complement inhibition mitigates neurodegeneration in an Alzheimer's disease mouse model. *Brain* 10.1093/brain/awae278

Publishers page: <http://dx.doi.org/10.1093/brain/awae278>

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# Brain-penetrant complement inhibition mitigates neurodegeneration in an Alzheimer's disease mouse model

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## Abstract

Complement activation is implicated in driving brain inflammation, self-cell damage and progression of injury in Alzheimer's disease and other neurodegenerative diseases. Here, we investigate the impact of brain delivery of a complement-blocking antibody on neurodegeneration in an Alzheimer's mouse model. We engineered a brain-penetrant recombinant antibody targeting the pro-inflammatory membrane attack complex. Systemic administration of this antibody in APP<sup>NL-G-F</sup> mice reduced brain levels of complement activation products, demonstrating successful brain entry and target engagement. Prolonged treatment decreased synapse loss, amyloid burden and brain inflammatory cytokine levels, concomitant with cognitive improvement compared to controls. These results underscore the potential of brain-penetrant complement-inhibiting drugs as promising therapeutics, targeting downstream of amyloid plaques in Alzheimer's disease.

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11  
12 **Running title:** Complement inhibition mitigates dementia

13 **Keywords:** neuroinflammation; therapy; drug delivery; blood–brain barrier; mouse model

## 15 **Introduction**

16 Alzheimer’s disease (AD) is the most prevalent neurodegenerative disease (NDD) and commonest  
17 cause of dementia. To date, only a handful of drugs are approved for treatment of AD; most provide  
18 only symptomatic relief and have no impact on disease course<sup>1-5</sup>. In the quest for disease-  
19 modifying drugs, most attention has focussed on removing amyloid, culminating in the recent FDA  
20 approval of the anti-A $\beta$  antibodies aducanumab, lecanemab and donanemab, despite their limited  
21 impact on rate of disease progression and toxicity issues.<sup>6-9</sup> There remains an overwhelming need  
22 for new drugs that slow or stop progression in AD and this requires the identification of new  
23 targets. Numerous recent reports, including genetic, biomarker and animal model studies, have  
24 implicated neuroinflammation as a driver of pathology in AD;<sup>10-12</sup> therefore, targeting  
25 neuroinflammation early in the disease process is an attractive strategy.

1 Complement is a key component of immune defence and a critical driver of inflammation in health  
2 and disease; indeed, modulating complement activation has proven effective in therapy of diverse  
3 inflammatory diseases.<sup>13</sup> Complement comprises three main pathways, the classical pathway  
4 activated by immobilised antibody, the lectin pathway activated by specific sugars, and the  
5 alternative pathway activated spontaneously or by the other two pathways to amplify activation.  
6 All activation pathways converge on C3 cleavage which initiates terminal pathway activation.  
7 Inflammatory products (C3a, C5a) and opsonins (C4b, C3b) are generated from the activation  
8 pathways while the terminal pathway yields the cytolytic membrane attack complex (MAC).  
9 Several lines of evidence implicate complement in AD pathogenesis. The genes encoding the  
10 complement system proteins complement receptor 1 (CR1) and clusterin are top hits in AD  
11 genome-wide association studies (GWAS),<sup>14,15</sup> while fluid (plasma and cerebrospinal fluid (CSF))  
12 and tissue complement biomarker studies in AD provide evidence of complement dysregulation  
13 early in the disease course.<sup>10,16,17</sup> Complement deficiency in animal models of AD prevented or  
14 ameliorated disease.<sup>18-21</sup> Collectively, the data implicate complement as a driver of  
15 neuroinflammation and resultant neurodegeneration in AD.

16 Complement offers many opportunities for drug inhibition;<sup>18,19,22</sup> however, complement provides  
17 critical defence against bacterial infections and is crucial to immune complex handling and  
18 immune defence with roles including priming innate and adaptive immunity, regulating  
19 metabolism, and tailoring neural development.<sup>23-25</sup> Potential impact on these protective and  
20 homeostatic roles influences choice in selecting drug targets, particularly for long-term therapy.  
21 Targeting in the terminal pathway, responsible for MAC formation, spares most protective and  
22 homeostatic functions, including opsonic and chemotactic activities. Indeed, a drug targeting MAC  
23 formation at the stage of C5, eculizumab, has been in the clinic for nearly twenty years.<sup>13,26</sup> Several  
24 other complement blockers have recently been FDA approved. The majority of these are antibodies  
25 (~150 kDa) or other large molecules, unable to penetrate the blood-brain barrier (BBB) efficiently  
26 because of their size; a few are small molecules but are not designed to be BBB permeable.<sup>13,18</sup>  
27 For effective inhibition of complement in the brain we need new complement-blocking drugs that  
28 can penetrate the BBB.

29 We have developed a toolbox of novel monoclonal antibodies (mAb) that block MAC formation  
30 by targeting C7; these are efficient inhibitors of human and rodent complement *in vitro* and *in*  
31 *vivo*.<sup>27</sup> Here we describe the generation and characterisation of a recombinant version of one of

1 these mAbs, clone 73D1, chosen for strong inhibition of mouse C7 and modified for efficient BBB  
2 penetrance. The recombinant mAb was expressed fused to a nanobody (Nb62) against the  
3 transferrin receptor (TfR); Nb62 has previously been shown to facilitate brain entry of cargo  
4 peptides and proteins.<sup>28,29</sup> The Nb62-linked recombinant mAb (Nb62-r-mAb) retained full  
5 complement blocking activity and was BBB penetrant *in vivo* in mice. When administered  
6 systemically to AD model (APP<sup>NL-G-F</sup>) mice, Nb62-r-mAb reduced complement activation in brain,  
7 protected from synapse loss, reduced brain inflammation and amyloid load and improved cognition  
8 when compared to treatment with a BBB non-penetrant control comprising the same r-mAb linked  
9 to an irrelevant nanobody (control-r-mAb). The data suggest that MAC blockade in brain might  
10 be an effective therapy for AD.

11

## 12 **Materials and methods**

### 13 **Reagents and Sera**

14 All chemicals, except where otherwise stated, were obtained from Fisher Scientific UK  
15 (Loughborough, UK) or Sigma Aldrich (Gillingham, UK) and were of analytical grade. All tissue  
16 culture reagents and plastics were from Invitrogen Life Technologies (Paisley, UK). Sheep  
17 erythrocytes in Alsever's solution were from TCS Biosciences (Claydon, UK). Human and animal  
18 sera were prepared in house from freshly collected blood. For human serum, blood was clotted at  
19 room temperature (RT) for 1 hour (h), then placed on ice overnight for clot retraction before  
20 centrifugation and harvesting of serum. For mouse serum, blood was placed on ice immediately  
21 after harvest and clotted for 2 h on ice before serum harvest. Sera were stored in aliquots at  $-80^{\circ}\text{C}$   
22 and not subjected to freeze-thaw cycles.

### 23 **Animals**

24 Animals were group housed in open topped cages with 12 h light-dark cycles and food and water  
25 available *ad libitum*. Heterozygous C7 deficient mice (C57BL/6NJ-C7<sup>em1(IMPC)</sup> J/Mmjax C7<sup>+/-</sup>;  
26 Jackson ImmunoResearch, Baltimore, USA) were bred in house to obtain homozygous C7-  
27 deficient mice.<sup>27</sup> APP<sup>NL-G-F</sup> knock-in mice carrying the APP Swedish (KM670/671NL), Iberian  
28 (I716F), and Arctic (E693G) mutations were kindly donated by Dr. Takaomi Saido under a

1 materials transfer agreement.<sup>30</sup> These mice develop aggressive amyloidosis with plaques,  
2 surrounded by activated microglia and astrocytes, present from ~2 months; synapse loss is present  
3 from 4 months and cognitive impairment apparent from 6 months  
4 (<https://www.alzforum.org/research-models/app-nl-g-f-knock>). For all the experiments a single  
5 sex (males) was used because the well-described differences in complement activity between male  
6 and female mice adds a confounder in mixed sex studies.<sup>31,32</sup> All animal procedures were  
7 performed in accordance with UK Home Office Animals Scientific Procedures Act 1986 and local  
8 institutional guidelines. At appropriate time points, mice were humanely sacrificed with increasing  
9 CO<sub>2</sub> concentration and death confirmed by permanent cessation of circulation. Whole blood was  
10 collected by transcardial puncture and processed as described above. Mice were perfused  
11 intracardially with phosphate buffered saline (PBS), brains removed and cut sagittally, one half  
12 fixed in paraformaldehyde (PFA; 1.5%) for immunocytochemistry and DiOlistic spine labelling  
13 and the other half snap frozen for protein analyses.

## 14 **Generation of recombinant monoclonal antibodies (r-mAb)**

15 Monoclonal antibodies (mAb) against C7 protein were produced by immunisation of C7 deficient  
16 mice as described previously.<sup>27</sup> The mAb 73D1, a strong blocker of mouse C7, was selected for  
17 generation of the r-mAb. The variable light and heavy chain of the mAb were sequenced  
18 (<https://absoluteantibody.com/>) and fused respectively to a mouse kappa light constant domain and  
19 mouse IgG2a framework in which the D265A mutation had been introduced to disable FcγR  
20 binding.<sup>33</sup> One heavy chain of the r-mAb was modified at the carboxy terminus to include a  
21 nanobody, Nb62, that binds to TfR, expressed on brain endothelium and shown to confer shuttle  
22 delivery to brain.<sup>28,29</sup> A control non-brain penetrant r-mAb was generated by substituting an anti-  
23 GFP (green fluorescent protein) nanobody for Nb62. Both r-mAbs incorporated a 6-His tag and an  
24 ALFA tag for use in purification and detection respectively. A cartoon of the control and brain-  
25 penetrant r-mAbs is shown (Fig. 1A). The r-mAbs were produced in house by transient expression  
26 in Expi293F cells (A14527, ThermoFisher Scientific, Loughborough, UK) for 5 days according to  
27 the manufacturer's instructions. Briefly, plasmid DNA (1 µg/ml) and ExpiFectamine™293 reagent  
28 (A14524, ThermoFisher) were diluted with Opti-MEM™ reduced serum medium (ThermoFisher)  
29 and incubated for 5 min at RT. The plasmid DNAs were mixed in 1:1:1 ratio of the mAb light  
30 chain: heavy constant domain chain: heavy variable chain. The diluted ExpiFectamine™293

1 reagent was mixed with diluted plasmid DNA, incubated at RT for 20 min, and then slowly added  
2 with shaking to Expi293F cells ( $3 \times 10^6$  cells/60ml). Cells were then incubated in a humidified  
3 incubator (37°C, 5% CO<sub>2</sub>) on an orbital shaker. After 20 h, ExpiFectamine™293 Transfection  
4 Enhancers 1 and 2 (A14524, ThermoFisher) were added. Culture medium was harvested 5 days  
5 post transfection and diluted 1:1 with PBS pH 7.4 prior to affinity purification of the r-mAb on a  
6 protein G column (17-0405-01, Cytiva, Amersham, UK).

## 7 **Characterising purified r-mAb by SDS-PAGE and ELISA**

8 The control and brain-penetrant r-mAb (5 µg) were resolved by SDS-PAGE under reducing (R)  
9 and non-reducing (NR) conditions on 7.5% PAGE gels. Gels were stained with Coomassie Blue  
10 dye (NB4500078-11, Generon, Slough, UK). Direct ELISA was used to test binding of the r-mAb  
11 to human and mouse C7 immunoaffinity purified in house as previously described.<sup>27,34</sup> Maxisorp  
12 (ThermoFisher) 96-well plates were coated with C7 (mouse or human, 0.5 µg/ml in bicarbonate  
13 buffer, pH 9.6) at 4°C overnight, blocked (30 minutes (min) at 37°C) with 2% bovine serum  
14 albumin (BSA) in PBS and washed in PBS containing 0.05% Tween 20 (PBS-T). Dilutions of  
15 purified parent mAb or r-mAb, 1000 – 0 ng/ml (stock concentrations of all proteins measured using  
16 the Bicinchoninic Acid (BCA) assay (23235, ThermoFisher)) in 0.2% BSA-PBS, were added in  
17 triplicate to C7-coated wells and incubated for 1 h at 37°C. Wells were washed with PBS-T then  
18 incubated (1 h, 37°C) with secondary antibody (donkey anti-mouse-horseradish peroxidase (HRP);  
19 715-035-150, Jackson ImmunoResearch, Ely, UK). After washing, plates were developed using  
20 O-phenylenediamine dihydrochloride (OPD, SigmaFast<sup>α</sup>™ ; Sigma-Aldrich) and absorbance (492  
21 nm) measured.

## 22 **SPR measurement of binding affinity of native mAb and r-mAb to** 23 **mouse and human C7**

24 Surface plasmon resonance (SPR) binding analyses were carried out on a Biacore T200 instrument  
25 (Cytiva). All protein reagents used were of high purity and polished by size exclusion  
26 chromatography immediately before use to ensure removal of aggregates. Human or mouse C7  
27 was immobilized directly onto the CM5 sensor chip by amine coupling (#29-1496-03; Cytiva) to  
28 approximately 250 RU. Control or brain-penetrant r-mAb or native mAb in HBS (10 mM HEPES,  
29 pH 7.4, 150 mM NaCl) containing 0.05% surfactant P20 (HBS-EP) was flowed over the

1 immobilised C7 in a concentration series from 16 to 0.5 nM and interactions with the immobilised  
2 C7 were measured. For kinetic analysis, the flow rate was maintained at 30  $\mu$ l/min and data were  
3 collected at 25°C. Data from a reference cell were subtracted to control for bulk refractive index  
4 changes. The Rmax was kept low and the flow rate high to eliminate mass transfer. Data were  
5 evaluated using BIAcore Evaluation software (Cytiva).

## 6 **Haemolysis assays**

7 The complement inhibitory activities of the native mAb and r-mAb in normal human serum (NHS)  
8 and normal male mouse serum (NMS) were investigated using classical pathway (CP) haemolysis  
9 assays (CH50). Sheep erythrocytes (ShE) were sensitised with rabbit anti-ShE antiserum  
10 (#ORLC25, Siemens Amboceptor; Cruinn Diagnostics, Dublin, Ireland; ShEA), then suspended at  
11 2% (vol:vol) in HEPES-buffered saline (HBS) containing  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . For measurement of CP  
12 activity in NMS, ShEA were additionally incubated with mouse anti-rabbit IgG (#3123;  
13 Invitrogen; 25  $\mu$ g/mL) for 30 min at 37°C before washing and re-suspending in HBS.<sup>32,35</sup> Serum  
14 dilutions for each species were selected in preliminary experiments to give near complete  
15 haemolysis in the CP assay in the absence of test mAb, typically 2.5% for NHS and 25% for NMS  
16 (using the double-sensitized cells). A serial dilution series of native mAb or r-mAb (100 – 0  $\mu$ g/ml)  
17 was prepared in HBS and aliquoted in triplicate into a 96-well round-bottomed plate at 50  $\mu$ l/well,  
18 then serum at the appropriate dilution and 2% ShEA (50  $\mu$ l/well of each; double-sensitized for  
19 mouse as above) added. Plates were incubated at 37°C for 30 min, centrifuged and haemoglobin  
20 in the supernatant was measured by absorbance at 405 nm. For each assay, percentage lysis was  
21 calculated according to: % Lysis = Absorbance (Abs) sample – Abs background)/(Abs max – Abs  
22 background) x 100%. GraphPad Prism (v. 9.0) was used for data analysis.

## 23 **Preparation of total brain homogenate (TBH), brain tissue bound** 24 **protein (TBP) and peripheral organ lysates**

25 TBH were prepared by homogenising individual snap-frozen brain hemispheres in 0.3 ml of ice-  
26 cold RIPA buffer (R0278-50ML, Sigma Aldrich, Gillingham, UK) supplemented with 1x EDTA-  
27 free protease inhibitor (4693159001, Roche cOmplete mini EDTA-free, Sigma Aldrich) for 5 min  
28 on ice using a motorised homogeniser (431-0100, VWR, Lutterworth, UK). Lysed samples were  
29 centrifuged at 13000 rpm for 20 min at 4°C, and supernatant (TBH) collected. The remaining pellet



1 was further processed as described previously<sup>36</sup> to prepare the brain tissue bound protein (TBP)  
2 fraction containing aggregated A $\beta$ , and tissue-fixed complement proteins. Briefly, 0.3 ml 8M  
3 Guanidine-HCl (24115, ThermoFisher) was added (final concentration of Guanidine-HCl in the  
4 sample was 5M), the pellets homogenised on ice as described above, incubated on ice for 30 min  
5 then centrifuged; the resultant supernatant (TBP) was collected for ELISA measurements. Total  
6 protein concentration in the extracts was measured using the Pierce Micro BCA Protein Assay Kit  
7 (23235, ThermoFisher). TBH and TBP samples were adjusted to 1mg/ml, aliquoted and frozen  
8 immediately after preparation, stored at -80°C until testing and not subjected to freeze-thaw.

9 Lysates of other peripheral organs (liver, spleen, kidney, heart, lung, eye and muscle) were made  
10 to explore tissue distribution of the FPs. Organs were harvested, diced and homogenised in PBS  
11 (3:1 v/w ratio) containing 1% Triton X-100 supplemented with protease inhibitors (cOmplete,  
12 Sigma) using a motorised homogeniser as described above and incubated for 30 min on ice. Lysates  
13 were spun down at 13000 rpm for 20 min at 4°C, the supernatant collected, protein measured,  
14 standardised as above and stored in aliquots at -80°C prior to analysis.

## 15 **Detection of r-mAb in brain and peripheral organ lysates and serum**

16 Levels of control-r-Mab or Nb62-r-mAb in brain lysates (TBH), organ lysates and serum from the  
17 treated mice were measured using an ELISA. TBH and organ lysates were used neat or diluted 1:2  
18 in PBS; serum was diluted 1:500 in PBS. The test samples were incubated on nickel-coated plates  
19 (ThermoFisher, 15142) for 2h at RT to capture the r-mAb via the His tag, wells washed in PBS-T  
20 then HRP-labelled detection mAb (1G5 anti-ALFA tag-HRP; N1502-HRP-SY, 2BScientific,  
21 Göttingen, Germany) added (1:3000 in PBS; 1 h at RT). Plates were washed, developed using OPD  
22 and absorbance (492nm) measured. Standard curves were generated using the respective r-mAbs  
23 (500 - 0 ng/ml) enabling quantification in the samples. The intra- and inter-assay precision was  
24 calculated (%CV; <5%).

25 The presence of r-mAb in TBH from the treated mice was confirmed using western blotting (WB).  
26 TBH were diluted 1:2 in PBS, resolved on SDS-PAGE under non-reducing conditions and  
27 electrophoretically transferred to 0.45 $\mu$ m nitrocellulose membranes (GE Healthcare); membranes  
28 were blocked with 5% BSA in PBS-T, washed in PBS-T, incubated for 1h at RT with HRP  
29 conjugated anti-ALFA tag antibody (2.5 $\mu$ g/ml; Nanotag Biotechnologies, N1501-HRP) in 5% BSA

1 PBS-T, washed, developed with enhanced chemiluminescence (GE Healthcare) and visualized by  
2 autoradiography.

### 3 **Detection of IL-1 $\alpha$ and IL-1 $\beta$ by ELISA in TBH**

4 Levels of IL-1 $\alpha$  and IL-1 $\beta$  in TBH were measured using commercial ELISA kits (respectively, 88-  
5 5019-22, Invitrogen; DY401-05, R&D Systems) in accordance with the manufacturer's  
6 instructions. Briefly, wells were coated with capture antibody (1:250 in PBS, 100  $\mu$ l/well) O/N at  
7 RT, washed, blocked, then incubated with 100  $\mu$ l of TBH (1:2) or standards (serial dilutions: 500  
8 – 0 pg/ml). After washing, detection antibody was added followed by streptavidin-HRP. The assay  
9 was developed using TMB, absorbance at 450 nm was recorded, and IL-1 $\alpha$  or IL-1 $\beta$  levels in TBH  
10 samples read from the standard curve and expressed as pg/mg total protein.

### 11 **Detection of complement activation products in brain and serum by** 12 **sandwich ELISA**

13 The C3 fragment (C3b/iC3b/C3c) and terminal complement complex (TCC; fluid phase marker of  
14 terminal pathway activation) assays were performed as described previously (38). For TCC  
15 detection, Maxisorp (ThermoFisher) 96-well plates were coated with rabbit anti-rat/mouse C9 IgG  
16 (10 $\mu$ g/ml in bicarbonate buffer pH 9.6) at 4 $^{\circ}$ C overnight; wells were blocked (30 min at 37 $^{\circ}$ C)  
17 with 3% BSA in PBS-T and washed in PBS-T. TBH or TBP were diluted 1:100, sera 1:20 in 0.3%  
18 BSA PBS-T containing 10mM EDTA (PBS-T-EDTA), added in duplicate to ELISA wells and  
19 incubated overnight at 4 $^{\circ}$ C. Wells were washed with PBS-T, then HRP-labelled detection mAb  
20 12C3 anti-mouse TCC-neo added at 5 $\mu$ g/ml in PBS-T-EDTA and incubated for 1.5 h at RT. Plates  
21 were washed, developed using OPD and absorbance (492 nm) measured. Standard curves were  
22 generated using serial dilutions of in-house activated normal male mouse serum (Act-NMS)  
23 prepared as described previously.<sup>27</sup> Results were expressed as Units (U) per mg total protein. Two  
24 Act-NMS samples (1 in 800) were included as inter-assay controls across all plates and assays to  
25 calculate intra- and inter-assay precision (%CV; <10%).

26 To measure mouse C3b/iC3b/C3c, plates were coated with 2/11 mAb anti-mouse C3b/iC3b/C3c  
27 (5 $\mu$ g/ml, HM1065, Hycult Biotech, Uden, Netherlands), blocked in 3% BSA-PBS-T and washed  
28 in PBS-T. TBH or TBP were diluted 1:800, sera 1:20000 in 0.3% BSA-PBS-T-EDTA, added in  
29 duplicate to ELISA wells, incubated overnight at 4 $^{\circ}$ C, washed and bound C3 fragments detected

1 using in house HRP-labelled rabbit anti-human C3 (cross-reactive with mouse), 1:500 in 0.3%  
2 BSA-PBS-T-EDTA for 1.5 h at RT. Plates were washed, assays developed with OPD and  
3 absorbance measured as above. Standard curves were generated using Act-NMS from a starting  
4 dilution of 1:5000 in 0.3% BSA-PBST-EDTA in duplicate and results expressed as U/mg total  
5 protein. The intra- and inter-assay precision was calculated from internal standards as above  
6 (%CV; <10%).

## 7 **Detection of amyloid load by ELISA and immunofluorescence in** 8 **APP<sup>NL-G-F</sup> mouse brain**

9 To measure human A $\beta$ 42 levels in TBP extracts of APP<sup>NL-G-F</sup> brains, a commercial assay  
10 (KHB3442, ThermoFisher) was used according to the manufacturer's protocol. In brief, TBP  
11 samples (1:4000) or standard (2000 – 0 pg/ml) were added in duplicate (50 $\mu$ l/well) into the capture  
12 antibody-coated wells, incubated, washed then biotinylated detection antibody (50 $\mu$ l/well) added  
13 and incubated 3 h at RT with shaking. After washing, 100  $\mu$ l of Streptavidin-HRP was added  
14 (1:100), incubated 30 min at RT, washed, developed with TMB and absorbance (450nm) measured.  
15 Concentrations of A $\beta$ 42 in the TBP were calculated from the standard curve and results expressed  
16 as pg/mg total protein.

17 For assessment of amyloid plaque load, fixed brains were sectioned using a Leica VT1200S  
18 vibratome (Leica Biosystems); free-floating fixed brain sections were incubated in 100  $\mu$ M X-34  
19 (Sigma, SML1954) in 40% Ethanol, 60% dH<sub>2</sub>O, 0.05 M NaOH for 20 min at RT, washed in 40%  
20 Ethanol, 60% dH<sub>2</sub>O for 5 minutes and then twice in PBS-T before mounting in FluorSave  
21 (Millipore).

22 To further characterise plaque load and type, immunofluorescence was performed. Fixed free-  
23 floating sections were incubated in 70% formic acid for 15 min at RT on a shaker, washed in dH<sub>2</sub>O  
24 for 2 mins at RT then in PBS for 20 min at RT. Sections were incubated in blocking solution (5%  
25 normal goat serum (Vector labs, S-1000-20) in PBS-T) for 2 hrs at RT, then with anti-A $\beta$  antibodies  
26 (residues 17-24, 4G8-Alexa Fluor 488; residues 1-16, 6E10-Alexa Fluor 594; BioLegend numbers  
27 800714 and 803019 respectively; both at 1:250 in blocking solution) for 48 h. Sections were  
28 washed in PBS-T, incubated in 1% Sudan black in 70% ethanol for 20 min at RT then washed

1 sequentially in 70% ethanol, 50% ethanol, and PBS. Sections were counterstained in DAPI and  
2 mounted in Vectasheild vibrance (VectorLab; H1700).

3 Images from the hippocampus CA1 region and overlying cortex were taken on a Leica SP8  
4 Lightning confocal microscope (10x objective; 60  $\mu\text{m}$  stack with z-axis interval 8.5  $\mu\text{m}$ ; image  
5 size 1550  $\mu\text{m}^2$ ). Excitation was set at 405 nm (HyD laser power 5%; gain 10%) with detection  
6 window set at 450 nm -600 nm. Plaque images were batch analysed in Imaris (version 10.0,  
7 Bitplane, Zurich, Switzerland) by combining into a time-series and isolating the relevant regions  
8 using the Surface and Masking function. Images were then normalised across the batch using the  
9 Imaris XTensions "Normalise Time Points" tool followed by automatic plaque quantification using  
10 the Surface function.

## 11 **Dendritic spine labelling, imaging and analyses**

12 For measurement of dendritic spines we used unbiased ballistic labelling by DiOlistics on fixed 60  
13  $\mu\text{m}$  free-floating fixed brain sections. Ten brain hemisphere sections per mouse covering the dorsal  
14 hippocampal field were transferred to histology slides and subjected to neuronal DiOlistic labelling  
15 as previously described.<sup>37,38</sup> Tungsten microcarrier particles coated with 1,1'-Dioctadecyl-3,3,3',3'-  
16 Tetramethylindocarbocyanine Perchlorate (DiI; Life Technologies) were fired at a pressure of 80  
17 psi onto tissue sections through an inverted cell culture insert (8.0  $\mu\text{m}$ ; BD Falcon, BD  
18 Biosciences). Dye was allowed to diffuse by incubating sections at RT for 1 h in PBS; sections  
19 were fixed with 4% PFA then mounted in FluorSave (Millipore). Dye-labelled CA1 hippocampal  
20 neurons were confocally imaged using a Leica SP8 Lightning confocal microscope and  
21 deconvolved using Leica Lightning Deconvolution (63x objective; z-axis interval 0.189  $\mu\text{m}$ ).  
22 Labelled dendritic spines were captured from CA1 secondary apical dendrites within the CA1  
23 stratum radiatum field. Images were blind analysed using Imaris FilamentTracer module (version  
24 9.2, Bitplane, Zurich, Switzerland) and quantified as one batch to minimise operator bias. Spine  
25 subtypes (stubby, mushroom, thin) were classified based on predefined morphology using the  
26 SpineClassifier MATLAB extension.

27 For assessment of synaptic puncta, free-floating brain sections were subjected to epitope retrieval,  
28 permeabilised in Triton X-100 (1% (v/v) in PBS), blocked (5% NGS in PBS-T) and incubated with  
29 primary antibodies against PSD95 (Abcam ab18258, rabbit) and Bassoon (Synaptic Systems  
30 141004, guinea pig) at 1:500 in blocking buffer for 48 h at 4°C. Species-specific Alexa Fluor Plus

1 secondary antibodies (Invitrogen, 1:500) were then added for 2 h at RT. Endogenous  
2 autofluorescence was quenched with Sudan Black, and nuclei were stained with DAPI. Sections  
3 were mounted in Vectashield Mounting Medium and stored at 4 °C in the dark until imaged using  
4 a Leica SP8 Lightning confocal microscope. Sections from four mice per group were imaged, Z  
5 stacks ( $Z=0.6\ \mu\text{m}$ , interval  $0.12\ \mu\text{m}$ ) generated and 12 fields per mouse imaged and analysed in  
6 Imaris by combining maximum projections into a ‘timeseries’ allowing batch analysis using the  
7 Imaris XTensions "Normalise Time Points" tool to standardise the image intensities, followed by  
8 automated quantification using the Imaris Surface function with a predefined threshold cutoff  
9 relative to the background signal.

## 10 **Brain penetrance of r-mAb after systemic delivery and impact on** 11 **complement activation and neurodegeneration**

12 To test the capacity of the r-mAb to access the brain following systemic administration we used  
13 C7-deficient (C7<sup>-/-</sup>) mice; this choice eliminated the complication of ligand (C7) binding to the r-  
14 mAb in the periphery or brain. C7<sup>-/-</sup> mice (age 4-6 weeks) were administered either Nb-62-r-mAb  
15 or control-r-mAb by IP injection (0.1 mg in PBS, 4 mg/kg dose), then sacrificed at 2, 4 and 24 h  
16 post-injection (4 mice per timepoint for each agent), blood harvested for serum, perfused  
17 intracardially with PBS then brains harvested and snap frozen. Serum and TBH were generated  
18 and levels of the r-mAb measured in ELISA as described above.

19 To test impact of the r-mAbs on complement activation and AD pathology, male APP<sup>NL-G-F</sup> mice  
20 age 5 – 6 months (“young mice”; n=15) or 11 – 13 months (“old mice”; n=11) were first injected  
21 subcutaneously (SC) with a saturating dose of mAb 73D1 (40 mg/kg in PBS) to swamp the  
22 peripheral C7; 2 h later either Nb62-r-mAb or control-r-mAb were delivered by IP injection at a  
23 dose of 4 mg/kg to “young mice” (Nb62-r-mAb, n = 8; control-r-mAb, n = 7) and “old mice”  
24 (Nb62-r-mAb, n = 6; control-r-mAb, n = 5). A second set of injections, 73D1 (40mg/kg SC)  
25 followed by the relevant r-mAb (IP, 2mg/kg for all mice), was given on day 3. Mice were tail bled  
26 on days 0, 3 and 7 prior to any injections, serum harvested and tested for haemolytic activity as  
27 described previously.<sup>27</sup> Mice were sacrificed on day 7, PBS perfused, brains harvested and split  
28 sagittally; one half was snap frozen for preparation of TBH and TBP extracts for measurement of  
29 levels of agent, complement activation markers and A $\beta$ , the other half fixed in 1.5% PFA for  
30 measurement of spine density by DiOlistics and amyloid plaque load by IHC.

1 In a separate experiment, APP<sup>NL-G-F</sup> mice aged 6 – 9 months were randomly assigned to two groups  
2 of 12. All mice received anti-C7 mAb twice weekly (sc) while group 1 additionally received  
3 control-r-mAb and group 2 Nb62-r-mAb with dosing and schedules as for the 7-day study but  
4 extended for 13 weeks. Mice were tail-bled at intervals and serum harvested for measurement of  
5 complement activity. Prior to sacrifice, mice were subjected to behavioural testing using  
6 burrowing, open field and novel object recognition tests as described below. Mice were sacrificed  
7 on day 91, PBS perfused, brains harvested and split sagittally; one half was snap frozen for  
8 preparation of TBH and TBP extracts for measurement of levels of r-Mab, complement activation  
9 markers, inflammatory cytokines and A $\beta$ , the other half fixed in 1.5% PFA for measurement of  
10 spine density by DiOlistics and plaque load by IHC.

## 11 **Burrowing, Open-field, and novel object recognition behavioural** 12 **tests**

13 All tests were performed with mice randomised and the operator blinded to group assignments. To  
14 minimize stress, animals were acclimatised to the experimental room in their home cages for a  
15 week prior to testing. All experiments were conducted in the same dimly lit room illuminated with  
16 red lights to ensure consistency. For assessment of burrowing activity, the mouse was placed  
17 overnight in a fresh cage (90cm x 60cm x 120cm) containing a tube filled with 200 grams of pea  
18 gravel. The next morning the remaining pea gravel in the tube was weighed and the percentage  
19 burrowed was calculated.<sup>39</sup>

20 For open field test, mice were placed in the centre of an opaque box (40x40x40 cm). Every  
21 experiment commenced with the mouse positioned in the centre; movements were recorded using  
22 a fixed overhead camera for seven minutes. The percentage of time spent in a marked central zone  
23 was determined by reviewing the videos and measuring the time spent in both peripheral and  
24 central zones.<sup>40</sup>

25 The novel object recognition (NOR) test was conducted in the same box but with visual cues (star  
26 and cross) on the walls. To acclimatise, mice were placed in the box for 10 min on two consecutive  
27 days without any object cues. On the third day (Day 1; training phase), two identical objects (A  
28 and A') were introduced for ten minutes, with exploration behaviour recorded on video and time  
29 spent examining each object noted. The following day (Day 2; test phase), mice were presented

1 with two objects: one from the training phase (A) and a novel object (B) for ten minutes,  
2 exploration behaviour was recorded and analysed as before.<sup>41</sup> Cages and objects were meticulously  
3 cleaned between experiments to eliminate scent clues and ensure unbiased results.

## 4 **Statistics**

5 Statistical calculations were performed with GraphPad Prism Software version 9.4.1 (San Diego,  
6 California, USA). Data are presented as means  $\pm$ SD. Statistical significance of differences between  
7 two groups was obtained using the unpaired t-test and for multiple groups using one-way ANOVA  
8 after testing for normality. For all animal studies, all animals survived to the planned endpoint,  
9 enabling all data points to be included in analysis. Behavioural tests, and all histopathologic  
10 analysis and quantification, including DiOlistics, were performed blinded to experimental groups.

11

## 12 **Results**

### 13 **The r-mAb bind C7 and inhibit complement *in vitro*.**

14 The control-r-mAb and Nb62-r-mAb derived from the 73D1 mAb were expressed in Expi293 cells  
15 and aseptically purified to homogeneity on protein G; each r-mAb ran as a single band of ~150  
16 kDa (intact r-mAb) in non-reduced SDS-PAGE and three bands under reducing conditions; ~55  
17 kDa, ~70 kDa (r-mAb heavy chains; one with the nanobody attached) and ~25 kDa (r-mAb light  
18 chain) (Fig. 1A,B). Direct ELISAs showed that both r-mAb and the native mAb 73D1 recognised  
19 human and mouse C7 (Fig. 1C). The capacity of r-mAb to inhibit complement was compared with  
20 that of the parent mAb 73D1; both r-mAb inhibited complement activity in human and mouse  
21 serum with the same efficiency as the benchmark 73D1 mAb (Fig. 1D). Strong binding of r-mAb  
22 and 73D1 to mouse and human C7 was confirmed by SPR analysis on immobilised mouse or  
23 human C7 (Fig. 1E). The r-mAbs displayed very slow off-rates as previously reported for clone  
24 73D1, an efficient inhibitor of complement *in vivo*.<sup>29</sup> The calculated KDs (not true KDs because  
25 of antibody avidity but valid comparators) were similar for the two r-mAb and the parent 73D1  
26 mAb, demonstrating that the cloning process had not adversely impacted binding of r-mAb to C7  
27 ligand (Fig. 1F).

## 1 **The Nb62-r-mAb penetrates mouse brain *in vivo***

2 To test BBB penetrance *in vivo*, C7<sup>-/-</sup> mice (4-6 weeks) were treated with a single dose of Nb62-  
3 r-mAb or control-r-mAb (4mg/kg, IP); groups of mice were sacrificed at 2, 4 and 24h (n = 4 for  
4 each r-mAb at each time point), blood and brains harvested. Nb62-r-mAb was detected by ELISA  
5 in brain homogenates (TBH) at each time point, albeit with sharply reducing levels with time (2 h,  
6 0.448 ng/mg; 4 h, 0.087 ng/mg; 8 h, 0.008 ng/mg); in contrast, control-r-mAb was not detected in  
7 TBH at any timepoint, demonstrating that levels in brain did not reach the assay detection limit  
8 (<0.005 ng/mg) at any time point (Fig. 2A). Presence of Nb62-r-mAb in TBH was confirmed by  
9 western blotting at the 2h timepoint; no control-r-mAb was detected in TBH westerns at this  
10 timepoint (Fig. 2B). Both proteins were strongly detected in serum across the study duration;  
11 however, while levels of the control-r-mAb were unchanged over the 24h period, serum levels of  
12 Nb62-r-mAb fell sharply between 2 and 24 h, (Fig. 2C). These data suggest that the TfR-targeting  
13 Nb62 nanobody mediates enhanced clearance through binding to TfR, expressed ubiquitously in  
14 peripheral organs. To test this, C7<sup>-/-</sup> mice were given a single IP dose of Nb62-r-mAb or control-  
15 r-mAb (4mg/kg; 4 per group), sacrificed at 4h and r-mAb concentrations in various organs  
16 measured by ELISA (Fig. 2D). Nb62-r-mAb was present at significantly higher levels compared  
17 to control-r-mAb in brain, muscle, spleen and eye of treated animals at 4h, confirming enhanced  
18 peripheral uptake. In contrast, significantly more control-r-mAb was detected in kidney and lung.

## 19 **C7 Inhibition acutely reduces complement activation, synapse loss** 20 **and A $\beta$ levels in AD mouse brain**

21 APP<sup>NL-G-F</sup> mice, either 5-6 months (young) or 11-13 months (old), were treated on days zero and  
22 3 with the parent mAb delivered subcutaneously to swamp systemic C7 followed 2 hours later by  
23 Nb62-r-mAb or control-r-mAb delivered IP. Complement haemolytic activity in serum was  
24 reduced to background levels throughout the experiment, confirming that no free C7 remained in  
25 the circulation (Fig 3A, B). Mice were sacrificed on day 7, blood collected and perfused brains  
26 harvested and split sagittally. Brain tissue extracts (TBH; TBP) were prepared from one half and  
27 assayed in sandwich ELISAs for r-mAb levels, complement activation products, and A $\beta$ 42 levels;  
28 the other half was reserved for imaging. Neither Nb62-r-mAb nor control-r-mAb were detected in  
29 TBH at day 7 (data not shown), expected given the rapid clearance observed in the preliminary



1 study and the limited brain penetration respectively. Despite this, levels of complement activation  
2 products, both C3 fragments and terminal complement complex (TCC), were significantly reduced  
3 in TBH from Nb62-r-mAb treated mice compared to controls in both young and old groups (C3  
4 fragments; Fig. 3C;  $p=0.0002$ ,  $p=0.0012$ , respectively; TCC; Fig. 3D;  $p < 0.0001$ ,  $p=0.0462$ ). A $\beta$ 42  
5 levels in TBP samples were significantly reduced in Nb62-r-mAb treated young and old APP<sup>NL-G-F</sup>  
6 mice compared to control-r-mAb ( $p=0.0318$ ;  $p=0.0363$  respectively; Fig. 3E). Amyloid plaque  
7 coverage, assessed in cortex and hippocampus by staining with anti-A $\beta$  antibodies 6E10 (vs.  
8 residues 4-10; Fig. 3F) or 4G8 (vs. residues 18-23; Fig. 3G), was not different between Nb62-r-  
9 mAb and control-r-mAb treated mice in either site or age group.

10 To test the impact of 7-day treatment with Nb62-r-mAb on neurodegeneration, synapse loss was  
11 assessed using DiOlistics on fixed brain sections; representative images are shown in Fig. 3H.  
12 Spine numbers from CA1 hippocampal neurons (pooled dendrites per treatment mouse; average  
13 of at least 10 dendritic segments per mouse) and morphological subtypes were automatically  
14 quantified. In both young and old APP<sup>NL-G-F</sup> mice treated with Nb62-r-mAb, overall spine density  
15 was higher compared to control-r-mAb treated age-matched APP<sup>NL-G-F</sup> mice, significant in the old  
16 group ( $p = 0.0035$ , Fig. 3I). Comparison of different morphological spine subtypes showed that  
17 thin spine density was significantly different between Nb62-r-mAb and control-r-mAb treated  
18 mice in young ( $p = 0.0355$ ) and old ( $p = 0.0048$ ) groups (Fig. 3J).

## 19 **Prolonged C7 inhibition reduces pathology and improves cognition in** 20 **AD mice**

21 Male APP<sup>NL-G-F</sup> mice aged between 6 and 9 months were randomised into two groups of 12; all  
22 mice received anti-C7 mAb twice weekly (SC) to block systemic C7 while group 1 additionally  
23 received control-r-mAb and group 2 Nb62-r-mAb for 13 weeks with dosing and schedules as for  
24 the short treatment study. Systemic complement activity was completely inhibited throughout the  
25 time course (Fig. 4A). Mice were subjected to behavioural testing at 13 weeks, then sacrificed,  
26 brains harvested, and parameters of complement activation and pathology measured in lysates and  
27 sections as for the short treatment study. Complement activation products in TBH were  
28 significantly reduced in Nb62-r-mAb treated APP<sup>NL-G-F</sup> mice compared to control-r-mAb (C3  
29 fragments,  $p = 0.0206$ ; TCC,  $p = 0.0014$ ; Fig. 4B, C), demonstrating target engagement by Nb62-  
30 r-mAb. To investigate impact on neuroinflammation, the pro-inflammatory cytokines IL1- $\alpha$  and

1 IL-1 $\beta$  were measured in TBH; both were significantly reduced in Nb62-r-mAb treated mice  
2 compared to controls (Fig. 4D, E;  $p = 0.0039$ ,  $p = 0.0106$ ). Levels of A $\beta$ 42 measured in TBP were  
3 markedly reduced in Nb62-r-mAb treated mice compared to controls (Fig. 4F;  $p = 0.0469$ ).

4 The impact of Nb62-r-mAb administration on amyloid plaque coverage was assessed by staining  
5 with the well-characterised plaque marker X34 and anti-A $\beta$  antibodies 6E10 and 4G8; percent  
6 coverage, plaque number and average plaque size in hippocampus and cortex were automatically  
7 calculated in IMARIS. Although A $\beta$  antibody (6E10 and 4G8) staining in hippocampus was lower  
8 in Nb62-r-mAb treated mice compared to controls, there were no significant differences between  
9 Nb62-r-mAb and control-r-mAb groups in any of these markers of plaque load in either location  
10 (Fig. 4G-I).

11 Dendritic spines were measured using DiOlistics on fixed brain slices as described in the short  
12 treatment study; spine numbers (from at least 10 pooled dendrites per treatment mouse) and  
13 morphological subtypes were quantified from CA1 hippocampal neurons. Representative images  
14 from control-r-mAb and Nb62-r-mAb groups are shown in Fig. 4J. Overall spine density was  
15 significantly higher in Nb62-r-mAb treated APP<sup>NL-G-F</sup> mice compared to controls (Fig. 4K;  $p <$   
16  $0.0001$ ). Morphological analysis of spine subtypes showed that both thin and mushroom spines  
17 were significantly higher in Nb62-r-mAb mice (Fig. 4L; thin  $p < 0.0001$ ; mushroom  $p < 0.01$ ). To  
18 confirm impact of Nb62-r-mAb on synapses, synaptic puncta were quantified after  
19 immunofluorescence labelling with pre-synaptic (Bassoon) and post-synaptic (PSD95) markers  
20 (Fig. 4M); pre- and post-synaptic markers were higher in Nb62-r-mAb treated mice compared to  
21 controls, the latter reaching significance ( $p = 0.0231$ , Fig. 4N).

22 In behavioural tests conducted immediately prior to sacrifice, Nb62-r-mAb treated mice performed  
23 significantly better than controls on all three tests, burrowing (Fig. 4O;  $p = 0.0041$ ), OF (Fig. 4P;  
24  $p = 0.0022$ ) and NOR (Fig. 4Q;  $p < 0.0001$ , testing). These three tests interrogate different aspects  
25 of cognition: Burrowing exploits a natural rodent behaviour to test hippocampal integrity; OF tests  
26 exploratory behaviour, locomotor activity and anxiety, and has been reported as an early index of  
27 cognitive impairment in APP<sup>NL-G-F</sup> mice;<sup>42</sup> NOR provides a simple test of learning and working  
28 memory, widely used in drug testing in AD models. Together, these results demonstrate that  
29 prolonged treatment with Nb62-r-mAb had a marked effect on cognition, reducing cognitive  
30 decline and rescuing other behavioural deficits in APP<sup>NL-G-F</sup> mice.

## 1 Discussion

2 Complement is an attractive therapeutic target in neurodegenerative diseases. We here provide  
3 proof-of-concept that an antibody blocker of the pro-inflammatory complement effector MAC can  
4 be delivered to the brain using an anti-TfR nanobody shuttle in a mouse model of AD. We  
5 demonstrate target engagement in brain, effective protection against neurodegeneration  
6 downstream of amyloid plaque formation and protection against cognitive decline in the model.

7 Drugs targeting complement are increasingly used for treatment of systemic inflammatory  
8 diseases, but use in dementia requires efficient brain penetrance, lacking in current anti-  
9 complement drugs, most of which are monoclonal antibodies (mAb) with minimal blood-brain  
10 barrier penetrance.<sup>22</sup> To address this deficit, we generated a brain-penetrant anti-complement drug  
11 based on our novel anti-C7 mAb that specifically inhibits assembly of MAC, the cytolytic complex  
12 of complement responsible for triggering inflammatory pathways, including the NLRP3  
13 inflammasome, in diverse cell types.<sup>43,44</sup> We targeted MAC because we have demonstrated that  
14 MAC drives neuroinflammation, neurodegeneration and synapse elimination in AD models.<sup>38</sup> C7  
15 is essential for MAC formation, less abundant than C5, the target of Eculizumab and other drugs  
16 in the clinic (~2-fold lower plasma concentration) and is not an acute phase reactant,<sup>45</sup> properties  
17 that make it a better drug target. Inhibiting C7 may also confer less infection risk compared to C5  
18 because C5a-mediated neutrophil recruitment is unimpaired.<sup>46</sup>

19 The selected anti-C7 mAb, 73D1, an efficient cross-species inhibitor of C7 and of comparable  
20 efficacy to the benchmark C5-blocking mAb BB5.1 in models of peripheral inflammation,<sup>27,47,48</sup>  
21 was used as template to generate a recombinant mAb (r-mAb) tagged with a nanobody (Nb62) that  
22 binds TfR on brain endothelial cells to engage receptor-mediated transcytosis. Nb62 was shown to  
23 deliver a barrier-impenetrant neuropeptide into brain,<sup>28</sup> and a modified version mediated brain  
24 delivery of anti-BACE1 mAb in mice expressing human TfR.<sup>29</sup> Others have demonstrated the  
25 capacity of TfR shuttles to deliver antibodies and other large cargoes into the brain.<sup>49-50</sup> We  
26 confirmed that Nb62-r-mAb administered systemically in healthy C7 deficient mice crossed the  
27 BBB; however, brain clearance of the agent was rapid as previously reported for other TfR-  
28 delivered cargoes.<sup>28,51,52</sup> Plasma levels of Nb62-r-mAb fell sharply over the 24-hour period; we  
29 showed that this was a consequence of enhanced uptake of TfR-targeted cargoes in the periphery,  
30 as reported elsewhere.<sup>53,54</sup> The Nb62 nanobody was engineered for reduced TfR affinity to

1 minimise peripheral uptake;<sup>28</sup> nevertheless, rapid clearance remained, highlighting the need for  
2 further modifications for efficient delivery and sustained impact.

3 The APP<sup>NL-G-F</sup> mouse contains an endogenous promoter-driven single knock-in of human APP  
4 incorporating three familial AD associated mutations that together promote toxicity and plaque  
5 formation by increasing A $\beta$  production and aggregation; it is a robust model for studying plaques  
6 and glial responses to plaques but lacks other components of AD pathology. We selected this model  
7 based on practicalities; synapse loss, plaque accumulation and cognitive impairment occur  
8 relatively early and in a consistent manner. Importantly, we recently showed that complement  
9 activation products are markedly increased in APP<sup>NL-G-F</sup> mice and increase further with age,  
10 evidence of increasing complement dysregulation with progressing pathology.<sup>38</sup> Systemic C7 was  
11 first saturated with the parent mAb to ensure that the Nb62-r-mAb retained C7-binding capacity;  
12 we chose to use the parent mAb for this purpose rather than Nb62-r-mAb to reduce the risk of  
13 swamping the brain uptake pathway. Systemic delivery of Nb62-r-mAb over 7 days reduced brain  
14 levels of TCC, a marker for MAC formation, confirming target engagement; C3 fragments,  
15 markers of complement activation, were also significantly reduced, likely because MAC inhibition  
16 reduces injury and resultant complement activation, as previously shown in a demyelination  
17 model.<sup>55</sup> Nb62-r-mAb also reduced A $\beta$ 42 levels in APP<sup>NL-G-F</sup> brains, while hippocampal synaptic  
18 spine density was significantly increased. These results demonstrate a clear and rapid effect of  
19 shuttle delivery of anti-C7 r-mAb on neurodegeneration.

20 Having confirmed target engagement, we extended the treatment period to 13 weeks; Nb62-r-mAb  
21 treatment inhibited MAC formation, reduced brain inflammation and protected from complement-  
22 driven synapse loss, reflected in a significant increase in spine density that translated to improved  
23 cognition; the relationship between spine density and cognition has been reported in other mouse  
24 AD models.<sup>56,57</sup> Notably, these effects of Nb62-r-mAb treatment occurred in the absence of an  
25 impact on the number of amyloid plaques, although A $\beta$ 42 levels in brain extracts were significantly  
26 reduced, implying an effect on plaque amyloid fibril content.<sup>58</sup> While the role of complement and  
27 inflammation on amyloid fibril formation and plaque accumulation deserves further attention, our  
28 findings suggest that the beneficial effects on cognition are likely downstream of amyloid plaques;  
29 hence, plaque removal is not a prerequisite for synapse rescue and improved cognition. Indeed,  
30 others have reported that C3 deficiency improved cognition but increased plaque load in an AD  
31 model.<sup>59</sup> Levels of inflammatory cytokines, including IL-1 $\beta$ , a marker of inflammasome

1 activation, were markedly reduced in Nb62-r-mAb treated mice. Microglia express the NLRP3  
2 inflammasome and are the primary source of IL-1 $\beta$  in brain,<sup>60,61</sup> while we and others have shown  
3 that MAC directly triggers inflammasome activation in diverse cell types.<sup>43,44</sup> Together, these  
4 observations suggest that activation of microglia by MAC contributes to neuroinflammation.

5 This work provides proof-of-concept that anti-complement drugs can be delivered to the brain  
6 using shuttles to reduce complement dysregulation, neuroinflammation and neurodegeneration,  
7 and confirm our recent demonstration that MAC is an important player in complement-mediated  
8 synapse loss in AD models,<sup>38</sup>. The work supports and extends our recent demonstration that  
9 treatment with the unmodified mAb 73D1 in 9 month old APP<sup>NL-G-F</sup> mice reduced brain  
10 complement activation, amyloid load and synapse loss.<sup>62</sup> In this context, brain penetrance of the  
11 mAb was dependent on BBB impairment, seen only late in the disease course; therefore,  
12 developing a brain-penetrant molecule is essential for early treatment and prevention of AD in  
13 models and man. Taken together, these studies consolidate our hypothesis that complement  
14 dysregulation, and specifically MAC formation, contribute to neuroinflammation and  
15 neurodegeneration in AD and the corollary that brain-targeted MAC inhibition may provide a  
16 novel therapeutic approach in AD and other dementias. While targeting complement always carries  
17 risk of infection, MAC inhibition carries much reduced risk compared with early pathway  
18 inhibition because it does not impair pathogen tagging and opsonisation.<sup>22,23</sup> For anti-C5 therapies  
19 already in the clinic, risk of *Neisseria* infection is managed using vaccination and prophylactic  
20 antibiotics;<sup>22</sup> C7 inhibition would require a similar approach to prophylaxis.

21 The work has several limitations. First, we used a single AD mouse model, albeit one of the best  
22 for study of amyloid pathology; replication in other models, including those with tau pathology, is  
23 now needed. Second, peripheral inhibition of C7 was necessary to enable the Nb62-r-mAb to  
24 access the brain unimpeded by cargo; we are currently exploring several strategies to eliminate the  
25 need for this pre-treatment by modifying the r-mAb and by testing recombinant forms of other  
26 human C7-blocking antibodies from our toolbox for optimal delivery. Third, we used male mice  
27 in all experiments, a pragmatic choice to minimise the effects of known sex differences in  
28 complement activity in mice on study outcomes; testing in females should be prioritised in future  
29 confirmatory studies. Finally, we do not include a comprehensive analysis of the impact of C7  
30 inhibition on glial activation state or neuronal survival in the model; this work is ongoing.

1

## 2 **Data availability**

3 The data that support the findings of this study are available from the corresponding author, upon  
4 reasonable request.

5

## 6 **Funding**

7 This work is supported by the UK Dementia Research Institute [award number UK DRI-3002]  
8 through UK DRI Ltd, principally funded by the Medical Research Council, Alzheimer's Research  
9 Race Against Dementia Fellowship [award number 520488], Alzheimer's Research Race Against  
10 Dementia Fellowship Award grant 520488, Interne Fondsen KU Leuven/Internal Funds KU  
11 Leuven grant, UKRI-MRC (MR/Y014847/1), European Research Council (grant agreement no.  
12 ERC 834682 CELLPHASE\_AD), and the Grand challenges program of VIB grant.

13

## 14 **Competing interests**

15 The authors declare no competing interests.

16

## 17 **Supplementary material**

18 Supplementary material id available at *Brain* online.

19

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## 14 **Figure legends**

15 **Figure 1 Generation and characterisation of the brain penetrant anti-C7 r-mAb.** **A.** Cartoon  
16 representing the design of the Nb62-r-mAb (top) and control-r-mAb (bottom). Complementary  
17 determining regions (CDRs) from mAb 73D1 were grafted onto a mouse IgG2a framework  
18 modified (D265A) to ablate Fc receptor binding. Nanobodies, Nb62 against low-affinity  
19 transferrin receptor (TfR) for the test, anti-GFP (green fluorescent protein) for the control, were  
20 expressed at the carboxy terminus of one heavy chain. ALFA-tag and 6-His tag were included in  
21 each construct for use in detection and purification. **B.** SDS-PAGE of Nb62-r-mAb and control-r-  
22 mAb. Proteins were purified on protein G then run on 7.5% acrylamide gels, either non-reduced  
23 (NR), or reduced with 5%  $\beta$ -mercaptoethanol (R). Separated proteins were stained with Coomassie  
24 Blue. NR; ~150 kDa MW intact r-mAb; R; 55 kDa MW r-mAb heavy chain, 25 kDa MW r-mAb  
25 light chain, and ~70 kDa MW r-mAb heavy chain plus nanobody. **C.** Direct ELISA. Human and  
26 mouse C7 were immobilised on wells then native mAb, Nb62-r-mAb or control-r-mAb added in a  
27 dilution series (0 – 10  $\mu$ g/ml). Bound antibody was detected using labelled anti-mouse IgG.  
28 Binding curves for the three antibodies were superimposed and showed strong binding to both  
29 human and mouse C7. The assays were repeated three times with comparable results. The error

1 bars are standard errors of duplicates. **D.** Classical pathway haemolytic assays (CH50). Nb62-r-  
2 mAb and control-r-mAb were tested for inhibition of complement-mediated lysis in human and  
3 mouse serum. Both r-mAb inhibited mouse and human serum-mediated haemolysis as effectively  
4 as the native parent mAb 73D1. The experiments were repeated three times with comparable  
5 results. The error bars are standard errors of duplicates. **E.** Surface plasmon resonance (SPR) was  
6 used to determine the binding of Nb62-r-mAb and control-r-mAb to human and mouse C7. Human  
7 or mouse C7 was immobilised directly onto a CM5 sensor chip and the relevant r-mAb or native  
8 mAb 73D1 was flowed over the chip. Sensorgrams were collected and KDs calculated using the  
9 Langmuir 1:1 binding model (see Table 1). Representative sensorgrams are shown with fitted data  
10 in black (n=3).

11  
12 **Figure 2 In vivo testing of the r-mAb for delivery to the brain.** Entry of Nb62-r-mAb and  
13 control-r-mAb into brain was tested in 8-week-old C7-deficient mice. **A.** r-mAb in total brain  
14 homogenate (TBH) was detected by sandwich ELISA and expressed as ng/mg protein. Nb62-r-  
15 mAb was detected in TBH at 2, 4 and 24 h timepoints, whereas control-r-mAb was not detected at  
16 any timepoint. **B.** Western blotting confirmed the presence of Nb62-r-mAb but not control-r-mAb  
17 in TBH. **C.** r-mAb levels in serum were measured by ELISA; both Nb62-r-mAb and control-r-  
18 mAb were detectable at each timepoint but significantly less Nb62-r-mAb was detected at 4 and  
19 24h ( $p = 0.0044$ ,  $p < 0.0001$  respectively) suggesting rapid clearance. **D.** Distribution of the r-  
20 mAbs in other organs at 2 h was tested in tissue lysates using the ELISA; distribution patterns were  
21 different with the Nb62-r-mAb higher in muscle, brain and eye. All assays were repeated three  
22 times with comparable results. The error bars are standard errors of triplicates. Unpaired two-tailed  
23 t-test was used for the group comparison. Error bars correspond to SEM.

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26 **Figure 3 Impact of one week treatment with Nb62-r-mAb on brain parameters in APP<sup>NL-G-F</sup>**  
27 **mice.** APP<sup>NL-G-F</sup> mice were treated with anti-C7 mAb plus either Nb62-r-mAb or control-r-mAb  
28 over 7 days; systemic inhibition of complement, inhibition of complement activation in brain, brain  
29 inflammation, and neurodegeneration were assessed. **A,B.** Classical pathway haemolysis (CH50)  
30 assays confirm that systemic complement was inhibited in APP<sup>NL-G-F</sup> mice aged 5-6 months

1 (control-r-mAb: n = 7, Nb62-r-mAb: n = 8) or 11-13 months (control-r-mAb: n = 5, Nb62-r-mAb:  
 2 n = 6) over one week of systemic administration of 73D1 mAb and either r-mAb. **C-D.** Sandwich  
 3 ELISAs detecting mouse C3 fragments (**C**, C3b/iC3b/C3c) and terminal complement complex (**D**,  
 4 TCC) in TBH. Both C3 fragments and TCC levels were significantly lower in Nb62-r-mAb treated  
 5 mice at either age. Error bars are standard errors for each dataset. Groups were compared using an  
 6 unpaired two-tailed t-test. **E.** Sandwich ELISA to measure levels of A $\beta$  in TBP (tissue bound  
 7 protein) demonstrating significantly decreased A $\beta$  in TBP of Nb62-r-mAb treated APP<sup>NL-G-F</sup>  
 8 treated mice compared to controls in both age sets. **F-G.** Immunostaining of A $\beta$  plaques in  
 9 hippocampus and cortex using anti-A $\beta$  antibodies: 6E10 (**F**) and 4G8 (**G**) showed no significant  
 10 difference in plaque coverage between Nb62-r-mAb and control-r-mAb treated APP<sup>NL-G-F</sup> mice at  
 11 either age. **H.** Representative confocal images of DiOistics labelled CA1 hippocampal dendritic  
 12 segments in 5-6 month and 11-13 month APP<sup>NL-G-F</sup> mice treated with Nb62-r-mAb or control-r-  
 13 mAb. Scale bar 5  $\mu$ m. **I,J.** DiOistic labelled dendritic spines were analysed in prefixed coronal  
 14 brain slices. Overall spine density, analysed from dendritic segments of at least 30  $\mu$ m, was higher  
 15 in Nb62-r-mAb treated mice compared to controls in both ages, significant only in the 11-13 month  
 16 set. Analysis of spine subtypes showed that the number of thin spines was significantly increased  
 17 in Nb62-r-mAb treated groups in both age sets. Unpaired two-tailed t-test was used to compare  
 18 spine densities between groups. Error bars correspond to SEM.

19  
 20 **Figure 4 Impact of three months treatment with Nb62-r-mAb on brain parameters in APP<sup>NL-G-F</sup>**  
 21 **G-F mice.** APP<sup>NL-G-F</sup> mice were treated with anti-C7 mAb plus either Nb62-r-mAb (n=12) or  
 22 control-r-mAb (n=12) over three months; systemic complement activity, complement activation in  
 23 brain, brain inflammation, neurodegeneration and cognition were assessed. **A.** Classical pathway  
 24 haemolytic assays (CH50) assays demonstrate that systemic complement was inhibited in the  
 25 Nb62-r-mAb and control-r-mAb treated mice over the 3-month time course. **B-C.** Levels of C3  
 26 fragments (**B**; C3b/iC3b/C3c) and TCC (**C**) in TBH were significantly reduced at endpoint in  
 27 Nb62-r-mAb treated APP<sup>NL-G-F</sup> mice compared to controls. **D-E.** Levels of cytokines: IL-1 $\alpha$  and  
 28 IL-1 $\beta$  in TBH at endpoint were significantly decreased in Nb62-r-mAb treated APP<sup>NL-G-F</sup> mice  
 29 compared to controls. **F.** Levels of A $\beta$  in TBP (tissue bound protein) were significantly decreased  
 30 at endpoint in Nb62-r-mAb treated APP<sup>NL-G-F</sup> mice compared to controls. **G-I.** A $\beta$  plaques in  
 31 hippocampus and cortex were either stained with the plaque stain X34 (**G**) or immunostained with

1 anti-A $\beta$  antibodies 6E10 (**H**) and 4G8 (**I**); analysis revealed no significant differences in plaque  
2 coverage with any of these stains at endpoint between the Nb62-r-mAb and control-r-mAb treated  
3 APP<sup>NL-G-F</sup> mice. **J**. Representative confocal images of DiOlistics labelled CA1 hippocampal  
4 dendritic segments from APP<sup>NL-G-F</sup> mice treated with Nb62-r-mAb or control-r-mAb. Scale bar 5  
5  $\mu$ m. **K,L**. Quantification of DiOlistics-labeled dendritic spines in prefixed coronal brain slices.  
6 APP<sup>NL-G-F</sup> mice treated with Nb62-r-mAb showed significantly increased overall spine density  
7 compared to control-r-mAb treated mice (**K**). Analysis of spine subtypes showed that numbers of  
8 thin and mushroom spines was significantly increased in Nb62-r-mAb treated groups, most  
9 significantly for thin spines. **M**. Representative images of Bassoon (green) and PSD95 (red)  
10 immunoreactive synaptic puncta in the stratum radiatum of Nb62-r-mAb and control-r-mAb treated  
11 APP<sup>NL-G-F</sup> mice at endpoint; scale bar = 5  $\mu$ m. **N**. Synaptic puncta stained with Bassoon or PSD95  
12 were quantified (ROI; 20  $\mu$ m $\times$ 20  $\mu$ m, twelve per mouse) using Imaris Spot function; puncta were  
13 increased in Nb62-r-mAb treated mice compared with controls for both stains but significantly  
14 only for PSD95. **O-Q**. Comparison of Nb62-r-mAb treated and control-r-mAb treated APP<sup>NL-G-F</sup>  
15 mice in behavioural tests. In the burrowing test (**O**), Nb62-r-mAb treated mice burrowed  
16 significantly more of the gravel compared to controls. In the open field (OF) test (**P**), Nb62-r-mAb  
17 mice spent significantly more time exploring the central area of the box. In the novel object  
18 recognition (NOR) test (**Q**), Nb62-r-mAb treated mice spent significantly more time exploring the  
19 novel object. Each point represents one animal in these analyses. For all quantitative analyses, an  
20 unpaired two-tailed t-test was used to compare the two groups. Error bars correspond to SEM and  
21 p values are included where appropriate.

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1 **Table I Binding of Nb62-r-mAb and control-r-mAb to human and mouse C7 was measured using surface plasmon resonance**  
 2 **(SPR).**

Protein	mAb	ka(1/Ms)	kd (1/s)	KD (M)
Mouse C7	Nb62-r-mAb	$1.536 \times 10^6$	$6.910 \times 10^{-6}$	$4.499 \times 10^{-12}$
	control-r-mAb	$1.256 \times 10^6$	$1.350 \times 10^{-6}$	$1.075 \times 10^{-12}$
	mAb 73DI	$2.444 \times 10^6$	$1.621 \times 10^{-5}$	$6.63 \times 10^{-12}$
Human C7	Nb62-r-mAb	$9.556 \times 10^5$	$4.324 \times 10^{-4}$	$4.525 \times 10^{-10}$
	control-r-mAb	$4.703 \times 10^5$	$4.513 \times 10^{-4}$	$9.596 \times 10^{-10}$
	mAb 73DI	$1.041 \times 10^6$	$8.225 \times 10^{-4}$	$7.897 \times 10^{-10}$

3 Human or mouse C7 was immobilised directly onto a CM5 sensor chip and the relevant r-mAb or native mAb 73DI was flowed over the chip.  
 4 Sensorgrams were collected and KDs calculated using the Langmuir 1:1 binding model (n=3). Both the r-mAb and mAb 73DI strongly bound  
 5 mouse and human C7 (KDs  $\sim 10^{-12}$ ,  $\sim 10^{-10}$  respectively); the association constant (ka; on rate), dissociation constant (kd; off rate) and calculated  
 6 KD are stated in the table.

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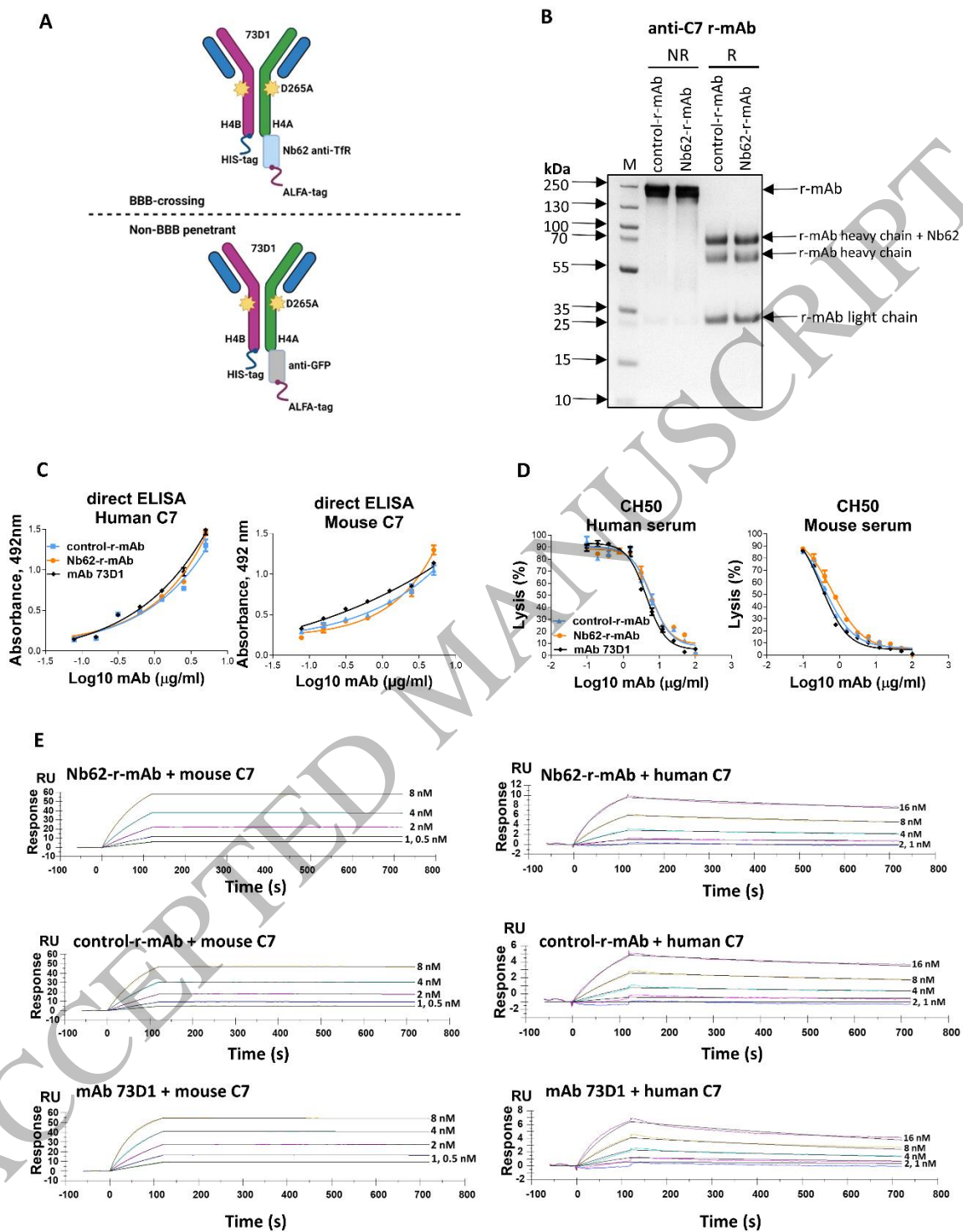


Figure 1  
190x240 mm (DPI)

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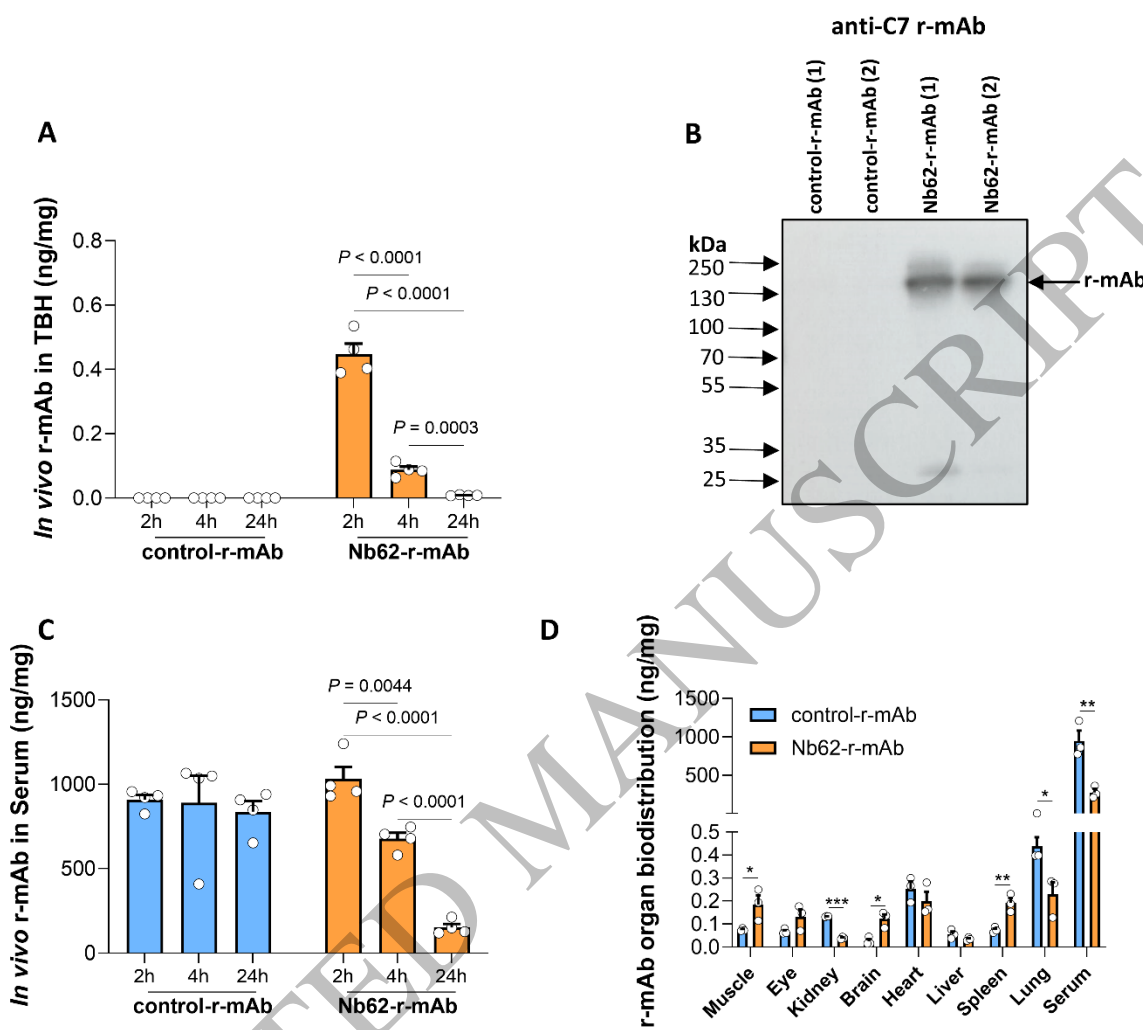


Figure 2  
156x143 mm (DPI)

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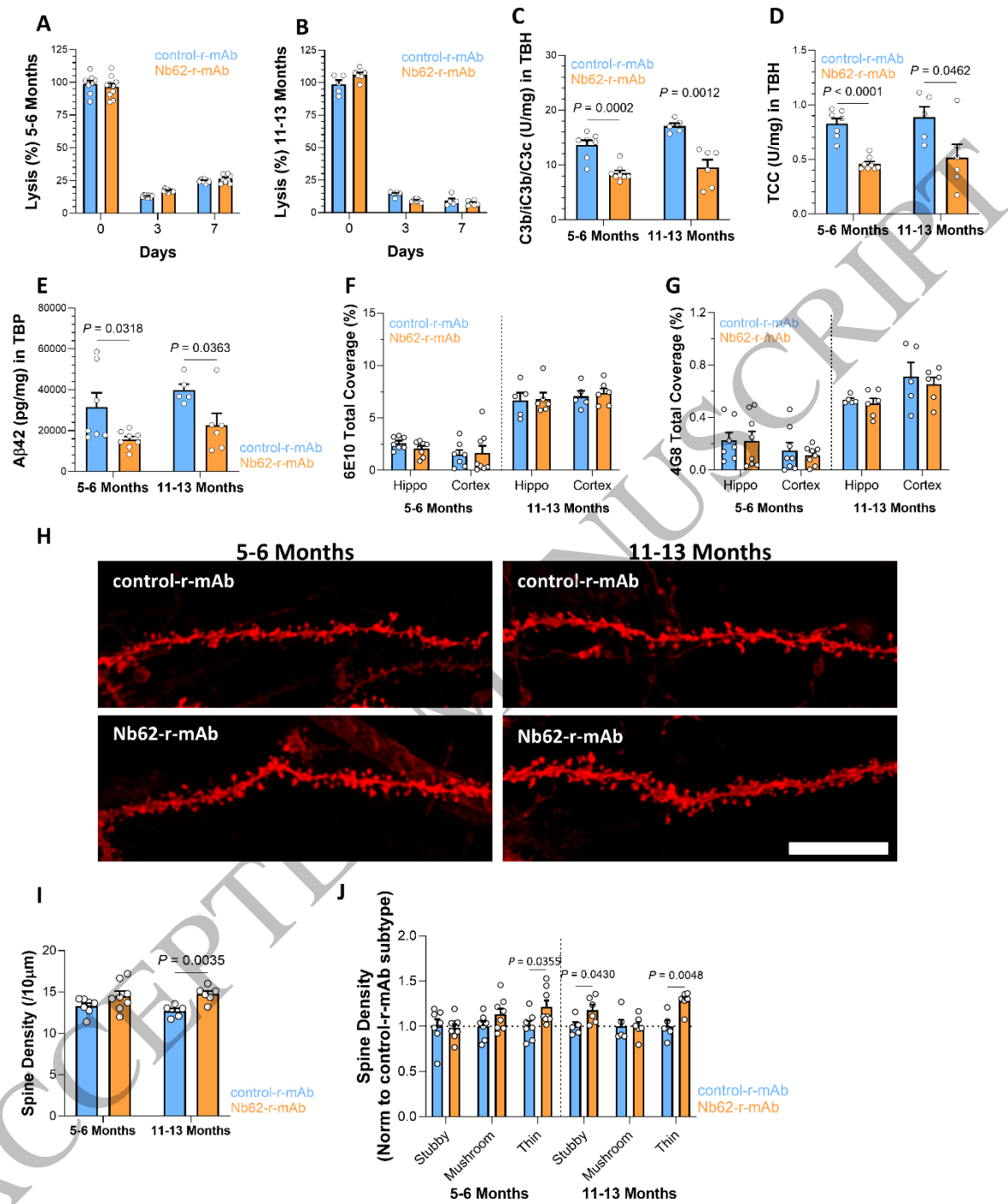
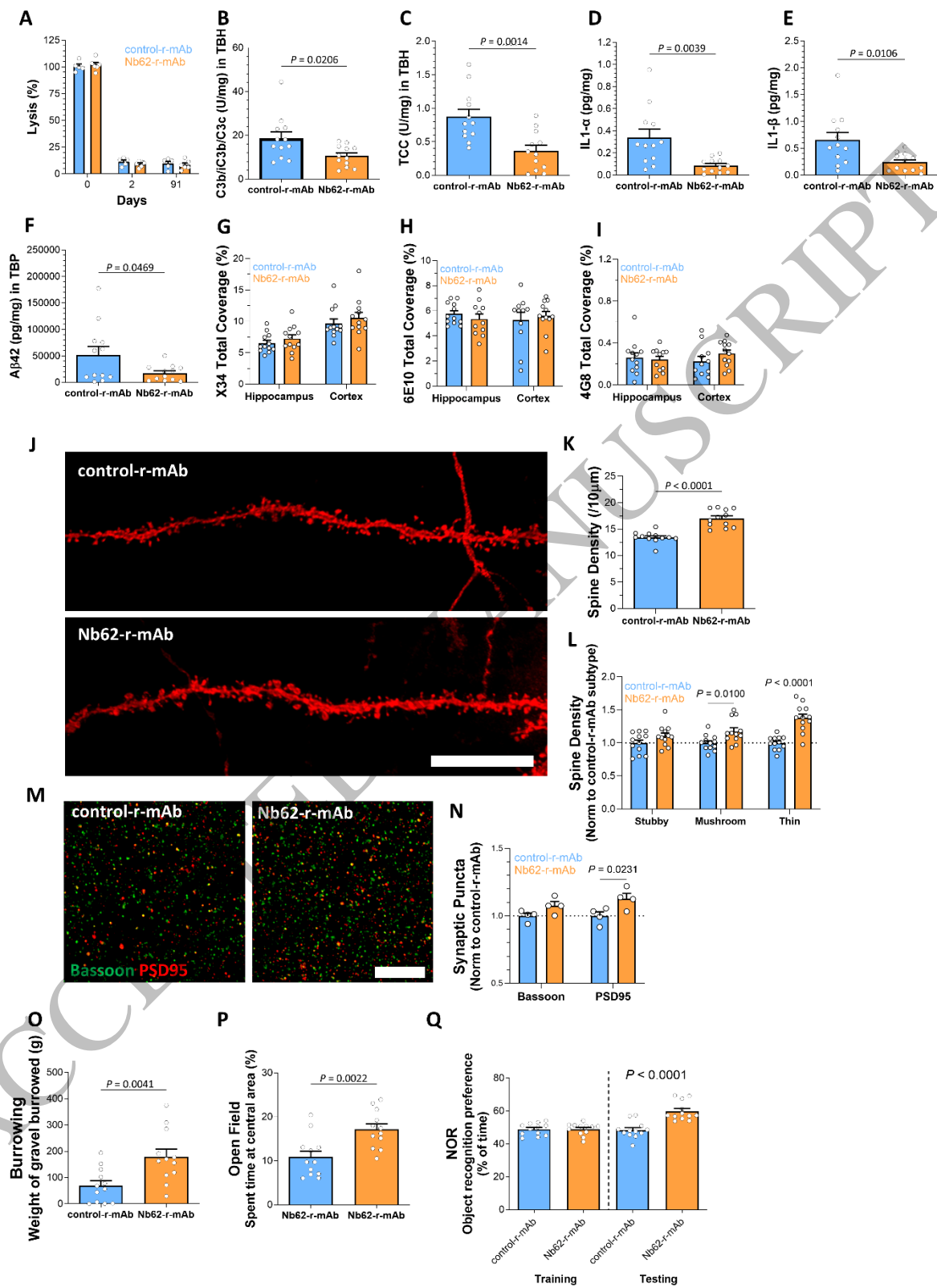


Figure 3  
176x213 mm (DPI)

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Figure 4  
182x246 mm (DPI)