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Neurotoxicity of Alzheimer disease Aβ-peptides is induced by small changes in the Aβ42 to Aβ40 ratio

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1st Editorial Decision   23 February 2010

Thank you for submitting your manuscript to the EMBO Journal. Your manuscript has now been seen by two referees and their comments to the authors are provided below. As you can see, both referees appreciate the analysis and support publication here pending satisfactory revision. They raise a number of specific concerns that I would like to ask you to address in a revised version.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for submitting your interesting work to the EMBO Journal

Yours sincerely,

Editor
The EMBO Journal

REFEREE REVIEWS

Referee #1 (Remarks to the Author):

The manuscript by Inna et al describes a number of important studies examining the "neurotoxic" properties of various Abeta assemblies generated by mixing different ratios of Abeta40 and Abeta42. Such studies have often been discussed in the field, but rarely executed. Importantly the group performs a number of careful controls to actually define the Abeta species before the studies. There are a number of studies included in the manuscript and the writing, the data itself and its interpretation is somewhat uneven. Indeed, this is the first time a group has systematically looked at 40:42 mixtures and their effect on synaptic function and toxicity. As noted below I think several of the conclusions in the manuscript are overreaching and it would be best to simply describe these studies and the data and then interpret them conservatively. In any case appropriately revised these studies represent a very important contribution to the field.
Concerns.
1. Both "pure" 10:0 Abeta42 and the 3:7 (40:42) mixture essentially behave the same in all assays tested. Except in figure 2 D where 10:0 shows enhanced fibrilization based on ThT. Given that the main novelty in the manuscript is the difference between 1:9 and 3:7 mixtures, might not the data support the notion that Abeta40 blocks the toxic conformational change, rather then claims of some "elusive" toxic oligomer that really cannot be isolated but can only be associated with various biophysical measurements. Indeed, I think the strength of the manuscript is that the 0:10 or 1:9 preparations are minimally toxic supporting that Abweta40 is in fact "protective".
2. Is the toxicity associated with a soluble Abeta form or insoluble? Centrifugation studies and testing of the insoluble versus soluble material would be very helpful here.
3. AFM studies might be very helpful in detecting spherical oligomeric intermediates. Given the state of the field inclusion of such studies would be highly appropriate
4. Arancio and colleagues show U shaped curves with respect to synaptic effects of certain Abeta preparations. Though excitatory at low concentrations they are inhibitory at higher concentrations. I would like to see a more extended dose response with the 3:7 mixture and the 0:10 mixture to see if these behave in that way. In other words one prediction is that 3:7 mixture when diluted would show an excitatory synaptic response.
5. A claim is made that the first aggregation species is a dimer. How can one claim this based on the data. Also although I am assuming that the mass-spec studies do in fact demonstrate dimer formation. I find the description of this data insufficient to understand how this conclusion is made. What is the charge state of the dimmers being detected? What about other charge states might they not show different ratios? I think this data might really be a mine field and really does not add anything to the manuscript. Perhaps it should be omitted.
6. Given the different kinetic properties of the 10:0 and 3:7 mixtures, one would think that they could be distinguished at least quantitatively with respect to the relative "toxicity" at some point. For example they are most different at 6 hours in the FTI spectra. So at this time point are they different biologically?
7. It is well known that different lots of Abeta behave differently and there is data suggesting that HFIP at very low concentrations actually alters the properties of Abeta. I think repeting critical data with Abeta obtained from another source and treated the same way would be improntat information.
8. The claim that the toxicity spreads from the synapse to the cell body at higher concentrations, would be better supported by studies where the neurons were grown in chambers that allow treatment of their processes without their cell-bodies (e.g. as performed in the recent Tessier-Levigne study). Its hard to spate when there is dose related toxicity. Also not really terribly important for this manuscript and could be published as a separate study.
9. The description of what statistical tests are used and when they are used seems somewhat haphazard. Please run appropriate statistical tests describe these tests and use asterisks to indicate all statistically significant changes.
10. The introduction is much too long and uneven. A good thorough editing would be helpful. Statements like "While Abeta42 seems more pathogenic....." in the abstract could be stated with more precision.
11. A thorough discussion of what acute toxicity studies show and what they do not show is important. For example direct neurotoxcity may not be the mediator of neuronal demise in AD. What about chronic inflammation. Until one develops a thepry targeting a particaulr toxic species and shows it cures or prevents AD I think it best to be parsimous and simply stae in the systems studies we find evidence for .. Rather then draw more sweeping generalizations.

12. Wogulis et al in J. Neurosci 2005 elegantly show that fibril growth may be a key link to toxicity. I think a discussion of the possibility of kinetics growth of fibrils influencing the results should be included in the discussion. Indeed, this study is often ignored or not even by many inthe fiedl but provides an alterantive conceptual framework for toxicity studies.

13. The preamble in the results regarding use of high concentrations of Abeta should be moved to the discussion or introduction.

14. The referencing is somewhat scattered. I think many landmark studies of 40 42 and there differences are not referenced in favor of more recent publications.

Referee #2 (Remarks to the Author):

The study by Kuperstein et al. addresses one of the very important questions in Alzheimer's disease (AD) research: what are the consequences of small changes in the Aβ42/40 ratio, and utilizes several complementary approaches to demonstrate what is the initial toxic impact of Aβ (or rather various Aβ42/40 mixtures), and how kinetics of Aβ aggregation are affected by changes in the proportion of Aβ40 and 42 in the Aβ mixtures. The hypothesis of the detrimental role of the increased Aβ42/40 ratio in the AD pathogenesis is not novel and is rather well accepted in the AD field, although the exact mechanism underlying increased Aβ42/40 toxicity is not entirely clear. The aggregation of Aβ peptides and the toxicity of different Aβ species have also been intensively studied by many research groups over the past 15-20 years. However, the current study undertakes a slightly different approach by focusing on aggregation dynamics and toxicity of Aβ mixtures, when Aβ 40 and Aβ 42 peptides are mixed in different ratios. Using well designed and elegant studies the authors show how shifts in the equilibrium between these two major Aβ species (e.g., small increase in the Aβ42 relative to Aβ40 as in 3:7 ratio) dramatically increases rate of nucleation and results in formation of very unstable intermediate (amorphous?) assemblies of the Aβ peptides that exerts strong neurotoxic effect. The highly dynamic and unstable nature of intermediate Abeta species has been reported previously. The authors add to and refine that knowledge by showing that (3:7) Aβ42/40 ratio prolongs the time window of existence of these highly toxic species, whereas (1:9) Aβ42/40 ratio nucleates late but once nucleated very rapidly converts into mature and rather benign amyloid fibrills. Importantly, the authors also show that toxic intermediate Aβ species initially binds to and impair synapses, whereas cell body pathology appears later. These are interesting and important findings, and although not unexpected and groundbreaking, this thorough study refines the model and demonstrates more accurately the dynamics and mechanism of elevated Aβ42/40 ratio toxicity. Moreover, results from in vivo experiments strongly support and strengthen the findings.

There are several concerns however that need to be addressed.

1. Fig.1 E,F- was any statistical analysis performed for the quantitative data presented here? "small but reproducible increase" (page 7), -not clear, is this a statistically significant increase? Please show p values.

2. Different Aβ mixtures were incubated at 25C before addition to the cell culture. Thus, we know in what state Aβ was at the moment when it is added to cells. But what happens to Aβ species when they are at 37C in tissue culture? What are the changes that occur to Aβ conformational state when Aβ is present at different ratios at 37C? Need to show what happens to Aβ mixtures after incubation at 37C during the time periods used to treat neurons. Unless, of course, Fig.3 TEM analysis was performed using Aβ incubated over different periods of time at 37C. Please clarify; there is no mention of the temperature given in the text, methods, or in the figure legend for TEM.

3. The authors used "spontaneous fire rate" and synaptophysin staining as read outs of synaptotoxicity caused by elevated Aβ42/40 ratio. Both are markers of presynaptic integrity. What happens to postsynaptic responses (could be measured by EPSC and/or IPSC)? Do they remain intact or are they also affected?

4. The authors report that 3:7 and 10:0 Aβ mixtures had negative effect on synapses and show A11 immunoreactivity at the synapses (Fig.2). On the contrary, it seems that (1:9) and especially (0:10) ratios enhance synaptic firing after 40 min incubation on cells, compared to that in control (Fig.2B,C). The authors claim that there was "no deposition of A11 staining at the synapse in (0:10) sample" but was there an Aβ (e.g. 6E10) staining? Is positive modulatory effect of the (0:10) induced by direct interaction of Aβ40 with the synapse? What is the difference between 0:10/1:9 (mainly Aβ40) "positive" and 3:7/ 10:0 "negative" modulation mechanisms? The authors should comment on this.

5. Western blots in Figure 3 B show the presence of small oligomeric species in (0:10) and (1:9) Aβ mixtures (x2, x3, x4 of mainly Aβ40 assemblies?). However, the authors report that there was no synaptic and/or cell toxicity detected with these samples. Moreover, Fig. 2B shows positive effect of (0:10) and (1:9) on synaptic firing. Thus, it is somewhat confusing since it has been shown by many research groups that small oligomeric Aβ species are toxic. Indeed, recent publication by Teplow's group (PNAS, 2009) showed that Aβ40 small order assemblies (dimers, and especially trimers and tetramers) are the most toxic Aβ species. Please clarify/comment. Are Aβ40 small oligomers inert or even beneficial for neurons in your experimental settings?

6. There are some discrepancies between the kinetics of aggregation data shown in Figure 1D-F, Figure 3 and Figure 4. For example, lag phase/nucleation time is longest for (0:10) and (1:9) in Fig.1 (ThT). However it seems not to be the case for (1:9) as detected by FTIS (Fig.4B). Fig. 1D,F show that elongation time is fastest in (0:10), i.e. that monomers/amorphous Abeta converts into fibrils very rapidly, but TEM shows that first fibrils are detected not in (0:10) but in (10:0, by 4 hrs). The authors couldn't see fibrils in 3:7 samples by TEM after 4 hr incubation period (Fig.3), but they detect "ThT positive oligomers" at 4hrs (Fig.1D) and "transient intermediate" in Fig.4E. The relationship between "oligomer" and FTIR "intermediate" is unclear and creates confusion. Also, the authors need to clarify whether in all these aggregation experiments the same concentration of Aβ was used (is it 50uM in Fig.1 D-F but 100uM in Figs. 3 and 4?). It will help if they present their results more clearly.

7. Figure 4 E and F: It is not clear and needs better explanation how the analysis was done and how curves (Fig 4E) were generated. What is "flat curve around zero"?

8. Figure 5. Please show western blot for cleaved caspase-3 since immunostaining is less accurate for subtle changes. Also, caspase immunorectivity in 1uM, (3:7), 12 hr treated cells in (A) and (E) is very different: no caspase IR in (A) but a lot of green dots in (E). Please address this discrepancy. It is also somewhat surprising that titer-blue viability assay for 3:7 and 10:0, 2hr, still detects ~25% of live neurons after 48 hours of treatment, since 12 hour treatment shows that pretty much every nucleus is PI-positive (red), which indicates that the cell membrane integrity is already jeopardized. Highly unlikely, that the neurons survived for another 36 hours after that.

1st Revision - authors' response   05 July 2010

Referee #1 (Remarks to the Author):

1.   Both "pure" 10:0 Abeta42 and the 3:7 (40:42) mixture essentially behave the same in all assays tested. Except in figure 2D where 10:0 shows enhanced fibrillisation based on ThT. Given that the main novelty in the manuscript is the difference between 1:9 and 3:7 mixtures, might not the data support the notion that Abeta40 blocks the toxic conformational change, rather then claims of some "elusive" toxic oligomer that really cannot be isolated but can only be associated with various biophysical measurements. Indeed, I think the strength of the manuscript is that the 0:10 or 1:9 preparations are minimally toxic supporting that Abeta40 is in fact "protective".

As we will also discuss further below, it is exactly one of the points of the current manuscript that toxicity is not necessarily associated with a precise stable oligomeric structure and that the current literature suggesting or stressing that toxicity can be associated with a particular Aβ oligomeric species is likely simplifying the actual situation. Techniques used to visualize Aβ oligomers, in particular SDS PAGE, have the tendency to promote certain conformations or aggregations (see below) and, importantly, do not necessarily reflect the actual situation of the oligomers in situ (see also the work of Hepler et al, Biochemisty, 2006 and Supplemental Figure 2 in which we compare the use of Native PAGE with SDS PAGE). We used therefore several biophysical approaches to describe the behavior of the different peptide mixtures, without making claims for precise oligomer sizes causing toxicity. Our data show indeed a complex interaction between Aβ40 and Aβ42 as both peptides influence each otherís behavior in various biophysical and biological assays. It is not so clear that Aβ40 is unequivocally protective. Actually the 3:7 ratio behaves at certain time points equally toxic as the pure Aβ42 10:0 ratio where no Aβ40 is present, and maintains in fact over longer time its ìtoxicî effect because its aggregation to inert fibrils is slower. We feel that we have not sufficiently explained our interpretation in the previous version of the manuscript and discuss

here below additional considerations and arguments for our interpretation and have also made changes in the manuscript as specified which support a more complicated interpretation of the protective effects of Aβ40.

1. Figure 1A shows the aggregation rates of Aβ40, Aβ42 and two ratios using Thioflavin T (ThT) fluorescence. Judging from these data, the behaviour of ratio 3:7 or ratio 1:9 are not simply the sum of the individual curves obtained with Aβ40 or Aβ42 separately. To further illustrate this, we attached a theoretical figure (Additional Figure 1 ñ only for referees). We made a theoretical calculation of the aggregation rates of 1:9 and 3:7 on the assumption that the aggregation rates of Aβ42 and Aβ40 are not affected by the presence of each other and that the effects are purely additive. Further details on the calculation of these values have been provided in the legend added to the figure. It is clear (additional figure 1 panel C) that the calculated and experimental curves differ from each other dramatically showing that the peptides influence each otherís behavior in a complex interactive way and that it is difficult to make predictions based on the behavior of the single peptides separately. We see this also in other assays as discussed in the paper and below.

2. In figures 1B and 1C, no linear dependence of Aβ ratio on aggregation properties (nucleation formation observed by the length of the lag phase in Figure 1B and the rate of fibril elongation in Figure 1C) is observed. A purely protective effect of Aβ40 against aggregation would show a linear dependence in both figures: i.e. the nucleation time of the 5:5 Aβ42:Aβ40 ratio would be derived from 50% of the contribution of Aβ42 and 50% of the contribution by Aβ40 and thus be 1.5 h instead of only 0.5 h as was experimentally determined.

3. Figure 2C shows that the toxic effect of 3:7 and Aβ42 (10:0) are both similarly strong at this particular time point (2 h of aggregation). A simple protective effect would show less toxicity for 3:7.

4. A second independent line supporting the concept that the 3:7 ratio causes dramatic changes in peptide behavior is provided in Figure 4A. TEM shows that aggregates are formed by the 3:7 ratio which are morphologically deviant from any of the other fibrils formed at any stage of the aggregation pathway. Also their rate of formation is delayed compared to Aβ40 which shows that there is not just a simple protection against aggregation. Please notice that we do not claim any particular toxicity associated to any particular morphology seen in these pictures.

5. Electrospray ionization mass spectrometry of Aβ40 and Aβ42 clearly shows that the two peptides can interact as masses are detected which are indicative for the formation of mixed dimers. The ratio between the peaks further shows that there is no preference to form homodimers (Aβ40 - Aβ40 and Aβ42 - Aβ42) over heterodimers (Aβ40 - Aβ42). These data are removed from the manuscript and are now depicted in figures S3 and S4 of the supplemental data. Please notice that we use these data only to demonstrate that Aβ40 and Aβ42 interact directly with each other.

2.   Is the toxicity associated with a soluble Abeta form or insoluble? Centrifugation studies and testing of the insoluble versus soluble material would be very helpful here.

We agree with the reviewer that solubility is indeed an important aspect to study, particularly since a number of reports appeared that supported the notion that specifically soluble Aβ is potently toxic (McLean et al. 1999, Ann Neurol 46:860-866; Lue et al. 1999, Am J Pathol 155:853-862). We hence investigated the solubility of the different Aβ42:Aβ40 ratios as a function of time (additional figure 2- only for referees). Details of the experiment can be found in the figure legend. We took these data one step further by evaluating the synaptotoxic response (using MEA) of the soluble fractions. It is clear that synaptotoxicity corresponds with a high level of soluble Aβ under the conditions tested: after 26 h Aβ42 exerts little synaptotoxicity while supernatant from the 3:7 ratio remains toxic. In order to further refine the results, we analyzed the supernatants of toxic ratios after 48 h of aggregation. As expected, Aβ42 did not exert any synaptotoxicity, while the supernatant of 3:7 ratio (14,000 rpm) was toxic. We have the impression that there is some reversibility in the process as we found in other experiments, when using high speed centrifugation (100,000 rpm/1.5 h) that the pellet was toxic when we added it to neuronal cultures. We decided not to further explore this issue as we are not particularly convinced that the toxicity is specifically associated with a particular species.

3. AFM studies might be very helpful in detecting spherical oligomeric intermediates. Given the state of the field inclusion of such studies would be highly appropriate.

In the light of studying oligomeric Aβ which is frequently cited as the toxic species AFM studies as suggested would indeed be highly valuable. As Ono et al (PNAS 2009) already suggested, the use of AFM and TEM can distinguish different morphologies as a result of the difference in substrates used for the visualization of Aβ features. We have therefore collaborated with the Faculty of Science and Technology of the University of Twente in The Netherlands who are experts in the visualization of oligomeric and fibrillar aggregates of proteins. We opted to use tapping-mode AFM in physiological solution to exclude artifacts induced by the drying of the sample or mechanical deformation of the aggregates as a result of the significant forces applied using continuous mode AFM. The results show that oligomers are present in all Aβ ratios after 1.5 h incubation. This is an interesting finding and it underlines that oligomeric Aβ per se is not toxic by definition. Earlier research already showed that also the HypF peptide can result in both benign and malign oligomeric species depending on the oligomeric structural properties. (Campioni et al. (2010). Nat. Chem. Biol. 6: 140-147). These data are added as panel B to figure 4 of the main manuscript.

4. A claim is made that the first aggregation species is a dimer. How can one claim this based on the data. Also although I am assuming that the mass-spec studies do in fact demonstrate dimer formation. I find the description of this data insufficient to understand how this conclusion is made. What is the charge state of the dimers being detected? What about other charge states might they not show different ratios? I think this data might really be a mine field and really does not add anything to the manuscript. Perhaps it should be omitted.

Apparently the way we presented these data created confusion. As we have already pointed out we do not think that toxicity is exclusively associated with a particular aggregate. We have rephrased the paragraph discussing the mass spectrometry analyses to make more clear that the reasons we observe only dimers is purely technical. We have preliminary evidence of the presence of (mixed) trimers and higher-n oligomers as well. We agree with the referee that using this technique does not allow making any conclusions with regard to the time-lapse of the aggregation formation. It is merely a snapshot of the complexes right after mixing. We have removed the statement that the dimers observed are the first step in the aggregation process of Aβ. Our suggestion that Aβ40 and Aβ42 interact directly and unequivocally (as suggested in our response to question 1 of this reviewer) is however only demonstrated directly by the mass spectrometry data. Indeed the Tht fluorescence, the cell biological toxicity assays and the Fourier transform infrared spectroscopy assays can only be explained by such interaction, but we felt it important to provide also direct evidence that the two Aβ peptides interact. We have therefore put the data in supplemental figure 4. Concerning the charge states of the dimers we incorporated supplementary figure 3. The mass spectra were obtained in positive mode. The charge state of the dimer is 7 as now labeled in the additional figure. Peaks for other charge states were also found in the scanned m/z mass range for charge state 9. The peaks were weak but showed the same ratio as for the observed dimers with charge 7. Even charge states show peaks at the same m/z as the monomers (e.g., the m/z of charge state 8 for the dimer equals to the m/z of charge state 4 of the monomer) and cannot be distinguished. A charge state of 7 is therefore ideal to study dimer formation. We have also added a technical description so that it is clear how these data were obtained.

5. Arancio and colleagues show U shaped curves with respect to synaptic effects of certain Abeta preparations. Though excitatory at low concentrations they are inhibitory at higher concentrations. I would like to see a more extended dose response with the 3:7 mixture and the 0:10 mixture to see if these behave in that way. In other words one prediction is that 3:7 mixture when diluted would show an excitatory synaptic response.

We add an additional figure 3 only for the referees. As can be seen, Aβ ratios 10:0 and 3:7, indeed, tend to slightly potentiate synaptic activity in cultures (n=2-3) at submicromolar concentrations. However, this effect is transient (in contrast to the stimulatory impact of Aβ40 at micromolar concentration). When we exposed the neuronal cultures to a low concentration of these ratios during an overnight incubation these preparations demonstrated a clear inhibition of synaptic activity.

6. Given the different kinetic properties of the 10:0 and 3:7 mixtures, one would think that they

could be distinguished at least quantitatively with respect to the relative "toxicity" at some point. For example they are most different at 6 hours in the FTIR spectra. So at this time point are they different biologically?

We agree with the reviewer that the 6 h time point is indeed a very interesting time point to study the toxicity of Aβ. We performed MEA assays at this time point and the results have been added to the results in the paper (Fig. 2E). At time point 6 h the 3:7 mixture is extremely toxic as expected, although considerable toxicity remains associated with 10:0 as well. The difference between 3:7 and Aβ42 is significant (*p<0.012) as indicated in the figure.

7. It is well known that different lots of Abeta behave differently and there is data suggesting that HFIP at very low concentrations actually alters the properties of Abeta. I think repeating critical data with Abeta obtained from another source and treated the same way would be important information.

Between-batch variation and the presence of compounds to aid the solubilization of hydrophobic Aβ peptide are indeed problems for this type of studies. We hence agree with the reviewer that these issues should be carefully considered before drawing conclusions from data sets obtained with these preparations. Currently we have a manuscript under consideration to discuss these issues and to propose an optimized solubilization procedure for Aβ to ensure that no interfering solubilization compounds are present in the final Aβ preparation. To inform the reviewer on this, we added a figure from this manuscript (Additional figure 4 for the referee only). We used fourier transform infrared spectroscopy (FTIR) to evaluate the presence of HFIP and DMSO which are both used in the Aβ solubilization procedure we developed. 1% solutions of these compounds have first been tested and additional figure panels 4A and B show that both have a characteristic fingerprint spectrum in FTIR. We use a desalting column to remove DMSO as we, and other authors have found in the past that DMSO affects the aggregation properties of Aβ. By collecting fractions of 150 L upon purification using the column, we determined the presence of both DMSO and HFIP in the different fractions. HFIP was absent in any of the fractions which means that the argon gas evaporation was sufficient to remove traces of HFIP. We also show that the concentration of DMSO increases with subsequent fractions of Aβ obtained from the column. Figure 4D shows the increase in typical intensity of DMSO in these fractions at 951 and 1011 cm-1. Up to fraction 8 (1200 L) no DMSO can be detected in the eluted Aβ from the column. We typically elute 500 - 1000 L Aβ solution from the column so we are confident that our samples are not contaminated with any of the solvents we use to aid dissolution of Aβ.

In the light of between-batch variation we performed all our assays with different batches (= lot numbers) of Aβ supplied by rPeptide. Approximately 2-4 different lot numbers have been used throughout this study consistently reproducing the results we presented in this manuscript. Before we started with the Aβ toxicity work we have tested also Aβ40 and Aβ42 batches from Sigma. We got very variable and inconsistent results with the peptides from this supplier, and have discontinued working with them, as the material from rPeptide appeared to give stable read outs over time. We have not taken the time to explore the reason for this problem with the Sigma peptides in further detail, as we stick to our current supplier. We have recently ordered Aβ42 from a third supplier (JPT Peptide Technologies GmbH,) which showed a similar aggregation profile (by Tht fluorescence and TEM) and synaptic toxicity as the peptide from rPeptide. We agree with the referee that it is extremely important to calibrate carefully the Aβ batch with one is working but once a good method of purification and reconstitution is obtained it is possible to obtain reproducible (and in our opinion reliable) results.

8. The description of what statistical tests are used and when they are used seems somewhat haphazard. Please run appropriate statistical tests describe these tests and use asterisks to indicate all statistically significant changes.

Referee 2 made a similar remark, in particular to figure 1. We have thus revised all figures and figure legends to indicate what tests were used and where signals reached statistical significance. We detail here a bit more the biophysical assays in figure 1. We performed triplicate experiments of separately prepared samples of Aβ. We averaged these data for the three different results and then displayed the calculated standard deviations over the average as error markers on the data bars shown in Figures 1B and C. P-values were derived from paired two-tailed student t tests. The time

resolution on ThT aggregation rates is approximately 6 min. The lag phase for example is calculated as the time at which the signal deviates more than 10% from the baseline signal. The difference between the repeats is often less than 6 min in time shift which then records as a standard deviation of 0. The actual standard errors calculated for the lag phases are therefore in many cases 0% as a result of the time resolution of the measurement (in fact this was the case for all ratios apart from ratios 0:10 and 1:9) and were always lower than 5%. We focused on the samples of interest: Aβ42:Aβ40 ratios 0:10, 1:9, 3:7, and 10:0 and have added the p-values to the bars representing these samples (see Fig. 1, B and C).

9. Wogulis et al in J. Neurosci 2005 elegantly show that fibril growth may be a key link to toxicity. I think a discussion of the possibility of kinetics growth of fibrils influencing the results should be included in the discussion. Indeed, this study is often ignored or not even by many in the field but provides an alternative conceptual framework for toxicity studies.

The publication by Wogulis et al (2005) concludes that, as long as the Aβ polymerization reaction is ongoing, there is toxicity. This finding is interesting as it suggests that, rather than a single defined Aβ oligomeric size the toxicity is related to the dynamics of the incorporation and rearrangement of Aβ into mature fibrils. The study is highly valuable as it shows that kinetic processes of Aβ rearrangements are of importance to induce Aβ-related toxicity, as well as for human amylin and non-A component of Alzheimer's disease amyloid plaques (NAC) and we added this reference to show that kinetics are supporting the relevance of studying time frames of Aβ behavior.

10. The preamble in the results regarding use of high concentrations of Abeta should be moved to the discussion or introduction.

We agree with the reviewer that the discussion is a place better suited for this remark and we have moved this paragraph therefore.

Referee #2 (Remarks to the Author):

1. Fig.1 E,F- was any statistical analysis performed for the quantitative data presented here? "small but reproducible increase" (page 7), -not clear, is this a statistically significant increase? Please show p values.

The same question was raised by referee 1 and we repeat here our answer. We performed triplicate experiments of separately prepared samples of Aβ. We performed triplicate experiments of separately prepared samples of Aβ. We averaged these data for the three different results and then displayed the calculated standard deviations over the average as error markers on the data bars shown in Figures 1B and C. P-values were derived from paired two-tailed student t tests. The time resolution on ThT aggregation rates is approximately 6 min. The lag phase for example is calculated as the time at which the signal deviates more than 10% from the baseline signal. The difference between the repeats is often less than 6 min in time shift which then records as a standard deviation of 0. The actual standard errors calculated for the lag phases are therefore in many cases 0% (in fact this was the case for all ratios apart from ratios 0:10 and 1:9) as a result of the time resolution of the measurement and were always lower than 5%. We focused on the samples of interest: Aβ42:Aβ40 ratios 0:10, 1:9, 3:7, and 10:0 and have added the p-values to the bars representing these samples (see Fig. 1, B and C).

2. Different Ab mixtures were incubated at 25C before addition to the cell culture. Thus, we know in what state Ab was at the moment when it is added to cells. But what happens to Ab species when they are at 37C in tissue culture? What are the changes that occur to Ab conformational state when Ab is present at different ratios at 37C? Need to show what happens to Ab mixtures after incubation at 37C during the time periods used to treat neurons. Unless, of course, Fig.3 TEM analysis was performed using Ab incubated over different periods of time at 37C. Please clarify; there is no mention of the temperature given in the text, methods, or in the figure legend for TEM.

We added a paragraph in the materials and methods section to explain how TEM figures were derived. The TEM data were collected at 25∫C. We did not repeat the experiments at 37∞C because the EM data are not directly relevant for what happens in the culture medium. The EM data are

simply a means to demonstrate the effects of the different peptides on fibril morphology. We addressed the issue raised by the referee however in figure 2D where we show that incubation of the peptides in culture medium at 37∞C for 40 min has no effect on the incorporation of ThT fluorescence.

3. The authors used "spontaneous fire rate" and synaptophysin staining as read outs of synaptotoxicity caused by elevated Aβ42/40 ratio. Both are markers of presynaptic integrity. What happens to postsynaptic responses (could be measured by EPSC and/or IPSC)? Do they remain intact or are they also affected?

Extracellular MEA-based recording can pick up APs, but it lacks sensitivity to detect a tiny postsynaptic activity in culture. Therefore we opted for the classic electrophysiological technique of whole-cell patch-clamp in order to see how Aβ mixtures affect EPSPs. The results are shown in Fig 3 in the manuscript. As the data obtained by means of patch-clamp consist of action potentials and EPSPs, we were able to quantify the effect of Aβ on both parameters. From our data it is clear that the Aβ (3:7, 2h) has a profound effect on the EPSPs frequency (Fig 3, C), indicating a fast block of post-synaptic depolarisations which eventually leads to a decrease in APs frequency (Fig 3, B). Similar pattern was observed for Aβ42 (10:0, 2h). Neither EPSPs nor APs frequency was altered by application of (1:9, 2h) ratio, what is again in line with MEA data. In contrast to (3:7, 2h) and (10:0, 2h), Aβ (0:10, 2h) significantly potentiated AP firing without affecting EPSPs. Our ongoing electrophysiological studies on mouse hippocampal slices aim on more detailed dissection of these mechanisms.

4. Western blots in Figure 3 B show the presence of small oligomeric species in (0:10) and (1:9) Ab mixtures (x2, x3, x4 of mainly Aβ40 assemblies?). However, the authors report that there was no synaptic and/or cell toxicity detected with these samples. Moreover, Fig. 2B shows positive effect of (0:10) and (1:9) on synaptic firing. Thus, it is somewhat confusing since it has been shown by many research groups that small oligomeric Ab species are toxic. Indeed, recent publication by Teplow's group (PNAS, 2009) showed that Aβ40 small order assemblies (dimers, and especially trimers and tetramers) are the most toxic Ab species. Please clarify/comment. Are Aβ40 small oligomers inert or even beneficial for neurons in your experimental settings?

This question by the reviewer is a heavily debated and a very controversial issue in the field. Several groups have suggested specific oligomer sizes to be responsible for Aβ toxicity, including dimers (Kluybin et al. J. Neurosci. 28: 4231-4237 (2008)), trimers (Townsend et al. J. Physiol. 572: 477-492 (2006)), Aβ species with a molecular weight of 56 kDa (LesnÈ et al. Nature 440: 352-357 (2006)), Aβ-derived diffusible ligands (ADDLs) (Gong et al. PNAS 100: 10417-10422 (2003)) and protofibrils (Kayed et al. J. Biol. Chem. 284: 4230-4237 (2003)). Others propose that toxicity is not directly correlated with a specific oligomer size but to certain Aβ conformations, and that toxicity is an evolving property in Aβ mixtures closely related to oligomerization and fibrillization, which are also dynamic processes (Wogulis et al. J Neurosci 25(5): 1071-1080, (2005). The experiments of Dr. Teplow (Ono et al, PNAS 2009) are an elegant study to tackle this difficult question at the molecular level. They use a cross linking approach and fractionation/SDS-PAGE to stabilize and purify specific intermediates along the equilibria that govern the progressive assembly of Aβ peptides into various oligomers and fibrils. They tested their toxicity in various assays. They find that dimers are about 3-fold and tetramers are about 13-fold more toxic, demonstrating a nonlinear relation between size and toxicity within the narrow frame they could actually investigate this question (oligomers larger that tetramers were, for technical reasons, not included). They also indicate in their manuscript that they cannot answer the question whether higher order assemblies could even be more toxic as they were not able to isolate sufficient cross linked oligomers of higher order. Important for our work is that these authors show that toxicity is distributed over different oligomeric Aβ species, an interpretation that we also propose. We find, similar to the work by Ono et al. (2009), that toxic conformations can be associated with different sizes. The FTIR experiments (sensitive to secondary structural organization) pick up these dynamic differences, while neither the SDS-PAGE/western blot techniques, nor AFM, apparently are able to show major differences between the different preparations. At least in our hands, we find in all preparations SDS resistant dimers and tetramers to similar extents (see supplemental figure S2). It is our experience that SDS PAGE seems to favor this type of separation and that the presence of these species is not necessarily correlated with what is present in solution (as determined for instance by Static Light Scattering (SLS), see also our previous paper Martins, Kuperstein et al, EMBO 2008). We conclude that Aβ

oligomer size per se does not dictate the toxic impact, but that structural parameters are determinative and we have now more clearly explained this in the new version of our manuscript.

5. There are some discrepancies between the kinetics of aggregation data shown in Figure 1D-F, Figure 3 and Figure 4. For example, lag phase/nucleation time is longest for (0:10) and (1:9) in Fig.1 (ThT). However it seems not to be the case for (1:9) as detected by FTIS (Fig.4B). Fig. 1D,F show that elongation time is fastest in (0:10), i.e. that monomers/amorphous Abeta converts into fibrils very rapidly, but TEM shows that first fibrils are detected not in (0:10) but in (10:0, by 4 hrs). The authors couldn't see fibrils in 3:7 samples by TEM after 4 hr incubation period (Fig.3), but they detect "ThT positive oligomers" at 4hrs (Fig.1D) and "transient intermediate" in Fig.4E. The relationship between "oligomer" and FTIR "intermediate" is unclear and creates confusion. Also, the authors need to clarify whether in all these aggregation experiments the same concentration of Ab was used (is it 50uM in Fig.1 D-F but 100uM in Figs. 3 and 4?). It will help if they present their results more clearly.

The reviewer correctly observes that all assays we performed have different lag phases. In addition to this, the order of the lag phase length is not the same over the different assays for the four Aβ42:Aβ40 ratios tested. This is in fact not worrying but rather informative as the different assays analyze different aspects of the aggregation process. E.g. TEM monitors the growth of mature fibrils while the FTIR probes for the formation of -sheet structure. Thioflavin T is thought to interact with -sheet like aggregates although the precise mode of interaction for Tht with fibrillar proteins remains to be confirmed. Nevertheless, this assay is high throughput and provides for rapid information on the aggregation kinetics of proteins. It is for this reason one of the most frequently employed means to obtain this kind of information. The use of various complementary approaches to study the aggregation process of Aβ allows us to study in much more detail the aggregation pathway and conversion processes involved toward the formation of mature aggregates. For example, ratio 1:9 has a lag phase of 3 h before ThT intensity starts to increase and it takes up to 6 h to start visually observing mature fibrils formed using TEM. In FTIR no lag phase is observed and the reaction is already halfway to completion before 1 h of incubation. This is highly informative as to the pathway of aggregation of ratio 1:9: it means that, first, Aβ in this ratio undergoes extensive conformational changes (FTIR), then it starts to form a Tht-positive assembly and only then it starts to form mature fibrils (TEM). For pure Aβ42 (ratio 10:0), the sequence of events is not dissimilar from ratio 1:9: only a very short lag phase in Tht after which a Tht-positive process initiates. Visual fibril formation is observed around 4 h (while small aggregates are formed already after 1.5 h incubation). FTIR shows no lag phase and very rapid formation of a -sheet-enriched conformation. For ratio 3:7 these observations deviate: the lag phase is, similar to Aβ42 very short (approx. 30 min). Then a slow start of Tht positive reaction is observed, as a little knick (shoulder) in the curve. However only after a lag phase of 1.5 h the actual polymerization reaction kicks in. By TEM no mature fibrils can be observed until 9 h of incubation and the conformational change observed by TEM is 50% completed at an incubation time of 2 h. This means that Tht-positive assemblies coincide with a conformational change in the sample but that this state is sustained for an extended time period until mature fibrils are formed which can be observed by TEM. Thus, smaller oligomeric species are present with a longer life time (so they are stabilized) for ratio 3:7. We include this information an discussion in the new version of the MS.
Concerning the differences in concentrations used for the various assays, unfortunately all biophysical assays have different requirements for Aβ concentrations to obtain optimal results so it is not possible to do all of them at similar concentrations. Again, the different assays are used to see how the different mixtures behave biophysically and are used to characterize the samples. We do not associate a specific biophysical property with toxicity, apart from the correlation we see with the intermediate structure observed in FTIR and the synaptotoxicity in MEA.

6. Figure 4 E and F: It is not clear and needs better explanation how the analysis was done and how curves (Fig 4E) were generated. What is "flat curve around zero"?

We have removed figure E from the manuscript as it is indeed confusing. This was a theoretical curve deduced from the curves in panels A-D to illustrate that in case of the non toxic ratios only two states are observed, with the gradual transition of unstructured to -pleated sheets, while in the toxic mixtures, some intermediary exists that we cannot capture in this assay. We have now attenuated this conclusion and rewritten the paragraphs hopefully in a more clear way.

Additional figures for the referees only

Additional figure 1: Thioflavin T fluorescence calculated versus experimental aggregation rates of Aβ42:Aβ40 ratios do not overlap suggesting interaction between Aβ40 and Aβ42.
(A). Experimental aggregation rates of Aβ42:Aβ40 ratios 0:10 (blue), 1:9 (green), 3:7 (red) and 10:0 (black) determined by Thioflavin T fluorescence (identical to Fig. 1A in main manuscript). (B). Experimental (open symbols) versus calculated (closed symbols) aggregation rates of Aβ42:Aβ40 ratios 0:10 (blue), 1:9 (green), 3:7 (red) and 10:0 (black). Calculated aggregation rates of Aβ42:Aβ40 ratios 1:9 and 3:7 have been derived by calculation of the proportional contribution of pure Aβ42 and Aβ40 aggregation rates based on the assumption that the aggregation rates of Aβ42 and Aβ40 are not affected by the presence of each other. (C). Combination from panel A and B: Experimental (open symbols) versus calculated (closed symbols) aggregation rates of Aβ42:Aβ40 ratios 1:9 (green), 3:7 (red) for direct comparison. Calculated rates have been obtained as explained for Figure 1 B. Equations used are: Calculated aggregation rate 1:9 = (0.1 x experimental aggregation rate Aβ42) + (0.9 x experimental aggregation rate Aβ40), Calculated aggregation rate 3:7 = (0.3 x experimental aggregation rate Aβ42) + (0.7 x experimental aggregation rate Aβ40)

Additional figure 2: Toxicity of Aβ42 10:0 and Aβ42:Aβ40 ratio 3:7 is related to soluble Aβ.
(A). Aβ was dissolved at a concentration of 50 M and vortexed for 1 min prior to collecting an aliquot for concentration determination. Two L samples were diluted in 98 L Bradford reagent, incubated for 10 min and the total concentration of Aβ in the sample was determined for absorbance at 595 nm using a spectrophotometer. To obtain supernatant, an aliquot of 30 L was centrifuged using an Eppendorf tabletop centrifuge at a speed of 13.4 x 1000 rpm for 1 min. The top 20 L was collected and 5 L supernatant was incubated for 10 min with 95 L Bradford reagent. The concentration of Aβ in the supernatant was determined spectrophotometrically using absorbance at 595 nm. Under the conditions used Aβ42 remains soluble for the shortest time, after 6 h there was no soluble Aβ42 anymore, while ratio 1:9 remained soluble for the longest time, after 46 h still 100% of Aβ was soluble after which solubility slowly declined. Aβ40 remains mostly soluble until 46 h and ratio 3:7 looses solubility between 20 and 30 h. (B). Supernatants were prepared in a similar manner as described for panel 2A: centrifugation at 13.4 x 1000 rpm for 1 min. The supernatant was then added to primary neurons for MEA and the spontaneous firing rate was determined and expressed as a % of the initial firing rate without addition of Aβ. At time=0 neither of the ratios tested, Aβ42 and ratio 3:7 are toxic, which is consistent with previously published data confirming that monomeric Aβ is not toxic. After 4 h both ratios are highly toxic and still soluble. After 26 h Aβ42 is not soluble anymore and the supernatant of this peptide is not toxic anymore. The ratio 3:7 is toxic at this time point as well as soluble.

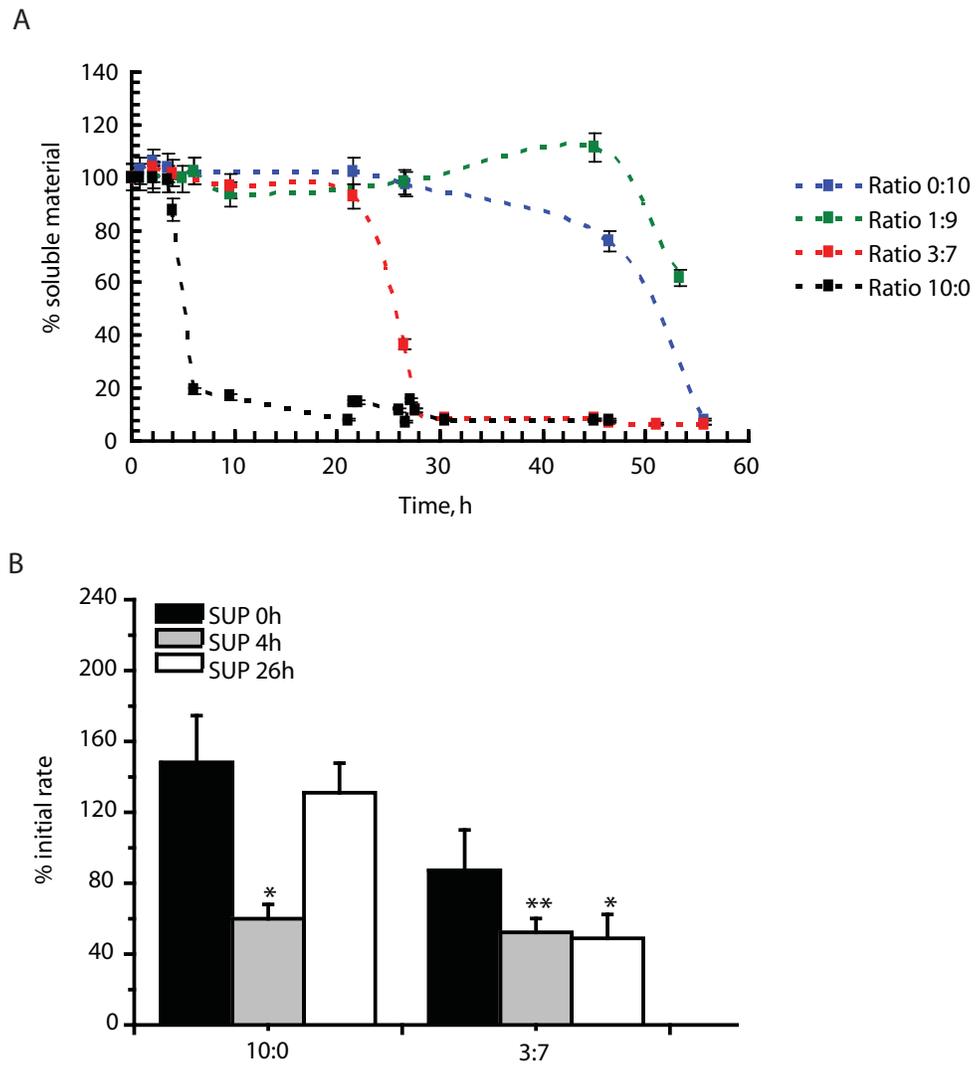Additional figure 3: Diluted concentrations of Aβ42 and ratio 3:7 and the effect on synaptic activity.
(A) Changes in spontaneous firing rates as a function of Aβ42:Aβ40 (10:0) (2 h) and 3:7 (2 h) concentrations applied to neurons on MEAs for 20 min; n=2-3. Notice a fast inhibitory effect produced by cytotoxic concentration (10 M) of Aβ. Slight potentiation of synaptic response by high nanomolar concentrations of Aβ oligomers did not reach statistical significance after 20 min, but was synaptotoxic after overnight bath application as illustrated by representative signal traces (B).

Additional figure 4: Validation of absence of HFIP or DMSO from the Aβ samples.
(A ñ B) Fourier Transform Infrared Spectroscopy (FTIR) of (A) 1% HFIP and (B) 1% DMSO shows that both compounds used for solvation of Aβ peptide have characteristic spectra which can be used to identify contamination of the eluted Aβ by HFIP or DMSO. (C) FTIR spectra of 150 L elution fractions from the desalting column. HFIP is efficiently removed from all fractions (no peak at 1190 cm-1 characteristic for the presence of HFIP) while with increasing fraction number a gradual increase in the concentration of DMSO is detected by the increasing absorbance intensities at 951 and 1011 cm-1. (D) Up to and including fraction 8 (1200 L) can be eluted from the desalting column without contamination by DMSO. The intensities of FTIR peaks characteristic for the presence of DMSO (1011 cm-1 and 951 cm-1) are plotted against eluted fraction number.
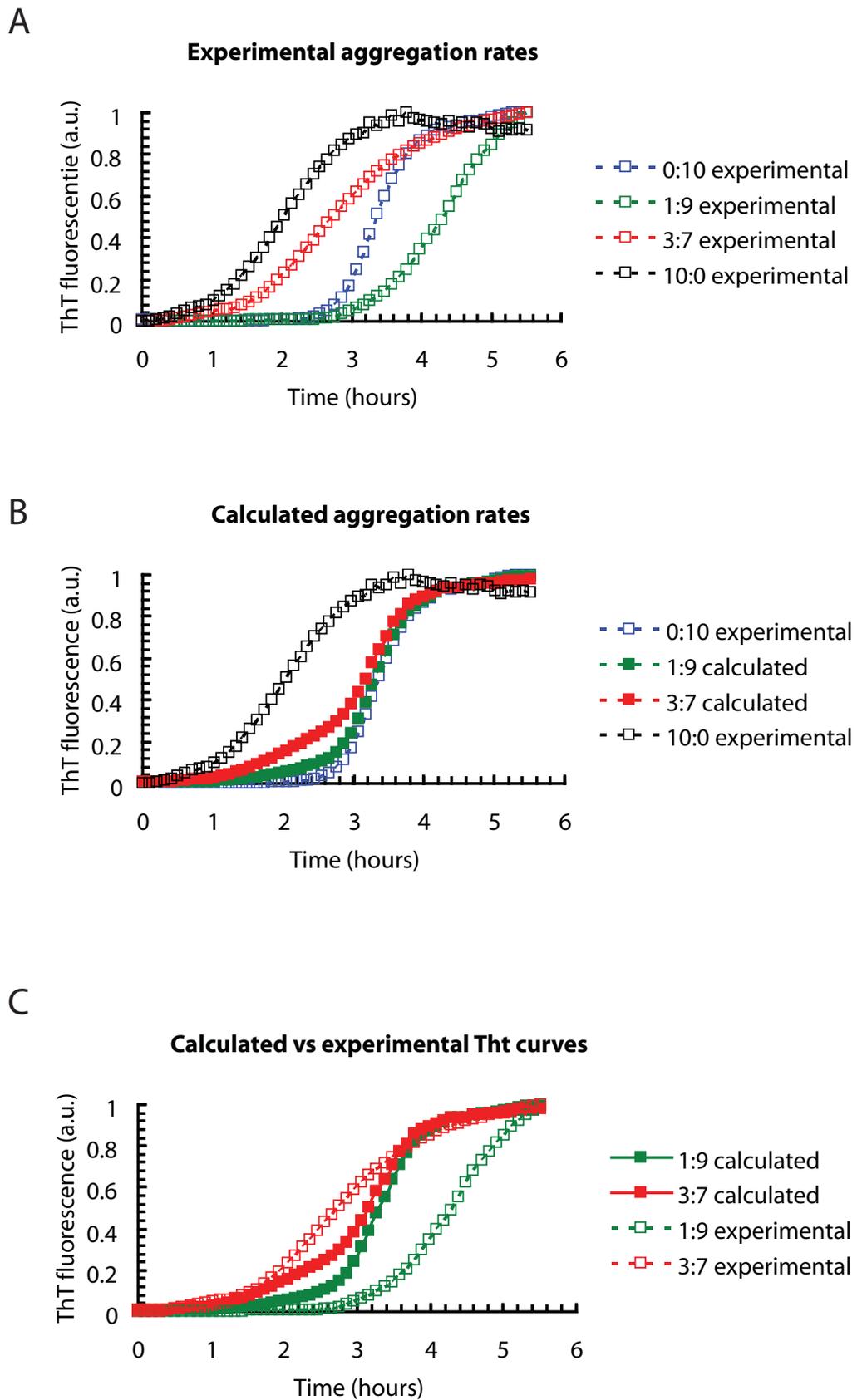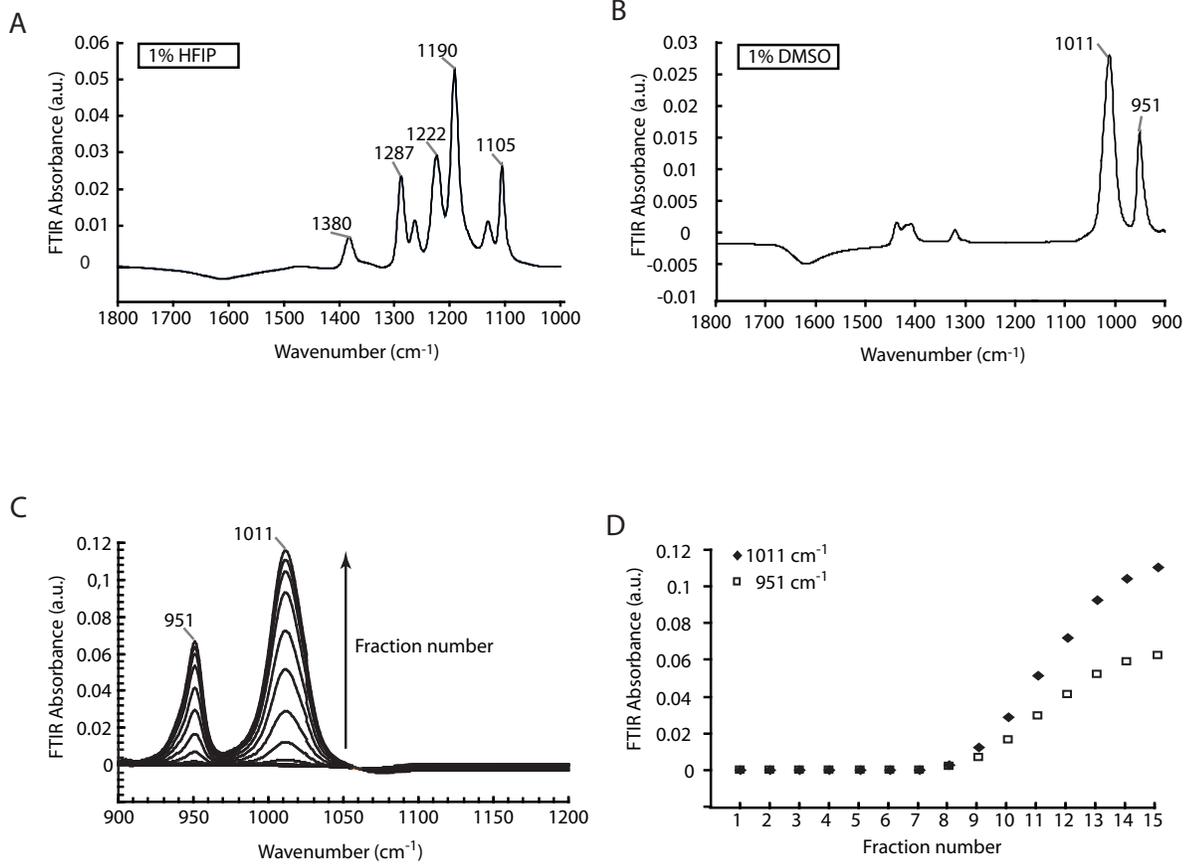
Figure 1 - Thioflavin T fluorescence calculated versus experimental aggregation rates of Aβ42:Aβ40 ratios do not overlay suggesting interaction between Aβ40 and Aβ42.

A

B

Figure 2 - Toxicity of Aβ42 and Aβ42:Aβ40 ratio 3:7 is related to soluble Aβ.

A

B
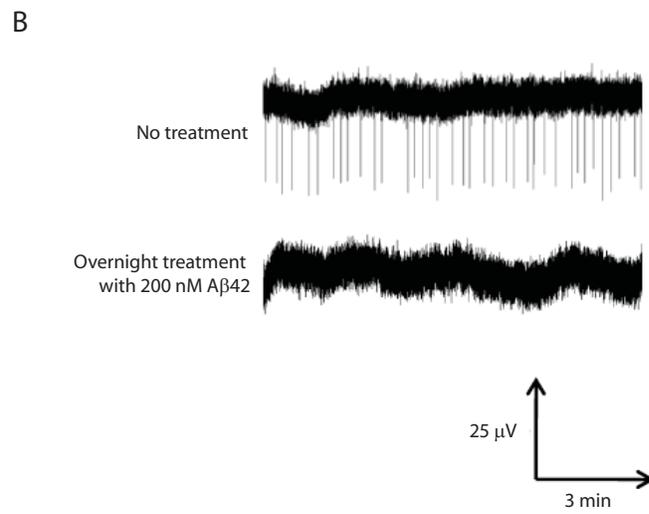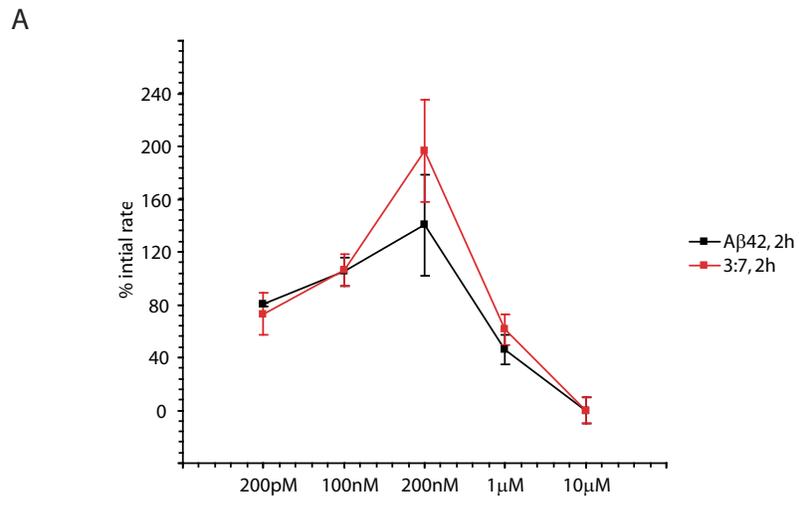
Figure 3 - Dilute concentrations of Aβ42 and ratio 3:7 and the effect on synaptic activity.

Figure 4 - validation of absence of HFIP or DMSO from the Aβ samples.

2nd Editorial Decision	28 July 2010

Thank you for submitting your revised manuscript to the EMBO Journal. I asked the original two referees to review the revised version and I have now received their comments. As you can see both referees are pleased with the introduced changes and appreciate the work that has been put into the revision. Referee #2 has a few remaining issues, that I would like to ask you to respond to in a final revision.

When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

I am looking forward to seeing the final version.

Best wishes

Editor
The EMBO Journal

REFEREE REVIEWS

Referee #1 (Remarks to the Author):

The authors did a superb job of responding to the critiques and have improved a strong manuscript further.

Referee #2 (Remarks to the Author):

The authors addressed most of my concerns except for the two issues (# 4 and #8 in the original comments to authors) that they have not provided any additional info and/or did not comment on. #4: The authors report that 3:7 and 10:0 Abeta mixtures had negative effect on synapses and show A11 immunoreactivity at the synapses (Fig.2). On the contrary, it seems that (1:9) and especially (0:10) ratios enhance synaptic firing after 40 min incubation on cells, compared to that in control (Fig.2B,C). The authors claim that there was "no deposition of A11 staining at the synapse in (0:10) sample" but was there an Abeta (e.g. 6E10) staining? Is positive modulatory effect of the (0:10) induced by direct interaction of Abeta40 with the synapse? What is the difference between 0:10/1:9 (mainly Abeta40) "positive" and 3:7/ 10:0 "negative" modulation mechanisms? The authors should comment on this.

#8 Figure 5 (now it is Figure 6). Please show western blot for cleaved caspase-3 since immunostaining is less accurate for subtle changes. Also, caspase immunorectivity in 1uM, (3:7), 12 hr treated cells in (A) and (E) is very different: no caspase IR in (A) but a lot of green dots in (E). Please address this discrepancy. It is also somewhat surprising that titer-blue viability assay for 3:7 and 10:0, 2hr, still detects ~25% of live neurons after 48 hours of treatment, since 12 hour treatment shows that pretty much every nucleus is PI-positive (red), which indicates that the cell membrane integrity is already jeopardized. Highly unlikely, that the neurons survived for another 36 hours after that.

The cleaved caspase immunostaining that the authors show is important to detect sub-cellular localization of the activated caspase. However, for any quantitative analysis of caspase activation Western blot detection is necessary.

Referee #1 (Remarks to the Author):

The authors did a superb job of responding to the critiques and have improved a strong manuscript further.

We thank the referee for this nice comment.

Referee #2 (Remarks to the Author):

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The referee is right that we have some indications for a positive modulating effect of the non-pathological ratios, as he/she already pointed out in the previous revision. We have responded to this remark with additional documentation of the effect in the current revised paper. Although we find the observation very interesting and plan to have a further look at this in further investigations, it should be said that other authors have already demonstrated such effects, i.e. Abeta peptides affecting electrical activity of the synapse. We feel that addressing the additional questions raised by the referee will require much more work than some simple immunohistochemical stainings and that further analysis of this issue is not within the scope of the current paper.

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The cleaved caspase immunostaining that the authors show is important to detect sub-cellular localization of the activated caspase. However, for any quantitative analysis of caspase activation Western blot detection is necessary.

Again, we agree with the referee that further work is possible and that by repeating the displayed experiments or performing variations of these experiments will probably result finally in the 'perfect' experiment. On the other hand, the quality of the immunostainings shown in the current version of the manuscript is high and should be compared with what is (and can be reasonably) expected in the field. Further detailing and fine tuning the observed effects will indeed not affect our conclusions in any way. We agree with the referee that the data in the criticized panels have to be interpreted in a qualitative way and we adapt our wording describing those results accordingly, but the experiments as they are displayed now clearly make the point we want to make: reproducible differences between 3:7 and 10:0 ratio's compared to 0:10 and 1:9 ratios. These effects are very consistent within experiments, but results differ quantitatively depending on the particular batch of primary

neurons used for the experiment. This explains why we see much more caspase staining in panel E than in panel B. Such batch to batch variation is not unusual when working with primary neuronal cultures, and it is therefore important to compare effects of any treatment between culture dishes from the same batch of cells. Within such experiments effects are very consistent and reproducible.