



# Systematic and standardized comparison of reported amyloid- $\beta$ receptors for sufficiency, affinity, and Alzheimer's disease relevance

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Oligomeric assemblies of amyloid- $\beta$  (A $\beta$ ) peptide (A $\beta$ o) in the brains of individuals with Alzheimer's disease (AD) are toxic to neuronal synapses. More than a dozen A $\beta$  receptor candidates have been suggested to be responsible for various aspects of the molecular pathology and memory impairment in mouse models of AD. A lack of consistent experimental design among previous studies of different receptor candidates limits evaluation of the relative roles of these candidates, producing some controversy within the field. Here, using cell-based assays with several A $\beta$  species, including A $\beta$ o from AD brains obtained by autopsy, we directly compared the A $\beta$ -binding capacity of multiple receptor candidates while accounting for variation in expression and confirming cell surface expression. In a survey of 15 reported A $\beta$  receptors, only cellular prion protein (PrP<sup>C</sup>), Nogo receptor 1 (NgR1), and leukocyte immunoglobulin-like receptor subfamily B member 2 (LilrB2) exhibited direct binding to synaptotoxic assemblies of synthetic A $\beta$ . Both PrP<sup>C</sup> and NgR1 preferentially bound synaptotoxic oligomers rather than nontoxic monomers, and the method of oligomer preparation did not significantly alter our binding results. Hippocampal neurons lacking both NgR1 and LilrB2 exhibited a partial reduction of A $\beta$ o binding, but this reduction was lower than in neurons lacking PrP<sup>C</sup> under the same conditions. Finally, binding studies with soluble A $\beta$ o from human AD brains revealed a strong affinity for PrP<sup>C</sup>, weak affinity for NgR1, and no detectable affinity for LilrB2. These findings clarify the relative contributions of previously reported A $\beta$  receptors under controlled conditions and highlight the prominence of PrP<sup>C</sup> as an A $\beta$ -binding site.

The pathophysiology responsible for the clinical signs and symptoms of AD<sup>2</sup> has been studied since Alois Alzheimer first

described the characteristic senile plaques and neurofibrillary tangles in the brain of a patient who suffered a deteriorating psychological condition that included sleep disturbances, memory impairment, and confusion (1). Today, AD is the leading cause of dementia worldwide and carries with it a tremendous economic burden projected to exceed 2 trillion United States dollars by the year 2030 (2). Despite more than a century of research, AD remains without a treatment that is capable of curing, preventing, or slowing the progression of disease. The role of senile plaques and neurofibrillary tangles in the etiology of Alzheimer's disease continues to be investigated and contested within the field. In 1998, Lambert *et al.* (3) described the self-assembly of synthetic  $\beta$ -amyloid monomers into soluble, multimeric, nonfibrillary aggregates dubbed A $\beta$ o. These oligomers were potentially neurotoxic and capable of inducing cell death, and they inhibited long-term potentiation in organotypic hippocampal slices. A $\beta$ o are immunologically distinct from monomers or fibrils, induce synapse loss, and are correlated with disease progression (4–8). Similar species of A $\beta$ o were identified in brains from human AD patients in 2003 (9). The observations that synthetic and AD brain-derived A $\beta$ o bound to neurons in a trypsin-sensitive manner gave rise to the search for cell surface receptors capable of binding extracellular A $\beta$ o and transducing their neurotoxic signal intracellularly. More than a dozen proteins have been reported as responsible for mediating the deleterious effects of A $\beta$ o on neurons (10–25) (reviewed in Ref. 26). These studies have been highly disparate in both the quality and nature of evidence used to qualify a candidate as a receptor for A $\beta$  (26). Variation in A $\beta$  preparations, experimental design, and model systems have led to a call

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This article contains Fig. S1.

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<sup>2</sup> The abbreviations used are: AD, Alzheimer's disease; A $\beta$ , amyloid- $\beta$ ; A $\beta$ o, amyloid- $\beta$  oligomer(s); PrP<sup>C</sup>, cellular prion protein; NgR1, Nogo receptor 1;

LilrB2, leukocyte immunoglobulin-like receptor subfamily B member 2; RAGE, advanced glycosylation end product-specific receptor; PGRMC1, membrane-associated progesterone receptor component 1; EphA1, ephrin type-A receptor 1; Fc $\gamma$ R1b, low-affinity immunoglobulin  $\gamma$  Fc region receptor II-b; SorLA, sortilin-related receptor; p75<sup>NTR</sup>, tumor necrosis factor receptor superfamily member 16; NLGN1, neuroligin 1; EphB2, ephrin type-B receptor 2; FZD5, frizzled-5; mGluR5, metabotropic glutamate receptor 5; EphA4, ephrin type-A receptor 4; CRMP2, collapsin response mediator protein 2; NRP1, neuropilin-1; BA $\beta$ , biotin amyloid- $\beta$ ; BA $\beta$ o, BA $\beta$  oligomers; A $\beta$ g, amyloid- $\beta$  globulomer(s); A $\beta$ m, amyloid- $\beta$  monomer(s); Pirb, paired immunoglobulin-like receptor b; nArchR $\alpha$ 7, neuronal acetylcholine receptor subunit  $\alpha$ -7; GluN2B, glutamate receptor ionotropic NMDA 2B; hPrP<sup>C</sup>, human PrP<sup>C</sup>; SEC, size-exclusion chromatography; HMW, high-molecular weight; RIPA, Radioimmunoprecipitation assay; TBS, Tris-buffered saline; HFIP, 1,1,1,3,3,3-hexafluoroisopropanol.

for a sharing of materials and validation of results between laboratories (26–28).

To address these discrepancies and better understand the relative contributions of each putative receptor to A $\beta$  neurotoxicity, we compared the potential of each receptor to confer A $\beta$  binding capacity to heterologous cells and neurons, the ability of each candidate to discriminate between nontoxic monomers and toxic oligomers, and the effect of different oligomer preparations on the binding profile. To determine whether synthetic preparations of A $\beta$  faithfully recapitulate the binding profile of A $\beta$  found in the brains of patients with AD, we also compared the ability of candidate receptors to bind soluble A $\beta$  extracted from the brains of patients diagnosed with AD. These insights are critical to clarifying the roles of these receptors in AD pathogenesis and their therapeutic value to drug development. Preventing the interaction of neurotoxic A $\beta$  with its receptors is an attractive drug target, and clinical trials targeting advanced glycosylation end product–specific receptor (RAGE), membrane-associated progesterone receptor component 1 (PGRMC1), and tumor necrosis factor receptor superfamily member 16 (p75<sup>NTR</sup>) are under way (NCT00141661, NCT00566397, NCT02916056, NCT02080364, NCT03522129, NCT03507790, and NCT03069014) (29–32).

## Results

### PrP<sup>C</sup>, LILRB2, and NgR1 bind oligomeric A $\beta$

Few descriptions of candidate receptors for A $\beta$  have included a demonstration of sufficiency for conferring A $\beta$  binding to live cells. To examine this attribute, we compiled a panel of putative receptors for A $\beta$  and subcloned the cDNA of each into expression vectors encoding a Myc epitope at the cytoplasmic terminus of transmembrane proteins or at the mature N terminus of glycosylphosphatidylinositol-anchored proteins, which included PrP<sup>C</sup> and NgR1. These orientations were selected so as to leave the extracellular A $\beta$ -binding domains undisturbed. The panel investigated here includes PrP<sup>C</sup>, LILRB2, NgR1, ephrin type-A receptor 1 (EphA1), low-affinity immunoglobulin  $\gamma$  Fc region receptor II-b (Fc $\gamma$ RIIb), sortilin-related receptor (SorLA), sortilin, p75<sup>NTR</sup>, PGRMC1, neuroligin 1 (NLGN1), RAGE, ephrin type-B receptor 2 (EphB2), frizzled-5 (FZD5), metabotropic glutamate receptor 5 (mGluR5), and ephrin type-A receptor 4 (EphA4) (10–25). We also included neuropilin-1 (NRP1) as a negative control (Fig. S1). To our knowledge, this protein has not been implicated in binding A $\beta$ .

We assessed the expression of each protein at the extracellular surface by cell surface biotinylation. Anti-Myc immunoprecipitation and immunoblotting confirmed that each protein was trafficked to the cell surface and of the expected molecular size (Fig. 1A). Furthermore, similar amounts of each receptor protein reached the plasma membrane and were subject to biotinylation by extracellular reagent. The Myc-tagged cytoplasmic protein collapsin response mediator protein 2 (CRMP2) was included as a control and demonstrated that only proteins localized to the cell surface were biotinylated.

For heterologous cell A $\beta$ -binding experiments, we chose COS-7 cells, as they exhibit minimal basal A $\beta$  binding. By

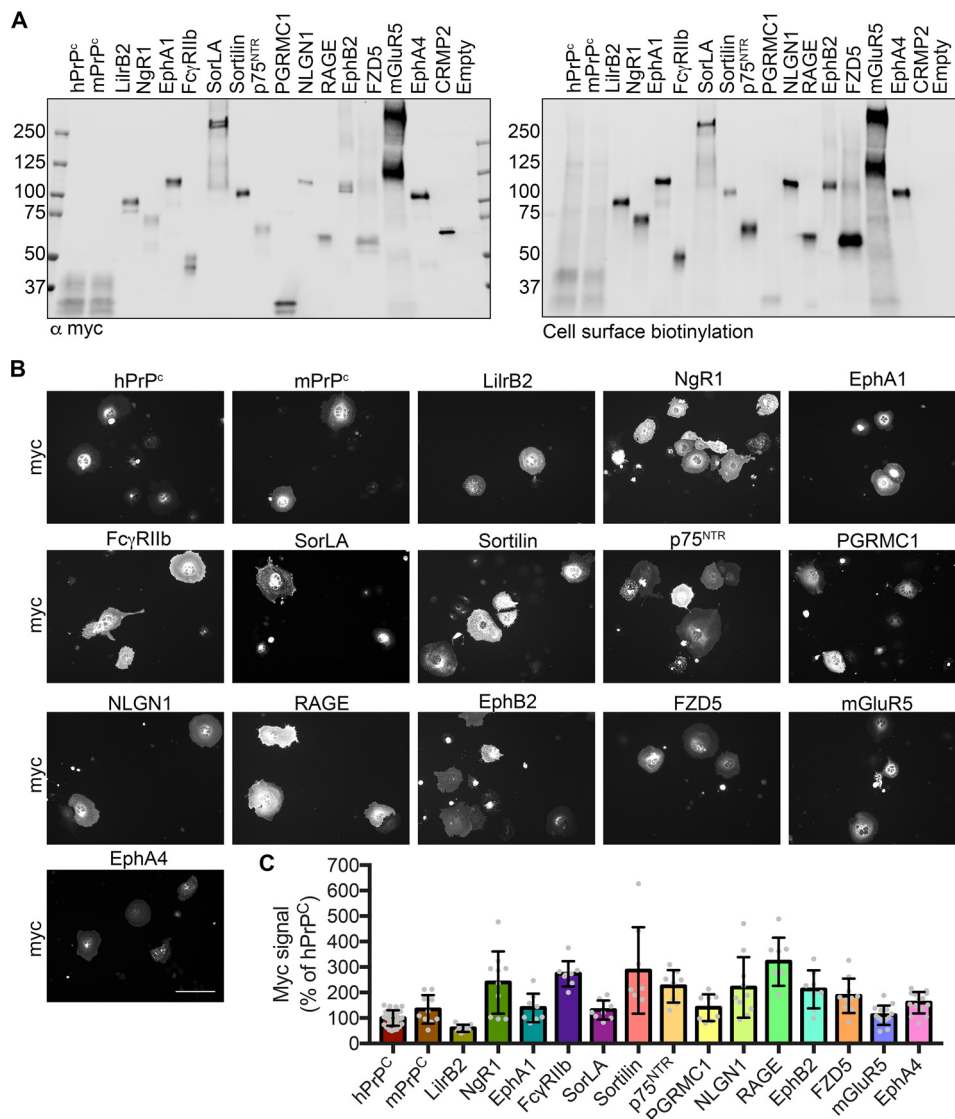
staining transfected cells with an anti-Myc antibody and quantifying the average intensity, we found that receptor expression was similar across the panel (Fig. 1, B and C). Utilizing a common epitope tag for expression normalization across the panel is critical to making direct comparisons of A $\beta$  binding. This allows us to account for any expression differences between constructs and experiments, without introducing variability from differences in the avidity of receptor-specific antibodies or potential interference with binding in the case of receptors whose A $\beta$  binding domains have not been identified.

The concentration of oligomeric A $\beta$  in AD patient brains has been reported to be  $\sim$ 2.6 nM monomer equivalent (7). To examine the ability of candidate receptors to bind neurotoxic oligomers of A $\beta$ , transfected cells were incubated with 1  $\mu$ M monomer equivalent ( $\sim$ 5 nM oligomer) biotinylated A $\beta$  oligomers (BA $\beta$ ) at 4  $^{\circ}$ C to allow binding but minimize receptor internalization following binding. A high concentration was utilized so as to allow detection of A $\beta$  binding even to low-affinity receptors. Following fixation and staining, the receptor expression–normalized A $\beta$  binding was calculated and normalized to that of human PrP<sup>C</sup> (hPrP<sup>C</sup>). Fig. 2 (A and B) shows that PrP<sup>C</sup>, LILRB2, and NgR1 exhibit A $\beta$  binding capacity. Among these, PrP<sup>C</sup> binds A $\beta$  more than either LILRB2 or NgR1 (Fig. 2B). None of the other receptors demonstrated detectable A $\beta$  binding with a lower limit of detection equal to 3% of hPrP<sup>C</sup> binding.

### PrP<sup>C</sup> and NgR1 preferentially bind synaptotoxic A $\beta$ species

An important characteristic of a receptor capable of transducing pathological signaling in response to A $\beta$  is an ability to discriminate between neurotoxic oligomers and nontoxic monomeric A $\beta$ . A $\beta$  monomers spontaneously and rapidly assemble into high-molecular weight species in physiological buffers. Samples incubated overnight yield preparations enriched for oligomeric assemblies, which can be resolved using size-exclusion chromatography (SEC) (Fig. 3A). Analysis of the relative abundance of oligomeric A $\beta$  in different preparations demonstrates that A $\beta$  freshly prepared in Ham's F12 medium is 7% high-molecular weight (HMW) A $\beta$ . Oligomeric preparations are 21% A $\beta$ . Oligomerization can be inhibited by the use of nonphysiological buffers. When A $\beta$  is dissolved in 0.1 N NaOH, only 3% of the preparation is HMW A $\beta$  (Fig. 3B). Incubating transfected cells with freshly prepared A $\beta$  in F12 is intended to provide a context in which monomeric A $\beta$  is more readily available for binding; however, the preparation cannot be considered purely monomeric. Fig. 3C shows the expression-normalized monomeric A $\beta$  signal for each receptor compared with oligomeric A $\beta$  binding to hPrP<sup>C</sup>. None of the receptors bind this monomeric preparation of A $\beta$  to a great extent (Fig. 3C). By comparing oligomeric and monomeric binding, we examined each receptor's ability to discriminate A $\beta$  species. PrP<sup>C</sup> and NgR1 demonstrate an ability to distinguish between oligomeric A $\beta$  and monomeric A $\beta$ . LILRB2 binds oligomeric and monomeric A $\beta$  to similar extents (Fig. 3D).

## Standardized comparison of reported A $\beta$ receptors



**Figure 1. Myc-tagged candidates are expressed at similar levels and traffic to the cell surface.** *A*, immunoblots of RIPA-soluble cell lysates following cell surface biotinylation and immunoprecipitation using anti-Myc-agarose beads. Candidate receptors were immunoblotted with an anti-Myc antibody (*left*) and fluorescently conjugated streptavidin (*right*). Empty, empty vector control. *B*, images of COS-7 cells transfected with the indicated candidate receptor and stained with anti-Myc antibody. Scale bar, 200  $\mu$ m. *C*, quantification of *B*. All samples are normalized to hPrP<sup>C</sup> Myc signal.  $n = 32$  experiments for hPrP<sup>C</sup> and 8–14 for all others. Individual data points indicate different experiments. Error bars, S.D.

### Neither temperature nor A $\beta$ oligomerization method alters the binding profile of the receptor panel

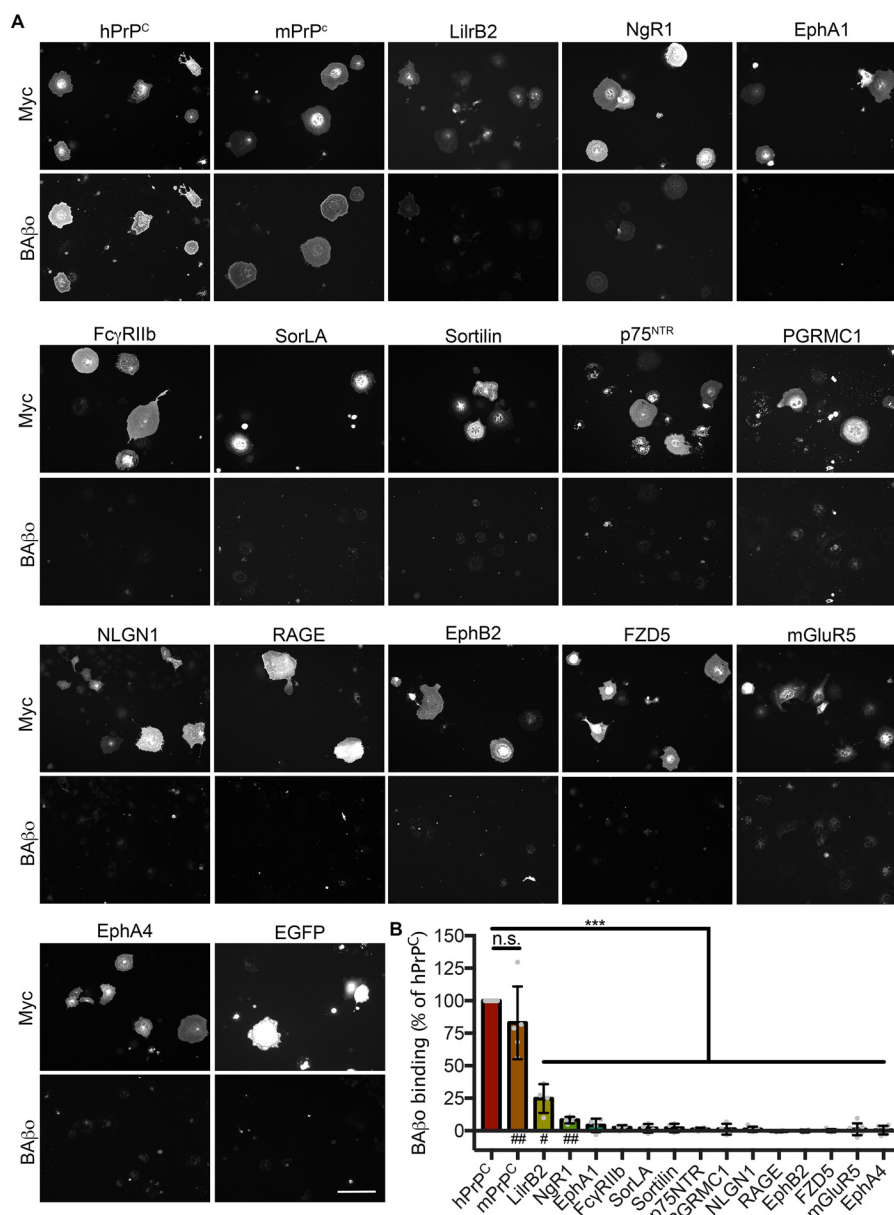
Conducting binding assays at 4 °C affords convenience but decreases the fluidity of the cell membrane and impacts the thermodynamics of receptor–ligand interactions. To determine whether temperature influences the binding profile of the receptor panel, we incubated transfected cells with 1  $\mu$ M monomer-equivalent BA $\beta$  at 37 °C. As with the 4 °C incubation, only PrP<sup>C</sup>, LirB2, and Ngr1 bind BA $\beta$  at 37 °C (Fig. 4A). In comparing binding at both temperatures, we found no effect of temperature on BA $\beta$  binding (Fig. 4B).

Multiple methods for the generation of oligomeric A $\beta$  assemblies from monomeric synthetic peptides have been reported (10, 33, 34). Differences in preparation lead to unique profiles with regard to the types and relative abundance of multimeric A $\beta$  assemblies as assessed under native conditions using SEC. Oligomers prepared in F12 culture medium, incu-

bated overnight, and separated from insoluble fibrillary species by centrifugation generate larger species of oligomers with masses in the range of 100 kDa and larger (7, 10). To account for differences in oligomeric species generated, we utilized a second preparation of soluble multimeric A $\beta$  termed globulomers (A $\beta$ g) that generates primarily a 60-kDa species by SEC (33). Atomic force microscopy confirms differences in sizes of soluble oligomers generated by the different preparations (Fig. 4C). Incubation of transfected cells with A $\beta$ g demonstrates a binding profile similar to that observed with the oligomeric preparations (Fig. 4D). When comparing binding of BA $\beta$  and A $\beta$ g to the binding-competent receptors, there is no effect of preparation on the binding (Fig. 4E).

### NgR1 and LirB2 are minor contributors to A $\beta$ binding to neurons

Our survey of putative receptors has revealed that of those reported, only PrP<sup>C</sup>, LirB2, and Ngr1 are sufficient for binding



**Figure 2. Only PrP<sup>C</sup>, LILRB2, and NgR1 confer A $\beta$ -binding capacity to cells.** A, COS-7 cells expressing the Myc-tagged candidate receptor of interest and incubated with 1  $\mu$ M biotin A $\beta$  at 4  $^{\circ}$ C. Scale bar, 200  $\mu$ m. B, quantification of biotin A $\beta$  binding to cells in A, normalized to candidate receptor Myc signal. For each experiment, Myc-normalized biotin A $\beta$  binding was expressed as a percentage of hPrP<sup>C</sup> in that experiment. *n.s.*, not significant; \*\*\*,  $p < 0.001$ , one-sided  $t$  test comparing with an expected value of 100 (% hPrP<sup>C</sup> binding). Significance of a one-sided  $t$  test comparing with an expected value of 0 (no binding) is denoted by a number symbol below the x axis. #,  $p < 0.05$ ; ##,  $p < 0.01$ .  $n = 4$ –7 experiments. Individual data points indicate different experiments. Error bars, S.D.

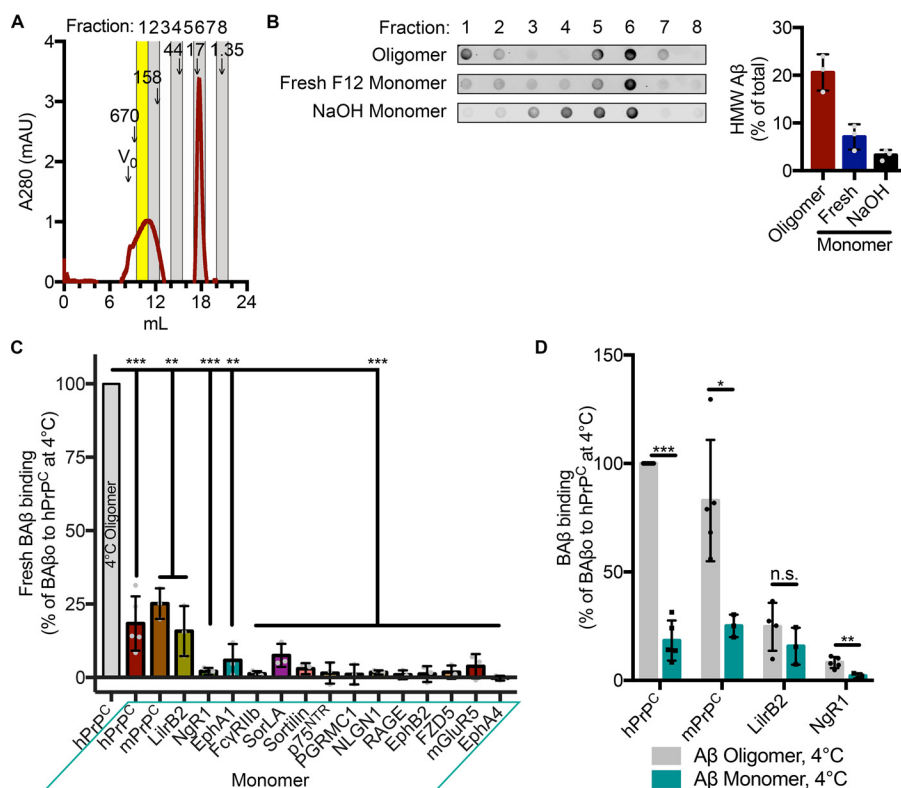
to neurotoxic preparations of synthetic A $\beta$ . PrP<sup>C</sup> has been demonstrated to be responsible for 50% of A $\beta$  binding to hippocampal neurons (10). To examine the contribution of the two additional A $\beta$  receptors confirmed here (11, 12), we examined the role of NgR1 and the murine homolog of LILRB2, Pirb, in double-knockout neurons under the same conditions used for PrP<sup>C</sup> (10). Loss of NgR1 and Pirb results in a 20% decrease in BA $\beta$ o binding to double-knockout neurons (Fig. 5, A and B).

#### PrP<sup>C</sup> is the highest-affinity receptor for A $\beta$

To better understand the relative affinities of the positive receptors, we implemented plate-based assays of A $\beta$  binding to purified protein. This assay is more sensitive and quantitative than the cell-based assays and allows for measurement of bind-

ing kinetics. We again utilized oligomeric, globulomeric, and monomeric A $\beta$ . Fig. 6 (A–F) shows the binding curves and Scatchard plots for A $\beta$ o, A $\beta$ g, and A $\beta$ m, respectively. As measured in the cell-based assays, hPrP<sup>C</sup>, LILRB2, and NgR1 bound neurotoxic oligomers and globulomers (Fig. 6). The plate assay faithfully recapitulated effects of A $\beta$  preparations on binding profiles. We observed highly similar binding of A $\beta$ o and A $\beta$ g to hPrP<sup>C</sup> and NgR1 (Fig. 6, G and H) and a preference of LILRB2 for A $\beta$ o over A $\beta$ g (Fig. 6I) as was also observed in Fig. 4E. Binding of A $\beta$ m to the receptors was minimal as was observed in the cell-based assay (Fig. 6, E and F). The dissociation constants and  $B_{\max}$  extracted from Fig. 6 are reported in Table 1. PrP<sup>C</sup> is the highest-affinity receptor for neurotoxic A $\beta$ o and A $\beta$ g, with affinities of 1.4 and 1.5 nM, respectively. The affinity of NgR1 for

## Standardized comparison of reported A $\beta$ receptors



**Figure 3. PrPC and NgR1 preferentially bind neurotoxic A $\beta$ .** *A*, SEC trace of 50  $\mu$ M BA $\beta$ o showing absorbance at 280 nm. Alternate vertical columns indicate fractions collected for analysis. Yellow fraction, HMW A $\beta$ . Elution times of various standards (kDa) are indicated by arrows.  $V_o$ , void volume as determined by blue dextran, 2,000 kDa. *B*, detection and quantification of the effect of the preparative method on the distribution of BA $\beta$  in different SEC fractions by dot blot analysis. *C*, quantification of expression-normalized A $\beta$  signal from COS-7 cells transfected with the indicated candidate receptor and incubated with 1  $\mu$ M monomeric biotin A $\beta$  at 4  $^{\circ}$ C. Values are normalized to oligomeric biotin A $\beta$  binding to hPrPC. One-sided *t* test comparing with an expected value of 100. *n* = 3–5 experiments. *D*, binding of receptors to monomeric A $\beta$  compared with A $\beta$ o from Fig. 2*B*. Shown are multiple *t* tests with Holm–Sidak correction for multiple comparisons. Individual data points indicate different experiments. Error bars, S.D. *n.s.*, not significant; \*, *p* < 0.05; \*\*, *p* < 0.01; \*\*\*, *p* < 0.001.

these preparations was ~3–4-fold lower than that of hPrPC. LilrB2 demonstrated the lowest affinity, with  $K_D$  of 42.3 and 207.2 nM. To quantify each receptor's ability to discriminate between neurotoxic and nontoxic preparations of A $\beta$ , we calculated a discrimination factor by dividing each receptor's  $K_D$  for A $\beta$ m by that of the indicated species. hPrPC was highly discriminate in its preference for oligomeric and globulomeric A $\beta$  with discrimination factors of 68.5 and 63.9, respectively. NgR1 also discriminated in binding, although to a lesser degree than hPrPC. LilrB2 exhibited a modest preference for A $\beta$ o compared with monomers and bound A $\beta$ g with the same affinity as for A $\beta$ m (Table 1).

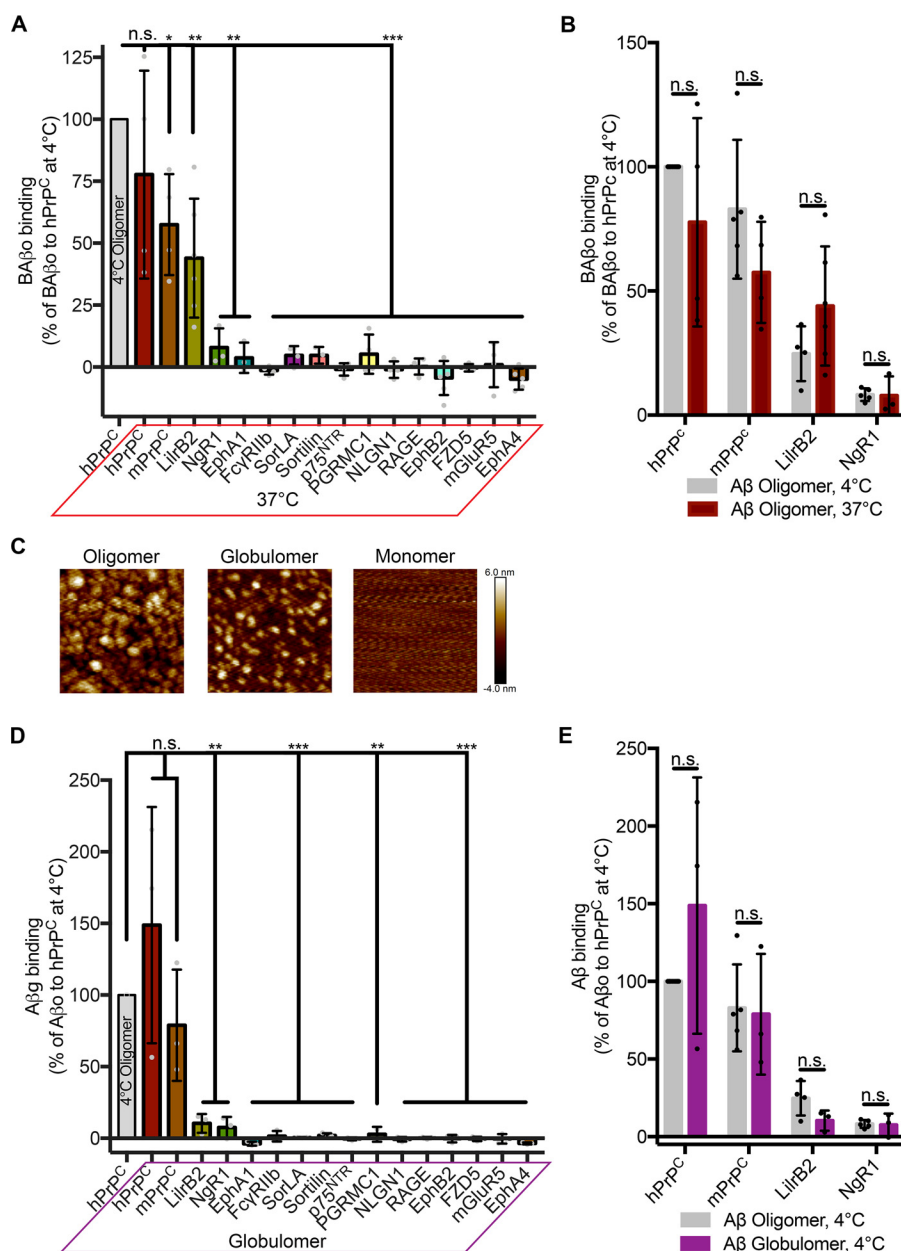
### PrPC and NgR1 bind A $\beta$ present in the brains of patients with AD

Despite the ability of preparations of synthetic A $\beta$  to generate neurotoxic species with masses similar to those found in the brains of patients with AD and to cause neurotoxicity *in vitro*, *ex vivo*, and *in vivo*, it is critical to examine each receptor's disease relevance using pathological A $\beta$  present in disease (10, 33, 35–37). Having validated the concordance of our plate-based assay with the observations made in living cells, we quantified binding of A $\beta$  present in TBS-soluble extracts of brains from 10 patients with autopsy-confirmed AD and compared it with signal from brains of 11 cognitively normal patients. The mean anti-A $\beta$  signal from wells coated with hPrPC was highest

and significantly greater than that from cognitively normal patients. LilrB2 exhibited no binding to A $\beta$  present in brains of patients with AD and no difference between AD and control brains. NgR1 modestly bound A $\beta$  present in AD patient brains, and this signal was significantly greater than that in brains from cognitively normal patients (Fig. 7).

### Discussion

Here, a panel of putative A $\beta$  receptors was expressed on the surface of nonneuronal cells and examined for sufficiency to mediate A $\beta$  binding. By accounting for differences in receptor expression using a common epitope tag across the receptors, we made direct comparisons of A $\beta$  affinity. Importantly, binding took place in living cells rather than cell-free systems. This allows for potential binding events to take place at the cell surface and in a physiological context with respect to the lipid and protein environment. Our experiments demonstrate that most of the reported receptors for A $\beta$  are not sufficient to confer A $\beta$  binding capacity to cells using three different preparations of synthetic A $\beta$ . It is possible that those receptors that failed to bind A $\beta$  in our experiments require a coreceptor that is not present in COS-7 cells. Whereas our cell surface biotinylation experiments demonstrate successful transport through the secretory system, which is typically associated with proper protein folding, they do not definitively exclude misfolding that could impact ligand binding. Whereas these negative proteins

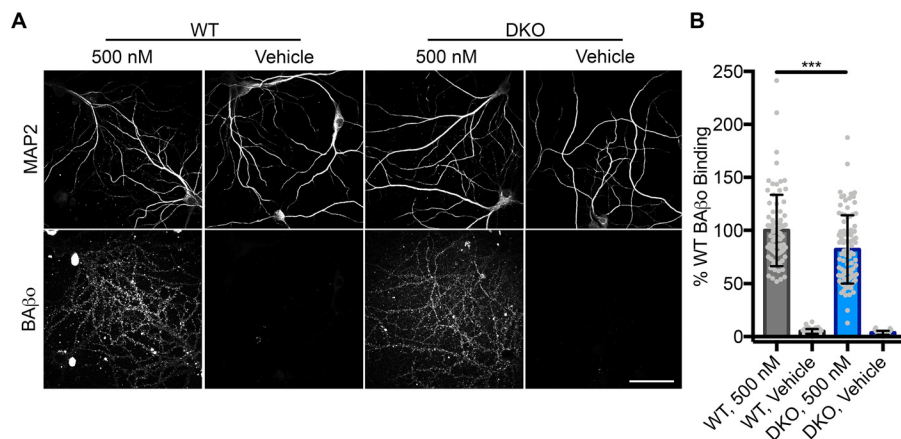


**Figure 4. Neither temperature nor oligomer preparation changes the binding profile of candidate receptors.** *A*, quantification of 1  $\mu$ M biotin A $\beta$ o binding to cells expressing candidate receptors at 37°C. Binding is normalized to that of hPrP $^C$  at 4°C. One-sided *t* test was used, comparing with an expected value of 100 (% hPrP $^C$  binding at 4°C). *n* = 3–6 experiments. *B*, comparison of the capacity of a candidate receptor to bind biotin A $\beta$ o at 4°C (Fig. 2*B*) versus 37°C. Shown are multiple *t* tests with Holm–Sidak correction for multiple comparisons. *C*, atomic force microscopy images showing differences in A $\beta$  generated by different preparations. Images are 200  $\times$  200 nm. *D*, Myc-normalized binding of 1  $\mu$ M A $\beta$  prepared using the globulomer protocol. Data are expressed relative to oligomerized A $\beta$  binding to hPrP $^C$ . One-sided *t* test was used, comparing with an expected value of 100 (% hPrP $^C$  binding); *n* = 3 experiments, 2 for PGRMC1. *E*, comparison of positive receptors' binding of oligomer (Fig. 2*B*) and globulomer A $\beta$ . Shown are multiple *t* tests with Holm–Sidak correction for multiple comparisons. Individual data points indicate different experiments. Error bars, S.D. n.s., not significant; \*, *p* < 0.05; \*\*, *p* < 0.01; \*\*\*, *p* < 0.001.

may have A $\beta$  affinity under some experimental conditions, in a cellular context, their binding affinity is clearly much less than that of the three positive receptors. By including an analysis of binding to monomeric A $\beta$ , we revealed that PrP $^C$  and NgR1 discriminate between pathological and nontoxic forms of A $\beta$ . It should be noted that neither oligomeric nor monomeric preparations are exclusive to a single species but that these designations are meant to reflect the assemblies for which the preparation is enriched. It is possible that the reduced but persistent binding of monomer preparations to PrP $^C$  and NgR1 may reflect the decrease in the abundance of A $\beta$ o. It is clear, how-

ever, that upon enrichment for monomeric A $\beta$ , none of the candidates that failed to bind A $\beta$ o showed an increase in binding. We also found that LiltrB2 does not differentiate in binding these oligomeric and monomeric A $\beta$  preparations. The promiscuity of LiltrB2 may indicate that an epitope present in both species of synthetic A $\beta$  mediates this interaction. Recently, Cao *et al.* demonstrated that LiltrB2 can bind minimal peptides comprising A $\beta$  amino acids 1–21, 15–35, and a tandem repeat of 16–21 without a specific oligomerization procedure (38). In contrast, PrP $^C$  has been shown to undergo structural changes specifically in the presence of oligomeric A $\beta$ g (39).

## Standardized comparison of reported A $\beta$ receptors



**Figure 5. Loss of NgR1 and the mouse LiltrB2 homolog Pirb decreases A $\beta$  binding to neurons.** A, images showing punctate biotin A $\beta$  binding to dendrites of hippocampal neurons. Scale bar, 50  $\mu$ m. B, quantification of mean A $\beta$  signal in A.  $n = 43$ –94 images from 5–7 dishes in four experiments. Unpaired  $t$  test was used. Error bars, S.D. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

Neuronal acetylcholine receptor subunit  $\alpha$ -7 (nAChR $\alpha$ 7) and NMDA receptor subunits 1 and 2B have also been reported to bind A $\beta$  (40–42). We sought to include these in our investigation; however, efficient transport to the cell surface as determined by cell surface biotinylation experiments was insufficient to make comparisons with the rest of the panel. Despite these data, and because we have observed that even very low expression levels of the high-affinity receptor PrP<sup>C</sup> can result in robust A $\beta$  binding, nArchR $\alpha$ 7 with and without the chaperone RIC-3 (resistance to inhibitors of cholinesterase 3) and GluN2B with and without GluN1 (glutamate receptor ionotropic NMDA 1) were included in our cell-binding experiments. No detectable A $\beta$ -binding signal was observed in nArchR $\alpha$ 7 or GluN2B experiments (data not shown).

Whereas the ability to bind A $\beta$  is a crucial characteristic for a receptor, we found it important to interrogate the necessity of confirmed receptors for A $\beta$  binding to neurons. Pirb is the mouse homolog of Liltrb2 and was shown to bind A $\beta$  (11). By generating double-knockout mice deficient in NgR1 and Pirb, we found that up to 20% of binding of synthetic A $\beta$  to neurons is mediated by these two receptors.

Preparations of synthetic A $\beta$  peptide provide a convenient source of neurotoxic A $\beta$  with species representative of those found in the brains of AD patients. When investigating the relevance of a potential receptor to human disease, the gold standard should be an ability to bind A $\beta$  present in the brains of patients diagnosed with AD. We first validated our plate-based assay by replicating the experiments done in mammalian cells and found the two to be in close agreement. We determined that hPrP<sup>C</sup> is the highest-affinity receptor for A $\beta$  ( $K_D = 1.4$ ), followed by NgR1 ( $K_D = 3.9$ ) and LiltrB2 ( $K_D = 42.3$ ). We then utilized this assay for the detection of binding to soluble A $\beta$  found in extracts of brains from patients diagnosed with AD. We found that hPrP<sup>C</sup> and NgR1 bound A $\beta$  present in AD patient brains and that hPrP<sup>C</sup> did so to the greatest extent. Finally, we found that despite binding synthetic A $\beta$  from multiple preparations and in cell-based and purified protein assays, LiltrB2 did not detectably bind the A $\beta$  species present in AD patient brains.

The experiments described here provide much needed clarity to the field regarding the nature of the interaction of neuro-

toxic A $\beta$  in the extracellular space with the cell surface. The observation made here that NgR1 and LiltrB2 account for up to 20% of A $\beta$  binding to hippocampal neurons, coupled with previous observations that PrP<sup>C</sup> is responsible for 50% of A $\beta$  binding to neurons and the knowledge that A $\beta$  binds neurons in a trypsin-sensitive manner, suggests that at least one additional receptor for A $\beta$  remains to be identified (3, 10). Future characterizations of novel A $\beta$  receptors should include experiments testing the necessity and sufficiency for cellular A $\beta$  binding. It will also be necessary to examine the disease relevance of observations made with synthetic peptides and transgenic animals by using materials obtained from patients with AD.

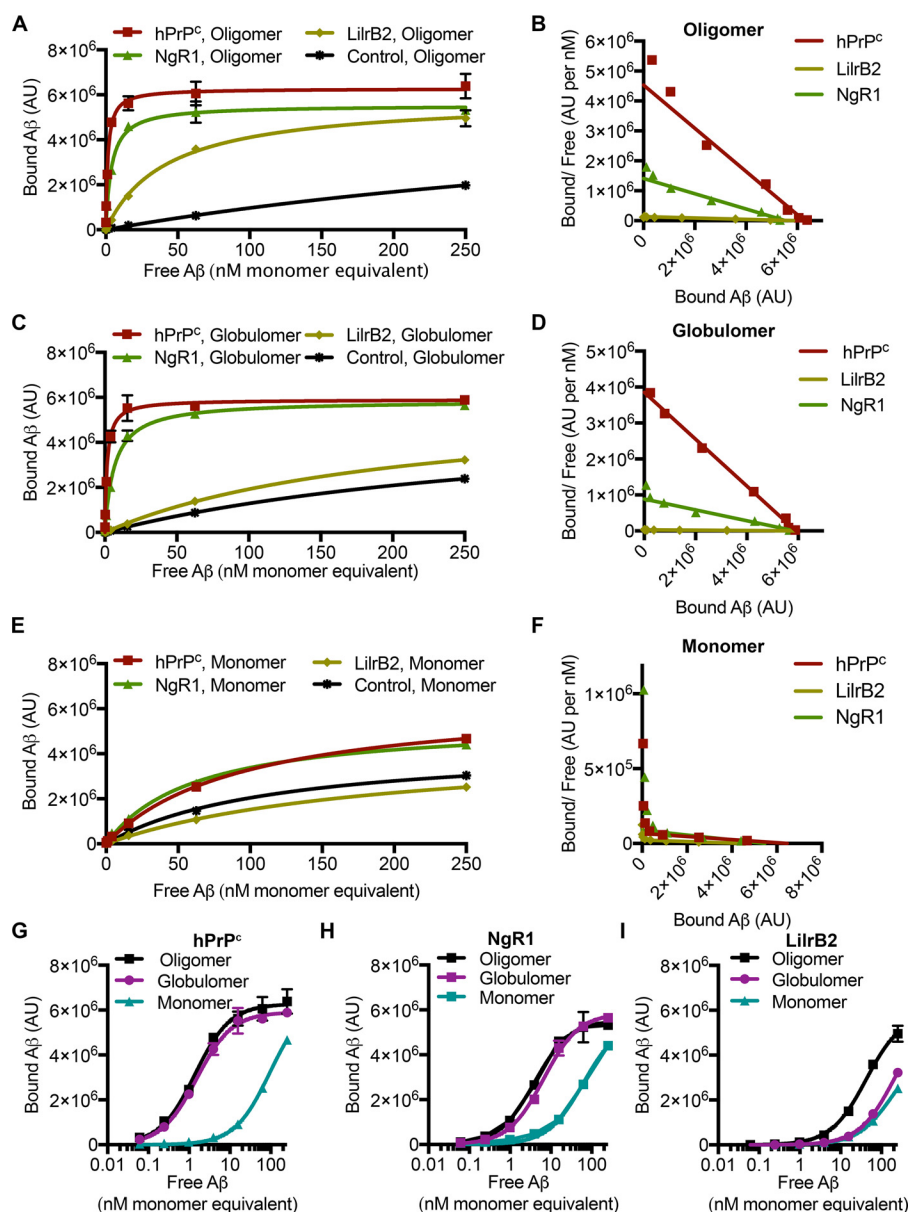
### Experimental procedures

#### Tissue culture

COS-7 or HEK293T cells were grown at 37 °C with 5% CO<sub>2</sub> on 100-mm tissue culture dishes (Fisher, 08-772E) in Dulbecco's modified Eagle's medium (Gibco, 11965) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Gibco, 15140). For A $\beta$ -binding experiments, COS-7 cells were seeded into 8-well chamber slides (Lab-Tek, 154941) and grown under the same conditions. Transfections were performed using Lipofectamine 3000 (Thermo Fisher Scientific, L3000015) diluted in Opti-MEM (Thermo Fisher Scientific, 31985).

#### Plasmids and cloning

cDNA encoding proteins of interest were obtained from Genecopoeia, Dharmacon, and Origene. cDNAs were subcloned into Myc-tagged expression vectors pcDNA3.1(+)/myc-His A (Thermo, V80020) or pSecTag2A (Thermo, V90020). Accession numbers for cDNA sequences are as follows: hPrP (NM\_000311.3), mPrP (NM\_011170.3), LiltrB2 (XM\_006726139.1), NgR1 (NM\_023004.5), EphA1 (BC\_130291.1), Fc $\gamma$ RIIb (BC031992.1), SorLA (NM\_003105.5), Sortilin (NM\_002959.5), p75<sup>NTR</sup> (NM\_002507.3), PGRMC1 (NM\_006667.4), NLGN1 (NM\_014932.3), RAGE (AB036432.1), EphB2 (BC146296.1), FZD5 (BC172518.1), mGluR5 (NM\_017012.1), EphA4 (BC026327.1), CRMP2 (NM\_001386). The construct encoding NRP1 has been described previously (43). Constructs were verified by DNA sequencing.



**Figure 6. hPrP<sup>c</sup>, NgR1-, and LiltrB2-coated plates recapitulate the cell-based assay.** A, binding of A $\beta$  to wells coated with hPrP<sup>c</sup>, NgR1, LiltrB2, or Fc control protein. B, Scatchard plot of data in A. C, binding of A $\beta$  globulomers to wells coated with hPrP<sup>c</sup>, NgR1, LiltrB2, or Fc control protein. D, Scatchard plot of data in C. E, binding of A $\beta$  monomers to wells coated with hPrP<sup>c</sup>, NgR1, LiltrB2, or Fc control protein. F, Scatchard plot of data in E. G, comparison of the binding of A $\beta$ , A $\beta$ g, and A $\beta$ m to hPrP<sup>c</sup>. H, comparison of the binding of A $\beta$ , A $\beta$ g, and A $\beta$ m to NgR1. I, comparison of the binding of A $\beta$ , A $\beta$ g, and A $\beta$ m to LiltrB2. AU, arbitrary units. Error bars, S.D.

### Cell surface biotinylation and immunoprecipitation

Dishes of transfected HEK293T cells were washed three times with ice-cold PBS, pH 8, and biotinylated with 10 ml of 0.48 mg/ml Sulfo-NHS-LC-LC-Biotin (Thermo Fisher 21338) in PBS, pH 8, and incubated at room temperature for 30 min. Cells were washed once with 50 mM Tris, pH 8, and then twice with ice-cold PBS, pH 8. Cells were harvested in 1 ml of ice-cold PBS and gently scraped to collect. Collected cells were transferred to a new tube and centrifuged at  $500 \times g$  for 3 min at 4 °C to pellet them. The cell pellet was resuspended in 1 ml of RIPA lysis buffer (Millipore 20-188) with PhosSTOP (Roche Diagnostics, 04906837001) and Complete Mini protease inhibitors (Roche Diagnostics, 11836170001) and sonicated. After sonication, samples were centrifuged at  $100,000 \times g$ , 4 °C for 30 min.

Supernatants were transferred to c-Myc beads (Pierce 20169) and incubated at 4 °C with endo-over-end mixing for 1 h. Beads were washed three times with RIPA buffer. Proteins were eluted with  $4 \times$  Laemmli sample buffer (Bio-Rad, 161-0747) and analyzed by immunoblotting using anti-Myc (Cell Signaling, catalog no. 2276, 1:1,000), donkey anti-mouse IRDye 680LT (LI-COR Biosciences, 926-68022, 1:20,000), and streptavidin IRDye 800CW (LI-COR Biosciences, 926-32230, 1:20,000) and imaged with the Odyssey infrared imaging system (LI-COR Biosciences).

### Synthetic A $\beta$ preparations

Lyophilized synthetic  $\beta$ -amyloid (residues 1–42) was purchased from The ERI Amyloid Laboratory, LLC. Vials of pep-

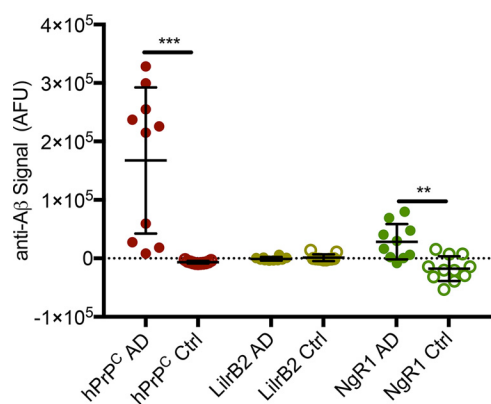


## Standardized comparison of reported A $\beta$ receptors

**Table 1**  
Binding affinities for positive receptor candidates and preparations of synthetic A $\beta$

The  $K_D$  and  $B_{max}$  of each of the receptors and the indicated A $\beta$  preparation are shown. Concentrations are expressed as monomer equivalents. Discrimination factor is the quotient of a receptor's  $K_D$  for monomeric A $\beta$  divided by that of the indicated preparation. Greater values indicate a preference for binding to neurotoxic preparations. AU, arbitrary units.

Parameter	A $\beta$ o	A $\beta$ g	A $\beta$ m
<b>PrP<sup>C</sup></b>			
$K_D$ (nM)	1.4 $\pm$ 0.2	1.5 $\pm$ 0.2	95.9 $\pm$ 6
$B_{max}$ (AU)	6.27 $\times$ 10 <sup>6</sup>	5.9 $\times$ 10 <sup>6</sup>	6.46 $\times$ 10 <sup>6</sup>
Discrimination factor	68.5	63.9	
<b>LilrB2</b>			
$K_D$ (nM)	42.3 $\pm$ 4.4	207.2 $\pm$ 18.2	192.4 $\pm$ 12
$B_{max}$ (AU)	5.84 $\times$ 10 <sup>6</sup>	5.9 $\times$ 10 <sup>6</sup>	4.45 $\times$ 10 <sup>6</sup>
Discrimination factor	4.5	0.9	
<b>NgR1</b>			
$K_D$ (nM)	3.9 $\pm$ 0.4	6.6 $\pm$ 0.5	61.6 $\pm$ 5.3
$B_{max}$ (AU)	5.53 $\times$ 10 <sup>6</sup>	5.85 $\times$ 10 <sup>6</sup>	5.46 $\times$ 10 <sup>6</sup>
Discrimination factor	15.8	9.3	



**Figure 7. hPrP<sup>C</sup> and NgR1 bind soluble A $\beta$  present in brains of AD patients.** Shown is binding of A $\beta$  in TBS-soluble fraction of brains of AD patients or control patients (*Ctrl*) to wells coated with hPrP<sup>C</sup>, NgR1, or LilrB2. Each dot represents an individual patient brain. Shown are multiple *t* tests with Holm-Sidak correction for multiple comparisons. Error bars, S.D. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . AFU, arbitrary fluorescence unit.

tide were reconstituted in 1,1,1,3,3,3-hexafluoroisopropanol at 10 mg/ml, boiled at 70 °C for 1 h, aliquoted into microcentrifuge tubes at 0.5 mg/tube, and allowed to dry overnight at room temperature. The next day, the aliquots were further dehydrated in a SpeedVac for 1 h. Oligomers were prepared by dissolving an aliquot of A $\beta$  in 40  $\mu$ l of DMSO, separating into two aliquots, diluting in 1 ml of F12 medium (Atlanta Biologicals, M15350) to 55  $\mu$ M, and incubating at room temperature overnight to allow oligomerization. The next day, samples were centrifuged in a tabletop centrifuge (Eppendorf 5430) at 20,817  $\times$  *g* for 15 min to precipitate fibrillary protein. The supernatant was treated as 55  $\mu$ M and diluted to working concentrations in F12 medium. Monomeric A $\beta$  was prepared by diluting the DMSO-dissolved peptide in F12 to working concentration as quickly as possible. Globulomer A $\beta$  was generated according to the previously published protocol (33). HFIP-processed synthetic A $\beta$  peptide films were resuspended in DMSO at 5 mM (22  $\mu$ l of DMSO, 0.5 mg of A $\beta$ ). Then 250  $\mu$ l of 1 $\times$  PBS, pH 7.4, was added to each tube, immediately followed by 31  $\mu$ l of 2% SDS. The resulting mix was incubated in the sealed tubes at 37 °C for

4–6 h and then diluted 4-fold with deionized water (resulting in 1.2 ml/tube final volume) and incubated at 37 °C overnight to yield mature globulomer A $\beta$ . The preparations were then centrifuged for 10 min at 10,000  $\times$  *g*, and the supernatants were concentrated using 30-kDa cutoff Amicon filters. Concentrated A $\beta$ g (1 mM A $\beta$  monomer) were then dialyzed against a 1000-fold excess of PBS diluted 4-fold with water (0.25  $\times$  PBS, pH 7.4) using 100  $\mu$ l of 20-kDa cutoff Slide-A-Lyzer mini cassettes. Dialysis buffer was changed three times over a course of 36 h. Dialyzed A $\beta$ g was then centrifuged at 100,000  $\times$  *g* for 1 h at 4 °C, and supernatants were aliquoted and flash-frozen in liquid nitrogen. Typically, 70% of initial A $\beta$  (dried HFIP film) was converted into A $\beta$ g as determined by absorbance at 280 nm using a ProtParam-derived extinction coefficient (1490 M<sup>-1</sup> cm<sup>-1</sup>). For atomic force microscopy and SEC, NaOH monomer samples were generated by dissolving HFIP-treated A $\beta$  in 440  $\mu$ l of 0.1 M NaOH, resulting in a 250  $\mu$ M stock, and processed for atomic force microscopy or SEC immediately.

### Immunocytochemistry and heterologous cell-binding assays

COS-7 cells were cultured in 8-well chamber slides as described above. For A $\beta$  binding experiments, the culture medium was removed from wells, leaving a volume sufficient to cover the cells, and washed with F12, and the indicated A $\beta$  preparation was added. Slides were then incubated for 2 h at the indicated study temperature. Wells were washed three times with PBS and fixed with 3.7% formaldehyde (J.T. Baker, 2106) in PBS with 6% sucrose (AmericanBio, AB01900) for 30 min at room temperature. The fixative was then removed, and cells were permeabilized by incubation in 0.2% Triton X-100 (Fisher Scientific, 42235-5000) in PBS for 30 min. After permeabilization, cells were blocked by incubation with 5% normal donkey serum (Jackson ImmunoResearch, 017-000-121) for 1 h at room temperature. After blocking, cells were incubated with the indicated primary antibodies for anti-Myc (Cell Signaling, catalog no. 2276, 1:5,000) or anti-A $\beta$  (Cell Signaling, catalog no. 8243, 1:1,000), diluted in 1% BSA (Sigma, catalog no. A9647) at 4 °C overnight. The next day, cells were washed three times with PBS with incubation periods of 0, 3, and 5 min. The indicated appropriate secondary antibodies diluted in 1% BSA in PBS (donkey anti-mouse Alexa Fluor 488, Life Technologies, catalog no. A21202, 1:500; donkey anti-rabbit Alexa Fluor 568, Life Technologies, catalog no. A10042, 1:500; Streptavidin Alexa Fluor 568, Life Technologies, S-11226, 1:500) were then added to wells and incubated for 1 h at room temperature. Wells were then washed four times with 0.02% Triton X-100 in PBS with incubation times of 0, 3, 5, and 15 min. After washing, slides were mounted using Vectashield with 4',6-diamidino-2-phenylindole (Vector Laboratories, H-1200) and imaged on an Axio Imager M2. CellProfiler was used for quantification of A $\beta$  binding to transfected cells (44). 4',6-Diamidino-2-phenylindole and anti-Myc signal were used for the identification of transfected cells, and the intensity of A $\beta$  and Myc inside of cell areas as well as background were measured in A $\beta$ -treated and nontreated wells. Background signal was subtracted from cellular signal, and from that value, mean A $\beta$  signal in cells transfected with cytoplasmic enhanced GFP was subtracted. The transfection-specific, background-subtracted A $\beta$  signal was

normalized by background-subtracted Myc signal and expressed as a percentage of that signal in hPrP<sup>C</sup>-transfected cells, which were included in every experiment for comparison. Limit of detection was calculated as the mean background-subtracted A $\beta$  signal in cells transfected with cytoplasmic enhanced GFP, expressed as a percentage of that in hPrP-transfected cells, plus two S.D. values.

### Size-exclusion chromatography

SEC was performed on the described preparations of biotinylated A $\beta$  using a Superdex 200 Increase 10/300 GL column (GE Healthcare, 28990944) and Akta Pure 25 M1 (GE Healthcare) with an F9-C fraction collector. SEC was performed at 4 °C. 1.5-ml fractions were collected in 2-ml deep 96-well plates (USA Scientific, 1896-2110). For the representative trace, 50  $\mu$ M BA $\beta$ o in Ham's F12 medium was loaded into a 500- $\mu$ l sample loop. For dot blot analysis, 1  $\mu$ M samples were loaded. Samples prepared in Ham's F12 were run using Ham's F12 as the mobile phase. NaOH monomer samples were run in a mobile phase of 0.25 $\times$  PBS, pH 7.4. Gel filtration standards used were blue dextran (Sigma, D4772), thyroglobulin, bovine  $\gamma$ -globulin, chicken ovalbumin, equine myoglobin, and vitamin B12 (Bio-Rad, 1511901). Fractions were concentrated using centrifugal filters with a 3,000 nominal molecular weight limit (Amicon UFC500324). Dot blot analysis was performed by applying 80% of concentrate volume to a nitrocellulose membrane using a Bio-Rad Bio-Dot apparatus. Samples were incubated on the membrane for 4 h at room temperature, washed twice with TBS, blocked for 1 h at room temperature with Rockland blocking buffer for fluorescent Western blotting (Rockland, MB070010TF), and probed overnight at 4 °C with streptavidin IRDye 800CW (LI-COR 926-32230, 1:1,000)

### Atomic force microscopy

AFM samples were prepared by placing 10  $\mu$ l of 0.25 mg/ml A $\beta$  preparations on freshly cleaved mica and allowing it to adsorb for 2 min. The sample was washed twice with 200  $\mu$ l of Milli-Q water and dried carefully. Images were taken in a Bruker Dimension Fastscan AFM in a tapping mode using silicon nitride cantilevers.

### Mouse breeding and care

Mice were cared for by the Yale Animal Resource Center, and all experiments were approved by Yale's institutional animal care and use committee. Ngr<sup>-/-</sup> (Rtn4r) and Pirb<sup>-/-</sup> mice have been described previously and were maintained on a C57/Bl6 background (45, 46). Pirb<sup>-/-</sup> mice were a generous gift of Dr. Toshiyuki Takai. Ngr<sup>-/-</sup> and Pirb<sup>-/-</sup> were bred together to generate double-heterozygous and eventually double-knockout mice.

### Neuron cultures and neuronal binding assays

Mouse hippocampal neurons from WT and RTN4R<sup>-/-</sup>, Pirb<sup>-/-</sup> double knockout animals were isolated from embryonic day 17 to postnatal day 0 pups and cultured on MatTek dishes (P35G-1.5-14-C) coated with poly-D-lysine (MP Bio, 0215017580) in Neurobasal medium (Gibco, 10888-022) supplemented with B-27 (Gibco, 17504-044), sodium pyruvate

(Gibco, 11360-070), and Glutamax (Gibco, 35050-061), with 1% penicillin and streptomycin (Gibco, 15140). Binding experiments were performed at 18–20 days *in vitro*. After washing, neurons were incubated with 500 nM monomer-equivalent (~2.5 nM oligomer) biotinylated A $\beta$  for 1 h at 4 °C. Following fixation, permeabilization, and blocking, dendrites were visualized using anti-MAP2 (1:2,000, Millipore, AB5622) and Alexa Fluor 488 donkey anti-rabbit IgG (1:1,000, Life Technologies, Inc., A21206), and biotinylated A $\beta$  was visualized with streptavidin Alexa Fluor 568 (1:500, Life Technologies, S11226). z-Stacks were collected at  $\times$ 40 using a spinning disc confocal microscope. z-Stacks were then max-projected using National Institutes of Health ImageJ (47). Quantification was performed using CellProfiler (44). Integrated punctate A $\beta$  signal was calculated after background subtraction and thresholding. Integrated signal was then normalized to the area positive for MAP2. When representing these data graphically, each data point represents a single region of interest from 5–7 dishes. The experiment was repeated four times.

### Plate-based assay

384-well MaxiSorp plates (Thermo, 460372) were coated overnight with 20  $\mu$ l/well of the indicated protein at 250 nM in 100 mM BupH carbonate-bicarbonate coating buffer (Thermo 28382) at 4 °C. Plates were washed twice with PBST (PBS, 0.05% Tween 20) and blocked with 25  $\mu$ l of Protein-Free T20 PBS Blocking buffer (Pierce 37573) for 4 h at room temperature. After washing three times with PBST, 20  $\mu$ l of either TBS fraction of human brain samples or synthetic A $\beta$  was applied to wells and incubated overnight at 4 °C. A $\beta$  binding was detected using D54D2 anti-A $\beta$  antibody (Cell Signaling, catalog no. 8243, 1:2,000) in PBSTB (PBS, 0.05% Tween 20, 0.5% BSA) for 2 h. After four washes with PBST, 20  $\mu$ l of Eu-N1 goat anti-rabbit IgG (PerkinElmer Life Sciences, AD105, 1:4,000) was diluted in DELFIA assay buffer (PerkinElmer Life Sciences) and incubated for 1 h at room temperature. After four washes with PBST, 20  $\mu$ l of DELFIA Enhancement Solution (PerkinElmer Life Sciences) was applied, and time-resolved europium fluorescence was measured with a Victor 3V plate reader (PerkinElmer Life Sciences).

Recombinant hexahistidine-tagged hPrP<sup>C</sup> was generated using a method described previously (7). The extracellular domain of LirB2 is encoded by amino acids 22–461. We subcloned cDNA encoding this region into pSecTag2A (Invitrogen) to include a C-terminal hexahistidine tag. HEK293T cells were transfected with this ecto-LirB2 construct, and 24 h later, the medium was replaced with serum-free medium. The next day, the conditioned medium was collected and purified using the same method described for hPrP<sup>C</sup>. Human Ngr-Fc decoy protein encodes amino acid residues 1–310 of human Ngr1 with the C266A and C309A substitutions fused to the Fc domain of human IgG1. Ngr(310)-Fc protein was produced as described previously and supplied by ReNetX Bio (48).

### Human brain samples

Post-mortem human tissue was collected in accordance with institutional review board protocols approved by Yale University. Samples of brain tissue were microscopically analyzed to

## Standardized comparison of reported A $\beta$ receptors

**Table 2**

**Characteristics of human brain tissue samples**

Patient characteristics for human brain tissue used are described. Values are  $\pm$  S.D.

	Sample size	Age	Sex (% female)	Post-mortem interval
		years	%	days
AD	10	78 $\pm$ 12	50	13 $\pm$ 9
Control	11	71 $\pm$ 14	64	20 $\pm$ 7

confirm the clinical diagnosis of AD (Braak Stage V or higher). Samples from neurologically healthy controls were required to have no or minimal histopathological signs of AD (Braak 0–II). Frozen prefrontal cortex was stored at  $-80^{\circ}\text{C}$  until used. Human brain was weighed and Dounce-homogenized in 3 volumes of TBS, pH 7.4 (Bio-Rad, catalog no. 170-6435) supplemented with Complete Mini Protease Inhibitors (Roche Diagnostics, 11836170001) and PhosStop phosphatase inhibitors (Roche Diagnostics, 04906837001). Samples were centrifuged at  $100,000 \times g$  at  $4^{\circ}\text{C}$  for 1 h, and the supernatant was collected, flash-frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  until assayed. The supernatant was referred to as the TBS-soluble fraction. Patient demographics for brains used are described in Table 2.

### Statistics

All results are presented as mean  $\pm$  S.D. unless otherwise stated. Prism version 7 was used for statistical analysis, and specific tests used are indicated in the figure legends.

**Author contributions**—L. M. S. and S. M. S. conceptualization; L. M. S. data curation; L. M. S. formal analysis; L. M. S., M. A. K., and S. L. investigation; L. M. S., M. A. K., and S. L. methodology; L. M. S. writing-original draft; L. M. S., M. A. K., S. L., and S. M. S. writing-review and editing; S. M. S. supervision; S. M. S. funding acquisition; S. M. S. project administration.

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