Antibody recognizing 4-sulphated chondroitin sulphate proteoglycans restores memory in tauopathy-induced neurodegeneration.

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Running title: Memory restoration with anti-CSPG antibody.

Number of pages : pages 27
Number of figures : 12
Number of words for
Abstract :; 254, Introduction:; 1076, Discussion: 1828

Abstract

Chondroitin sulphate proteoglycans (CSPGs) are the main active component of perineuronal nets (PNNs). Digestion of the glycosaminoglycan chains of CSPGs with chondroitinase ABC or transgenic attenuation of PNNs leads to prolongation of object recognition memory and activation of various forms of plasticity in the adult CNS. The inhibitory properties of the CSPGs depend on the pattern of sulphation of their glycosaminoglycans, with chondroitin 4-sulphate (C4S) being the most inhibitory form. In this study we tested a number of candidates for functional blocking of C4S, leading to selection of an antibody, Cat316, which specifically recognises C4S and blocks its inhibitory effects on axon growth. It also partly blocks binding of semaphorin 3A to PNNs and attenuates PNN formation. We asked whether injection of Cat316 into the perirhinal cortex would have the same effects on memory as chondroitinase ABC treatment. We found that masking C4S with the Cat316 antibody extended long-term object recognition memory in normal wild type mice to 24 h, similarly to chondroitinase or transgenic PNN attenuation. We then tested Cat316 for restoration of memory in a neurodegeneration model. Mice expressing tau with the P301S mutation showed profound loss of object recognition memory at 4 months of age. Injection of Cat316 into the perirhinal cortex normalised object recognition at 3 h in P301S mice. These data indicate that Cat316 binding to C4S in the extracellular matrix can restore plasticity and memory in the same way as chondroitinase ABC digestion. Our results suggest that antibodies to C4S could be a useful therapeutic to restore memory function in neurodegenerative disorders.

Key words: Perineuronal nets, CSPGs, Object recognition memory, Plasticity, Alzheimer's disease

1. Introduction

Memory loss is a largely unmet medical challenge. Halting brain degeneration would be the most effective form of treatment, but it has yet to be achieved. An alternative is to enable the brain to function despite the degenerative damage. Plasticity enables the brain to bypass damage, but it is limited in adults by inhibitory proteoglycans in the brain extracellular matrix situated in perineuronal nets (PNNs), which surround key regulatory neurons.

PNNs are dense pericellular extracellular matrix (ECM) structures with a structure similar to cartilage that develop at the time of closure of critical periods for plasticity. PNNs are a highly organised complex, mainly composed of hyaluronan, chondroitin sulphate proteoglycans (CSPGs), link proteins, and tenascin-R. The form of PNN most studied are those surrounding parvalbumin-positive (PV) interneurons recognised by the lectin WFA, although other forms of PNN exist around pyramidal and other neurons (Matthews et al. 2002). In the cortex PNNs surround PV interneurons which are known to influence plasticity and excitability, and PNNs are involved in the termination of plasticity at the end of critical periods (Fawcett, 2015; Pizzorusso et al., 2002; Sugiyama et al., 2009). PNNs are also involved in memory. Depletion of chondroitin sulfate (CS) with the CS digesting enzyme, chondroitinase ABC (ChABC) prolonged object recognition memory in normal mice (Romberg et al., 2013), restored lost memory in a neurodegeneration model in tauopathy mice and Abeta transgenic mice (Vegh et al., 2014; Yang et al., 2015), and affected fear memory extinction (Gogolla et al., 2009), demonstrating that CSPGs are the key molecules in regulating this form of memory, and that the CS glycosaminoglycan (GAG) chains digested by ChABC are their effectors. Animals deficient in the PNN component Crlt1 link protein have very attenuated PNNs and show the same changes in memory and plasticity as animals treated with ChABC despite having unaltered CSPGs in the CNS, demonstrating that it is CSPGs in PNNs that are controllers of plasticity and memory (Carulli et al., 2010; Romberg et al., 2013). However, in order to reactivate plasticity, ChABC has to be injected directly into the brain parenchyma where it digests a region of up to 1 mm across, and the effect lasts for around three weeks. ChABC is therefore not a practicable treatment for memory restoration in neurodegenerative diseases. This study investigates antibody treatment as an alternative.

CSPGs and PNNs are implicated in neurodegenerative conditions in various ways. CSPGs are abundant in amyloid and neurofibrillary tangles in Alzheimer's disease (AD) (reviewed in DeWitt et al., 1993; Kwok et al., 2011) and differentially sulphated

CS such as chondroitin 4-sulphate (C4S), chondroitin 6-sulphate (C6S) and unsulphated chondroitin were detected (DeWitt et al., 1993). A reduction of PNNs in brains affected by AD was shown by staining with lectin WFA (Baig et al., 2005; Kobayashi et al., 1989), although many markers were not affected in AD patients and animal models of AD (Morawski et al., 2012c; Morawski et al., 2010b). PV interneurons are largely unaffected by neurofibrillary tangles (Baig et al., 2005; Bruckner et al., 1999; Hartig et al., 2001; Morawski et al., 2010a; Morawski et al., 2012b). This suggests that PNNs may be protective, and there is evidence for regulation of ion homeostasis (Suttkus et al., 2014b), and neuroprotection against oxidative stress (Cabungcal et al., 2013; Suttkus et al., 2014a). Treatment with chondroitinase does not affect hyperphosporylated or filamentous tau (Yang et al., 2015), but an effect on Abeta quantity has been reported (Howell et al., 2015).

The functional effect and binding properties of CSPGs are heavily dependent on the patterns of sulphation of their GAG chains (Lin et al., 2011; Malavaki et al., 2008; Miyata and Kitagawa, 2015). The sugars in the GAG chain can be sulphated in the 4, 6, 2+6 and 4+6 positions on the CSs (C4S, C6S, C2,6S and C4,6S, also known as CS-A, CS-C, CS-D, CS-E) respectively, and different patterns of sulphation give motifs that can provide specific binding sites (Mikami and Kitagawa, 2013; Xu and Esko, 2014; Yabuno et al., 2015). C4S is inhibitory to neurite growth while C6S is more permissive to neurite growth and to plasticity (Lin et al., 2011; Miyata et al., 2012; Wang et al., 2008). Thus mice with a knockout of chondroitin 6-sulfotransferase 1 (C6ST1) showed worse regeneration and less plasticity than wild type animals (Lin et al., 2011) while C6ST1 overexpressing mice demonstrated enhanced plasticity into adulthood (Miyata et al., 2012). During embryonic development C6S predominates, but at the end of the critical period in the PNN fraction only 4.9% of CS-GAG is 6-sulphated while 80.6 % is the inhibitory 4-sulphated form (Carulli et al., 2010; Deepa et al., 2006). We have recently demonstrated that this trend continues into aging when memory deficits start to appear. Chondroitin 6-sulfate reduced almost to zero in aged rats (12- and 18-months old) when compared to young animals (Foscarin et al., 2017). Moreover, the sulphation pattern of PNN CS-GAGs (which make up 2% of total brain CS-GAG) is different from that of the general brain matrix, giving the PNN specific binding properties (Deepa et al., 2006). Thus semaphorin 3A (Sema3A) and OTX2 bind specifically to PNNs but not to the general matrix (Beurdeley et al., 2012; Dick et al., 2013; Miyata et al., 2012; Vo et al., 2013), where they are positioned to affect synapse dynamics and PV cell maturation. Both these molecules bind most strongly

to 4,6 disulphated CS-GAG. The major CSPG core protein of PNNs is aggrecan, which is necessary for PNN formation (Carulli et al., 2016; Dick et al., 2013; Giamanco et al., 2010; Morawski et al., 2012a) and contains specific glycan modifications in its linkage region (Yabuno et al., 2015).The CS-GAGs carried by aggrecan are probably the key component of PNNs that regulate memory and plasticity, because animals lacking aggrecan in the CNS show the same memory prolongation phenotype as animals with attenuated PNNs due to Crtl1 knockout or ChABC treatment (unpublished observations) (Romberg et al., 2013). Our hypothesis therefore was that masking C4S on aggrecan would make PNNs less inhibitory, and would therefore have a similar effect to ChABC in restoration of memory to brains damaged by neurodegeneration, or in prolonging memory in normal animals.

Seeking potential treatments for memory restoration, we have tested an inhibitory proteoglycan neutralising antibody. In this manuscript, we have identified Cat316 as a C4S binding antibody that can modulate the inhibitory properties of the glycan, reduce Sema3A binding, prolong object recognition memory in normal animals and restore lost memory in tauopathy animals.

2. Materials and methods

2.1. Screening of PNN blocking antibodies using PNN-HEK cells

PNN-HEK cells (~12,000 cells) were plated on poly-L-lysine coated coverslips for 2 h and cultured in low-serum medium (0.5 % fetal bovine serum in DMEM) in the presence of 5 % CO_2. Detached cells were then removed with a rinse in fresh warm medium. Potential PNN blocking antibodies (Cat316 is from Millipore; HAPLN1 is from R&D; 6B4 and 7B7 are from CosmoBio) were diluted into 5 µg/ml in cultured medium before incubation with the PNN-HEK cells for 48 h at 37°C with 5 % CO_2. Control treatment with ChABC (200 mU/ml for 30 min) was done after plating. Then cells were rinsed in warm culture medium, and stained with anti-aggrecan antibody (1:400, rabbit polyclonal, Millipore) for 30 min for PNNs. Cells were then fixed with 4 % paraformaldehyde (PFA) before incubation with secondary anti-rabbit antibodies. Cells were counterstained with Hoechst, coverslipped with anti-fading medium FluoroSave (CalBiochem) before visualised using Leica confocal microscope.

For experiments testing the efficiency of Cat316 in blocking Sema3A from binding to the PN-HEK cells, PNN-HEK cells were cultured as above and Sema3A (40ng/ml) was added to the culture medium for 2 h at 37°C. The cells were then rinsed with

warm DMEM twice for 10 minutes each at 37°C before being processed for immunocytochemistry of Sema3A using a protocol as above.

2.2. Quantification of optical density of aggrecan staining in PNN-HEK cells

All images were captured with the same settings using a Leica confocal microscope [with 1 frame at numerical aperture 1.15, 405 nm laser (12%), 488 nm laser (38%) and 532 nm laser (38%)]. The red channel corresponding to aggrecan staining of the PNNs was converted into black and white images. Optical density of the images was measured using ImageJ software (1.49v, National Institutes of Health, USA). The data was then normalised first to a negative control (omitting primary antibodies), before further normalisation against the positive control (i.e. PBS). For each condition, 5 random images were taken from each coverslip with 4 coverslips per experiment. Each experiment was repeated three times.

2.3. Neurite outgrowth assay

Dorsal root ganglia (DRGs) were dissected in cold DMEM from adult Sprague-Dawley rats (~ 3 months). The DRGs were dissociated in collagenase (0.2 % w/v in Hanks-DMEM buffer) and trypsin (0.1% w/v in PBS) before being reconstituted in culture medium (1x insulin-tryptophan-selenium, 10 ng/ml nerve growth factor in DMEM). The neurons were then plated onto laminin (1 µg/ml) or aggrecan-laminin (25 µg/ml:1 µg/ml) coated coverslips. The DRG neurons were cultured for 24 or 48 h with or without the addition of different PNN blocking antibodies (5 ng/ml) including HAPLN1, Cat316, 6B4 and 7B7. For the control, the coverslips were treated with ChABC for 30 minutes before the plating of the DRGs. The neurons were then fixed using 4% PFA. The neurites were stained with anti-beta III tubulin antibody (1:1000, chicken, AbCam) and IB4-fluorescein lectin (1:250, Sigma). The longest neurite per neuron was measured using ImageJ software (1.49v, National Institutes of Health, USA). Twenty neurons were measured per coverslip and 3 coverslips were prepared per condition in each experiment. Each experiment has been repeated 4 times. The number of neurons bearing neurites (i.e. the neurites were longer than the diameter of the cell bodies) was also counted in each coverslip under 20x magnification and 5 random views were counted per coverslip. The results were compared to the total number of neurons in the same visual field.

2.4. Slot blot assay for antibody specificity

Commercial CSs were purchased from Sigma Aldrich (for CS-A, -B, -C and aggrecan) and Seikagaku (CS-D and -E). Each CS was prepared as 2 and 10 µg/ml

in distilled water, then transferred to a nylon membrane using a slot blot cassette. The blots were blocked with 5 % skimmed milk before incubating with the anti-CS antibodies. After incubation, the blots were rinsed and probed with alkaline phosphatase conjugated secondary antibodies (Invitrogen, 1:500). The binding of antibodies was visualised using alkaline-phosphatase substrate (NBT/BCIP; Sigma).

2.5. Enzyme linked immunosorbent assay (ELISA)

The ELISA was modified from Dick *et al*. 2013. Biotinylated GAGs (0.5 g per well) isolated from loose extracellular matrix (B1) or PNN matrix (B4) were immobilized onto streptavidin- coated plates of 384 (Pierce/Thermo Scientific). Biotinylation of GAGs was performed by EDC and biotin-LC hydrazide conjugation (Pierce/Thermo Scientific). After GAGs were immobilized on the plates, the plates were blocked in 1% BSA and subjected to the binding of recombinant Sema3A-AP (150 ng/ml). Detection of Sema3A-AP was done by direct measurement of absorbance at 405 nm using p-nitrophenyl phosphate (Sigma). To detect binding of Sema3A-GFP, the wells were incubated with 1:2,000 rabbit anti-Sema3A antibody (Abcam), and subsequently with 1:10,000 AP-conjugated anti-rabbit antibody (Invitrogen). Detection was done by measuring the absorbance at 405 nm using p-nitrophenyl phosphate.

2.6. Mice

The normal wild type (WT) C57BL/6J (Charles river, UK) mice were used for Cat316 antibody treatment study and subjected to the long-term object recognition (OR) memory test with 24 h delay paradigm. Transgenic mice as a dementia model used for this manuscript were homozygous Tg P301S tau mice expressing human mutant P301S tau under the control of the murine *Thy1.2* promoter (Allen et al., 2002). Age- and sex-matched C57BL/6J mice (Charles river, UK) were used as WT controls. Male mice were used across the studies. Animals had unrestricted access to food and water, and were maintained on a 12 h light/dark cycle (lights off at 7:00 P.M.). All experiments were carried out in accordance with the UK Home Office Regulations for the Care and Use of Laboratory Animals and the UK Animals (Scientific Procedures) Act 1986.

2.7. Anti-CSPG antibody Cat316 injections

Anti-CSPG IgM antibody Cat316 (1mg/ml in PBS), or isotype control IgM (1mg/ml, Abcam) was stereotaxically injected to six different sites in the perirhinal cortex (PRh; 3 per hemisphere, 0.5 µl with a speed of 0.2 µl/min). Animal surgeries were

performed under isoflurane anesthesia. Injections were made with a 10 µl Hamilton syringe and a 33 gauge needle at the following sites (in mm from bregma and the surface of skull): 1. anteriorposterior (AP): -1.8; lateral (L): ± 4.6; ventral (V): -4.4. 2. AP: -2.8; L: ± 4.8; V: -4.3. 3. AP: -3.8; L: ± 4.8; V: -3.8. The needle remained in place at the injection site for 3 min before being slowly withdrawn over 2 min. After two days of first injections, additional injections were performed as described above.

2.8. Spontaneous Object Recognition task

The spontaneous OR task was performed three days after second injections of Cat316 or isotype control antibody. The protocol was slightly modified from the previously published protocol (Yang et al., 2015). All mice were habituated in two consecutive daily sessions in the empty Y-maze apparatus for 5 min prior to surgery. After recovery from surgery, animals received one day of habituation session for 5 min. Each test session consisted of a sample phase and a choice phase. In the sample phase, the animal was placed in the start arm and left to explore two identical objects, which were placed on the end of two arms for 5 min. The choice phase followed after a delay of either 3 h or 24 h, which the animal spent in the home cage. The choice phase was procedurally identical to the sample phase, except that one arm contained a novel object, whereas the other arm contained a copy of the repeated object. When the animals were assessed, a different object pair was used for each session for a given animal, and the order of exposure to object pairs were counterbalanced within and across groups. In the experiment using a P301S tauopathy model, a different object pair was used for each session for a given animal, and the test sessions were separated by a minimum of 48 h. The object exploration time was assessed from video recordings of the sample and choice phase. The direct nasal or head contacts only were regarded as an exploratory behaviour. A discrimination ratio was calculated by dividing the difference in exploration of the novel and familiar objects by the total object exploration time. Therefore, the discrimination ratio varies from 0 (equal exploration for novel and familiar objects) to 1 (exploration of the novel object only). The test sessions were separated by a minimum of 48 h. Group means were compared by ANOVA followed by Tukey post hoc test with a significance level of $p < 0.05$, using GraphPad Prism version 5.0. A two-tailed *t*-test was used for two-group comparisons.

2.9. Immunohistochemistry: DAB staining

Sample preparations and the general procedures of immunostaining have been described previously (Yang et al., 2015). In general, 30 µm free floating sections

were incubated with 10 % methanol, 3 % H_2O_2 in PBS for 20 min at room temperature for quenching endogenous peroxidase activity. Sections were rinsed three times in 0.2 % Triton-X in PBS (PBS-T) and were subsequently blocked with 5 % normal goat serum (NGS) or normal horse serum (NHS) in PBS-T for 1 h at room temperature. The primary antibodies (NeuN; 1:400, Millipore; AT8; 1:1000, Innogenetics; Cat316; 1:50, Millipore; WFA; 1:100, Sigma-Aldrich) were incubated over night at 4 °C following rinsing tissues for 5 min three times in PBS-T (0.2 % Triton-X in PBS). For Sema3A staining, as adapted from Vo et al, 2013, tissue sections were pre-incubated with 0.1 U/ml of ChABC (Seikagaku) in ChABC buffer (0.1 M Tris-HCl, 0.03 M sodium acetate, pH 8.0) for 2 h at 37 °C prior to quenching. Then Sema3A antibody (1:50; Santa Cruz Biotechnology) was incubated overnight at RT. Following 3 washes in PBS-T they were incubated for 2 h at RT with the appropriate biotinylated secondary antibody (Vector Laboratories) diluted 1:500 in PBS-T. For detecting the Cat316 antibody injection site, the biotinylated secondary antibody (biotinylated goat anti-mouse IgM, 1:500, Vector Laboratories) was incubated over night at 4 °C. Subsequently, sections were incubated with an avidin-biotin system (Vectastain; Vector Laboratories) for 1 h at RT and washed with PBS-T. The immunostaining was visualized with diaminobenzidine with 3 % H_2O_2 (DAB kit; Vector Laboratories) for 1-5 min at RT. For Sema3A staining the immunolabelling was performed with diaminobenzidine with 0.3 % H_2O_2 for 10-20 min at RT. The sections were mounted on gelatin-coated slides and air-dried. Following dehydrating tissue sections in ascending concentration of alcohols, they are cleared in xylene and coverslipped with DPX. The tissue sections were examined using a light microscope and photographed using a digital camera (Leica DM6000 Microsystems).

For quantification of WFA and Sema3A immunoreactivity, the images were captured under 20x object magnification using Leica DM6000 Microsystems. Four to six images per animal were randomly captured in the injection area of Cat316 or isotype control IgM (n=4 per group). These images, all acquired using the same microscope settings, were adjusted by threshold cutoff to 128 in Adobe photoshop CS5 (saved to a black and white image) in order to cut out the majority of background staining and black/white inverted. Then, ImageJ software (1.49v, National Institutes of Health, USA) was used to quantify the number of pixels brighter than the threshold.

3. Results

The aim of our experiments was to select and evaluate an antibody that can reactivate plasticity and restore memory through blocking inhibitory proteoglycans in PNNs. We tested several candidates for their ability to block inhibition by PNN

proteoglycans, from which we selected Cat316. The ability of Cat316 to bind to proteoglycans and PNNs and to compete with Sema3A binding to PNNs was then evaluated in more detail. We then asked whether Cat316 has the same effects on memory prolongation and restoration as chondroitinase digestion. Cat316 was injected into the PRh cortex, and its ability to prolong object recognition memory in normal mice and to restore memory in tauopathy mice was measured.

3.1. Selection of a chondroitin-4-sulphate blocking treatment

Our first task was to find a treatment that could block the inhibitory effects of C4S. In order to do this, we measured the binding of candidates to CS-GAGs, assayed for blocking inhibition of axon growth by aggrecan and tested for inhibition of PNN formation *in vitro*. Our candidates were antibodies that bind to CS-GAG, the CS-GAG binding lectin WFA and an antibody to the PNN component HAPLN1 link protein.

First, we assayed the binding of three antibodies and WFA to different sulphation forms of CS-GAG in a slot blot assay. Purified CSs of known sulphation type and aggrecan were immobilised on nylon membranes. Incubation with these reagents showed that only Cat316 had strong binding to C4S (CS-A) (at both 2 and 10 µg) and aggrecan (Fig. 1), but did not bind to C6S (CS-C), C2,6S (CS-D) or C4,6S (CS-E). Two other candidate antibodies CS56 (IgM anti CS-GAG) and AB1031 (polyclonal anti-aggrecan) did not show preferential binding to C4S (CS-A) (Fig. 1). We also tested the CS-GAG-binding lectin WFA, but this bound to aggrecan but not to the isolated CS-GAGs. The results show that of our candidates only Cat316 bound specifically to C4S.

To test the ability of our candidates to neutralize inhibition of neurite extension in the presence of inhibitory CSPGs, we cultured adult DRG neurons on a low concentration of laminin in the presence of the inhibitory CSPG aggrecan (Fig. 2). This assay has been widely used to assay for the inhibitory effects of CSPGs and CS-GAGs extracted from the CNS (Tan *et al*., 2012). DRG neurons cultured on mixed laminin/aggrecan substrates grew shorter (49.9% reduction) neurites compared to laminin control. The aggrecan inhibition was neutralised when the laminin/aggrecan substrates were pre-digested with ChABC. Of the various candidates Cat316 antibody and anti-HAPLN1 also neutralised the inhibition of aggrecan to the same extent as ChABC; the length of neurites cultured on laminin/aggrecan/Cat316 was similar to the laminin only condition (223.0 $\pm$ 30.6 µm

vs 199.8 ± 33.4 µm). The results demonstrate that the only CS-GAG-binding molecule of our candidates able to block inhibition by aggrecan was Cat316.

We tested the ability of our candidates to inhibit PNN formation. For this we used an *in vitro* model of PNN formation. PNN-HEK cells are surrounded by a PNN-like condensed matrix, whose formation is dependent on aggrecan, hyaluronan synthase and Crtl1 link protein (Kwok et al., 2010). We tested four antibodies, three of them bind to aggrecan (Cat316, 6B4 and 7B7) and one binds to Crtl1 link protein (anti-HAPLN1) for their ability to block the formation of the PNN-like matrix around PNN-HEK cells when placed in the growth medium for 48 h (Fig. 3). Aggrecan immunostaining and WFA staining were used as markers for PNNs on the cell surface. The binding of aggrecan to the PNN structures is partially dependent on its CS-GAG, because treatment of cells with ChABC for 30 minutes reduced aggrecan staining on the cell surface (Fig. 3B; 61.2% reduction). All the antibodies were able to reduce PNN formation, judged by surface aggrecan intensity, on the PNN-HEK cells but to different degrees; Anti-HAPLN1 by 28.2%, Cat316 by 55.1%, 6B4 by 17.4% and 7B7 by 17.7% (n=4, two-tailed paired *t*-test, ***$p < 0.001$). Measurement of the surface intensity of WFA showed a reduction when the cells were treated with chondroitinase, anti-HAPLN1 or Cat316 antibody. These results demonstrate that of the CS-GAG-binding antibodies, Cat316 antibody has the most powerful effect on blocking the formation of PNNs in PNN-HEK cells.

Based on the above results we selected Cat316 as the most promising treatment for blocking the inhibitory effect of C4S on PNN CSPGs, and further characterized its effects in two more *in vitro* assays.

3.2. Cat316 binds to PNN GAGs and inhibits semaphorin 3A binding

We tested the binding of Cat316 to GAGs purified from diffuse soluble brain ECM (B1) and from the PNN-enriched fraction (B4) using a modified glycan ELISA (Dick et al., 2013). CS-GAGs from brain ECM and PNNs were extracted from adult rat brains, using the sequential extraction method in which PNN-GAGs are enriched in the final 6 M urea buffer (B4) (Deepa et al., 2006; Kwok et al., 2015). These CS-GAGs were biotinylated and immobilized in ELISA wells. Cat316 was incubated with the immobilised B1-GAGs or B4-GAGs (Fig. 4). The results demonstrated that Cat316 bound to both B1 and B4 GAGs. This binding was greatly reduced when the GAGs were pre-treated with ChABC before the addition of Cat316. This suggests that

Cat316 binds to the ECM and PNN glycans with similar affinity and the binding is mediated through the CS-GAGs in the preparation.

A key effector of PNNs is Sema3A, which binds particularly to disulphated C4,6S (de Winter et al., 2016; Dick et al., 2013). We therefore tested the ability of Cat316 to bind to purified brain CS-GAGs from the PNN compartment, and for its ability to block Sema3A binding to these CS-GAGs. Both Cat316 (as in Fig. 4) and Sema3A bound strongly to these CS-GAGs (Fig. 5A). When Cat316 was added to the PNN-GAGs first, the subsequent binding of Sema3A was approximately halved. We then tested for the ability of Cat316 to inhibit binding of Sema3A to an *in vitro* model of PNNs. PNN-HEK cells were cultured to form PNNs, then Sema3A was added for 2 h. As shown in Fig. 5B, it bound strongly to the extracellular matrix of the cells. Cat316 was then incubated with the cells for 2 h before addition of the Sema3A, leading to a reduction (by 38.4%; n=3, paired *t*-test, ***$p < 0.001$) in binding of Sema3A to the PNN-HEK cell extracellular matrix Fig. 5, C and D). These experiments show that Cat316 binds to PNN-like CS-GAGs and blocks the binding of Sema3A to them.

3.3. Cat316 antibody blocks the inhibition of axon growth on Sema3A and PNN matrix

We next asked whether Cat316 affects axon growth on PNN-HEK cells to which Sema3A is bound. Dissociated DRG neurons were plated on coverslips coated with PNN-GAGs (B4-GAGs), then Sema3A for 2 h and cultured in the presence of Cat316 (Fig. 6). In the presence of Cat316 antibody, the length of neurites was increased on PNN-HEK-Sema3A matrix (Fig. 6, B and C). This suggests that Cat316 is efficient in blocking Sema3A-induced inhibition on PNN-HEK matrix.

3.4. Cat316 antibody binds to CS-GAGs in perineuronal nets *in vivo*

In order to identify the structures that bind Cat316 in mouse brain, sections of rat cortex were immunostained with the antibody. The antibody clearly stained PNNs throughout the cortex with a low level of diffuse staining to the matrix in the cortex in addition (Fig. 7). In order to confirm that Cat316 antibody binds to CSPGs in brain sections, the CS-GAGs were digested by pre-incubating the mouse brain sections for 1 h with ChABC at concentrations 0.01 U/ml, 0.1 U/ml, 1 U/ml. After the enzymatic degradation of CSPGs the tissues were immunostained with Cat316 (Fig. 7A). Immunoreactivity to Cat316 was gradually reduced as concentrations of ChABC increased, indicating that the epitope recognized by Cat316 antibody was removed by ChABC treatment and was therefore a CS-GAG (Fig. 7A). However, there was

still some diffuse staining of digested sections, in contrast to WFA staining which was completely removed by ChABC treatment (Fig. 7B). We saw a very similar pattern of PNN and diffuse matrix staining when the antibody was injected into the PRh cortex and detected with anti-IgM secondary (Fig. 8A,B). At high magnification the staining revealed a lattice-like matrix which enwrapped the soma and dendrites in a typical PNN fashion (Fig. 8B). Fig. 8A shows the spread of the antibody from a single injection, with the planes of section at bregma -1.8 mm, -2.8 mm and -3.8mm. The amount of antibody around the injection site decreased over time. At 5 days after injection there was strong staining with anti-IgM secondary, but by 10 days the staining was very weak. This decline in Cat316 concentration in the brain roughly correlates with the loss of effect in the behavioural object recognition memory test (see below). The antibody spread for approx. 1mm by diameter from the injection site.

3.5. Sema3A and WFA binding were reduced in Cat316 injected areas

Having shown above that Cat316 can inhibit the binding of Sema3A to PNNs in an *in vitro* assay, we asked whether we could see a similar inhibition *in vivo.* Four days after Cat316 injection Sema3A levels were measured by immunostaining of wild type mouse brain sections (Fig. 9A). Observation of the sections suggested that antibody injection reduced strong Sema3A staining of PNNs, but had little effect on weaker and diffuse staining. We therefore measured Sema3A staining intensity in the most strongly stained regions by thresholding all the images to a level that removed the diffuse stain and just left the intensely stained PNNs. We then measured the number of pixels in each section that exceeded the threshold. By this analysis, the intensity of Sema3A antibody immunostaining was reduced by 33 % (two-tailed paired *t*-test, **p*=0.0157, n=4), in the Cat316 injected area compared to the isotype control injection control (Fig. 9, B and C) We also measured binding of biotin-WFA by measuring staining intensity in the PRh cortex using the same thresholding method, and this was reduced by 27% in the injected region after Cat316 treatment to WT control mice compared to isotype control injection (two-tailed paired *t*-test, **p*=0.0243, n=4, Fig. 9D and E). These results indicate that Cat316 antibody binds competitively to the same binding sites as Sema3A and WFA.

3.6. Cat316 antibody enhanced long-term OR memory in wild type mice

Having demonstrated that Cat316 antibody binds to inhibitory C4S and blocks its inhibitory effect on axon growth, and also inhibits PNN formation and the binding of Sema3A to PNNs, we asked whether injection of the antibody to the PRh cortex has

the same effect on memory as ChABC. We therefore investigated object recognition memory, in which 24 h memory is greatly prolonged after ChABC injection or transgenic attenuation of PNNs (Romberg et al 2013, Yang et al 2015). We anticipated that blocking inhibitory CSPGs by Cat316 injection to the PRh cortex would mimic this ChABC-induced memory enhancing effect. The experimental animals were habituated in the Y-maze prior to the injections of Cat316 or an isotype control antibody. Cat316 antibody was delivered bilaterally to the PRh cortex (3 injections per hemisphere) twice with two days interval (Fig. 10A). The six injections of Cat316 were made to bregma AP: -1.8mm, -2.8mm, -3.8mm. WT mice infused with Cat316 or control antibody were tested for OR memory retention 5 days after the second injection. Animals treated with Cat316 antibody showed an enhancement of long-term (24 h) OR memory compared to controls (Isotype control injected wild type mice) at 5 days post injection (Isotype control 0.05 ± 0.02, n=9 *vs* Cat316 0.26 ± 0.05, n=11; two-tail unpaired *t*-test, ***p*=0.0049, Fig. 10B). We then asked whether effect would persist as the antibody gradually dispersed from the brain by testing animals at 8 or 11 days post injection. At both these time points there was no increase in persistent 24 h memory compared to isotype control, indicating that the antibody effect did not persist until day 8 after injection (Fig. 10C). Neither Cat316 nor control IgM injections to PRh cortex affected participation in the test, as measured by the motivation index (Fig. 10, B and C). These results show that Cat316 injected into PRh cortex has the same effect on prolongation of memory as ChABC and that the effect disappears as the antibody dissipates.

3.7. OR memory in P301S tau mice rescued by Cat316 antibody treatment

TgP301S tau mice express a mutant form of human tau which is responsible for genetically-determined neurodegeneration in humans (Allen et al., 2002). Mice expressing this gene show progressive tauopathy accompanied by progressive memory loss which can be corrected by ChABC treatment (Yang et al., 2015). This mouse line was re-derived into a C57BL/6J background, and OR memory assessed at 3, 4 and 5 months. TgP301S tau mice showed progressive memory decline with profound memory loss at 4M and 5M of age compared to age-matched WT mice whilst they have intact memory at 3M of age (Fig. 11A). We selected 4 month-old mice for further experiments since they showed consistent impairment in OR memory (WT *vs* P301S, unpaired *t*-test, ****p*=0.0008). This memory loss was seen even at the short delay time of 3 h. WT control and Tg P301S tau mice explored the sample objects to a similar extent without any side preference, indicating that there was no major difference in motivation between groups (Fig. 11A). At this age, approximately

15% neuronal cell loss detected by anti-NeuN antibody staining was present in the PRh cortex compared to the WT control mice (WT 51592 ± 1391 n=7, P301S 43458 ± 1381, n=6, unpaired *t*-test, *$p < 0.05$).

Based on our previous work showing restoration of memory in P301S mice after ChABC injection into the PRh cortex (Yang et al. 2015), and the results in this paper showing memory extension in control mice after infusion of Cat316, we next tested whether Cat316 infusion in the PRh cortex restores the OR memory deficit in TgP301S tau mice. Cat316 or isotype control IgM were bilaterally injected at three different sites into 4 month-old Tg P301S tau mice. Four and six days after injections animals were tested for short-term OR memory, measuring 3 h retention (Fig. 11B). The impaired OR memory shown in 4 month-old P301S mice was normalised to the level of control animals after Cat316 antibody treatment while animals treated with isotype control IgM showed the same deficit as untreated animals. This was comparable to the behavioural restoration as seen in ChABC treated animals (Isotype control 0.047 ± 0.09, n=8 v*s* Cat316 0.28 ± 0.04, n=14, ChABC 0.24 ± 0.05, n=6, One way ANOVA, *p=0.042)(Fig. 11C). However, animals injected with either Cat316 or ChABC did not show the enhanced 24 h OR memory retention seen in WT animals, which was at the same basal level of memory shown in the isotype control group (Fig. 11C). Following the Cat316 injection the immunostaining profile of tau pathology detected by AT8 antibody was not significantly altered (Isotype control 44 ± 17.9 n=6, Cat316 41 ± 13.7 n=6)(Fig. 12A,B). Moreover this treatment did not alter the progressive neurodegeneration in TgP301S mice, which still showed the same reduction in neuronal cell counts compared to WT control mice treated with isotype control antibody (WT:Isotype control 1.0 ± 0.03 n=3, P301S:Isotype control 0.84 ± 0.02 n=5, P301S:Cat316 0.81 ± 0.04 n=6)(Fig. 12C). These data show that Cat316 injections to PRh cortex restore memory in P301S mice to the same extent as ChABC treatment, and demonstrate that blocking inhibitory C4S CS-GAGs is sufficient to correct the adverse effect on memory of tauopathy-induced neurodegeneration.

4. Discussion

PNNs have been implicated in memory through several types of experiment. In OR memory, transgenic attenuation of PNNs or their digestion with ChABC leads to memory prolongation (Romberg et al., 2013). In fear memory ChABC digestion in the amygdala restores memory erasure (a form of learning) to the infantile pattern (Gogolla et al., 2009). Memory stimulated by environmental enrichment depends on

the formation of new inhibitory synapses on PV neurons, and this synapse formation is facilitated by ChABC digestion (Donato et al., 2013). We have focused on OR memory because it depends on a focal brain area which is affected early in Alzheimer's disease, PRh cortex, and because of its robust readouts that can be obtained in a time scale appropriate for testing treatment effects. Our previous work shows that ChABC injected to the PRh cortex can prolong OR memory in WT animals or restore memory to mice with memory loss caused by pathology due to transgenic expression of mutant tau (Romberg et al 2013, Yang et al 2015). ChABC injections can also restore memory in an animal model of Abeta pathology (Vegh et al., 2014) All of these experiments depend on the injection of ChABC into the brain, or transgenic attenuation of PNNs. In the present work we explored the possibility of using an antibody that recognizes and blocks the most inhibitory form of the CS-GAGs that are attached to the CSPGs of PNNs.

Inhibition of regeneration and plasticity by CSPGs is strongly influenced by the pattern of sulphation of CS-GAGs in PNNs. Most CS-GAG sugars are 4 or 6 mono-sulphated, with 6S being more permissive to axon growth and plasticity, 4S being more inhibitory (Lin et al., 2011; Miyata and Kitagawa, 2015; Miyata et al., 2012; Wang et al., 2008). In the adult CNS over 90% of CS-GAG is 4-suphated, this proportion increasing as plasticity reduces at the end of critical periods (Carulli et al., 2010; Deepa et al., 2006). The hypothesis behind this study was that blocking the inhibitory effects of 4-sulphated CS in PNNs would make the PNNs less inhibitory and consequently reactivate neuronal plasticity and affect memory in the same way as ChABC injections.

The first step was to select an appropriate C4S-blocking antibody. We tested a range of anti CSPG-antibodies and also the CS-GAG-binding lectin WFA for their ability to bind to C4S and to block its inhibitory effects and also to block binding of the PNN ligand and effector Sema3A (de Winter et al., 2016; Dick et al., 2013; Vo et al., 2013). We also tested for the ability of antibodies to inhibit aggrecan-dependent formation of PNN-like structures on PNN-HEK cells (Kwok et al., 2010). These PNN-HEK cells form PNN-like matrix with a similar chemical composition and hierarchical assembly as those found in the brain. From these tests, we selected Cat316 because it binds to C4S and aggrecan, inhibits PNN development around PNN-HEK cells, blocks the axon growth inhibitory effect of aggrecan, and also partly prevents the binding of Sema3A to PNNs. Cat316 was originally identified for its binding to aggrecan in PNNs in the visual cortex, the level of staining being affected by dark

rearing (Kind et al., 2013; Lander et al., 1997; Matthews et al., 2002). Surprisingly, WFA, which binds specifically to PNNs and is used to stain them for histology, did not bind to any of CS isoforms and only bound to aggrecan in a slot blot assay, and did not block aggrecan inhibition. This may indicate that WFA lectin binds to PNNs-CS in a conformation-dependent manner or recognises a specific glycan structure which is yet to be identified. WFA certainly recognises CS-GAGs because ChABC treatment, abolishes its ability to bind to mouse brain tissue sections. The binding of Cat316 *in vivo* was similar to our *in vitro* results, with Cat316 binding to PNNs and reducing the association of Sema3A with PNNs. Anti-CSPG antibody CS56 binds preferably to 6-sulphated CS, which is present in a very minute quantity in PNNs. Anti-aggrecan antibody AB1031 did not show preferential binding to CS-GAGs. And the other antibodies to aggrecan such as 6B4 and 7B7 had no effect in neurite outgrowth assay. Thus, we selected Cat316 to target C4S molecules in extracellular matrix including PNNs. There are unfortunately no other anti-C4S antibodies available, and in particular there is no IgG anti-C4S antibody that could be used for systemic treatment.

Having selected Cat316 as the candidate antibody we had to decide how to administer it. Because Cat316 is an IgM it presumably has very limited ability to cross the blood-brain-barrier and also limited ability to diffuse. We therefore decided for this proof of concept study to inject the antibody directly by stereotaxic injection into the PRh cortex. We infused Cat316 antibody twice to the same injection sites in order to build up a sufficient antibody level, and to ensure that Cat316 persists in the cortex during the 5 days that it takes to measure OR memory. Staining of Cat316 injections showed that the antibody spreads around 1mm from the injection site, and the amount remaining at the injection site declined to be barely detectable by 7 - 10 days. First, we tested for the ability of Cat316 to prolong memory in control mice. In normal mice OR memory does not persist beyond 12 h, but in our previous studies, ChABC treatment extended robust memory to 24 h, and memory was still detectable at 48 h (Romberg et al., 2013). We saw a similar effect with Cat316 antibody: treated normal WT animals showed strong object memory at 24 h, while in control IgM injected animals objects were forgotten by this time. However this treatment effect was of limited duration, because when we tested animals at 8 and 11 days after antibody injection they showed no prolongation of memory, reflecting the decline in antibody levels at the injection site with time.

The next experiment was to see whether we could restore OR memory in a neurodegeneration model. For these experiments we used a tauopathy model, in which mice express a mutant form of tau protein leading to a progressive loss of memory. By 4 months of age these animals can learn and remember objects for a short time, but by 3 h the memory has been lost. These animals have a normal number and appearance of PNNs (Yang et al., 2015). In previous work ChABC injections to the PRh cortex of tauopathy animals restored the deficit in 3 h memory retention to a normal level (Yang et al., 2015). In the P301S tauopathy model memory was restored to the same pattern as WT animals. As with ChABC, Cat316 in P301S animals did not cause the prolongation of memory to 24 hours and beyond that is seen when WT animals are treated. These results indicate that blocking inhibitory C4S CS-GAGs can restore memory function in adult animals with a neurodegenerative condition. Neutralizing CSPG by antibody in the human tauopathy mice P301S did not alter disease progression. At the time of testing both treated and untreated animals showed 15% neuronal loss, and the same extent of hyperphosphorylated tau staining.

What might be the mechanism through which Cat316 affects memory? The action is probably through PNNs. PNN-mediated CS-GAGs make up only 2% of total brain CS-GAG (Deepa et al., 2006), and both ChABC and Cat316 will therefore bind to CS-GAGs both on the PNNs and in the loose extracellular matrix, although injected Cat316 bound preferentially to PNNs rather than to the surrounding diffuse matrix. PNNs can be attenuated through transgenic deletion of the Crtl1 link protein without affecting the overall quantity or sulphation pattern of CSPGs in the CNS (Carulli et al., 2010). These animals with attenuated PNNs show exactly the same prolongation of OR memory that we obtained with Cat316 treatment (Romberg et al., 2013). Cat316 bound to extracellular matrix, predominantly PNNs when injected into the brain, suggesting that Cat316 is acting on PNNs. Much of the work on CSPGs and plasticity and memory has depended on ChABC digestion of CSPG CS-GAGs. This has effects via PNNs, but it also enhances sprouting and synapse dynamics generally (Donato et al., 2013; Fawcett, 2015; Orlando et al., 2012). In brains affected by tauopathy or other forms of neurodegeneration, where scattered neurons are non-functional, sprouting will enable some rewiring and formation of bypass circuits to restore function. It is possible, therefore, that both ChABC and Cat316 can restore function in neurodegeneration both through an effect on PNNs and through an effect on plasticity/sprouting. How might Cat316 affect PNN function? PNNs have a unique sulphation pattern which differs from that of the general CNS matrix, which

allows them to have specific binding properties (Deepa et al., 2006; Yabuno et al., 2015). This enables PNNs to bind specifically to Sema3A and Otx2, which are therefore concentrated in PNNs where they can affect synapse dynamics and PV cell maturity (Bernard and Prochiantz, 2016; Beurdeley et al., 2012; de Winter et al., 2016; Dick et al., 2013; Vo et al., 2013). We have shown previously that Sema3A binds most strongly to C4,6S disulphated CS-E (Dick et al., 2013), yet resent paper we are demonstrating that an antibody to C4S monosulphated CS-A blocks binding. The probable explanation is that C4,6S CS-E makes up less than 2% of the total CS-GAG while C4S CS-A is over 90%. Specific binding sites on CS chains are usually 8 or more sugars in length, so it is probable that a Sema3A binding site will contain both C4S and C4,6S disaccharides, enabling Cat316 to interfere with binding. Blocking the Sema3A receptors neuropilin 1 and 2 can reactivate ocular dominance plasticity in the visual cortex (Pizzorusso, unpublished observations). It is probable, therefore, that the ability of Cat316 to reduce the binding of Sema3A to PNNs is a significant part of its mechanism of action. Memory requires the formation of new inhibitory synapses on PV interneurons (Donato et al., 2015), and it is probable that CS-GAGs, mostly being consisted of C4S might attenuate the formation of these new connections, while Cat316 will reduce this inhibition and enhance the formation of new synapses on PV interneurons.

Our report shows that masking of the inhibitory 4-sulphated CS-GAGs on CSPGs using anti-CSPGs antibody can restore memory in a neurodegenerative condition. However, because Cat316 is an IgM we injected it directly into the brain. An antibody or blocking molecule with better ability to cross the blood-brain-barrier might be a useful treatment for memory loss when given systemically.

As with any therapeutic antibody there is a possibility of immune damage with systemic administration. The tissue of obvious concern is cartilage; however most of the CS in cartilage is 6-sulphated and CS in joint cartilage is not exposed to blood and only indirectly to synovial fluid (Lauder, et al., 2001,Lauder, et al., 2000). CS autoantibodies are found in most normal adults and may be up-regulated in autoimmune conditions, but there is no direct evidence that the antibodies are the cause of damage (Gyorgy et al., 2008).

Disclosure statement

J.W.F is a paid consultant for Acorda Therapeutics Inc., which is involved in the commercial development of chondroitinase. The other authors declare no other competing financial interests.

Acknowledgements

This work was supported by the ERC advanced grant ECM Neuro (294502), by a fellowship to SY from Alzheimer's Research UK (ARUK-RF2016A-1), and by the NIHR Cambridge Biomedical Research Centre. The authors are grateful to Dr Michel Goedert (MRC Laboratory of Molecular Biology, Cambridge UK) for providing the human P301S tau transgenic mice.

Figure legends

Fig. 1. Cat316 antibody bound to CS-A and aggrecan. Representative image of a slot blot demonstrating the binding specificity of Cat316. Cat316 antibody bound preferentially to CS-A (at both 2 and 10 µg/ml) and aggrecan (10 µg/ml).

Fig. 2. Cat316 antibody neutralised inhibition of DRG neurite outgrowth by aggrecan. Dissociated DRG neurons were cultured on laminin or laminin/aggrecan coated coverslips treated with PBS, ChABC or Cat316 antibody. The neurons were then stained with anti-betaIII tubulin antibody to reveal neurites. The length of the longest neurite per neuron was measured (20 neurons per coverslip, 3 coverslips per condition repeated 4 times). Data represents the mean ± S.D., n=4, one-way ANOVA with post-hoc analysis p **< 0.01 and p *** < 0.001.

Fig. 3. Cat316 blocked the development of PNNs in the PNN-HEK cells. Representative images of PNN-HEK cells cultured in the presence of anti-HAPLN1 (C) Cat316 (D) and the anti-aggrecan antibodies 6B4 (E) and 7B7 (F). For controls cells were grown in the absence of antibodies, then treated before staining with PBS (A) or with ChABC (B). The cells were then labelled with aggrecan (red) for PNNs and counterstained with Hoechst (blue). For quantification the optical density of aggrecan staining on the cell surface was measured and normalised to the PBS control (n=4, two-tailed paired t- test, *** p < 0.001) (G). Cell surface CSPGs were also stained with WFA lectin which binds to CS-GAGs which are digested by ChABC (H). All the antibodies reduced PNN formation, with anti-HAPLN1 and Cat316 having the strongest effects. (n=4, two-tailed paired t-test, ***p <0.001). Bar= 50 um.

Fig. 4. Cat316 binds to GAGs isolated from either diffuse ECM or PNNs. GAGs isolated from diffuse buffer-soluble ECM (B1) and the 6M urea soluble fraction enriched in PNN (B4) were biotinylated and immobilized on streptavidin-coated

ELISA plates. Cat316 demonstrated binding to both GAGs (bar 1 and 3). This binding was greatly reduced when the coated GAGs were treated with ChABC for 30 minutes before the Cat316. n=3, Student *t*-test, ***p <0.001.

Fig. 5. Cat316 inhibits binding of Sema3A to PNN GAGs and to PNN-HEK cells. (A) PNN-GAGs extracted from adult rat brain in buffer 4 of the progressive extraction method (Deepa et al., 2006), were biotinylated and adhered to streptavidin-coated ELISA plates. Binding of alkaline phosphatase-tagged Sema3A was measured in the presence and absence of Cat316. n=4, *** p<0.001. (B-D) Cat316 inhibits Sema3A binding to PNN-HEK cells. Sema3A-AP with or without Cat316 were added for 2 h to PNN-HEK cells, then immunostained. Optical density was measured and plotted in (D). n=4, ***p<0.001.

Fig. 6. Cat316 enhances DRG neurite growth on GAGs isolated from PNN-HEK and Sema3A. Dissociated DRG neurons were plated on coverslips first treated with laminin/PDL overnight, then coated with GAGs from PNN-HEK, followed by Sema3A-AP (1:10) for 2 h. Cat316 or control IgM was then added for another 2 h. Adult dissociated DRG neurons were plated on the substrate, and neurite length measured 24 h later. Immunostaining of β3-tubulin (red) was performed and the longest neurite per neuron was measured. (A-B) Representative images of the DRG neurons cultured on the GAGs with or without the presence of Cat316. (C) A graph showing the length of neurites. Cat316 antibody increased the length of the DRG neurites. Results were analyzed using two-tailed student *t*-test. Data presents the mean ± SEM from three experiments (n=3 per condition), * $p < 0.05$. Scale bar, 75 μm.

Fig. 7. Cat316-binding to brain tissue was prevented by ChABC treatment.
The free-floating tissues of mouse brains (retrosplenial cortex) were pre-incubated with or without ChABC at different concentrations (0, 0.01, 0.1 and 1 U/ml). They were stained with either Cat316 or biotin-WFA. (A) The immunostaining of Cat316 antibody without ChABC treatment showed typical structures of perineuronal nets around cell bodies and proximal dendrites as indicated by arrows. The PNNs labeling of Cat316 was gradually reduced with increasing concentrations of ChABC pre-treatment. Immunohistochemistry without Cat316 incubation showed no staining. (B) WFA labelling of PNNs and ECM was absent after ChABC digestion. Scale bar, 50 μm

Fig. 8. Injection of Cat316 into mouse brain. (A) The antibody bound areas were detected in C57BL/6J WT mice at 5, 7 and 10 days post-injection with anti-mouse IgM at three different levels; bregma -1.8 mm, bregma -2.8 mm, bregma -3.8 mm. (B) Cat316 binding on PNNs compared with isotype control IgM after injection to the cortex. Cat316 bound to PNNs at the edge of the injection site (a), and the centre of the injection area (c). Equivalent control IgM injections are in (b) and (d), showing diffuse stain only. (e) At high magnification (40x) typical PNN profiles of Cat316-labelled PNNs were seen. The binding pattern revealed the typical lattice-matrix around neuronal cell bodies and dendrites. Scale bar; 2mm (A); 30 µm (B)

Fig. 9. Staining for Sema3A and WFA was altered after Cat316 treatment *in vivo*. (A) Sema3A staining was measured 4 days post-injection in the Cat316 infused area, outlined in both sections. Representative images were captured at two different sites as boxed in the Cat316 injected area; I and II. (B) Images of the sites I and II in A were captured at 20 x magnification and I' and II' are the counterparts in isotype control IgM injections. The Cat316 injected area showed a reduced area of intense of Sema3A staining of PNNs compared to the sham injection. (C) Quantification of the pixel number per frame of intense Sema3A staining from the thresholded pictures, normalised to the isotype control injection (33 % reduction, n=4, 0.67 ± 0.06, two-tailed paired *t* test, **p*=0.0157). (D) WFA staining was reduced in the Cat316 infused area compared to sham. (E) Quantification of the number of thresholded pixels per frame of WFA staining was normalised to isotype control injection (27% reduction, n=4, 0.73 ± 0.073, two-tailed paired *t* test, * *p*=0.0243). Scale bar; 1mm (A); 30 µm (B, D)

Fig. 10. Cat316 treatment enhances long-term memory in WT control mice
(A) Experimental time line for the OR test after Cat316 or isotype control IgM injection. (B) Three month-old C57BL/6J WT mice were injected with Cat316 antibody or isotype control IgM. At 4 - 5 days post-injection, their long-term object recognition memory was tested in the spontaneous OR paradigm with 24 h delay. Cat316 injected WT mice showed a large enhancement of OR memory compared to controls (Control n=9, Cat316 n=11, two-tailed unpaired t-test, ** *p*=0.0049). Their exploratory behavior in the sample phase did not differ, indicating that motivation was not altered by Cat316 injection. (C) Cat316 treated mice were tested in the OR task at day 8 and day 11 post-injection to demonstrate the time scale of wash out of the effect (Day 8; Control 0.131 ± 0.08, n=8 *vs* Cat316 0.19 ± 0.07, n=10, Day 11; Control -0.006 ± 0.08, n=9 *vs* Cat316 0.033 ± 0.08, n=11). Motivation was not

affected by injection and there was no side preference. Data = mean ± SEM. ** p<0.01

Fig. 11. Cat316 treatment restored the memory deficit in transgenic P301S mice (A) The progression of the memory deficit in OR memory measured at 3 h was age-dependent in P301S mice. No deficit was present in 3 month-old mice compared to C57BL/6J WT mice (WT 0.37 ± 0.12, n=4 *vs* P301S 0.36 ± 0.06, n=5), while 4 and 5 month-old P301S mice showed a spontaneous OR memory deficit in the 3 h retention paradigm. The discrimination ratio is (novel - familiar object exploration time)/total exploration time). The OR memory deficit was significant in 4 and 5 month-old P301S mice compared to control mice (4M: WT 0.34 ± 0.05 n=8 *vs* P301S 0.07 ± 0.03, n=10, two-tailed unpaired t-test, *** p=0.0006, 5M: WT 0.32 ± 0.06 n=7 *vs* P301S 0.039 ± 0.06, n=5, two-tailed unpaired t-test, ** p=0.0049). There was no exploration deficit in sample phase and no side bias. (B) Experimental time line for the OR test after Cat316 or isotype control IgM injection. (C) Four month-old P301S mice were tested in the spontaneous OR task with 3 h delay following isotype control or Cat316 or ChABC treatment to the PRh cortex. The OR deficit exhibited in the P301S mice was restored by Cat316 treatment to the level of normal WT control (WT: Isotype control 0.316 ± 0.04 n=6, P301S: Isotype control 0.047 ± 0.09, n=8; Cat316 0.28 ± 0.04, n=14; ChABC 0.24 ± 0.05, n=6, One way ANOVA, *p=0.042). Long-term memory was measured in 24 h delay of OR paradigm after the same treatment in P301S mice. Data are presented as mean ± SEM.

Fig. 12. Tauopathy after Cat316 treatment in Tg P301S mice.
(A) Hyperphosphorylated tau was visualized by AT8 antibody immunostaining in the PRh of isotype control IgM or Cat316 antibody injected mice at 7 days post-injection. (B) Quantification of AT8-positive tau tangles in the PRh cortex presented no difference (Istotype control *vs* Cat316, unpaired *t*-test, n.s. p=0.925) (C) NeuN-positive cells were stereologically quantified in the PRh cortex of P301S mice. No significant influence of Cat316 treatment on AT8 staining or neuronal cell number was present in the Tg P301S mice. Tg P301S: Isotype control n=6, Cat316 n=6, WT: Isotype control n=4 for NeuN staining (WT: Isotype control *vs* P301S: Isotype control, unpaired *t*-test, **p=0.0056, P301S: Isotype control *vs* P301S: Cat316 n.s. p=0.415). Scale bar; 100 µm

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