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Quantitative modeling of amyloidogenic processing and its influence by SORLA in Alzheimer's disease

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1st Editorial Decision 14 April 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. Let me first of all apologise for the delay in getting back to you with a decision. Unfortunately, we experienced difficulties in finding suitable and willing referees for this manuscript. In addition, two of the referees were not able to return their reports as quickly as initially expected.

Three referees have now evaluated the manuscript, and their comments are shown below. As you will see, while referees 2 and 3 are more positive and would support publication here after appropriate revision referee 1 is not in favour of publication of the study here. Essentially, he/she raises two types of concerns: issues regarding the physiological significance of and the conceptual advance provided by your findings. On balance, we have decided to follow the positive majority vote here. We will therefore be able to consider a revised version of the manuscript that addresses the issues raised by the referees in an adequate manner. It will be important, however, to address the major point 1 of referee 1 by further experimentation. Referee 1's second major point should be addressed by text changes.

I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version as well as the final assessment by the referees.

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We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

Schmidt and colleagues used classical models of enzyme biochemistry and kinetics to quantitatively model the proteolytic processing of the Alzheimer protein APP either in the presence or the absence of the SORLA receptor. Previous work from the same lab and from others implicated SORLA as a genetic risk factor for Alzheimer's disease and demonstrated that SORLA forms complexes with APP and limits the proteolytic processing of APP.

The current work is a beautiful and novel approach to apply quantitative measures to describe APP processing and to derive mechanistic insights into the stoichiometry of this proteolytic cleavage. However, my major criticism is two-fold: the lack of truly novel insights into the mechanism of APP cleavage and the use of overexpression systems, which may not be physiologically relevant. The work is of more specialized interest to scientists studying APP processing or the biophysics of membrane protein catalysis.

Major points:

1. The authors strengthen the importance of studying SORLA, because reduced SORLA expression is considered a risk factor for Alzheimer's disease. However, CHO cells with overexpression of APP and SORLA are used. Expression levels of both proteins are much higher than under endogenous conditions (in particular for APP). Thus, at least the critical findings of the quantitative measurements need to repeated in more relevant cells, such as neuronal cell lines, where the endogenous SORLA expression is reduced, for example by knock-down approaches.

2. The conclusions are not truly novel. It has been known for a long time that APP is a dimer and that the beta-secretase is a dimer and that dimerization of both proteins strongly affects APP processing. It has also been clear from previous work by the authors how SORLA affects APP processing.

Minor points:

3. ADAM10 expression should be shown in addition to TACE expression, given that several studies last year, but also Buxbaum et al. JBC 1998, ruled out TACE as alpha-secretase.

4. Figure 1B: Show endogenous APP and possibly SORLA levels as a control. Otherwise, it remains unclear how leaky the system is and where the 0% base line is. If the expression at 77 /78 hours was down to 0% (instead of 20-40%), the effects would even be stronger than described.

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5. Figure 2: I do not see how the data in panel A lead to the strong differences in the quantification in B. The fixed protein levels of APP or SORLA in A seem to remain constant in all lanes, whereas the regulated APP and SORLA levels decrease to a similar extent. Thus, the ratios in B. should not differ strongly. The authors should include a quantification of the APP and SORLA levels from all conditions in A and not only show the ratios.

6. The ELISA for APP measurements needs to be described in the methods' section. How do the authors distinguish between full-length and soluble APP in the cell lysate? Antibody 6E10 would detect both as well as C-terminal APP fragments.

7. Figure 3: Is velocity of sAPP generation linear over the 24 hours or how was the 24 hour time point chosen?

8. Figure 4: Are these the same data as in figure 3? This should be indicated.

9. Figure 5: Why is the reduction in Abeta stronger than the reduction of sAPPbeta?

Referee #2

This paper presents a quantitative model for the proteolytic processing of amyloid precursor proteins (APPs) and the regulation of this process by SORLA, a receptor protein which inhibits this process and has been shown to be a genetic risk factor for certain forms of Alzheimer's disease. The quantitative model consists of two main parts: an experimental expression system in which the levels of APP and SORLA can be varied continuously with the application of tetracylcline, and a mathematical model that is used to encode and test mechanistic hypotheses based on the experimental data. Secretase enzymes catalyze the proteolysis of APPs in the initial step of process that can lead to either amyloidogenic or nonamyloidogenic products. The quantitative expression system developed here enables detailed characterization of the kinetics of this process inside live cells over 1-2 orders of magnitude in APP concentration - a wide enough range to establish the cooperative or noncooperative character of the activity. Interestingly, it is found that these enzymes have sigmoidal dose dependence in the absence of SORLA, but that the response switches to hyperbolic in the presence of the inhibitor. The experimental evidence for this switch is clear and convincing. Further characterization of the enzyme mechanism shows that 1) secretase activity itself is not demonstrably effected by SORLA and 2) the presence of SORLA inhibits the oligomerization of APP through binding to a region of the protein that prevents dimerization. These observations suggest a mechanism in which the secretase enzymes, which themselves are known to dimerize, act preferentially on dimerized APPs. This mechanistic hypothesis is tested by the construction of a mathematical model in the form of ODE's, which is shown to fit the data well, in contrast to models that allow secretase to act only on the monomeric or dimeric form of the substrate. Although this does not prove the model is correct, it does provide strong support for the model, which embodies the most complete description of the biochemistry of this clinically-relevant process to date. Beyond its interest to those studying the biochemistry related to Alzheimer's disease, this study represents a very nice example of combining quantitative experimental approaches with modeling to discover important biochemical mechanisms. The model could also form the basis for future extended models of this important system that consider other possible sources of regulation.

Overall, the paper is clearly written and the results shown in the figures provide strong support for the claims made in the paper. The main weakness of the paper, from my point of view, is that the model is not described in much detail and in particular it is difficult to determine which aspects of the model are clearly supported by the current fits to experimental data and which aspects are more uncertain. I have some more specific comments below.

1. The process of fitting the parameters to model data should be more completely described. The last line of the supplement indicates that a substantial number of replicates was performed in the process: "For the local estimates, the switch of the mode of secretase action from dimers to monomers under the influence of SORLA was reproducible for 80% of the fits." Thus, it should be possible to indicate which qualitative aspects of the model are well-supported by the data and which aspects may not be. This would be very helpful in evaluating the mechanistic choices made in

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developing the model and which of those choices needs to be further tested. My questions below raise a few such possibilities.

2. What is the significance of secretase dimerization or of the model restriction that the monomeric form of the enzyme acts only on monomeric substrates and the dimeric form acts only on dimeric substrates?

3. How well can the affinity of SORLA binding can be estimated? In particular it would be interesting if the model could be used to identify an intermediate level of SORLA expression at which the dose-dependence of the kinetics would be intermediate between the cooperative and non-cooperative regimes shown in Figs. 3 and 4.

Referee #3

This is a fascinating manuscript that applies a highly novel analysis to the question of how SorLA modulates metabolism of the Alzheimer's amyloid precursor protein.

SorLA (SorL1/LR11) is genetically associated with late onset Alzheimer's disease (LOAD) and decreased protein levels have been reported in the brains of patients with mild cognitive impairment and LOAD.

Previous work by Schmidt and colleagues has demonstrated a role for SorLA in the intracellular trafficking of APP and regulation of Aβ generation. It is proposed that SorLA has pleiotropic functions in APP trafficking and regulates Aβ generation through the following mechanism; 1) retromer dependent retrieval of APP from endosomes to the trans Golgi network TGN, 2) competitive interaction with BACE thereby preventing APP-BACE interaction and 3) as a retention factor for APP within the TGN preventing APP transit into the amyloidogenic and non-amyloidogenic pathways.

Schmidt and colleagues provide further novel insight here into SorLA regulation of APP processing. Through Doxycylcine regulatable expression of SorLA and APP and mathematical modeling, the authors demonstrate that SorLA inhibits APP oligomerization and thereby decreases the affinity of both the α and β but not γ- secretase machinery for APP. The authors are to be commended for having employed appropriate caution in the titling of the paper (i.e., they use the word "modeling").

Issues to be addressed by the authors:

1) The authors state that their data demonstrate that the α and β secretases are allosteric enzymes that depend on APP oligomers for efficient processing. This statement suggests that APP oligomerization is necessary for efficient activity of α/β secretases. The authors should make it clear that APP oligomerization increases α/β secretase affinity for APP but not other α/β secretase substrates.

2) The authors use CHO cells for experiments in the absence of SorLA. Is SorLA endogenously expressed in CHO cells? Confirmation or reference to these experiments is required or make it clear that they mean in absence of human SorLA.

3) In figure 2 the molar ratio decrease of SorLA detected by ELISA following doxycyline treatment is described as only 2.5 fold reduction. This doesn't appear to be representative of the western blot which shows almost complete reduction of SorLA protein expression. Is there any explanation why the western and ELISA don't correspond especially since ELISAs are used from there on in to quantify SorLA expression levels.

4) Figure 9 demonstrates that in the presence of SorLA, APP oligomers do not form. The text also states that SorLA can reversibly form a complex with monomeric APP to reduce the formation of substrate oligomers, the authors should reference this interaction appropriately from previous observations.

5) The authors previously demonstrated that SorLA interaction with BACE decreases association of

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BACE with APP and reduction in BACE cleavage of APP, this should be included in the discussion where they discuss that SorLA doesn't functionally interact with the α or β secretases.

1st Revision - authors' response 26 June 2011

COMMENTS TO REFEREE #1:

Major point 1: *" Thus, at least the critical findings of the quantitative measurements need to repeated in more relevant cells, such as neuronal cell lines, where the endogenous SORLA expression is reduced, for example by knock-down approaches. "*

We agree with the reviewer that appropriate caution should be used when interpreting cellular overexpression systems for APP processing. We had indicated this caveat of overexpression systems in the introduction section (page 3, last sentence of first paragraph). However, the generation of quantitative data sets for systems biology approaches critically requires the use of recombinant expression systems to enable modulation of target gene expression at will.

As requested, we now confirm critical findings of the quantitative measurements on endogenous proteins in the neuronal cell line SH-SY5Y by knockdown approaches. As shown in figure 7, application of siRNA enabled us to modulate the levels of endogenous SORLA in this cell line and to quantify the consequences for endogenous APP processing over a wide concentration range. This new data set fully confirms our previous conclusion from CHO cell experiments that subtle changes in SORLA expression directly affect APP processing rates.

In addition to the experiments in SH-SY5Y cell lines suggested by the reviewer, we also performed several new experiments that fully substantiate the relevance of our findings for the *in vivo* situation.

Thus, we demonstrate the ability of SORLA to interfere with APP dimerization in the mouse brain *in vivo*. As shown in figure 10B, two immuno-reactive bands corresponding to monomeric and oligomeric forms of APP are seen by native PAGE in wild-type mouse brain extracts. In contrast, only the oligomeric APP complex is present in tissues from SORLA knockout mice, demonstrating increased APP complex formation in the absence of this receptor. To the best of our knowledge, this is the first experimental proof of the existence of APP dimers *in vivo*, and, of course, for the ability of SORLA to prevent complex formation in the brain.

In addition, we accurately determined the molar ratio of APP and SORLA in the human brain using autopsy material. These studies demonstrate a physiological molar ratio of APP to SORLA of 1.2 : 1 in this tissue. This ratio compares to the molar ratios of APP : SORLA in our CHO cell lines that vary from 17.7 : 1 to 1.4 : 1. Thus, our overexpression systems even underestimate the relevance of SORLA for APP processing *in vivo* as significantly less receptor molecules are present per APP molecule in CHO cells as compared to the human brain. We have revised the text in the discussion section to more clearly convey this information (page 17, line 4 from above).

Taken together, we strongly believe that perfect agreement of our theoretical simulations with a host of biochemical data on recombinant proteins in CHO cells, on endogenous proteins in neuronal cell lines, and on the respective proteins in the mouse and human brain fully substantiate the physiological relevance of our systems biology approach to Alzheimer's disease research.

Major point 2: "*The conclusions are not truly novel. It has been known for a long time that APP is a dimer and that the beta-secretase is a dimer and that dimerization of both proteins strongly affects APP processing. It has also been clear from previous work by the authors how SORLA affects APP processing."*

We respectfully disagree with the reviewer on the assumption that our conclusions lack novelty for the following reasons:

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1) While the existence of APP and BACE dimers has been described before, the physiological relevance of APP dimerization remained disputed. Interpretation of previous data was complicated by the fact that in some cases APP peptides rather than the full-length protein were used. Alternatively, mutant forms of APP have been studied where dimerization motifs had been disrupted or, in the converse situation, where amino acid changes were introduced to force APP dimerization by artificial intermolecular disulphide bridge formation. Potentially, mutagenesis of the juxtamembrane region of APP (where dimerization occurs) may also affect the interaction of APP with secretases and alter processing independent of whether or not dimers are formed. Our study is the first to address the consequences of dimer formation of the wild-type full-length APP in the context of an intact cell. More importantly, our study is the first to report the existence of APP dimers in the brain *in vivo*, and the ability of SORLA to prevent dimer formation (Fig. 10B).

2) Concerning the relevance of APP dimer formation, one of the most widely discussed hypothesis states that dimerization is pathophysiologically relevant because APP dimers are the substrate to produce dimeric C99 and, subsequently, dimeric Aß species, neurotoxic variants of the amyloid-ß peptide. Our study now uncovered an alternative reason for APP dimer formation inasmuch as it enables secretases to act as allosteric enzymes in a cooperative mode of action. This observation has fundamental implications for our understanding of Alzheimer's disease (AD) that reaches far beyond the mere concept of enzyme kinetics. As opposed to enzymes following normal Michaelis-Menten kinetics, allosteric enzymes act by an on-off switch mechanism enabling rapid adaptation of enzyme activity to even subtle changes in substrate concentration. This notion is exemplified $Aß_{40}$ production in this study where a rise in enzyme activity from 10% of V_{max} to 75% of V_{max} is achieved with only 4.8-fold rise in substrate concentration. This unique finding may hold the key to understanding how even subtle changes in APP metabolism may have such dramatic consequences as seen in patients with sporadic AD.

3) With respect to SORLA action in AD, the ability of this receptor to bind to APP and to prevent proteolytic processing has been well established by several studies. Yet, the mode of action(s) of this important risk factor remained poorly understood. Our study now identified the ability of SORLA to block formation of APP dimers, thereby eliminating the preferred secretase substrates and to impair processing. Most importantly, revisions to our manuscript now substantiate this mode of action in the mouse brain (Fig. 10B), identifying control of APP dimerization as a new paradigm in regulation of amyloidogenic processing *in vivo.*

4) Further to the role of SORLA as one of the most important risk genes in sporadic AD, our study provides important new data to support this hypothesis. Thus, epidemiological and histopathological studies have suggested a mere 25% reduction in SORLA mRNA and/or protein in patients with sporadic AD. Whether such minimal changes in SORLA activity may bare any relevance for disease progression remained strongly disputed by many and has been the topic of editorials and discussions in the ALZ forum. Our study now provides functional proof of a clear dose-dependent action of SORLA with a linear correlation of receptor activity with amyloidogenic processing over a wide range of concentrations. As suggested by this reviewer, we now have confirmed this relationship not only in transgenic cell lines but also on endogenously expressed proteins in neurons (Fig. 7).

5) Irrespective of all the considerations above, we strongly believe an important advance to science made by this study is the application of mathematic modeling to evaluate the contribution of genetic risk factors to Alzheimer's disease. Perhaps more than any other disease entity, AD is a biological process controlled by quantitative aspects of protein functions. In particular, subtle changes in efficiency of APP metabolism and Aß formation are believed to be the main mechanism whereby genetic risk factors over the course of a life-time affect onset and progression of disease. In this study, we now have developed the first mathematical description of the influence of SORLA, a major risk factor in AD, on APP cleavage by α and ß secretases. As suggested by reviewer 2, we significantly extended our modeling to intermediate concentrations of SORLA (as seen in patients with AD) enabling us to identify critical concentrations for the inhibitor when secretases switch between efficient cooperative and inefficient non-cooperative mode of actions. Good agreement of our mathematical model with experimental data from recombinant proteins in CHO cells, from endogenous proteins in neurons, and from findings in the murine and human brain in this manuscript strongly support the notion that our systems biology approach represents a major conceptual advance to AD research likely to affect the way we study AD processes in the future.

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Minor point 3: *"ADAM10 expression should be shown in addition to TACE expression, given that several studies last year, but also Buxbaum et al. JBC 1998, ruled out TACE as alpha-secretase*."

We appreciate this helpful comment and we now included Western blot analysis of ADAM10 in the inset to revised figure 8A. No difference in expression levels of ADAM10 are seen comparing CHO cell lines with or without SORLA.

Minor point 4: "*Figure 1B: Show endogenous APP and possibly SORLA levels as a control. Otherwise, it remains unclear how leaky the system is and where the 0% base line is."*

As suggested, we now included additional data demonstrating lack of expression of endogenous SORLA and APP in parental CHO cells (Suppl. figure 2). Thus, the residual expression of SORLA and APP seen after 77 hours of 1 ng/ml doxycycline application in figures 1B and of 10 ng/ml of doxycycline treatment in Fig. 2A represents leaky expression of the human proteins from the respective pTet constructs.

We have more clearly stated this fact now in the revised result section (page 5, line 3 from below). The text reads… The residual levels of APP and SORLA seen in transfectants at 10 ng/ml of doxycycline (Fig. 2A) represented leaky expression from the Tet-off constructs as no signals corresponding to the endogenous proteins were seen in parental CHO cells (Suppl. figure 2).…

Minor point 5: *"Figure 2: I do not see how the data in panel A lead to the strong differences in the quantification in B. …The authors should include a quantification of the APP and SORLA levels from all conditions in A and not only show the ratios."*

As suggested, we have recalculated all APP and SORLA levels and expressed them as % of untreated control cells. These data are seen in revised figure 2B and fully confirm our previous conclusion that down-regulation of protein expression works best for pTet-APP with an overall reduction to 10% of normal APP levels. Down-regulation for pTet-SORLA reduces receptor expression to approximately 40-50% of normal levels.

The quantitative data shown in figure 2B represent values determined by ELISA measurement. Obviously, another concern of this reviewer relates to the apparent discrepancy in SORLA protein reduction seen by ELISA (Fig. 2B) and as judged from Western blotting (Fig. 2A).

Western blot analyses using ECL are semi-quantitative at best as there is an inherent problem of correlation of signal strength with signal perception. For example, strong immuno-signals in one lane tend to further quench the signal intensity of weaker bands in neighboring lanes of the same blot. Also, ECL signals are typically not linear over a wide-range of intensities. This is why we solely rely on ELISA measurements for accurate quantitative data acquisition. The Western blots shown in Fig. 2A were merely intended to visually support the conclusion by ELISA that doxycycline application regulates expression of pTet-APP and pTet-SORLA in a dose-dependent manner.

Minor point 6: *"The ELISA for APP measurements needs to be described in the methods' section. How do the authors distinguish between full-length and soluble APP in the cell lysate? Antibody 6E10 would detect both as well as C-terminal APP fragments."*

For determination of APP in cell lysates and of processing products in the media, we applied commercial ELISA kits that are commonly used in the field. The kits for quantification of sAPPß and Aß are specific for the respective peptides. As correctly stated by the reviewer, the ELISA used for detection of APP in cell lysates (Invitrogen #KHB0051) potentially also recognizes residual soluble APP products in cells. Also, the ELISA used for detection of sAPPα in the media (IBL, #27734) may detect full-length APP.

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To control for specificity of our measurements, we tested the amount of soluble APPα in cell lysates and of full-length APP in media using specific antisera. As shown in the novel supplementary figure 3, no sAPPα was detected by Western blot analysis in CHO cell extracts using specific antiserum IgG 2B3, even when overexposing the blots. Also, no full-length APP was seen in cell supernatants using IgG1227. This control experiment confirms that the immuno-reactivity measured by anti-APP ELISA in cell extracts and by sAPPα ELISA in media represents the anticipated proteins. We have now revised the supplementary method section on "Quantification of proteins in CHO cells" to discuss these considerations.

Minor point 7: *"Figure 3: Is velocity of sAPP generation linear over the 24 hours or how was the 24 hour time point chosen?"*

We performed a time course of doxycycline application to follow APP expression levels. As seen in Fig. 1B, stable APP levels are seen after 24 h of drug application. That is why we subsequently conditioned cell supernatants from 24 to 48 hours post doxycycline addition. This range is linear for APP production as shown in Fig. 1B. We now have more clearly indicated the time point of sample collection in all relevant figure legends (e.g, Figs. 2 through 5).

Minor point 8: *"Figure 4: Are these the same data as in figure 3? This should be indicated."*

The reviewer is correct inasmuch as the data points in figure 4 are the same as in figure 3. However, linear instead of logarithmic presentation of data points was chosen in the latter figure to better illustrate the dramatic decrease in soluble APP in the presence of SORLA and to calculate half-maximal velocity ($V_{0.5}$). We have more clearly stated this fact in the legend to figure 4 now.

In fact, the same applies to figure 5B (Aß production in logarithmic presentation to demonstrate differences in Hill versus Michaelis-Menten kinetics) and figure 5C (Hill kinetic presented in linear scale to calculate $V_{0.5}$). This fact had been stated in legend to figure 5 in the original manuscript.

Minor point 9: *"Figure 5: Why is the reduction in Abeta stronger than the reduction of sAPPbeta?"*

We agree with the reviewer that data points for production of sAPPα and sAPPß in Fig. 4 and for Aß in Fig. 5C suggest a more dramatic effect of SORLA on α- and γ -secretase activities than on ß-secretase processing. However, this difference is modest as deduced from determination of enzyme kinetics. As stated in the result section, at half maximal velocity ($V_{0.5}$) of a-secretase, the amount of sAPPα produced from 106.9 nM APP was reduced from 97.2 fmol/h to 2.7 fmol/h in the presence of 120 nM SORLA (97% reduction) (Fig. 4A). The amount of sAPPß produced from 62.6 nM APP ($V_{0.5}$ of b-secretase) was reduced from 4.6 fmol/h to 1.1 fmol/h in the presence of 120 nM SORLA (75% reduction) (Fig. 4B). $Aß_{40}$ production at 35.4 nM APP ($V_{0.5}$) was reduced from 18.7 fmol/h in to 0.77 fmol/h in the presence of 120 nM SORLA (96% reduction) (Fig. 5C).

The reason for the slight difference in extent of inhibition of 75% for sAPPß versus 96% for Aß is not entirely clear. We believe that the reason is technical by nature given the different half-life and stability of the processing products in the media. For example, the amount of Aß may be affected by degrading enzymes acting on the peptide during 24 hour sampling.

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COMMENTS TO REFEREE #2:

General comment: *"The main weakness of the paper, from my point of view, is that the model is not described in much detail and in particular it is difficult to determine which aspects of the model are clearly supported by the current fits to experimental data and which aspects are more uncertain."*

Following the reviewer's comments, we have included the following texts in the supporting information under "Mathematical modeling of APP processing":

…The biochemical network consists of monomer processing (upper panel of Fig. 11A) and dimer processing (lower panel of Fig. 11A). The two modules are connected by the reversible dimerization-dissociation of APP and secretases. Two identical monomeric forms of APP and secretases are dimerised to give the corresponding dimeric forms of APP and secretases. In the reverse direction, the dimeric forms of APP and secretases give the respective identical monomeric forms of APP and secretases. In both modules, the interaction of APP with α- and β-secretases leads to the formation of non-amyloidogenic (sAPPα and C83) and amyloidogenic (sAPPβ and C99) products. However, the reactants APP and secretases are in monomeric form within the monomer processing and in dimeric form within the dimer processing. The interaction between SORLA and monomeric APP lessens the amount of APP monomers available for processing and prevents the formation of APP dimers. We focused our model in such a way that the dimeric forms of the secretases act only on the dimeric forms of APP and the monomeric forms of the secretases act only on monomeric forms of APP. Descriptions of the variables used in the biochemical network are provided in Table A.1. All the parameter values of the model are estimated by optimization from dose-response series for sAPPα and sAPPβ as a function of APP_{Tot} for cells with or without SORLA.…

Please also refer to our response to the specific comments for additional details on the model and its validation discussed below.

Specific comment 1: *"The process of fitting the parameters to model data should be more completely described. Thus, it should be possible to indicate which qualitative aspects of the model are well-supported by the data and which aspects may not be."*

In agreement with the reviewer's suggestions, we now have included the following additional descriptions in the supporting information "Model parameter estimation":

…We used equations for $\dot{sAPP\alpha}$, $\dot{sAPP\beta}$ and $\dot{sAPP\alpha^*}$, $\dot{sAPP\beta^*}$ from monomer and dimer processing, and estimated the parameter values from dose response series. APP is calculated from the equation for APP_{Tot} using the fzero() function in MATLAB. The estimation has been performed by the following steps:

a. The initial values for the parameters are randomly assigned using the rand() function in MATLAB.
b. The initial values described in (a) are used for the lsqnonlin() function in MATLAB to estimate the parameter values of the mathematical equations.
c. Repeat (a) and (b) iteratively.
d. If the parameter values of the best fit are outside the boundaries of rand() then increase the boundaries and go back to (a).

We have also included the following text in the supporting information "Parameter values":

…In the combined model, where all parameters are estimated globally, about 75% of the fits show that alpha cleavage prefers dimer processing, while beta cleavage prefers monomer processing with and without SORLA. As shown experimentally, SORLA does not interact directly with the α- and β-

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secretases. It is for this reason that complex formation with the secretases is not considered in the model…

…We performed 300 global estimates and 300 global-local estimates to compare the quality of both estimates. In the global-local fit, all parameters except $K_{M\beta}$ and $K_{M\beta_d}$ are estimated globally. Out of the 300 global-local estimation runs ($K_{M\beta}$ and $K_{M\beta_d}$ are estimated locally) and 300 global estimation runs, 214 and 216 fits are generated, respectively, satisfying the condition that all parameter values are positive. Note that none of the parameter values were taken from the literature due to the differences in the experimental methods applied. Thus, most kinetic data available in the literature on APP processing by a/b secretases were obtained in cell free assays with purified enzyme and artificial peptide substrate. In contrast, our model is based on quantitative data obtained on APP processing in intact cells.…

…The model, where all parameters except $K_{M\beta}$ and $K_{M\beta_d}$ are estimated globally, supports the hypothesis of a switch from dimer processing in the absence of SORLA to monomer processing in the presence of SORLA in 80% of the global-local fits. This is in good agreement with our experimental finding that SORLA disrupts oligomerization of APP. The other 20% of fits show beta cleavage prefers monomer processing in both, the absence and presence of SORLA.…

…The goodness of a fit was quantified by calculating the residual value, i.e. the sum of the squared differences between the data and model (see supporting information "*Model parameter estimation*"). Comparing global-local and purely global parameter estimates, the residual values for the best global fit (i.e. 30.26) are about 50% worse than the residual values of all the global-local estimation (as observed in Figure A.4). The residual values of all the 214 global-local estimations are ranked from the lowest residual value of 21.5. A fit with a residual value smaller than 22 is considered a "good fit" (cf. Figure A4). Moreover, the global-local fits, where $K_{M\alpha}$ and $K_{M\alpha_d}$ are estimated locally, are a bit weaker (residual value 23.50) than the model presented in the manuscript, where $K_{M\beta}$ and $K_{M\beta_d}$ are locally estimated (residual value of 21.50), but better than the model where all parameters are fitted globally. This implies that the model, where all parameters except $K_{M\beta}$ and $K_{M\beta_d}$ are estimated globally, provides the best match to the experimental data. This observation also suggests a yet unidentified biological process whereby SORLA might indirectly affect the β-secretase, but not other secretases. This activity needs to be investigated in future experimental studies.…

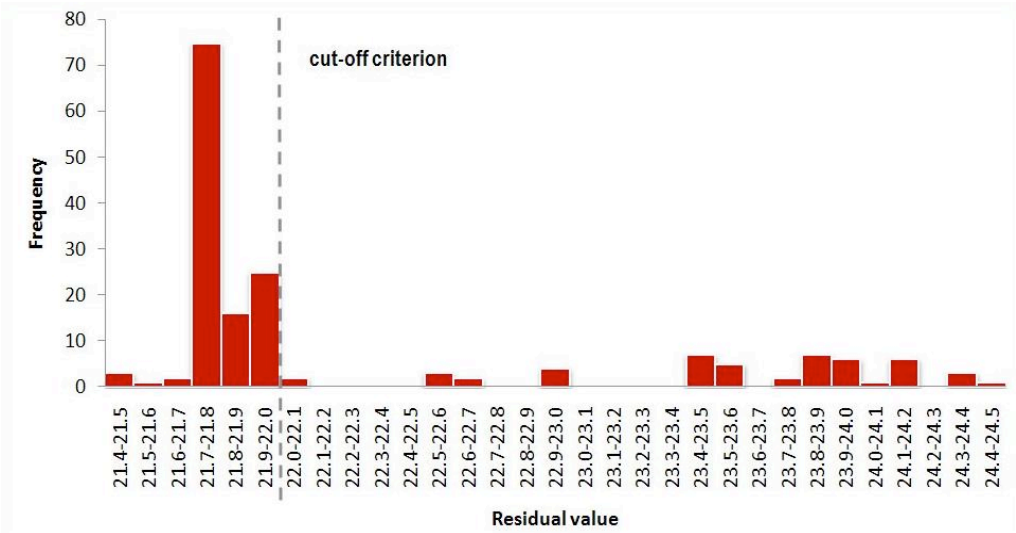

Figure A.4. Frequency distribution of the residual values of the 214 global –local fits. We have ranked the 214 global-local fits and have chosen 146 "good fits" starting from the lowest residuals.

The distribution of residuals suggests an empirical cut-off criterion at 22. A fit with a residual value smaller than 22 is then considered a "good fit".

Specific comment 2: *"What is the significance of secretase dimerization or of the model restriction that the monomeric form of the enzyme acts only on monomeric substrates and the dimeric form acts only on dimeric substrates?"*

With respect to the biochemistry of SORLA and APP interaction we considered two fundamental scenarios: one being a preformed APP dimer binding to a preformed BACE dimer resulting in positive cooperativity of APP processing. This situation preferentially occurs in the absence of SORLA. The other scenario represents a monomer of APP binding to a monomer of BACE resulting in non-cooperative binding and inefficient processing. This scenario preferentially occurs in the presence of SORLA.

From a biochemical point of view, one could also envision other interactions whereby an APP monomer interacts with a BACE dimer or an APP dimer binding to a BACE monomer. Whether or not these interactions also result in cooperativity of processing is unknown and virtually impossible to test experimentally. One would expect these alternative interactions to show weaker cooperativity, if at all. Because all experimental data in this study and previous work on APP and secretase dimer formation consider "all dimer" or "all monomer" interactions only, we focused on these two principal concepts for modeling.

In support of our approach, the cooperativity in associating monomeric and dimeric proteins has also been mathematically modeled and discussed by Levitzy and Schlessinger (Biochemistry,**13**(25),1974). In this study, the authors also conclude that an accumulation of the dimer-dimer complexes results in the highest possible positive cooperativity.

Specific comment 3: "*How well can the affinity of SORLA binding be estimated? In particular it would be interesting if the model could be used to identify an intermediate level of SORLA expression at which the dose-dependence of the kinetics would be intermediate between the cooperative and non-cooperative regimes shown in Figs. 3 and 4."*

We agree with the referee that a more detailed simulation study of the model will lead to more insights into the biochemistry of APP processing. In particular, identification of intermediate concentrations of SORLA whereby a switch from cooperative to non-cooperative interactions may be particularly interesting in light of its pathophysiological consequences for AD progression. We now have successfully addressed the reviewer's suggestion in the following way:

Due to the fact that the two parameters, $K_{M\beta}$ and $K_{M\beta_d}$ are fitted locally, the global-local estimation describes an indirect influence of SORLA. The binding affinity of SORLA cannot be shown directly by performing simulations for an intermediate level of SORLA. However, we have followed the referee's suggestion and we have calculated the intermediate level of SORLA expression between the cooperative and non-cooperative regimes in an indirect manner. We tried different approaches (i.e. linear, hyperbolic and exponential curves) to determine the dependencies of $K_{M\beta}$ and $K_{M\beta_d}$ on the intermediate levels of SORLA.

Figure A.5 shows the empirically derived (almost exponential) dependencies of $K_{M\beta}$ and $K_{M\beta_d}$ on the intermediate levels of SORLA, where it is ensured that the simulations of the intermediate curves for $sA\dot{P}P\alpha$ and $sA\dot{P}P\beta$ (blue dashed curves Figure 12 A and B) stay smoothly in between the curves with and without SORLA curves (black curves in Figure 12 A

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and B). As we decreased the amount of SORLA, the intermediate curves stay smoothly in between with and without SORLA curves.

The simulations of the dose response curves of $sA\dot{P}P\alpha$ and $sA\dot{P}P\beta$ for intermediate levels of SORLA expression are shown in Figure 12 (C-H). Interestingly, the switch between the cooperative and non-cooperative regimes occurs at low SORLA concentrations, i.e. ~0.12 x $SORLA_{Tot}$.

Motivated by the reviewer's suggestions, we have generated an additional figure (Fig. 12) included in the main text that visualizes the dose dependence of the switch between the cooperative and non-cooperative regimes on the intermediate levels of SORLA.

We also included a new section, "A.5.Intermediate levels of SORLA expression", in the supporting information with the following text and figure:

A.5. Intermediate levels of SORLA expression
We calculated the intermediate levels of SORLA expression between the cooperative and non-cooperative regimes in an indirect manner. Since the two parameters, $K_{M\beta}$ and $K_{M\beta_d}$ are fitted locally, the global-local estimation describes an indirect influence of SORLA.

Figure A.5 shows the empirically derived (almost exponential) dependencies of $K_{M\beta}$ and $K_{M\beta_d}$ on the intermediate levels of SORLA, where it is ensured that the simulations of the intermediate curves for $sAPP\dot{\alpha}_{Tot}$ and $sAPP\dot{\beta}_{Tot}$ (blue dashed curves Figure 12 A and B) stay smoothly in between the curves with and without SORLA (black curves in Figure 12 A and B). As we decreased the amount of SORLA, the intermediate curves stay smoothly in between with and without SORLA curves.

The simulations of the dose response curves of $sAPP\dot{\alpha}_{Tot}$, $sAPP\dot{\beta}_{Tot}$ (total processing in black curves), $sA\dot{P}P\alpha$, $sA\dot{P}P\beta$ (monomer processing in red curves) and $sA\dot{P}P\alpha^*$, $sA\dot{P}P\beta^*$ (dimer processing in green curves) for intermediate levels of SORLA expression are shown in Figure 12 (C-H). Interestingly, the switch between the cooperative and non-cooperative regimes occurs at low SORLA concentrations, i.e. ~0.12 x $SORLA_{Tot}$ (where $SORLA_{Tot}$ = 5.13 x 10^5 fmol, as shown in Table A.4).

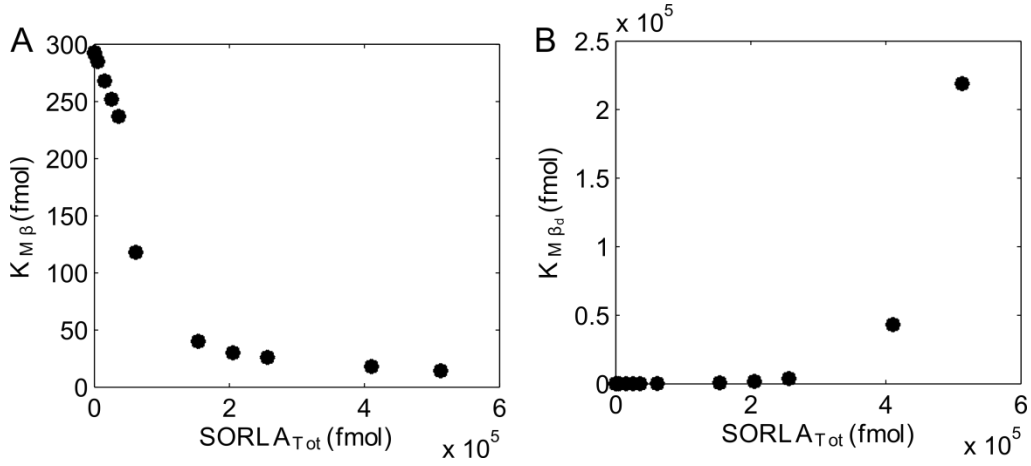

Figure A.5. Dependence of $K_{M\beta}$ and $K_{M\beta_d}$ on SORLA. The intermediate levels of SORLA expression are calculated in an indirect manner by empirically determining the dependencies of $K_{M\beta}$ (A) and $K_{M\beta_d}$ (B) on the intermediate levels of SORLA. These parameter values are used

to simulate the dose-response kinetics of $sAPP\dot{\alpha}_{Tot}$, $sAPP\dot{\beta}_{Tot}$, $sA\dot{P}P\alpha$, $sA\dot{P}P\beta$ and

$sA\dot{P}P\alpha$* , $sA\dot{P}P\beta$ * in dependence on intermediate levels of SORLA expression presented in Figure 12.

COMMENTS TO REFEREE #3:

Point 1: *"The authors should make it clear that APP oligomerization increases alpha/beta secretase affinity for APP but not other secretase substrates."*

We now have specifically mentioned in the discussion section that our model only refers to APP but not to other secretase substrates, some of which are also know to dimerize (page 16, line 11 from below).

The new text reads: …Whether or not the dependence on dimerization shown for APP here also applies to other physiological α- or ß-secretase substrates remains to be shown…

Point 2: *"Is SorLA endogenously expressed in CHO cells? Confirmation or reference to these experiments is required or make it clear that they mean in absence of human SorLA."*

CHO cells do not express endogenous APP or SORLA. We now have included a Western blot to demonstrate this fact (Supplementary figure 2). Thus, all biochemical and modeling data discussed here, relate to the interaction of human APP and SORLA. We now also specifically state this fact in the result section (page 6, line 4 from above).

The text reads: …no signals corresponding to the endogenous proteins were seen in parental CHO cells (Suppl. figure 2). Accordingly, all analyses in this study relate to the cellular interaction of human APP and human SORLA.…

Point 3: *"In figure 2 the molar ratio decrease of SorLA detected by ELISA following doxycyline treatment is described as only 2.5 fold reduction. This doesn't appear to be representative of the western blot which shows almost complete reduction of SorLA protein expression."*

Western blot analyses using ECL are semi-quantitative at best as there is an inherent problem of correlation of signal strength with signal perception. For example, strong immuno-signals in one lane tend to further quench the signal intensity of weaker bands in neighboring lanes of the same blot. Also, ECL signals are typically not linear over a wide-range of intensities. This is why we solely rely on ELISA measurements for accurate quantitative data acquisition. The Western blots shown in Fig. 2A were merely intended to visually support the conclusion from ELISA that doxycycline application regulates expression of pTet-APP and pTet-SORLA in a dose-dependent manner.

Point 4: *"Figure 9 demonstrates that in the presence of SorLA, APP oligomers do not form. The text also states that SorLA can reversibly form a complex with monomeric APP to reduce the formation of substrate oligomers, the authors should reference this interaction appropriately from previous observations."*

As suggested by the reviewer, we have properly referenced previous studies that demonstrated the ability of APP and SORLA to interact. We have cited earlier work that demonstrated interaction of the two proteins by coIP (Andersen et al, 2005; Offe et al, 2006; Schmidt et al, 2007) and FLIM (Spoelgen et al, 2006). We referenced previous publications that showed interaction of both proteins via their extracellular domains (Andersen et al., 2006). Last but not least we mentioned a

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study that demonstrated the ability of APP and SORLA to interact with a 1:1 stoichiometry indicating monomer interaction (Andersen et al, 2005).

The relevant text can be found in the discussion section (page 15, line 4 from below). It reads… That SORLA blocks formation of APP dimers is supported by findings that the interaction between both proteins proceeds via the cluster of complement type repeats in SORLA that bind to the carbohydrate-linked domain in the E2 region of APP (Andersen et al, 2006), the region essential for APP dimerization; and by the fact that APP and SORLA interact in a 1:1 stochiometric complex (Andersen et al, 2005)…

Point 5: "*The authors previously demonstrated that SorLA interaction with BACE decreases association of BACE with APP and reduction in BACE cleavage of APP, this should be included in the discussion where they discuss that SorLA doesn't functionally interact with the secretases."*

As suggested by this reviewer, we now discuss pervious work by Spoelgen et al. suggesting functional interaction of SORLA and BACE. The relevant text can be found in the discussion section (page 17, line 5 from below).

It reads: …Also, the observation that our model requires a local parameter estimate for b-secretase activity in the presence or absence of SORLA to closely resemble the experimental data (see supplementary methods for details), suggests that additional indirect affects of the receptor on this enzyme contribute to regulation of amyloidogenic processing in the context of an intact cell. Whether these effects entail interference of SORLA with the ability of BACE to bind APP as shown by Spoelgen and colleagues (Spoelgen et al, 2006) or other yet unknown mechanisms remains to be elucidated.…

2nd Editorial Decision 05 August 2011

Thank you for sending us your revised manuscript. After some delay due to the on-going summer holiday period, our original referees have now seen the manuscript again, and I have had a chance to look into the matter in depth and to discuss the case within our editorial team.

As you will see, while referees 2 and 3 now support publication of the manuscript here, referee 1 is still not convinced that you have made a sufficient case for the physiological significance of your findings, which - in his/her view - precludes publication of the study here. Now, obviously this is an important point that was also one of the main conditions that needed to be addressed during revision, and that we need to take very seriously. Clearly, this issue needs to be addressed. At this point, I would like to ask you to look into the two points that the referee still raises, the issue of physiologically relevant SORLA levels and the issue of APP dimerization, and to write a point-by-point response with suggestions how these two points (in particular point 1) could be addressed in your view. I will then look into the case again and may seek further independent expert advice if needed. We will then take it from there.

Furthermore, there are a number of editorial issues that would need further attention in case a solution to the problem discussed above can be found. First, an author contribution section and a conflict of interest statement is needed in the main body of the text, after the acknowledgement section. Second, statistical details such as the number of independent repeats are needed in the legends of figures 2B, 8 and 9B. Finally, a scale bar together with an explanation is needed in supplementary figure 1.

For now, we are looking forward to receiving your detailed letter of response.

Yours sincerely,

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Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The authors have addressed many of my previous points and have added a substantial amount of new data. It is largely carried out carefully and provides an excellent approach to mathematically describing a complex enzymatic and cellular process. This is a big achievement. While the kinetic analysis and its potential implication for dimerized substrate cleavage is intriguing, I am still not convinced of the physiological relevance of the data reported.
The idea of the authors - as stated clearly in the introduction - was to analyze whether mild changes in SORLA expression (as they occur in AD brains) are able to modulate APP amyloidogenic processing. Initially the authors compare two CHO cell lines with and without overexpression of SORLA. Overexpression of SORLA leads to an at least 50-fold difference in APP secretion compared to control cells. This was achieved using two differently selected cell populations of CHO cells. In contrast, by varying the concentration of SORLA in the APP overexpressing cells or by RNAi in the SH-SY5Y cells, only a very moderate change in APP secretion was obtained. In fact, the data in figure 6 are somewhat misleading, as the y-axis does not start at zero, but at 70 or 1100. Thus, the change of sAPPalpha levels is minor, when physiological SORLA levels are changed over a large concentration range - much larger than what is seen in AD. In these settings (figure 6 and 7), the kinetic curves are linear and do not allow a conclusion about the Hill equation at physiological SORLA expression levels (in contrast to SORLA overexpression in figure 3). Thus, the exciting part from figure 3 remains suggestive and cannot be proven under physiological conditions.
Another point is the dimerization of APP. I agree with the authors that previous evidence for APP dimerization is not fully conclusive. But the same is true for the experiments in figure 10. APP dimerization was shown for GFP-tagged APP. GFP itself is known to artificially dimerize proteins. For the endogenous APP a cross-linker was used, as far as I understand it from the methods section. This is another artificial situation and does not finally resolve the question of whether APP is truly dimeric or not. Moreover, APP was immunoprecipitated. Thus, the immunoglobulins are also part of the APP-containing complex in Figure 10B. Molecular weight markers are missing. Thus, it remains unclear whether the upper band has the double molecular weight of the lower band. While SORLA is clearly affecting the levels of the upper and lower bands, it is also possible that proteins other than APP are associated in both complexes (upper and lower one) and thus explain the different apparent molecular weights. Taken together, the data in figure 10 remain suggestive about APP dimerization and its SORLA dependence.

Referee #2 (Remarks to the Author):

The authors have more than adequately addressed the points raised in my previous review.

Referee #3 (Remarks to the Author):

The authors have adequately responded to my concerns, and I would recommend the current version of the manuscript for publication in EMBO Journal.

Additional correspondence (author) 26 August 2011

Point-by-point response

Point 1: Physiologically relevant levels of SORLA

"The idea of the authors - as stated clearly in the introduction - was to analyze whether mild changes in SORLA expression (as they occur in AD brains) are able to modulate APP amyloidogenic processing."

We agree that this is one of the main ideas to be tested in this study as it provides proof that subtle changes in SORLA as in patients with Alzheimer's disease (AD) are pathophysiologically relevant.

In the initial manuscript, we have used a CHO cell line with Tet-regulatable SORLA transgene expression to induce subtle alterations in receptor levels and to mimic the situation seen in sporadic AD (Fig. 6). Following the specific request by reviewer 1, we repeated the very same study on endogenous SORLA using siRNA knockdown in neuronal SY5Y cells. These new data fully support our initial findings in CHO cells as they document a strictly inverse linear relationship of SORLA concentration with APP processing rates over a range of physiologically relevant receptor levels (Fig. 7).
Thus, the change of sAPPalpha levels is minor, when physiological SORLA levels are changed over a large concentration range - much larger than what is seen in AD.
As shown both for CHO cells (Fig. 6) and for SY5Y cells (Fig. 7), changes in SORLA levels are small and vary between 2-fold (Fig. 6, Fig. 7A/B) and 4-fold (Fig. 7C). Similar variations in receptor levels are seen in patients with sporadic AD (2-fold) as published by Scherzer CR, et al (Loss of apolipoprotein E receptor LR11 in Alzheimer disease. Arch Neurol 61: 1200-1205, 2004).

Still, subtle changes in SORLA expression in CHO and in SY5Y cells translate into highly significant alterations in APP processing rates over the entire concentration range as documented by linear regression analysis. According to the graphs for endogenous SORLA in SY5Y cells (Fig. 7), a 2-fold reduction in receptor levels translates into a 30% increase in sAPPalpha and a 40% increase in sAPPbeta production. Remarkably, identical increases in APP processing rates are observed in patients with sporadic AD (45% increase in sAPPalpha and 40% increase in sAPPbeta production; Lewczuk P et al., Molecular Psychiatry (2010) 15, 138–145).
In conclusion, the reviewer's claim that changes in physiological levels of SORLA in our cell lines are (i) much larger than in AD and (ii) result in minor changes in APP processing are clearly incorrect. In fact, the changes of SORLA levels in CHO and SY5Y cells are within the observable range *in vivo* and result in changes that are pathophysiologically relevant in sporadic cases of AD.

We suggest including a more detailed description of these considerations and the relevant references in a revised version of the discussion section to more clearly indicate these facts.

In fact, the data in figure 6 are somewhat misleading, as the y-axis does not start at zero, but at 70 or 1100.

The data in Fig. 6 and 7 represent linear regression analyses. The interpretation of the data solely depends on the mathematically description of the slope of the trend lines and their statistically significant deviation from a horizontal line. Obviously, the steepness of the line is independent of the start and end points of the y-axe. In all data in Fig. 6 and 7, a highly significant decrease in APP processing rated with increase in physiological SORLA concentration has been documented ($p<0.001$).
If considered helpful, we would be happy to adjust the y-axes to start at 0 for all graphs.

In these settings (figure 6 and 7), the kinetic curves are linear and do not allow a conclusion about the Hill equation at physiological SORLA expression.

The reviewer confuses the methodology to analyze enzyme kinetics. To determine enzyme kinetics according to Michaelis-Menten equations, levels of substrate APP have to be varied and correlated with product rates (sAPPalpha, sAPPbeta, Abeta). The experiment to modulate SORLA levels by siRNA in neuronal cell line SY5Y

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specifically requested by him/her cannot be used to determine kinetics of secretases.

Point 2: APP dimerization

APP dimerization was shown for GFP-tagged APP. GFP itself is known to artificially dimerize proteins. For the endogenous APP a cross-linker was used, as far as I understand it from the methods section. This is another artificial situation and does not finally resolve the question of whether APP is truly dimeric or not.

In our study, we document the presence of oligomeric forms of APP both in cultured cells (Fig. 10A) and in the mouse brain (Fig. 10B) using native PAGE. The formation of such oligomers is blocked by SORLA suggesting that the receptor acts as inhibitor of amyloidogenic processing by eliminating the preferred form of the substrate APP and forcing secretases to switch to a less efficient non-allosteric mode of action. The ability of APP to dimerize has been documented in transfected cells be others before. However, the reasons for dimerization remained obscure. Our data provide the first experimental proof for the existence of APP oligomers in the brain, and they document the physiological relevance of oligomerization for efficient precursor processing.

In the reviewer's opinion, our data do not rule out with absolute certainty that high molecular weight forms for APP seen in cells or *in vivo* represent complexes of APP with an unknown protein rather than APP homomers. This concern can easily be addressed by additional data that we generated while the revised manuscript had been under review. In detail, we have applied fluorescence correlation spectroscopy (FCS), an advanced method for studying molecular dynamics in living cells, to demonstrated molecular interaction of discrete APP variants (APP-RFP and APP-GFP) in intact neuronal cells, and the ability of SORLA to prevent dimerization.

I have attached these data as supplementary information for your consideration. If approved by the editorial board and you, I would include these additional data in the manuscript to fully address the reviewer's concern.

Moreover, APP was immunoprecipitated. Thus, the immunoglobulins are also part of the APP-containing complex in Figure 10B. Molecular weight markers are missing. Thus, it remains unclear whether the upper band has the double molecular weight of the lower band.

Obviously, native PAGE analysis cannot be used to identify the exact molecular weight of proteins as the migration in native gels is not only determined by molecular weight but also by charge and tertiary structure. Accordingly, the native PAGE data in Fig. 10A (CHO cells) and 10B (mouse brain) demonstrate the existence of higher molecular APP complexes that are affected in their abundance by the presence or absence of SORLA. However, these experiments cannot resolve the issue whether these complexes represent dimeric or oligomeric forms of the precursor. That is why we have carefully referred to this complex as APP oligomers throughout the text. For example, the relevant text in the abstract reads:

"We also show that SORLA prevents APP oligomerization both in cultured cells and in the brain *in vivo*, eliminating the preferred form of the substrate and causing secretases to switch to a less efficient non-allosteric mode of action"

Also, in the discussion section, the relevant subheading reads:

"SORLA blocks APP oligomerization and prevents cooperativity in processing. (see page 14).

Taken together, our biochemical data in cell lines and in the mouse brain combined with the mathematical modeling strongly suggest (i) that oligomeric forms of APP represent the preferred substrate for secretases as they support cooperativity in

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enzyme binding and (ii) that SORLA prevents such APP oligomerization. Because of previous data demonstrating dimerization of APP and BACE in vitro, it is intriguing to speculate that such APP oligomers represent dimers. However, this fact cannot be concluded with certainty. Clearly, the issue of APP dimers vs. oligomers does not affect the overall conclusion and the impact of this study.

We suggest to even more clearly emphasize this fact in the manuscript.

Additional editorial points:

We would be happy to address the remaining editorial issues (author contribution section, conflict of interest statement) as well as statistical details in a revised version of the manuscript.

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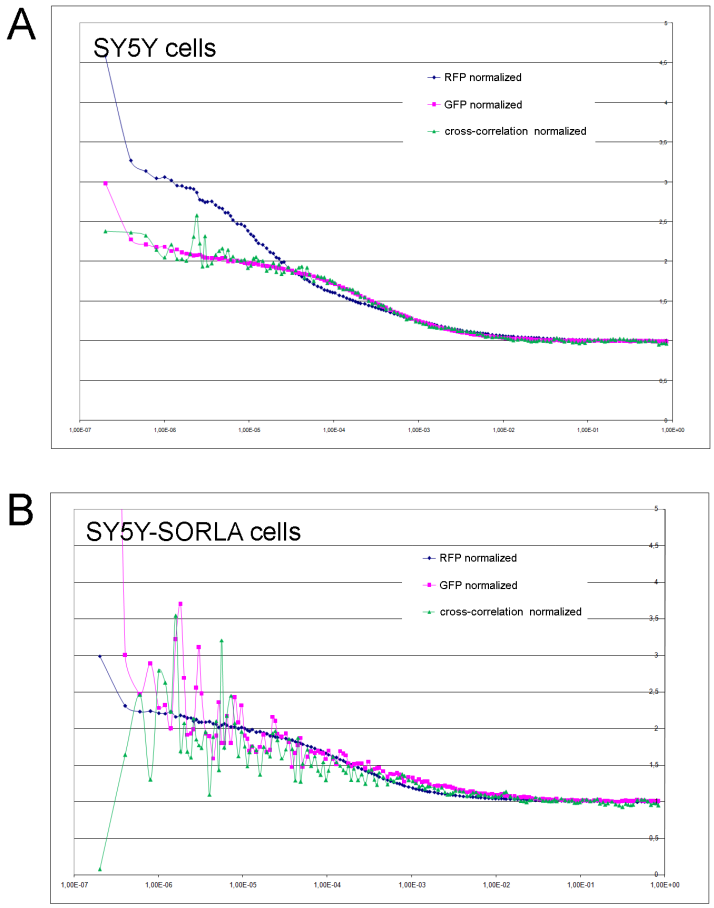
Figure: Fluorescence correlation spectroscopy of APP dimerization in SY5Y cells in the presence (B) or absence of SORLA (A).



Replicate layers of the neuronal blastoma cell line SH-SY5Y were co-transfected with expression constructs for APP-GFP and APP-RFP. Two days later, unperturbed cells were subjected to live cell imaging using Fluorescence Correlation Spectroscopy (FCS). FCS is an analytic tool that enables both qualitatively and quantitatively examining the molecular dynamics of protein-protein interactions by determining the fluctuation in fluorescence intensities of moving fluorescent molecules (here APP-GFP and APP-RFP) in a small light cavity with a defined volume. Such fluctuations are caused by subtle changes in the number and orientation of fluorescent APP-GFP and APP-RFP molecules diffusing in and out of the detection area. This movement can be measured by the autocorrelation for the respective proteins (Panels A and B; APP-RFP (blue line), APP-GFP (purple line)). If the two protein species form a dimer they will fluctuate in concert leading to a smooth cross-correlation curve (green line). This situation can be observed in the parental SY5Y cell line lacking SORLA (Panel A). In contrast, in SY5Y cells expressing a SORLA trasngene a more widespread cross-correlation curve (Fig. B, green line) is obvious indicating independent fluctuation (less dimerization) of the two proteins. Using the ZEN Software 2009 (Zeiss) we quantified the percentage of cross-correlation. Panel C shows approximately 30% of APP-GFP/APP-RFP dimerization in SY5Y cell line without SORLA whereas cells expressing SORLA exhibit significantly reduced APP dimmer formation (p=0.0067). In conclusion, these data provide independent experimental proof in live neuronal cells that APP forms dimers and that this process is blocked by SORLA.

Additional correspondence (editor) 29 August 2011

Thank you for your letter and your point-by-point response. I have now had a change to go through it in detail. I would suggest including your considerations with respect to physiologically relevant levels of SORLA as well as APP dimerization into the discussion section of the manuscript and to also include the additional data you put forward. In addition, and as pointed out earlier, the additional editorial issues should be addressed. I will then formally accept the manuscript.

Thank you very much again for considering our journal for publication of your work.

Yours sincerely,

Editor
The EMBO Journal

2nd Revision - authors' response 02 September 2011

My co-author and I are delighted about your decision to accept our manuscript for publication in the EMBO journal.

Please find enclosed the final revised versions of the main text, supplementary information, and figures. As suggested by you, we have made the following changes to the manuscript. They are highlighted in yellow in the document for your approval.

1) The additional data on analysis of APP dimer formation in SH-SY5Y cells using fluorescence correlation spectroscopy have been included as Fig. 10B and C, and are discussed in the result section on page 10, line 11 from top.

2) The considerations concerning physiologically relevant changes in SORLA expression levels in our cell models as compared to the human brain have been included in the revised discussion section on page 17, line 8 from below.

3) Numbers of independent repeats have been added to legends for figures 2B, 8 and 9B (3 – 4 independent repeats each).

4) Scale bars have been added to supplementary figure 1 (25 μm).
5) Statements concerning author contributions and lack of conflicting interest have been added to the main text following the acknowledgments.

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