

Published in final edited form as:

Neuron. 2014 April 16; 82(2): 308–319. doi:10.1016/j.neuron.2014.02.027.

Soluble A β oligomers are rapidly sequestered from brain ISF *in vivo* and bind GM1 ganglioside on cellular membranes

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SUMMARY

Soluble A β oligomers contribute importantly to synaptotoxicity in Alzheimer's disease, but their dynamics *in vivo* remain unclear. Here, we found that soluble A β oligomers were sequestered from brain interstitial fluid onto brain membranes much more rapidly than non-toxic monomers and were recovered in part as bound to GM1 ganglioside on membranes. A β oligomers bound strongly to GM1 ganglioside, and blocking the sialic acid residue on GM1 decreased oligomer-mediated LTP impairment in mouse hippocampal slices. In a hAPP transgenic mouse model, substantial levels of GM1-bound A β ₄₂ were recovered from brain membrane fractions. We also detected GM1-bound A β in human CSF, and its levels correlated with A β ₄₂, suggesting its potential as a biomarker of A β -related membrane dysfunction. Together, these findings highlight a novel mechanism whereby hydrophobic A β oligomers become sequestered onto GM1 ganglioside and presumably other lipids on neuronal membranes, where they may induce progressive functional and structural changes.

INTRODUCTION

The most prevalent neurodegenerative disorder, Alzheimer's disease (AD) impairs episodic declarative memory and executive function early in its clinical phase. Enhanced synapse loss, particularly in the temporal and frontal cortices, helps distinguish AD from normal brain aging (West et al., 1994) and serves as a strong correlate of cognitive decline (Terry et al., 1991). Research in many laboratories has provided extensive evidence that AD is initially a disorder of selected synapses in which soluble, low molecular weight (LMW)

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Competing Interests Statement. DJS is a consultant to Elan and a director of Prothena.

oligomers of amyloid β -protein ($A\beta$) can act as prime synaptotoxic agents (reviewed in Mucke and Selkoe, 2012). In one example, mice expressing an APP mutation that causes AD in humans underwent rapid formation and stabilization of $A\beta$ oligomers accompanied by profound synaptic and neuronal loss in the absence of fibrillar amyloid plaques in the cortex (Tomiya et al., 2010). In light of numerous such studies implicating a pathogenic role of soluble $A\beta$ oligomers and the evidence that lowered $A\beta_{42}$ monomer levels in CSF represents the earliest biomarker for AD (Bateman et al., 2012; Craig-Schapiro et al., 2009; Fagan et al., 2009; Golde et al., 2011; Morris and Selkoe, 2011), the search for such oligomers in biological fluids, primarily in human cerebrospinal fluid (CSF), has intensified in recent years (Benilova et al., 2012). The recent report that a Phase 3 trial of the $A\beta$ -specific monoclonal antibody solanezumab produced a small but significant cognitive benefit in patients with mild AD (Doody, 2012) has made it even more critical to understand the earliest changes in the economy of synaptotoxic $A\beta$ oligomers in the brain and biological fluids. A few reports of the detection of apparent $A\beta$ oligomers in CSF and plasma have appeared (Fukumoto et al., 2010; Gao et al., 2010; Klyubin et al., 2008; Villemagne et al., 2010); however, the interpretation of these reports has been clouded by failure to define the precise oligomeric unit the assays are detecting, an inability to exclude definitively the detection of $A\beta$ monomers, and in some cases, the lack of validating the assays on natural oligomers in biological samples.

In this context, we have systematically examined the steady-state levels of LMW $A\beta$ oligomers in aqueous compartments of the central nervous system (CNS), i.e., the brain interstitial fluid (ISF) and CSF of a well-characterized mouse model of AD, the J20 hAPP transgenic (tg) line (Mucke et al., 2000). While we readily detected monomers, we failed to detect LMW (<35 kDa) $A\beta$ oligomers in the CNS fluid compartment using several biochemical methods. We then hypothesized that due to their increased hydrophobicity, $A\beta$ oligomers may bind to cell membranes, pre-existing $A\beta$ aggregates (in plaque-containing brains) or other hydrophobic surfaces much more rapidly than monomers do. If so, this could help clarify potential mechanisms behind observations that $A\beta$ oligomers, but not monomers, can exert synaptotoxicity (Gong et al., 2003; Klyubin et al., 2005; Lacor et al., 2004; Lambert et al., 1998; Lesné et al., 2006; Shankar et al., 2007; 2008; Walsh et al., 2002), alter tau and other cytoskeletal proteins (Gotz et al., 2001; Jin et al., 2011; Lewis et al., 2001; Oddo et al., 2004; Zempel et al., 2010), and induce other cytotoxic effects. To address this concept, we quantified the *in vivo* half-life of microinjected $A\beta$ dimers vs. monomers in the ISF of healthy, wild-type (wt) mice using microdialysis and ISF injections of synthetic $A\beta$ dimers and natural oligomers isolated from human (AD) brain tissue. We followed the fate of the microinjected $A\beta$ and found that most of it became tightly bound to endogenous GM1 ganglioside on membrane extracts of the brains. *In vitro* binding assays indicated that $A\beta$ dimers bind to GM1 ganglioside much more strongly than monomers. Masking the sialic acid residue on GM1 decreased $A\beta$ oligomer-mediated LTP inhibition in wt mouse hippocampal slices. Next, we examined brain membrane extracts of J20 tg mice prior to plaque formation and recovered a significantly higher level of endogenous $A\beta_{42}$ than $A\beta_{40}$ complexed with GM1. The levels of GM1- $A\beta$ complexes obtained from brain membrane extracts were inversely proportional to the levels of free ISF $A\beta$ with age, suggesting that the formation of the GM1- $A\beta$ complexes is a dynamic equilibrium. Finally,

using a sensitive immunoprecipitation (IP) technique, we discovered low levels of GM1-bound A β directly in human CSF, and its levels correlated significantly with the CSF levels of A β ₄₂. The implications of these new findings for understanding the dynamic economy of soluble A β oligomers in the brain are discussed.

RESULTS

Steady-state levels of LMW A β oligomers in CNS fluids are very low or undetectable

We initially assessed the levels of soluble A β oligomers in the hippocampal ISF and CSF. We define A β oligomers here as soluble LMW oligomers (dimers, trimers, tetramers, etc.) that remain in the supernatant after high speed ultracentrifugation, as opposed to high molecular weight, prefibrillar A β aggregates that can often be pelleted by ultracentrifugation. To obtain hippocampal ISF, we performed *in vivo* microdialysis in awake, behaving J20 tg mice using microdialysis rates (0.2-0.4 μ l/min) low enough to allow ready recovery of soluble A β (Cirrito et al., 2003; Hong et al., 2011). CSF was collected from the cisterna magna of anesthetized J20 mice (DeMattos et al., 2002), a method which, in contrast to microdialysis, does not limit the recovery of soluble molecules according to their size. Anti-A β immunoprecipitates of hippocampal ISF and CSF yielded the 4 kDa monomer and a 5 kDa A β -immunoreactive band migrating just above it, but no SDS-stable A β dimers (which run at 6.5 kDa in these gels) (Figures 1A and S1A). This apparent lack of A β oligomers in both tg ISF and CSF was not due to an inability of our two polyclonal A β antisera (AW8 (Hong et al., 2011) or R1282 (Podlisny et al., 1998)) to immunoprecipitate oligomers, as each effectively pulled down both the endogenous A β dimers present in Tris-buffered saline (TBS) extracts of 20 mo tg mouse brains and the synthetic wt A β and disulfide-crosslinked (A β ₁₋₄₀ S26C)₂ dimers (Shankar et al., 2008) (Figures 1A, S1A and S1B).

Next, we used a recently developed A β oligomer-ELISA (o-ELISA) that specifically recognizes natural A β oligomers of a wide range of sizes (dimers up to prefibrillar soluble oligomers), but not A β monomers, and detects small amounts (sensitivity \approx 6 pg/ml) of both synthetic and natural A β oligomers isolated from AD cortex (Yang et al., 2013). However, the o-ELISA failed to detect any A β oligomer signal in 3 mo and 20 mo tg ISF or 3 mo tg CSF (Figure 1B). Using a conventional ELISA that recognizes all A β _{1-x} species including monomers, we confirmed that the tg mouse ISF and CSF indeed contained ample amounts of A β (Figure S1C). We excluded the possibility that molecules in ISF or CSF interfere with the o-ELISA technique, as spiking these CNS fluids with low amounts of pure S26C A β dimers gave positive signals in the expected range (Figure 1B). We next eluted ISF of 3 mo and 20 mo tg mice off a Superdex 75 size exclusion chromatography (SEC) column and subjected the resultant SEC fractions to a triplex ELISA that can detect low-femtogram amounts of endogenous A β ₃₈, A β ₄₀ and A β ₄₂ (detection antibody: 6E10). Whereas SEC fractions #14-16 in which A β monomers elute (i.e., the \sim 4 kDa fractions) gave abundant signals for all three A β peptides, fractions #12 and 13 in which dimers elute (i.e., the \sim 8 kDa fractions) (Figure S1D) yielded no detectable signal above background from ISF at either age (Figures 1C and D). We confirmed that the A β triplex ELISA is capable of detecting SEC fractions of synthetic dimers and monomers to commensurate degrees (Figures S1D and

S1E). Collectively, these results from multiple sensitive biochemical assays – IP/WB, o-ELISA, and SEC followed by ultrasensitive A β triplex ELISA – suggest that steady-state levels of LMW A β oligomers in brain ISF or CSF of J20 tg mice are very low and thus below the detection limits of the methods used here (Figure S1C).

A β oligomers are rapidly sequestered away from hippocampal ISF *in vivo*

In light of this failure to detect significant amounts of LMW oligomers in brain ISF and CSF, we asked whether the half-life of A β oligomers in ISF is much shorter than that of monomers. We acutely administered exogenous A β dimers or monomers into hippocampal ISF of behaving wt mice using a cannula attached to the microdialysis probe. To distinguish the acutely administered A β from endogenous A β , we injected A β of human sequence into ISF of wt mice and quantified the recovery of injected human A β in ISF microdialysates using A β _{1-x} ELISA, which recognizes human but not mouse A β . As our monomer source, we used wt A β ₁₋₄₀ monomers, and as the A β dimer-rich source, we used the S26C dimer, which was almost all dimeric and stayed as such throughout the time of our *in vivo* microdialysis (Figures S2A and S2B). We first empirically determined the concentrations of monomers and dimers that would allow equal percentages of these species to flow across the microdialysis membrane *in vitro*; the concentrations turned out to be 8 nM for monomers and 40 nM for dimers (see Supplemental Experimental Procedures; Figure S2C). In stark contrast to these *in vitro* test-tube experiments where the recoveries of A β dimers and monomers were equivalent (Figure 2A), the *in vivo* recovery of the injected dimers in the ISF dialysate was <10% of that of the injected monomers (Figure 2B). This large difference in the relative recoveries of A β dimers vs. monomers between *in vitro* (102 \pm 11.4%) and *in vivo* (8.9 \pm 2.8%) microdialysis was highly significant (p<0.0001) (Figure 2C). Importantly, the disappearance of the injected A β dimers from wt ISF occurred rapidly (Figure 2D). The low recovery of injected A β dimers vs. monomers in the ISF was not due to a general limit on *in vivo* diffusion, nor did we obtain evidence of acute perturbations of several non-A β analytes in the respective microdialysates (Figures S2D-F). The rapid disappearance of A β oligomers from brain ISF was confirmed using microinjection of a second source of synthetic A β dimers, namely, non-crosslinked wt dimers (Figures S2H and S2I).

A β dimers injected into hippocampal ISF *in vivo* are principally recovered in the membrane fraction as monomers bound to GM1 ganglioside

To follow the fate of A β oligomers released into ISF, we injected A β dimers or monomers in low, endogenous amounts (320 pg) into the hippocampal ISF of wt mice and immediately (<1 min) sacrificed the mice for brain fractionation. The cytosolic/extracellular (TBS) and membrane-associated (TBS with 1% Triton-X100 (TBS-Tx)) fractions were promptly immunoprecipitated for human A β . A much higher percentage of the injected A β was immunoprecipitated from brain membrane fractions of the dimer-injected mice than those of the monomer-injected mice (Figures 2E and 2F). Surface biotinylation experiments in intact primary hippocampal neurons demonstrated the surface retention of A β dimers, but not monomers, on the neuronal plasma membrane; this surface binding occurred prior to lysing the biotinylated cells (Figure S3A). The latter finding, together with the rapid sequestration of A β dimers away from ISF and to the brain membrane fraction (Figure 2), suggests that A β

dimers bind cellular membranes more avidly than monomers do, and may explain their very low or undetectable levels in CNS fluids (ISF and CSF) at steady state.

In contrast to the *in vitro* microdialysis experiments where A β dimers remained stable as dimers for 24–36 h (Figure S2B), the A β S26C dimers we injected into ISF *in vivo* were recovered as reduced monomers in both the ISF microdialysates (1 h post injection) (Figure S2G) and the brain TBS and TBS-Tx fractions (Figures 2E and 3A: see S26C 320 pg *in vivo*), except when a much higher concentration of A β S26C dimers was injected (Figure 3A: see S26C 320 ng *in vivo*). The conversion of the disulfide crosslinked synthetic dimers to monomers when injected *in vivo* could be explained by the brain providing a reducing environment. We therefore decided to isolate natural A β dimers directly from TBS cortical extracts of typical AD brains using SEC (Shankar et al., 2008) and injected these human A β dimers into the ISF of wt mice. Even this largely dimeric material was recovered by IP/WB from the brain homogenates as 4- and 5-kDa A β monomers (Figure 3A: see huAD TBS dimers *in vivo*). Almost all of the injected A β dimers (whether synthetic or from human brain) were recovered in the TBS- and TBS-Tx-soluble extracts (Fig. 3A), and none was recovered in the TBS-Tx-insoluble pellets (data not shown); the latter suggests that the injected dimers did not accumulate into large insoluble aggregates during the short time course of our experiment.

Intriguingly, whereas the injected A β immunoprecipitated from TBS extract ran at the size of monomers (4 kDa), the injected A β immunoprecipitated from TBS-Tx extract ran slightly higher as a 5-kDa species (Figure 3A: see S26C 320 pg *in vivo*, and huAD TBS dimers *in vivo*). Based on a prior report of a GM1 ganglioside-bound form of human A β monomer occurring as a 5-kDa species in diffuse plaque-rich cortex of early AD or Down syndrome (DS) cases (Yanagisawa et al., 1995), we probed the microinjected, immunoprecipitated A β species with the β -subunit of cholera toxin (CT β), which specifically binds GM1 ganglioside (van Heyningen, 1974). The immunoprecipitated 5-kDa A β species recovered from the TBS-Tx extract was reactive with CT β , indicating that the A β in the membrane fraction was bound to GM1 ganglioside (Figure 3B: 1st and 5th panels). High-resolution imaging of the hippocampal CA1 region of such wt mouse that received *in vivo* A β injection demonstrated that most of the retained A β was co-localized with endogenous GM1 ganglioside (Figure S3B). This result on brain sections suggests that the association of GM1 with A β occurs *in vivo*, that is, it can be seen without tissue homogenization. Likewise, the above-mentioned surface biotinylation of A β on intact neurons (Figure S3A) supports this conclusion. Importantly, we failed to recover the 5 kDa A β species when we performed the acute A β injections into mice lacking gangliotetraose gangliosides (i.e., with a disrupted gene for GM2/GD2 synthase (Sheikh et al., 1999)), thus providing confirmatory evidence that the 5 kDa A β species is GM1 ganglioside-bound A β (Figure S3C). Interestingly, we did not observe a 5 kDa GM1-bound A β species when we spiked the A β S26C dimers *ex vivo* into postmortem wt mouse brain homogenates (Figure 3: see 320 pg *ex vivo*), suggesting that the binding of A β to endogenous GM1 on membranes required the intact environment of the living brain. Furthermore, when we injected a high level of A β S26C *in vivo*, we recovered from the TBS-Tx fraction the A β S26C as intact dimers bound to GM1, namely, a ~10 kDa CT β -positive A β species (Figures 3A and B: see 320 ng *in vivo*).

In light of the reported co-localization of PrP^c to GM1 ganglioside on membranes (Sanghera et al., 2008; Théry et al., 2009) and recent reports that PrP^c acts as a key binding receptor for A β oligomers (Chen et al., 2010; Laurén et al., 2009), we asked whether PrP^c is required for the observed binding of the acutely injected A β to endogenous GM1 ganglioside. After similar acute injection of a high amount (320 ng) of A β S26C dimer into the hippocampus of *PrnP*^{-/-} (null) mice, the animals were sacrificed immediately for brain homogenization. We recovered A β S26C as bound to endogenous GM1 in *PrnP* null mice (Figure S3D) in a manner indistinguishable from the injection results in wt mice (Figure 3: 320 ng *in vivo*). Therefore, PrP^c is not required for the binding of A β to GM1 ganglioside on brain membranes.

A β oligomers preferentially bind to GM1 ganglioside

We next performed isothermal titration calorimetry (ITC), measuring A β binding to or incorporation into lipid membranes by detection of concomitant heat release (Seelig, 2004). To use ITC to quantitatively assess membrane binding *in vitro*, we titrated suspensions of small unilamellar vesicles composed of POPC/GM1 (9:1) into freshly prepared A β S26C dimer solutions and measured the observed heat release via ITC. Analysis of the partition equilibrium revealed an apparent binding constant K_p of the A β S26C interaction with GM1 of $\sim 10000 \text{ M}^{-1}$ per lipid molecule bound (Figure 4A). As a control for the specificity of the interaction of A β S26C with GM1, we repeated the ITC experiments titrating suspensions of either pure POPC or POPC/SM (9:1) into A β S26C dimer solutions. These experiments yielded no detectable heat release during the titration, indicating a lack of interaction between A β S26C and POPC alone or POPC plus sphingomyelin (Figure 4A). Similar negative results were obtained upon titrating POPC/GM1 into solutions of either wt A β 40 monomer or A β S26C solutions with reducing agent (1% β ME) present (i.e., monomers) (Figure 4B). These ITC experiments suggest that the dimeric state of the A β protein binds GM1 much more avidly than the monomeric state, and that the monosialotetrahexosyl head group of GM1 may be necessary for efficient A β binding to lipid membranes, at least *in vitro*.

Blocking the GM1 sialic acid moiety prevents A β oligomer-mediated LTP impairment

We asked whether the binding of A β dimers to GM1 ganglioside has functional consequences, that is whether it plays a role in the known ability of the dimers to potently inhibit long-term potentiation (LTP) (Li et al., 2011; Shankar et al., 2008). The sialic acid of GM1 has been shown to be required for the *in vitro* binding of A β to GM1 (Ariga et al., 2001; Choo-Smith et al., 1997; McLaurin and Chakrabarty, 1996). We therefore asked whether masking the sialic acid with CT β , which binds to the two terminal sugars of GM1 including sialic acid (Merritt et al., 1994), may interfere with the binding of A β oligomers to GM1 in hippocampal slices and thus reveal any functional consequences of the binding. At a dose (0.4 μM) at which CT β by itself caused a slight decrease of LTP magnitude in wt mouse hippocampal slices ($140 \pm 6\%$, $n=8$; Figure 5A, blue circles), pre-treatment with CT β fully prevented the impairment of hippocampal LTP by soluble S26C A β dimers ($153 \pm 3\%$, $n=9$, green triangles) (Figure 5). These data suggest that the binding of A β oligomers to GM1 ganglioside plays a required role in mediating A β -induced synaptic dysfunction.

Endogenous A β , particularly A β ₄₂, is complexed to GM1 ganglioside on membranes of young hAPP transgenic mice

We next asked whether *endogenous* A β occurs in a complex with GM1 ganglioside on brain membranes. Using an A β antiserum (R1282), we immunoprecipitated the brain TBS-Tx extracts of 3 mo J20 tg, 20 mo J20 tg, or non-tg littermate mice and found that the TBS-Tx extracts of the plaque-free 3 mo tg mice contained CT β -positive GM1-bound A β (Figure 6A). In contrast, the plaque-rich 20 mo tg contained very little GM1-bound A β in the membrane extract despite having far more total A β (Figure 6A). We failed to detect other brain lipids such as galactocerebroside (GCB) or phosphatidylethanolamine (PE) in the A β immunoprecipitates of 3 mo tg TBS-Tx (Figure S4B). Moreover, thin-layer chromatography of the R1282 A β immunoprecipitates showed lack of enrichment in the major brain lipids detectable by the lipid dye primulin (Figure S4C), whereas the A β immunoprecipitate of the 3 mo tg TBS-Tx (but not that of the non-tg) yielded a CT β -positive spot (Figure S4D). Next, we took advantage of a monoclonal antibody (4396C) specifically raised against GM1-bound A β isolated from membrane fractions of human brains rich in diffuse plaques (Hayashi et al., 2004; Yanagisawa et al., 1997). We confirmed that 4396C immunoprecipitates neither A β nor GM1 alone (Figure S4A), but that it immunoprecipitates the GM1-bound A β complex from 3 mo tg TBS-Tx, leaving behind in the supernatant an A β species that is not CT β -positive, as recovered by a subsequent R1282 IP (Figures 6B and S4A). The 4396C immunoprecipitate from 3 mo tg TBS-Tx contained a CT β -positive 5 kDa A β species (Figure 6B, lane 1) with a characteristic cap-like shape akin to that of the 5 kDa A β from wt TBS-Tx that received *in vivo* A β injection (Figures 2E and 3). As in the R1282 A β immunoprecipitates of 20 mo tg TBS-Tx (Figure 6A), 4396C brought down very low levels of CT β -positive 5-kDa A β from these old, plaque-rich animals (Figures 6B). Using C-terminal specific A β antibodies for IP, we found substantially more A β ₄₂ than A β ₄₀ to be GM1 ganglioside-bound, despite there being comparable levels of total A β ₄₀ and A β ₄₂ recovered from the membrane extracts of 3 mo tg (Figures 6C and D). Collectively, these various findings in J20 tg mice support the hypothesis that more hydrophobic A β species (oligomers>monomers; A β ₄₂>A β ₄₀) are sequestered onto neuronal membranes, primarily onto GM1 ganglioside.

In contrast to the A β acute microinjection experiments in 3 mo wt mice, where most of the exogenous A β was recovered as bound to GM1 ganglioside in the membrane extracts (Figure 3), a smaller portion of *endogenous* A β (~5-30%) was recovered as bound to GM1 ganglioside in the 3 mo J20 tg brains (Figures 6B and S4D). Furthermore, the higher recovery of endogenous GM1-bound A β in 3 mo tg brain (pre-plaque, high ISF A β ₄₂) vs. 20 mo tg brain (plaque-rich, low ISF A β ₄₂) may be associated with the levels of available ISF A β at these two ages (Hong et al., 2011). To assess whether it is predominantly the endogenous A β which is actively released into the ISF that binds GM1, we treated the 3 mo tg mice with Compound E, a strong inhibitor of γ -secretase that induces a rapid decline in brain ISF A β ($t_{1/2}$ of ~2 h) (Hong et al., 2011). Compound E decreased the levels of GM1-bound A β in the 3 mo tg TBS-Tx in a manner comparable to its reduction of ISF A β levels (Figures 6E and F), suggesting that there is a rapid equilibrium between soluble (ISF) and membrane-bound (TBS-Tx) pools of A β . We confirmed that Compound E had no effect on total GM1 levels in the brain (data not shown). Collectively, all of the results in this section

suggest that newly generated A β , and especially A β ₄₂, upon being released into the extracellular fluid (i.e., ISF), readily binds to GM1 ganglioside on adjacent membranes, and this can apparently occur early in the AD pathogenic process, i.e., before plaques form. Finally, whereas we recover dimers in the 20 mo tg TBS-Tx, they do not seem to be associated with GM1, as evidenced by the negative CT β blotting in the R1282 A β immunoprecipitates (Figure 6A). The dimers we observe in the TBS-Tx extract of plaque-rich 20 mo tg may not necessarily be associated with membranes per se but may represent A β oligomers that are extracted from plaques during tissue homogenization in TBS-Tx (Hong et al., 2011).

GM1-bound A β is detectable in human CSF and correlates with CSF A β ₄₂ levels

Using the 4396C antibody that is specific for the GM1-A β complex (Figure S4A), we recovered a soluble GM1-bound A β species in normal human CSF (n=10 samples), as evidenced by positive reaction for A β and GM1 (CT β +) at the ~4.5 - 5 kDa position (Figure 7A: 4396C IP/A β WB first, then stripped and blotted with CT β ; Figure 7B: 4396C IP directly blotted with CT β). The levels of GM1-bound A β in human CSF showed a significant direct correlation ($r^2=0.72$) with levels of A β ₄₂ in the same CSF samples (Figures 7C and 7D) and to a lesser but still significant degree ($r^2=0.49$) with levels of A β ₄₀ (Figure 7E).

DISCUSSION

Despite the extensive genetic and biochemical evidence for a pathogenic role of soluble A β oligomers in the most common cognitive syndrome of late life, our understanding of the dynamics of A β oligomers in the CNS *in vivo* is limited. To address key mechanistic and biomarker-related questions of whether A β oligomers are present in biological fluids and the nature of their dynamics, we first searched for oligomers in the two CNS aqueous compartments, ISF and CSF, of tg mice that express substantial levels of hAPP and undergo progressive AD-like cerebral deposition of human A β . Despite combining three sensitive biochemical methods, IP-WB, o-ELISA, and SEC followed by triplex ELISA, we failed to detect soluble LMW (<35 kDa) A β oligomers in hippocampal ISF or CSF of this AD mouse model. These new results are consistent with two previous ELISA reports that failed to detect A β oligomers in human CSF (Esparza et al., 2013; Yang et al., 2013), one of which used two highly specific A β o-ELISAs in samples of CSF from 90 humans (including elderly subjects with either presymptomatic or early clinical AD) (Yang et al., 2013). Based on these studies and the current work, we conclude that the steady-state levels of LMW A β oligomers in the brain's aqueous compartments are very low (<0.02% of total CSF A β levels) and thus below the detection limits of the specific methods used here.

To explore this dearth of soluble oligomers mechanistically, we performed *in vivo* A β microinjection experiments into the hippocampal ISF of wt (plaque-free) mice and observed far lower recovery of injected dimers than of injected monomers, raising the hypothesis that soluble A β dimers released into the extracellular space are unlikely to remain very long in aqueous fluids. In accord, their relative hydrophobicity appeared to drive the sequestration of the dimers away from the extracellular fluid and onto local membranes. A much higher

proportion of the injected dimers was recovered in the membrane (TBS-Tx) extract than in the soluble (TBS) fraction of the brain. Surface biotinylation experiments on intact hippocampal neurons and a quantitative *in vitro* binding assay using isothermal calorimetry likewise demonstrated that A β oligomers bind membranes much more avidly than A β monomers do. We therefore conclude that any newly generated and released A β oligomers may be rapidly sequestered from the extracellular fluid (ISF) onto plasma membranes of surrounding cells in the brain parenchyma.

In mimicking newly formed A β oligomers in the brain, the *in vivo* injected dimers rapidly became membrane-bound, in contrast to the monomers. Intriguingly, both synthetic dimers and natural dimers isolated from human AD cortex that were injected at low levels into wt mouse ISF were recovered in membrane extracts as monomers bound to GM1 ganglioside, thus shifting their electrophoretic migration to ~5 kDa. This, along with the ITC binding assays and the microinjection experiments in GM1 KO mice, suggests a relative propensity for A β dimers to bind to GM1-containing lipids on membranes. This realization led us to use pan-A β , C-terminal specific, and GM1-A β -specific antibodies to probe A β species in a hAPP tg mouse model, and we observed substantial levels of endogenous GM1-bound A β , in particular the A β ₄₂ peptide, in the brain membrane fraction at an early age (3 mo), before plaques form in this model. This result is reminiscent of an earlier study showing that A β ₄₂ can be found associated with GM1 ganglioside in the brains of AD and DS subjects who had abundant diffuse plaques but little to no mature plaques or neurofibrillary tangles (Yanagisawa et al., 1995). The disassociation of dimers to monomers (unless given at high ng levels) seems to occur after the dimers' bind to membranes, as suggested by both the surface biotinylation experiment in primary hippocampal neurons and the ITC. The use of a covalently crosslinked form of A β oligomers would be insightful to further understand this process. In addition, it would be interesting to measure the half-life and fate of oligomers in brains already having very abundant A β plaques, as we previously did with the monomers (Hong et al., 2011).

To date, we have failed to detect an enrichment of other brain lipids bound to A β in our assays, including certain phospholipids, ceramides and phosphosphingolipids that we probed for and that may be present at higher levels than GM1 in the CNS. Moreover, preliminary data using GM2/GD2 synthase KO mice suggested a possible gene-dosage effect of GM1 in mediating the sequestration of A β dimers onto membranes: the less GM1 the brain had, the lower the percent of injected A β dimers that we recovered in the membrane fraction (data not shown). However, caution is needed in using this mouse model, as these mice now have robustly increased levels of GM3 and GD3 in the CNS (Wu et al., 2001); GM3 has also been shown *in vitro* to bind to and facilitate the aggregation of A β (Ariga et al., 2001; Grimm et al., 2012; Yahi et al., 2010; Yamamoto et al., 2005). Given these confounding factors, we do not regard this GM1 KO model as ideal for the study of A β effects. It is important to emphasize that although our data using an array of techniques uncover a preferential binding of A β oligomers to GM1 gangliosides and that this A β -GM1 binding survives denaturation by SDS and heat, there may well be other lipid targets to which A β binds. Lipidomic mass spectrometry is currently being pursued to examine various lipid candidates. The key finding that we can recover a GM1-A β complex in fresh human CSF samples suggests that the

GM1-A β complex exists *in vivo* in the human brain (i.e., in the absence of tissue homogenization) and that it reaches the CSF in small amounts. Likewise, immunohistochemistry of mouse brain cryostat sections post-*in vivo* A β injection showed that the remaining injected A β was principally co-localized with endogenous GM1 ganglioside, again eliminating tissue homogenization as an artifactual reason for this association. Finally, the spiking of A β oligomers into postmortem brain membrane extracts *ex vivo* failed to induce any binding of the exogenous A β to endogenous GM1 gangliosides, suggesting that the formation of the GM1-A β complex that we recover both in wt *in vivo* microinjection studies and in hAPP tg mice requires the environment of the living brain.

Individual laboratories have reported various potential protein receptors for A β oligomers, including $\alpha 7$ nicotinic receptors, NMDA, mGluR and AMPA receptors, insulin receptors, PrP^c and LirB2/PirB (De Felice et al., 2007; 2009; Kim et al., 2013; Laurén et al., 2009; Wang et al., 2000; Zhao et al., 2010). Although it is possible that A β oligomers bind to specific cell-surface proteins such as these and that this stabilizes their presence on the plasma membrane, the amphipathic hydrophobicity of the self-aggregating A β_{42} peptide suggests a biochemically more plausible scenario: that the hydrophobic A β_{42} oligomers released into the extracellular fluid (ISF) become rapidly sequestered onto hydrophobic surfaces of adjacent lipid membranes. Our data here suggest that GM1 ganglioside could be a principal lipid target of A β_{42} oligomers early in the course of AD-type β -amyloidosis. Gangliosides are particularly enriched in the nervous system compared to other tissues, and the subcellular localization of GM1 has been mapped out to be mostly in “raft-like domains” enriched in cholesterol and sphingomyelin on plasma membranes of neuronal processes, including pre- and postsynaptic membranes, and on certain normal lipid vesicles such as exosomes (Hansson et al., 1977; Pernber et al., 2012; Sonnino et al., 2007; Svennerholm and Gottfries, 1994; Théry et al., 2009; Yamamoto et al., 2008). Concentrations of GM1 and to a lesser extent GM2 have been reported to be increased in detergent-resistant membranes from frontal cortices of early AD brains (Molander-Melin et al., 2005) and the A β -GM1 complexes were found in the cortical neuropil and not on amyloid plaques *per se* in early AD and DS brains (Hayashi et al., 2004). Both our *in vivo* ISF studies and *in vitro* ITC binding data indicate that A β dimers can rapidly and avidly associate with GM1-containing lipid membranes. We note that hydrophobicity alone may not be the key factor for the A β oligomers’ preferential binding to GM1, in particular given our current ITC and previous *in vitro* data that the negatively charged sialic acid of gangliosides may be a prime binding site (Ariga et al., 2001; Choo-Smith et al., 1997; McLaurin and Chakrabarty, 1996).

Collectively, our results elucidate the complex economy of newly secreted A β oligomers as regards their membrane association and progressive loss of solubility over time. Our findings here help explain the apparently very low levels of A β oligomers as soluble, freely diffusible species in ISF and CSF at steady state *in vivo*, and they support the hypothesis that due to their increased hydrophobicity, A β_{42} oligomers primarily bind to GM1 ganglioside (and potentially other surface lipids) on neuronal membranes. Blocking the specific GM1 receptor site using CT β rescues the well-documented impairment of hippocampal LTP by A β oligomers, suggesting that the binding of A β to GM1 may be a step which leads to downstream synaptotoxic effects of A β . Thus, A β , upon binding to GM1 ganglioside, may

then mediate subtle but progressively detrimental biophysical alterations of neuronal membranes—for e.g., by disrupting membrane fluidity or ion permeability—and thus lead secondarily to aberrant function and turnover of a variety of transmembrane receptors.

Finally, we show that a GM1-bound A β species can be detected, albeit in low amounts, in human CSF and that its levels are significantly correlated with the levels of A β ₄₂ (and less so of A β ₄₀). Reduced CSF levels of A β ₄₂ in subjects with presymptomatic or early AD are a widely validated biomarker of the disease process, and these levels appear to relate inversely to amyloid plaque burden, degree of brain atrophy and severity of cognitive deficits in humans (Bateman et al., 2012; Fagan et al., 2009; Motter et al., 1995; Shaw et al., 2009). Interestingly, there has been a report of increased GM1 gangliosides in the CSF of AD patients vs. healthy controls (Blennow et al., 1991); however, the level of total gangliosides may not necessarily reflect the portion of A β -bound GM1. Accordingly, it will now be important to examine whether the GM1-bound A β complexes in CSF we describe could serve as an early biomarker in presymptomatic subjects, especially in light of the challenges of quantifying A β oligomers in CSF.

EXPERIMENTAL PROCEDURES

Animals

All animal procedures were approved by the Harvard Medical School Institutional Animal Care and Use Committee. See Supplemental Experimental Procedures for details.

In vivo microdialysis

Microdialysis was performed in the hippocampus of awake and normally behaving mice housed in a “Raturn” cage system (Bioanalytical Systems) as previously described (Cirrito et al., 2003; Hong et al., 2011). 1.5% bovine serum albumin in artificial CSF (in mM: 1.3 CaCl₂, 1.2 MgSO₄, 3 KCl, 0.4 KH₂PO₄, 25 NaHCO₃, and 122 NaCl, pH 7.35) was perfused using BR-2 probes with 35-kDa MWCO membranes (Bioanalytical Systems) at 0.2 or 0.4 μ l/min using an infusion syringe pump (Stoelting).

CSF collection

CSF was collected from cisterna magna of deeply anesthetized mice (DeMattos et al., 2002). Midline incision was made from top of the skull to dorsal thorax, followed by excision of muscles and excess tissue from base of the skull to first vertebrae. A small hole was made to arachnoid membrane covering the cistern using tip of 29 G 1/2” needle and CSF exiting compartment was collected using a gel-loading pipet.

Half-life studies of A β in ISF in vivo

3 μ l of 8 nM A β monomers or 40 nM dimers was injected into the hippocampus of adult C57BL6xDBA2 mice through a combination infusion cannula and microdialysis probe (IBR-2, Bioanalytical Systems) at 0.2 μ l/min. ISF was collected hourly at 0.4 μ l/min, paused during the injection, then restarted 1 min after injection. ISF was analyzed for levels of A β by A β _{1-x} ELISA or o-ELISA or for levels of lactate, pyruvate, urea and glucose (Yale Center for Clinical Investigation).

Intracortical injection of A β

Synthetic A β or natural A β isolated from AD cortex (1-2 μ l vol.) was injected at 0.5 μ l/min via a Hamilton syringe into left hippocampus of anesthetized mice (Hong et al., 2011). Mice were sacrificed <1 min post end of injection for biochemical analysis.

Mouse brain sample preparation for biochemical analyses

Brains were homogenized using a mechanical Dounce homogenizer with 20 strokes at 4000 rpm in ice-cold TBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.4) and protease inhibitors at 4:1 TBS volume:brain wet weight. Homogenate was centrifuged for 30 min at 175,000 g in a 4 °C TLA100.2 rotor on Beckman TL100 (resulting supernatant="TBS extract"). Pellet was homogenized in ice-cold TBS with 1% Triton-X (TBS-Tx) and protease inhibitors at 4:1 TBS-Tx volume:brain wet weight and centrifuged for 30 min at 175,000 g in a 4°C TLA100.2 rotor (resulting supernatant="TBS-Tx extract"). TBS-Tx pellet was incubated with 88% formic acid at RT for 2 h and lyophilized ("FA extract").

Immunoprecipitation and Western blot for A β and GM1 ganglioside

IP/WB was performed as previously described with minor modifications (Walsh et al., 2000). See Supplemental Experimental Procedures for details.

A β ELISA

Sandwich ELISAs for A β were performed using the MULTI-ARRAY® 96-well Plate platform (MesoScale Discovery) as described previously (Hong et al., 2011; Yang et al., 2013). See Supplemental Experimental Procedures for details.

Size-exclusion chromatography

ISF sampled at 0.4 μ l/min (250 μ l) or synthetic A β (2 ng) were eluted at 0.5 ml/min from a Superdex 75 10/300GL column (GE Healthcare) with 50 mM ammonium acetate, pH 8.5. Resulting 1-ml fractions were lyophilized then reconstituted in 60 μ l perfusion buffer and analyzed using 6E10 A β Triplex ELISA. For WB analysis, samples were subjected to WB using 3D6 and ECL Plus WB Detection Reagent.

Preparation of A β isolated from AD cortex

A β from TBS extract of human AD cortical tissue was prepared as previously described (Yang et al., 2013). Briefly, frozen temporal or frontal cortex samples were dissected then homogenized as described above for mouse brain sample preparation. TBS extract was then subjected to IP with 3D6 (Elan, plc) and the A β immunoprecipitate was eluted from a Superdex 75 SEC column in 50 mM ammonium acetate, pH 8.5. The SEC elution profile for A β was verified by SDS-PAGE. The corresponding fractions where A β dimers were eluted were used for the injection studies.

Isothermal titration calorimetry

For ITC, a Microcal ITC-200 instrument with a motor driven syringe was used. Volume of the cell was 204 μ l. Small volumes of small unilamellar vesicles, obtained by sanitation (40 mM total lipid) were injected into the protein solution (15 μ M) of calorimetry cell at 20 °C.

Buffer used was 25 mM ammonium bicarbonate, pH 8.4, with 1 vol% β -mercapto ethanol added in case of the S26C monomer titrations. Monomeric or dimeric forms of A β used for experiments were freshly eluted off a Superdex 75 SEC column and the corresponding fractions were kept on ice before use. For the experiments, 19 injections of 2 μ l were used, after a pre-injection of 0.4 μ l to account for diffusion of titrant from the syringe. Integration of the heat signals and correction of the base line was done in Microcal Origin 5.0. Control titrations of lipid suspension into buffer alone were subtracted from the experimental data. The apparent partition constant was calculated from the initial heat signals where the equilibrium seems to be independent from electrostatic interaction between the protein molecules (Seelig, 1997).

Electrophysiology on acute hippocampal slices

Hippocampal slices were prepared as previously described (Li et al., 2013). Briefly, 350 μ m thick transverse slices from hippocampus of 6-8 wk old C57BL/6x129 were incubated for >90 min in aCSF containing (in mM): 124 NaCl, 2 KCl, 2 MgSO₄, 1.25 NaH₂PO₄, 2.5 CaCl₂, 26 NaHCO₃, 10 D-glucose, pH 7.4, 310 mOsm, then were submerged beneath continuously perfused aCSF saturated with 95% O₂ and 5% CO₂ for stimulation at RT (~24 °C). For electrophysiology, standard field excitatory postsynaptic potentials (fEPSP) were recorded in the CA1 region of hippocampus as previously described (Li et al., 2013). Briefly, stimulating electrode was placed in Schaffer collaterals to deliver test and conditioning stimuli, and borosilicate glass recording electrode filled with aCSF was positioned 200-300 μ m from the stimulating electrode in stratum radiatum of CA1. To induce LTP, two consecutive trains (1 s) of stimuli at 100 Hz separated by 20 s, a protocol that induces LTP lasting ~1.5 h in wt mice of this genetic background, were applied to the slices. The field potentials were amplified 100x using Axon Instruments 200B amplifier and digitized with Digidata 1322A. The data were sampled at 10 kHz and filtered at 2 kHz. Traces were obtained by pClamp 9.2 and analyzed using the Clampfit 9.2.

Statistical analysis

Data was analyzed using PRISM (Graphpad Software) for one-way or two-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test if means were significantly different by ANOVA, or by two-tailed Student's *t* test, as appropriate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are very grateful to R. Ledeen (NJMS) for GM2/GD2 synthase KO mice, D. Harris (BU) for *PrnP* KO mice, R. Sperling (BWH) for human CSF samples, L. Mucke (UCSF) for J20 mice, DM Walsh (BWH) for AW8 antibody, and Elan, plc (South San Francisco, CA) for 3D6, 2G3 and 21F12 antibodies. We thank DR Podlisny and WT Cavanaugh for technical assistance, MJ LaVoie and TL Young-Pearse for critical reading of manuscript, and members of the Selkoe laboratory for helpful comments. Supported by NIH grant AG027443 (DJS), PPG P01 AG036694 (Sperling/DJS), a Harvard NeuroDiscovery Center Pre-doctoral Training Fellowship (SH) and a Jerome L. Rappaport Fellowship at HMS (SH).

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HIGHLIGHTS

- A β oligomers are rapidly sequestered away from hippocampal ISF *in vivo*
- A β oligomers bind to GM1 on neuronal membranes (oligomer>monomer; A β ₄₂> A β ₄₀)
- Blocking the GM1 sialic acid moiety decreases A β oligomer-mediated LTP inhibition
- GM1-bound A β can be recovered in human CSF and correlates with CSF A β ₄₂ levels

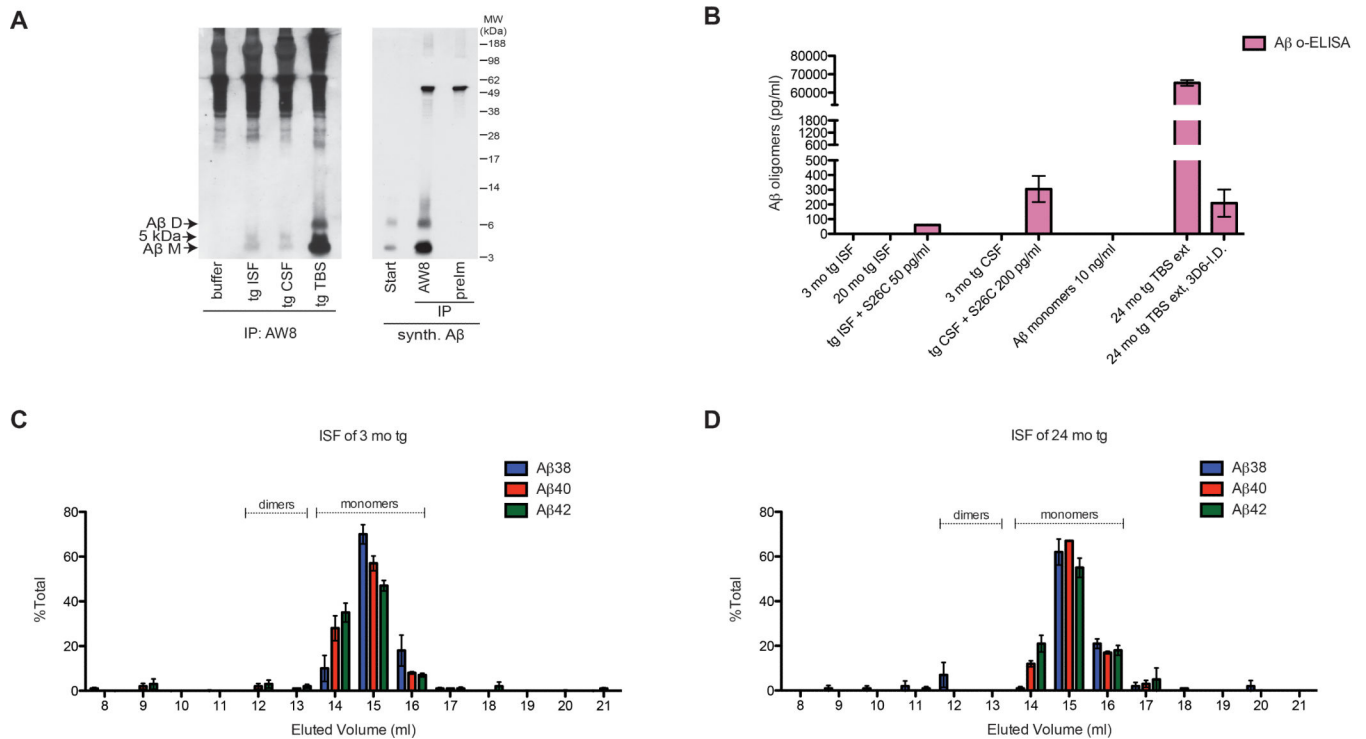


Figure 1. Aβ oligomers are not detected in J20 hAPP tg brain ISF or CSF

(A) IP/WB of buffer (1.5% BSA in aCSF), 3 mo tg hippocampal ISF and CSF, 20 mo tg TBS extract, and synthetic Aβ. IP: AW8 anti-Aβ sera (1:100) or pre-immune AW8 sera (preIm, 1:100); WB: 3D6+2G3+21F12. D=dimer, M=monomer, MW=Molecular weight. Representative of 10 experiments. (B) ISF of 3 mo tg (N=4 mice) and 20 mo tg (N=7 mice) and CSF of 3 mo tg (N=7 mice) were assayed using Aβ o-ELISA. Sensitivity and specificity of o-ELISA were tested using tg ISF and CSF spiked with low levels of synthetic Aβ dimers or monomers and 20 mo tg TBS extract and its corresponding 3D6 Aβ-immunodepleted sample. (C-D) 250 μl hippocampal ISF from 3 mo tg (C) and 20 mo tg (D) were loaded onto Superdex 75 SEC column and resultant fractions were subjected to 6E10 Aβ triplex ELISA. Value=normalized mean ± SEM; N=3 mice each. Dotted lines indicate where synthetic Aβ dimers and monomers were eluted using the same SEC column. See also Figure S1.

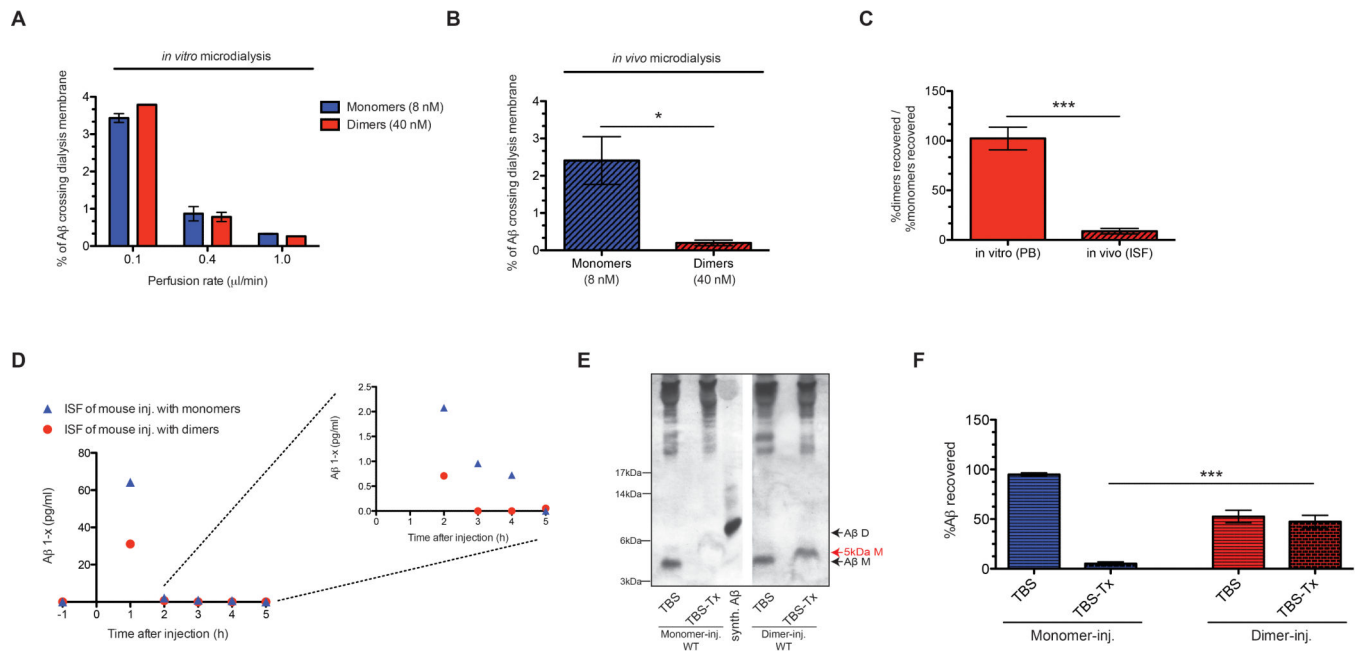


Figure 2. A β dimers are rapidly sequestered away from the hippocampal ISF *in vivo* to bind membranes

(A) (A β_{1-40} S26C)₂ dimers (40 nM) or A β_{1-40} monomers (8 nM) in test tubes were microdialyzed at 0.1, 0.4 and 1.0 μ l/min and the resulting microdialysates were assessed using A β_{1-x} ELISA. N=3 per group. (B) A β dimers or monomers were administered to living wt hippocampal ISF via a microdialysis cannula and post-injection ISF was collected at 0.4 μ l/min for 1 h and analyzed for A β_{1-x} . N=6 mice and N=5 mice for monomer- and dimer-injected. *: $P=0.0126$ by two-tailed Student's t test. (C) *In vivo* recovery of injected dimers was $8.85 \pm 2.81\%$ of that of injected monomers (N=5 pairs), in contrast to the *in vitro* paradigm ($102 \pm 11.4\%$ (N=3 pairs)). ***: $P<0.0001$ by two-tailed Student's t test. (D) Representative hourly monitoring of injected A β monomers (blue triangle) or S26C A β dimers (red circle) in ISF of wt mice before and after injection. Inset shows values using a finer y-scale for $t=2-5$ h. (E) 320 pg A β monomers or S26C dimers were injected into hippocampi of wt mice and their brain homogenates were immediately fractionated for R1282 A β IP. WB: 3D6+6E10. Both monomers and dimers were recovered as monomers in the TBS and TBS-Tx. Representative of 4 experiments. (F) IP/WB quantification by ImageJ of N=8 mice injected side by side with monomers or dimers. Proportion of A β recovered in TBS-Tx vs TBS extracts post A β dimer injection was 23 ± 6.4 fold than that of A β monomer injection. ***: $P<0.001$ by 2-way ANOVA, followed by Bonferonni post-test. Value=mean \pm SEM. See also Figure S2.

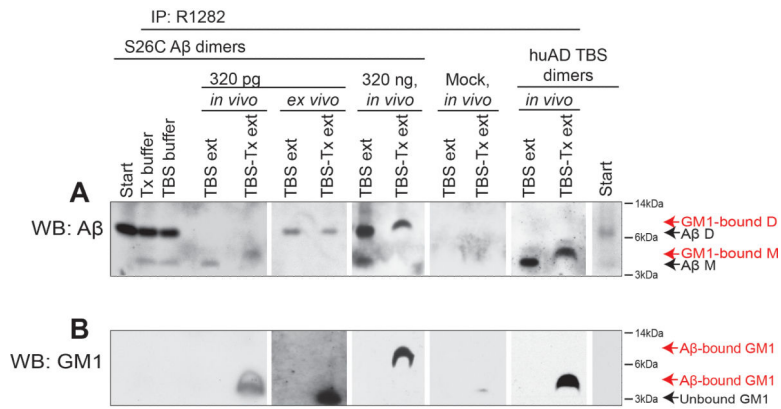


Figure 3. Aβ injected to wt hippocampus *in vivo* is recovered from the membrane-associated fraction as bound to GM1 ganglioside
 320 pg or 320 ng synthetic S26C Aβ dimers, huAD TBS dimers or buffer (mock) were injected to anesthetized wt mice, then the brains were immediately harvested for brain fractionation. The *in vivo* injected brain fractions (TBS and TBS-Tx extracts) or S26C Aβ spiked *ex vivo* to extracts or to buffer alone were immunoprecipitated for Aβ using R1282. WB: 3D6+6E10 for Aβ (**A**) or CTβ for GM1 (**B**). Representative blots of N=5, 3, 5 and 5 mice for 320 pg S26C, 320 ng S26C, mock or huAD TBS dimer injections, respectively. See also Figure S3.

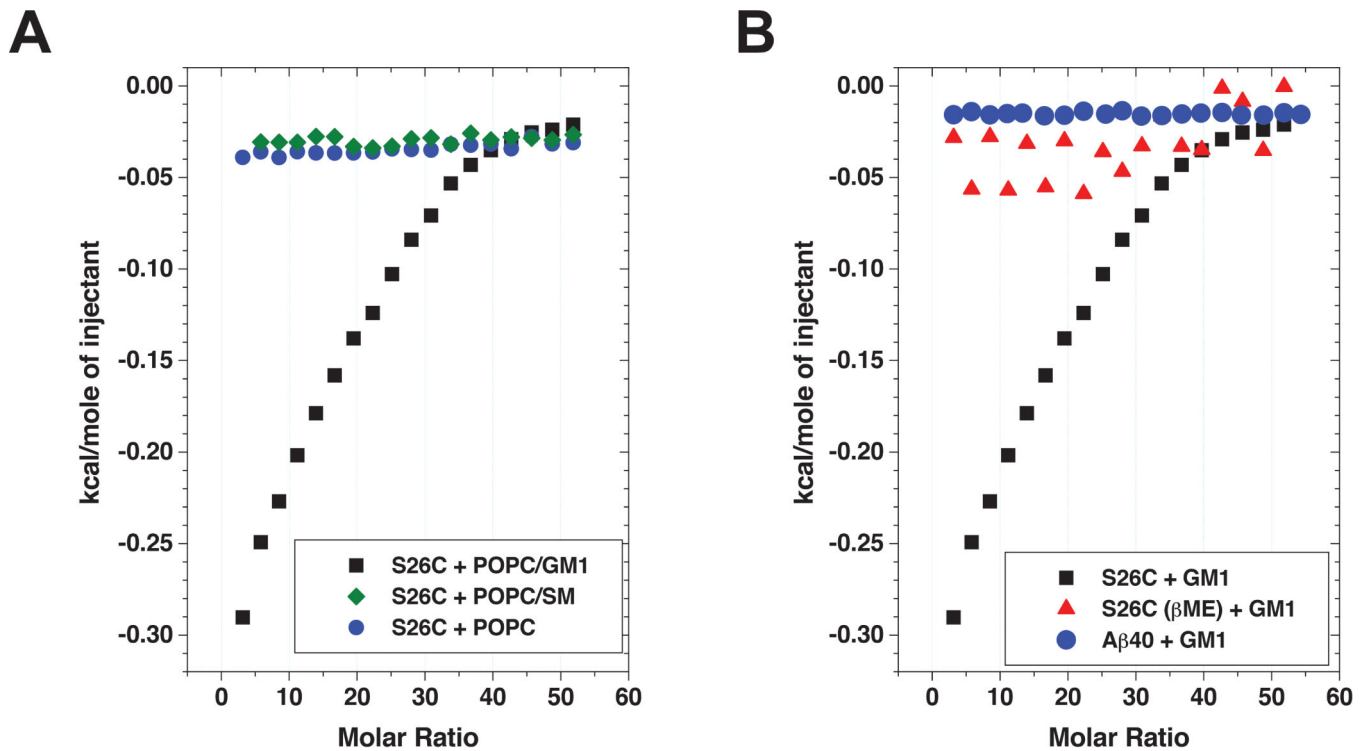


Figure 4. Aβ dimers bind to GM1 ganglioside much more avidly than Aβ monomers

(A) Suspensions of POPC/GM1 (9:1), POPC/sphingomyelin (SM) (9:1) or pure POPC small unilamellar vesicles at 40 mM were titrated into freshly prepared Aβ S26C dimer solutions (15 μM) and subsequent heat release was measured. The hyperbolic shape of the titration curve in case of POPC/GM1 (9:1) indicates a partition equilibrium of Aβ S26C dimers into the lipid membrane, while titrations of POPC/sphingomyelin (SM) (9:1) or pure POPC vesicles yielded no detectable heat release. (B) Titrating suspensions of POPC/GM1 (9:1) into solutions containing wt Aβ40 monomers or Aβ S26C with 1% βME present (i.e., reduced monomers) yielded no detectable heat release during the titration when compared to Aβ S26C dimers. Representative of 3 experiments each using two different preparations of Aβ S26C and vesicles.

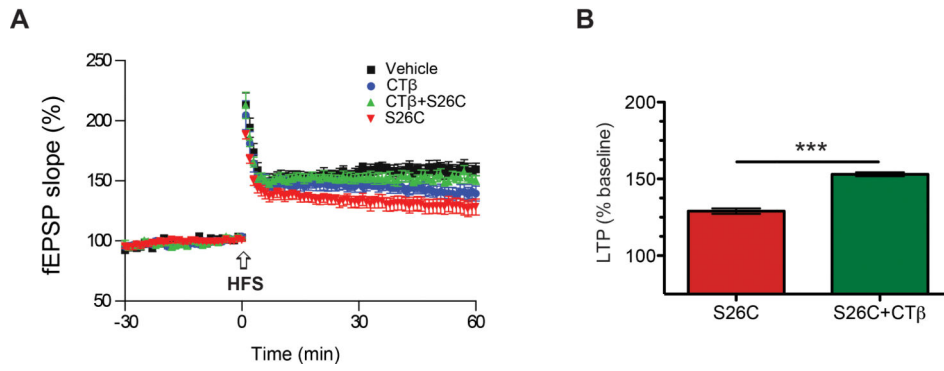


Figure 5. Pre-treatment of hippocampal slices with CTβ blocks Aβ oligomer-mediated LTP inhibition

(A) S26C dimers (10 nM) alone blocked LTP induction ($129 \pm 6\%$, $n=12$, red inverted triangles) compared to vehicle alone (aCSF) ($159 \pm 6\%$, $n=12$, black squares). Whereas CTβ (0.4 μM) alone slightly decreased LTP ($140 \pm 6\%$, $n=8$, blue circles), it fully restored the S26C dimer-impaired hippocampal LTP ($153 \pm 4\%$, $n=9$, green triangles). n =slices. (B) The effect of S26C alone vs. S26C on slices pretreated with CTβ is compared. Value=mean \pm SEM at 55 min of LTP induction. ***: $P<0.0001$ by two-tailed Student's t test.

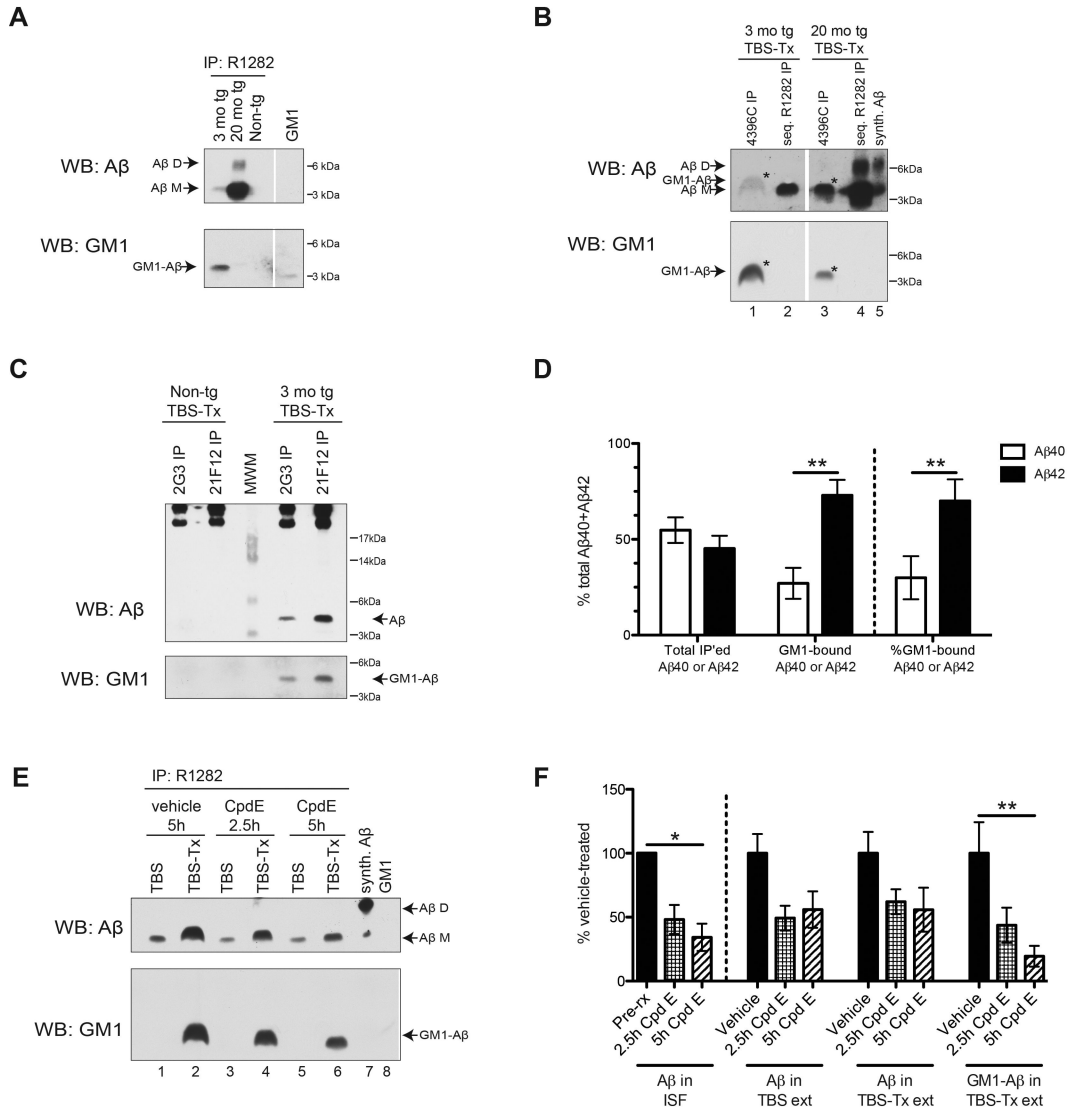


Figure 6. Aβ in membrane-bound fraction of pre-plaque J20 tg mice is associated with GM1 ganglioside

(A, B, C and E) IP: As indicated, WB: 3D6+6E10+266+2G3+21F12 for Aβ or CTβ for GM1. (A) R1282 Aβ IP of 3 mo tg, 20 mo tg or non-tg littermate control TBS-Tx extracts. (B) 3 mo tg and 20 mo tg TBS-Tx were first subjected to 4396C IP, then the immunodepleted supernatants were subjected to R1282 IP. Asterisk=GM1-Aβ. Representative of 3 experiments. (C, D) 2G3 or 21F12 IP of 3 mo tg and littermate control TBS-Tx extracts. (D) Quantification of total, GM1-bound or % GM1-bound Aβ in 3 mo tg TBS-Tx pulled down by 2G3 (Aβ₄₀) or 21F12 (Aβ₄₂). N=6 mice. (E, F) 3 mo tg were injected with Compound E or vehicle; 2.5 h or 5 h later, their brains were harvested and their TBS and TBS-Tx extracts were subjected to R1282 IP. (F) Quantification of total or GM1-bound Aβ recovered from TBS and TBS-Tx with or without treatment. N=4, 3 and 4 mice for vehicle-, 2.5 h, and 5 h Compound E-treated, respectively. (D, F) Value=mean ± SEM. *: P<0.05 or **: P<0.01 by 2-way ANOVA, followed by Bonferonni post-test. See also Figure S4.

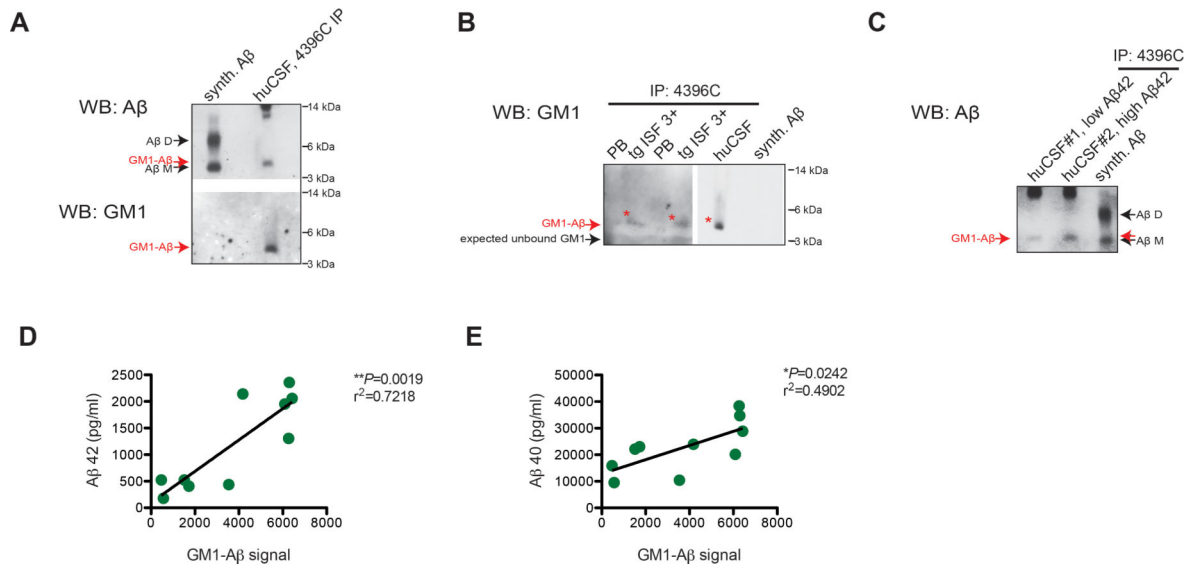


Figure 7. Low levels of GM1-bound Aβ are recovered from hAPP tg mouse ISF and human CSF (A-C) IP: 4396C, WB: CTβ for GM1 ganglioside or WB: 3D6+6E10+266+2G3+21F12 for Aβ. (A, B) 4396C pull-down of human CSF probed for Aβ then stripped and reprobed for GM1 (A) or GM1 alone on a naïve IP blot (B; Red asterisk=GM1-Aβ). (C) CSF from 2 humans with differing levels of Aβ₄₂ was immunoprecipitated for GM1-bound Aβ. Representative blots of at least 3 experiments. (D, E) Levels of GM1-bound Aβ recovered using 4396C in ten human CSF samples showed a strong correlation to their levels of Aβ₄₂ (D; 21F12/3D6B ELISA) and less but still significant to levels of Aβ₄₀ (E; 2G3/3D6B ELISA).