The stress response neuropeptide CRF increases amyloid-β production by regulating γ-secretase activity

Hyo-Jin Park, Yong Ran, Joo In Jung, Oliver Holmes, Ashleigh R Price, Lisa Smithson, Carolina Ceballos-Diaz, Chul Han, Michael S. Wolfe, Yehia Daada, Andrey E Ryabinin, Seong-Hun Kim, Richard L Hauger, Todd E Golde and Kevin Felsenstein

Corresponding author: Kevin Felsenstein, University of Florida

Review timeline:

<table>
<thead>
<tr>
<th>Event</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Submission date</td>
<td>28 April 2014</td>
</tr>
<tr>
<td>Editorial Decision</td>
<td>21 May 2014</td>
</tr>
<tr>
<td>Revision received</td>
<td>19 November 2014</td>
</tr>
<tr>
<td>Editorial Decision</td>
<td>16 December 2014</td>
</tr>
<tr>
<td>Revision received</td>
<td>10 April 2015</td>
</tr>
<tr>
<td>Accepted</td>
<td>15 April 2015</td>
</tr>
</tbody>
</table>

Editor: Karin Dumstrei

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 21 May 2014

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by two referees and their comments are provided below.

As you can see both referees find the analysis interesting and suitable for publication in The EMBO Journal. However, they also find that the analysis has to be strengthened and that some of the work should be confirmed in more physiological relevant conditions. The referees provide very constructive comments and the issues raised are clearly outlined below. Given these comments, I would like to invite you to submit a suitably revised manuscript for our consideration. I should add that it is EMBO Journal policy to allow only a single major round of revision, and it is therefore important to address the raised concerns at this stage.

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, please visit our website: http://emboj.embopress.org/about#Transparent_Process

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

REFEREE REPORTS

Referee #1:

In this manuscript the authors try to characterize molecular events that potentially link stress to amyloid peptide production in AD. They focus on corticotrophin releasing factor and the interaction of CRF1 receptor with γ-secretase. They first confirm that stress and CRF affects Abeta generation in mice and primary cell cultures of neurons. They then provide evidence that CFR1 binds to γ-
secretase complex, that CRF/CRF1 influences the localization of γ-secretase in the cell membrane and that CRF itself affects γ-secretase directly in an in vitro assay. It is puzzling that CRF1 antagonists do not block CRF action on γ-secretase. What is lacking is "closure" of the cycle. The work on the molecular mechanisms is mainly performed in overexpressing cells. At least some important parts of this work should be confirmed in more physiological relevant conditions. A second major criticism is that the mechanism is only very superficially explored.

We make a series of concrete suggestions for additional experiments below

Specific points:

Fig. 2A: Oakley, RH et al. (2007) have previously nicely demonstrated the internalization of the CRF1 receptor following stimulation with CRF. A shorter time course (e.g. 30s-2 min is sufficient to stimulate β-arrestin recruitment). Demonstration of the intracellular time-dependent accumulation of CRFR1 would be more informative in such time course than the flat curve shown now.

Given that CRF1 is endogenously expressed in a number of other cells lines (Fig. S1A), it would be more convincing to perform these studies under physiological conditions.

Fig 2B: The authors state in the text that they label only cell surface nicastrin (as a marker for matured complex). I am not sure whether this can claimed as such: how do the authors get only surface nicastrin labeled? Moreover the internalization experiment shows that also internal nicastrin is stained by the protocol. Please clarify.

It would be nice to confirm the colocalization with at least one other γ-secretase subunit and also using superresolution to confirm their close proximity (Fig. 2C).

Fig. 3A: The IP experiments are performed under mild conditions using CHAPSO, which preserves the integrity of the γ-secretase complex but probably allows also additional proteins in the membrane patches to be co-precipitated. Therefore this experiment does not show a direct interaction with γ-secretase. These studies should be repeated under more stringent conditions eg to which component of the complex does CRF1 bind? Controls for the specificity of the binding need to be included.

The question is also whether the interaction can be detected at endogenous levels of expression for the γ-secretase and CRFR1. Overexpression could artificially induce the coprecipitation and in any event increases the chance of aspecific interactions (eg binding could be induced after solubilisation).

The IP experiments are performed under basal conditions, indicating that CRFR1 and the γ-secretase complex associate in the absence of receptor stimulation. One premise of the manuscript relies on modulation of CRFR1 function and γ-secretase activity following treatment with agonists and antagonists. Therefore, the effect that CRF (and possibly, antagonist) treatment has on the interaction between CRFR1 and the γ-secretase complex should be addressed as well.

The authors state "...CRFR1 interacts with the active, γ-secretase complex." However, in Fig. 3A, CRFR1 co-precipitates with both mature and immat. NCT. Could they be traffickin to the cell surface together?

Fig. 3B. In the current manuscript, the authors say that "Δ386/IC3 and Δ386 mutations impair the ability of the CRFR1 to recruit and bind β-arrestin 2, these studies suggest that the interaction between CRFR1 and γ-secretase may require β-arrestin2." The authors should formally demonstrate that the interaction between CRFR1 and β-arrestin 2 is impaired in their cellular assay system.

What about confirming this speculative interpretation by adding additional siRNA experiments to show that the interaction is indeed beta-arrestin dependent?

Mechanistically this could also be better explored: Does beta-arrestin 2 interact with CRFR1 under these assay conditions? Does it interact with the γ-secretase complex? Do the CRFR1 mutants affect the interaction between the γ-secretase complex and beta-arrestin? Does CRF treatment affect the
interaction between the γ-secretase complex and beta-arrestin?

The experiment would gain from additional controls: i.e. how is the reverse interaction (IP with CRF1 and detection of gamma-secretase subunits) affected by the mutations? Can the authors exclude that the interaction is lowered by differential targeting of the CRF1 mutants? It is for instance possible that the mutated receptor is no longer or less expressed at the cell surface compared to CRF1 WT.

The authors demonstrate that the Δ386 deletion mutant and the (Δ386-IC3-5ST/A) deletion mutants drastically reduce the interaction with the PS1 NTF. Does CRF treatment affect this interaction? What effect do these mutants have on interaction with the other γ-secretase complex components? How do the mutants and CRF treatment affect this interaction?

The authors mention that the IP was done with endogenous PS1 - what cell line? Under what conditions?

Addressing the issues mentioned above would provide insight into the mechanism of action of the CRFR1, βbeta-arrestin 2, and γ-secretase modulation of Abeta generation and would greatly improve the quality of the manuscript.

Fig. 3C. Based on the supplemental figure (Fig. S3), overexpression CRFCR1 increases localization of the γ-secretase in lipid rafts. Fig. 3 shows increased localization of the γ-secretase in lipid rafts in cells that are treated with CRF. Does treatment increase localization above the co-expression levels? How do these conditions affect localization of βbeta-arrestin 2? What is the effect on the other γ-secretase complex components? How do the mutants of CRF1 affect this distribution?

Fig. 3D. The authors demonstrate that CRF treatment stimulates Abetaβ40, Abetaβ42, and Abetaβtotal release in CRFR1 WT and CRFR1 Δ386 deletion mutant and stimulates Abetaβ42 release in the Δ386-IC3-5ST/A deletion mutant. Oakley et al. (2007) demonstrated that the Δ386 deletion was important for beta-arrestin recruitment and that "...combining the IC3 and Δ386 deletions (Δ386-IC3-5ST/A) had no significant impact on βbeta-arrestin-2 recruitment compared with the Δ386 deletion alone." How can the authors reconcile the lack of an effect on Abetaβ42 release in the mutant cell lines given the perturbation of the interaction between βbeta-arrestin 2 and CRFR1 and CRFR1 and PS1 NTF under these conditions based on the previous experiments? Are the βbeta-arrestin 2/CRFR1 and CRFR1/PS1 NTF described interactions not important for modulation of Abetaβ generation? Does the Δ386-IC3-5ST/A mutant affect Abetaβ ratio - if so, how?

Fig. 4. These series of experiments suggest a direct interaction of CRF with the gamma-secretase complex. First, I wonder whether the concentrations needed to see these effects are physiological relevant, and why the authors believe that these could be relevant for the stress response. Second, what controls were used to demonstrate that this effect is specific? Three, what mechanism do the authors envisage to explain these results?

Fig. 5. Antalarmin treatment alone has no effect on Aβ levels. Was the efficacy of the Antalarmin validated in the animal study (Fig. 5B)?

"Further, Antalarmin-treated mice displayed higher levels of Aβ40 and Aβ42 levels in both no stress and restraint stress groups, compared to vehicle-treated mice." The statement was not supported by the data in Fig. 5B.

Fig. 5C and 5D show very different changes in Abetaβ40 and Abetaβ42 levels with Antalarmin and NBI-27914 treatment. What are the possible explanations?

Fig S2A. Does the expression of Aph1, the α-secretase (e.g. ADAM10), and BACE1 change? Total sAPP levels have been measured, but are there changes in the direct cleavage products of the α- and βbeta-secretases, sAPPα and sAPPβ? This would convincing establish an effect of CRF on the γ-secretase-mediated cleavage of APP.
The study by Park and colleagues addresses how the stress response mediator CRF influences Abeta production. Using primary cultures and cell culture systems (HEK, N2a, SH-SY5Y), with a range of assays, the study shows that both acute stress and CRF cause increased Abeta levels in mice/mouse cultures. CRF treatment of cell lines causes increases in Abeta and internalization of both the CRF receptor 1 (CRFR1) and gamma-secretase. CRFR1 and presenilin via nicastrin can be co-immunoprecipitated. Using CRFR1 mutants (that need to be better specified) they show that it is the carboxy-terminal domain that mediates the effect of CRF on Abeta levels. In support (using three assays) they further show that CRF increases gamma-secretase activity in vitro. Surprisingly however Abeta levels are increased when (again this needs to be better specified) a set of CRFR1 antagonists is employed.

The study has been well performed. My main questions relate to the dosing, the role of arrestin, and how part of the information is provided in the manuscript.

Specific comments:
(1) Abstract: I would slightly reword the sentences 'Unexpectedly ...' and 'These data mechanistically link ...' because 'these data' refers to the entire study and not to the unexpected data in the preceding sentence that are harder to explain. One could write something like: 'While, unexpectedly, CRFR1 antagonists caused increases in Abeta (would add: in a dose-dependent manner), both in vivo and in vitro, our data collectively demonstrate ...'
(2) Page 8: Unless one reads the discussion (page 12) first, there is no rational provided for what the role is of GPR3, of beta-adrenergic receptor and arrestin or what the different CRFR1 mutants (Delta412 etc) are and why they have been generated. A scheme with functional domains and what the mutation elicits would be useful. I would suggest rewording the second half of page 8.
(3) Similarly, on page 10, C100, but more so Astressin, CRF9-41 (these two molecules are labeled differently in Figure 5) as well as Antalarmin need to be better introduced especially as the authors in the last sentences of the discussion suggest which types of molecules might be better CRF antagonists that really suppress Abeta formation. When the authors discuss the option of direct targeting of CRF or a G-protein-biased CRFR1 agonist that does not result in beta-arrestin2 recruitment to CRFR1 the problem again is that it is not clearly laid out how the different antagonists work and to what they exactly bind.
(4) The antagonist study would indicate that there are different effects for different doses: Abeta can be reduced when Antalarmin goes up to 25 uM. This needs to be better discussed and also possibly be rephrased in the discussion.
(5) Figure 3: Panel B please add Delta386 to the quantification. A pull-down with tagged versions of CRFR1 and PS1NT and knocking-down arrestin2 would clarify the question raised in the second-last sentence page 8.
(6) Figures 4/5: Different CRF concentrations have been used. What is the rationale for this especially for using a much lower concentration for the experiment shown in Figure 5? (4A: 25 uM CRF, 4B: 25,60,90, 4C: 10, 20, 50, Fig 5: only 1 uM)

Minor comments (typos, etc):
(1) Although 'corticotropes' is the correct term, the sentence on page 4 might be easier to read when 'corticotropic cells' is used.
(2) It would help to explain the broken cell assay mentioned on page 9.
(3) Page 11, third line (C99 stable lines): mention cell-type
(4) Page 13, line 4: 'the system' and 'UCN ligands' need to be defined.
(5) Discussion page 11, second-last line; Typo: 'in some studies, we preferential...'
We thank the reviewers for their constructive criticisms. Major concerns from both reviewers centered on the role of β-arrestin in the association of CRFR1 to γ-secretase and our CRFR1 mutants data. We have added new co-IP studies of CRFR1 and γ-secretase in β-arrestin 2 KO, β-arrestin 1/2 KO, and wild type mouse embryonic fibroblasts. As shown in Figure 3D, we observed significant difference of their association in WT and β-arrestin 2 KO MEF, suggesting that β-arrestin 2 has a role in the association of CRFR1 to γ-secretase. Furthermore, the reduction of the CRFR1-γ-secretase interaction is considerably greater in the MEF cells with the double β-arrestin 1 and 2 KO compared to the single β-arrestin 2 KO, indicating that not only β-arrestin 2 but also β-arrestin 1 can contribute to the association of CRFR1 and γ-secretase. We also have provided more data on the CRFR1 mutant (Figure 3B and 3C).

A point by point response is provided below.

Referee #1:

In this manuscript the authors try to characterize molecular events that potentially link stress to amyloid peptide production in AD. They focus on corticotrophin releasing factor and the interaction of CRF1 receptor with γ-secretase. They first confirm that stress and CRF affects Abeta generation in mice and primary cell cultures of neurons. They then provide evidence that CFR1 binds to γ-secretase complex, that CRF/CRF1 influences the localization of γ-secretase in the cell membrane and that CRF itself affects γ-secretase directly in an in vitro assay. It is puzzling that CRF1 antagonists do not block CRF action on γ-secretase. What is lacking is "closure" of the cycle. The work on the molecular mechanisms is mainly performed in overexpressing cells. At least some important parts of this work should be confirmed in more physiological relevant conditions. A second major criticism is that the mechanism is only very superficially explored.

We make a series of concrete suggestions for additional experiments below

Specific points:

(1) Fig. 2A : Oakley, RH et al. (2007) have previously nicely demonstrated the internalization of the CRF1 receptor following stimulation with CRF. A shorter time course (e.g. 30s-2 min is sufficient to stimulate beta-arrestin recruitment). Demonstration of the intracellular time-dependent accumulation of CRFR1 would be more informative in such time course than the flat curve shown now.

- Previously Drs. Oakley and Hauger (Oakley et al, 2007) reported a rapid recruitment of β-arrestin2 to membrane CRFR1 during a 2 minutes CRF exposure. CRFR1 recruitment of β-arrestin1 was slower. They also found that strong CRFR1 internalization did not become maximal until cells were stimulated with CRF for at least 30 minutes. In our study, we have detected significant internalization of CRFR1 after 10 to 30 minutes of CRF treatment, at which time CRFR1 internalization appeared to reach a maximum. We agree with Referee #1 that a shorter exposure of CRF would induce less internalization of CRFR1, however we believe that Figure 2A is a sufficient evidence to show that CRF treatment induces the internalization of cell surface CRFR1 in our system.

(2) Given that CRF1 is endogenously expressed in a number of other cells lines (Fig. S1A), it would be more convincing to perform these studies under physiological conditions.

- Referee #1 suggests a study of endogenously expressed CRFR1 with γ-secretase under physiological conditions. This approach would require, however, using a high affinity and high specificity antibody capable of recognizing the endogenous CRFR1. Unfortunately, a long-standing problem in the CRF field is that most of CRFR1 antibodies have not been confirmed to be selective. Both Santa Cruz’s V14 antibody (sc-12381) and Sigma’s AA102-117 antibody (SAB2500272) that we tried, did not even recognize overexpressed CRFR1 as well as endogenous CRFR1. Also, Dr. Hauger’s study (Oakley et al, 2007) had to transfec
arrestin2 to membrane CRFR1, pointing out how difficult it can be to demonstrate specific binding and interactions of non-tagged proteins.

(3) Fig 2B: The authors state in the text that they label only cell surface nicastrin (as a marker for matured complex). I am not sure whether this can claimed as such: how do the authors get only surface nicastrin labeled? Moreover the internalization experiment shows that also internal nicastrin is stained by the protocol. Please clarify. It would be nice to confirm the colocalization with at least one other γ-secretase subunit and also using superresolution to confirm their close proximity (Fig. 2C).

- For the live-staining experiment, we labeled unfixed and unpermeabilized cells with a NCT antibody. Since the antibody cannot get inside the cells without fixation and permeabilization, the epitope of the target protein has to be exposed to extracellular region to be labeled with the antibody. For this reason, NCT is the only molecule that can be used for live-staining of γ-secretase complex in our system. The antibodies that we used for other γ-secretase complex subunits, recognize intracellular regions of the proteins. Cells were incubated with NCT antibody at 10 degrees, the condition that blocks endocytosis so that extracellular reagents do not get into the cells. After labeling NCT with the primary antibody, cells were washed and incubated at 37 degrees with or without CRF for internalization. After this step, the labeled NCT on cell surface was internalized with CRFR1. Then cells were fixed, permeabilized, and stained with secondary antibodies. All the visualized NCT on cell surface or intracellular region on Figure 2C was surface NCT before CRF treatment.

(4) Fig. 3A: The IP experiments are performed under mild conditions using CHAPSO, which preserves the integrity of the γ-secretase complex but probably allows also additional proteins in the membrane patches to be co-precipitated. Therefore this experiment does not show a direct interaction with γ-secretase. These studies should be repeated under more stringent conditions eg to which component of the complex does CRF1 bind? Controls for the specificity of the binding need to be included.

- Although co-IP results could have resulted from nonspecific precipitation of the membrane patches; this phenomenon has generally not been an issue that has arisen in numerous studies of PSEN interacting proteins. The new studies on β-arrestin KO MEF (see response comment 8 below) largely address this issue, as the result would not be predicted if the IP was due to non-specific interactions.

(5) The question is also whether the interaction can be detected at endogenous levels of expression for the γ-secretase and CRFR1. Overexpression could artificially induce the coprecipitation and in any event increases the chance of aspecific interactions (eg binding could be induced after solubilisation).

- Referee #1 suggests a study of endogenously expressed CRFR1 interactions with γ-secretase. As we explained above, this approach requires a high affinity and high specificity antibody of recognizing the endogenous CRFR1 which is not available. We have however shown the interaction between overexpressed CRFR1 and endogenous γ-secretase by immunoprecipitating endogenous PS1 in Figure 3C. Also, our co-IP assay utilizing deletion mutants of CRFR1, CRFR1 Δ386/IC3 showed significantly reduced interactions with PS1 (Figure 3C), supporting our hypothesis that recruitment and binding of β-arrestin to CRFR1 is required for the interaction between CRFR1 and γ-secretase.

(6) The IP experiments are performed under basal conditions, indicating that CRFR1 and the γ-secretase complex associate in the absence of receptor stimulation. One premise of the manuscript relies on modulation of CRFR1 function and γ-secretase activity following treatment with agonists and antagonists. Therefore, the effect that CRF (and possibly, antagonist) treatment has on the interaction between CRFR1 and the γ-secretase complex should be addressed as well.

- Although this suggestion could be interesting, we believe that these experiments are beyond the scope of the current study and would not change our conclusions. CRFR1 and γ-secretase components are co-internalized and localized at early endosomes upon CRF treatment. Generally after internalization, GPCRs are either recycled or degraded depending on receptor- and cell-specific mechanisms. The interaction
between CRFR1 and γ-secretase may be an acute response to agonist-induced CRFR1 activation and signaling. However, agonist treatment often does not increase co-IP of a GPCR with a cellular protein. Recently a CRFR1 interacting protein, SAP97, was shown to co-immunoprecipitate with CRFR1 by binding to its 4-amino acid C-terminal, PDZ site. This interaction was not increased when CRFR1 was activated by CRF treatment (Dunn et al, 2013)

(7) The authors state that "...CRFR1 interacts with the active, γ-secretase complex." However, in Fig. 3A, CRFR1 co-precipitates with both mature and immat. NCT. Could they be trafficking to the cell surface together?

- In the cell lines overexpressing γ-secretase, majority of NCT in total lysate (Figure 3A, left, lane 1) is immature NCT. However, when we isolate active γ-secretase by immunoprecipitating PS1, the ratio of mature to immature NCT markedly increases (Figure 3A, left, lane 4). After CRFR1 immunoprecipitation, the ratio of mature to immature NCT interacting with CRFR1 also increases similar to that in active γ-secretase (Figure 3A, left, lane 3 and 4). CRFR1 co-immunoprecipitated PEN2 as well (Figure 3A, left, lane 3) which is the last molecule to involve in the γ-secretase complex and promote a mature and active γ-secretase. Although active γ-secretase localizes preferentially at the late secretory compartments, active γ-secretase can be assembled and localized in early secretory compartments (Kim et al, 2004). Therefore, some of γ-secretase and CRFR1 complex could be possibly trafficking to the cell surface together, but the localization that makes the complex functional would be in late secretory compartments. Again, we don’t believe this to be an issue since our conclusions would not be altered even if a small amount of immature NCT associates with CRFR1.

(8) Fig. 3B. In the current manuscript, the authors say that "Δ386/IC3 and Δ386 mutations impair the ability of the CRFR1 to recruit and bind beta-arrestin 2, these studies suggest that the interaction between CRFR1 and γ-secretase may require beta-arrestin2." The authors should formally demonstrate that the interaction between CRFR1 and beta-arrestin 2 is impaired in their cellular assay system. What about confirming this speculative interpretation by adding additional siRNA experiments to show that the interaction is indeed beta-arrestin dependent?

Mechanistically this could also be better explored: Does beta-arrestin 2 interact with CRFR1 under these assay conditions? Does it interact with the γ-secretase complex? Do the CRFR1 mutants affect the interaction between the γ-secretase complex and beta-arrestin? Does CRF treatment affect the interaction between the γ-secretase complex and beta-arrestin?

The experiment would gain from additional controls: i.e. how is the reverse interaction (IP with CRF1 and detection of gamma-secretase subunits) affected by the mutations? Can the authors exclude that the interaction is lowered by differential targeting of the CRF1 mutants? It is for instance possible that the mutated receptor is no longer or less expressed at the cell surface compared to CFR1 WT.

- As Dr. Hauger has previously reported, the β-arrestin2 recruitment is significantly less for the Δ386 and Δ386/IC3 CRFR1 mutants. However, the mutant CRFR1s are expressed on the cell membrane and β-arrestin2 clearly translocates to mutant CRFR1s at the cell surface, indicating that mutation of CRFR1 did not affect the localization of the receptor (Oakley et al, 2007, Figure 10 and 12). Moreover, Dr. Hauger did not find major differences in membrane agonist binding between the wild type and mutants CRFR1s (Oakley et al, 2007, Table 1)

The authors demonstrate that the Δ386 deletion mutant and the (Δ386-IC3-5ST/A) deletion mutants drastically reduce the interaction with the PS1 NTF. Does CRF treatment affect this interaction? What effect do these mutants have on interaction with the other γ-secretase complex components? How do the mutants and CRF treatment affect this interaction?

- As discussed in response to point 6 above, agonist treatment often does not increase co-IP of a GPCR with a cellular protein. Recently a CRFR1 interacting protein, SAP97, was shown to co-immunoprecipitate with
CRFR1 by binding to its C-terminal 4 amino acids, PDZ site. This interaction was not increased when CRFR1 was activated by CRF treatment (Dunn et al, 2013).

The authors mention that the IP was done with endogenous PS1 - what cell line? Under what conditions?
- In Figure 3C, co-IP was performed in HEK293 cells and CRFR1 mutants are overexpressed. After cells are lysed in 1% CHAPS co-IP buffer (see materials and method), endogenous PS1 was immunoprecipitated.

Addressing the issues mentioned above would provide insight into the mechanism of action of the CRFR1, beta-arrestin 2, and γ-secretase modulation of Abeta generation and would greatly improve the quality of the manuscript.
- To define a role of β-arrestin in interaction between γ-secretase and CRFR1, we have performed co-IP experiments in wild type, β-arrestin2 KO, or β-arrestin1 and 2 KO mouse embryonic fibroblasts (MEF). In the condition of PS1 and CRFR1 overexpression, we observed significant difference of their association in WT and β-arrestin 2 KO MEF, suggesting that β-arrestin 2 has a role in the association of CRFR1 to γ-secretase (Figure 3D, graph). The interaction in β-arrestin 1 and 2 KO MEF is markedly reduced, indicating that not only β-arrestin 2 but also β-arrestin 1 contributes to the association of CRFR1 and γ-secretase. In addition, we have detected interaction between endogenous PS1 and overexpressed CRFR1 in wild type and β-arrestin 2 KO cell lines but not in β-arrestin 1 and 2 cell lines (data not shown). These results show that the interaction between γ-secretase and CRFR1 is specific and the expression of β-arrestins is required for the interaction. We have incorporate this new data that provides stronger confirmation of our hypotheses in the Results and Discussion sections of our manuscript.

Fig. 3C. Based on the supplemental figure (Fig. S3), overexpression CRFcr1 increases localization of the γ-secretase in lipid rafts. Fig. 3 shows increased localization of the γ-secretase in lipid rafts in cells that are treated with CRF. Does treatment increase localization above the co-expression levels? How do these conditions affect localization of beta-arrestin 2? What is the effect on the other γ-secretase complex components? How do the mutants of CRF1 affect this distribution?
- Both CRFR1 overexpression and the receptor activation increase γ-secretase in raft. CRFR1 is activated and internalized by agonist but probably a small portion of CRFR1 is constitutively internalized with no agonist-induced activation (see Figure 2A, solvent treated). Also, CRFR1 interacts with γ-secretase even without agonist-induced activation (see Figure 3A, 3C, 3D), so the increase in γ-secretase in raft in Figure. S3 could be due to increase in constitutive internalization of CRFR1/γ-secretase in stable cell lines. We detected PS1N1TF which represent active γ-secretase. The change in other γ-secretase components should be same.

Fig. 3D. The authors demonstrate that CRF treatment stimulates Abeta40, Abeta42, and Abeta total release in CRFR1 WT and CRFR1 Δ386 deletion mutant and stimulates Abeta42 release in the Δ386-IC3-5ST/A deletion mutant. Oakley et al. (2007) demonstrated that the Δ386 deletion was important for beta-arrestin recruitment and that "...combining the IC3 and Δ386 deletions (Δ386-IC3-5ST/A) had no significant impact on beta-arrestin-2 recruitment compared with the Δ386 deletion alone." How can the authors reconcile the lack of an effect on Abeta42 release in the mutant cell lines given the perturbation of the interaction between beta-arrestin 2 and CRFR1 and CRFR1 and PS1 NTF under these conditions based on the previous experiments? Are the beta-arrestin 2/CRR1 and CRFR1/PS1 NTF described interactions not important for modulation of Abeta generation? Does the Δ386-IC3-5ST/A mutant affect Abeta ratio - if so, how?
- We have observed a significant decrease in the interaction of CRFR1Δ386 and CRFR1Δ386/IC3 mutants with γ-secretase. CRFR1Δ386/IC3 showed lowest interaction with γ-secretase (Figure 3C, graph) although this effect was not significantly different from CRFR1Δ386. CRF treatment increases Aβ through at least two different mechanisms; 1) CRFR1-mediated γ-secretase activation and 2) direct activation of γ-secretase by the agonist or antagonist. Based on our study (Figure 4 and 5), the increase of Aβ42 levels in CRFR1Δ386/IC3 expressing cells (Figure 3F), could be a direct effect of CRF on γ-secretase, similar to the effect of inverse γ-
secretase modulators (iGSM). Future experiments on CRFR1 antagonist association to \(\gamma\)-secretase will answer how the agonist or antagonist directly increases \(\gamma\)-secretase activity.

(11) Fig. 4. These series of experiments suggest a direct interaction of CRF with the gamma-secretase complex. First, I wonder whether the concentrations needed to see these effects are physiological relevant, and why the authors believe that these could be relevant for the stress response. Second, what controls were used to demonstrate that this effect is specific? Three, what mechanism do the authors envisage to explain these results?

- Previous studies have shown a direct effect of CRF on A\(\beta\) levels in vivo using microdialysis techniques (Kang et al, 2007); we believe this study established the relevance of our current mechanistic studies. To dissect out which of the many in vivo processes may be involved in this effect is well beyond the scope of our study since we have focused on defining the cellular and molecular mechanisms mediating CRFR1-\(\gamma\)-secretase interaction. Brain CRFR1 signaling in vivo is most likely causing the increase in A\(\beta\) formation based on the previous findings with tau phosphorylation CRF receptor KO mice subjected to stress (see (Rissman et al, 2007; Rissman et al, 2012)). However, considering the complex effects of CRFR1 antagonists on A\(\beta\), determining the role of CRFR1 is not going to be easy to assess. We have over the years tested numerous compounds and peptides in in vitro \(\gamma\)-secretase assays; many are inactive. The dose response and the vehicle controls are appropriate for these studies, and we make sure that the CRF or other peptides do not interfere with the ELISA assay. Determining an answer to the third part of this question is also certainly beyond the scope of our current study. \(\gamma\)-Secretase remains an enigmatic protease and our data show a direct effect on the complex. These effects are consistent with an allosteric mechanism. However, proving this will require extensive word that we will pursue in our future research.

(12) Fig. 5. Antalarmin treatment alone has no effect on Abeta levels. Was the efficacy of the Antalarmin validated in the animal study (Fig. 5B)?

"Further, Antalarmin-treated mice displayed higher levels of Abeta40 and Abeta42 levels in both no stress and restraint stress groups, compared to vehicle-treated mice." The statement was not supported by the data in Fig. 5B.

- We used the standard 20 mg/kg dose confirmed to strongly antagonize CRFR1 binding and G protein-coupled signaling in many studies. Thus, there is every reason to assume Antalarmin was active in cell culture. We also used the same lot of Antalarmin for our in vivo experiments. Measurements of endogenous A\(\beta\) in mouse brain are challenging. We have removed the statement about effect in non-stressed group as these are trends that not reach significance.

(13) Fig 5C and 5D show very different changes in Abeta40 and Abeta42 levels with Antalarmin and NBI-27914 treatment. What are the possible explanations?

- They are different compounds and we believe the effect is likely attributable to both receptor dependent and independent actions. Thus, in these cell culture studies, we would suggest that both mechanisms are active.

(14) Fig S2A. Does the expression of Aph1, the \(\alpha\)-secretase (e.g. ADAM10), and BACE1 change? Total sAPP levels have been measured, but are there changes in the direct cleavage products of the \(\alpha\)- and \(\beta\)-secretases, sAPP\(\alpha\) and sAPP\(\beta\)? This would convincingly establish an effect of CRF on the \(\gamma\)-secretase-mediated cleavage of APP.

- In SHSY5Y cells, CRH treatment did not change the levels of APP, sAPP, APP-CTF\(\beta\), and APP-CTF\(\alpha\) (Supplementary Figure 2A and 2B), suggesting that there is no significant change in \(\alpha\)-secretase or \(\beta\)-secretase activity and levels. We only showed total levels of endogenous sAPP, since it is technically not available to separate sAPP\(\alpha\) and sAPP\(\beta\). Since SHSY5Y cells show at least 2-3 specific endogenous mature APP bands (due to different sizes of APP isoforms) and the size range of sAPP\(\alpha\) and sAPP\(\beta\) from different APP isoforms are overlapped each other, it is technically very difficult to separate each of sAPP\(\alpha\) and sAPP\(\beta\) bands by western blotting.
- Aph1, NCT, PS1, and PEN2 assemble and form γ-secretase complex; the γ-secretase subunits stabilize each other. It is widely accepted that the level of each subunit (especially mature form of NCT and PS1) represents the level of γ-secretase. We detected no change in the levels of three endogenous γ-secretase subunits (Supplementary Figure 2B), suggesting that the γ-secretase levels did not change.

(15) On page 9, Fig. S2A and S2B are mislabeled. It should be Fig. S3.
- We have changed it to Fig S3 (page 9, line 18, highlighted).

Referee #2:

The study by Park and colleagues addresses how the stress response mediator CRF influences Abeta production. Using primary cultures and cell culture systems (HEK, N2a, SH-SY5Y), with a range of assays, the study shows that both acute stress and CRF cause increased Abeta levels in mouse/mouse cultures. CRF treatment of cell lines causes increases in Abeta and internalization of both the CRF receptor 1 (CRFR1) and gamma-secretase. CRFR1 and presenilin via nicastrin can be co-immunoprecipitated. Using CRFR1 mutants (that need to be better specified) they show that it is the carboxy-terminal domain that mediates the effect of CRF on Abeta levels. In support (using three assays) they further show that CRF increases gamma-secretase activity in vitro. Surprisingly however Abeta levels are increased when (again this needs to be better specified) a set of CRFR1 antagonists is employed.

The study has been well performed. My main questions relate to the dosing, the role of arrestin, and how part of the information is provided in the manuscript.

Specific comments:

(1) Abstract: I would slightly reword the sentences 'Unexpectedly ...' and 'These data mechanistically link ...' because 'these data' refers to the entire study and not to the unexpected data in the preceding sentence that are harder to explain. One could write something like: 'While, unexpectedly, CRFR1 antagonists caused increases in Abeta (would add: in a dose-dependent manner), both in vivo and in vivo, our data collectively demonstrate ...'
- We have reworded the sentences in abstract as referee #2 suggested (page 2, line 11, highlighted).

(2) Page 8: Unless one reads the discussion (page 12) first, there is no rational provided for what the role is of GPR3, of beta-adrenergic receptor and arrestin or what the different CRFR1 mutants (Delta412 etc) are and why they have been generated. A scheme with functional domains and what the mutation elicits would be useful. I would suggest rewording the second half of page 8.
- We have added an explanation what the CRFR1 mutants (page 8, line 17, highlighted) are. Also we have added a schematic diagram of CRFR1 mutants in Figure 3B.

(3) Similarly, on page 10, C100, but more so Astressin, CRF9-41 (these two molecules are labeled differently in Figure 5) as well as Antalarmin need to be better introduced especially as the authors in the last sentences of the discussion suggest which types of molecules might be better CRF antagonists that really suppress Abeta formation. When the authors discuss the option of direct targeting of CRF or a G-protein-biased CRFR1 agonist that does not result in beta-arrestin2 recruitment to CRFR1 the problem again is that it is not clearly laid out how the different antagonists work and to what they exactly bind.
- C100 is the APP C-terminal fragment and the exogenous substrate of γ-secretase that we used for our in vitro study. To purify the substrate protein in bacterial system, a methionine is added to the N-terminus of C99. We have addressed it (page 10, line 16 for C99, page 11, line 3 for antagonists). About the future approach to target CRF directly, we have tried to elaborate to address this concern.
(4) The antagonist study would indicate that there are different effects for different doses: Abeta can be reduced when Antalarmin goes up to 25 uM. This needs to be better discussed and also possibly be rephrased in the discussion.

- See the response to point 3 above. We have found that γ-secretase modulators and reverse modulators tend to inhibit overall Aβ production at high concentrations of treatment. This finding is typical in both cell-based and cell-free assay systems. Cellular toxicity caused by modulator treatment in case of cell–based system or limited accessibility of the protease to substrate caused by saturation of modulator molecules in the system may be involved.

(5) Figure 3: Panel B please add Delta386 to the quantification. A pull-down with tagged versions of CRFR1 and PS1NT and knocking-down arrestin2 would clarify the question raised in the second-last sentence page 8.

- This quantification is now provided in Figure 3 C. As noted at the beginning of the response to the reviewers, and in point 8 response to reviewer 1 above we have performed studies in β-arrestin KO MEFs.

(6) Figures 4/5: Different CRF concentrations have been used. What is the rationale for this especially for using a much lower concentration for the experiment shown in Figure 5? (4A: 25 uM CRF, 4B: 25,60,90, 4C: 10, 20, 50, Fig 5: only 1 uM)

- The experiment for Figure 5A was performed in cell culture system (SHSY5Y cells). When we tested the effects of CRF in SHSY5Y cells in cell cultures, Aβ levels increased in a dose dependent manner by 1-10 μM of CRF treatment (Figure 1D). To see the inhibitory effects of CRFR antagonists on CRF-induced Aβ peptide increases, we selected 1 μM CRF. On the other hand, for the in vitro cell free γ-secretase activity assay systems (Figure 4, 5C, 5D, and 5E), it was important to use saturating CRF concentrations to see the maximum effect of CRF or CRFR antagonists on γ-secretase.

Minor comments (typos, etc):

(1) Although 'corticotropes' is the correct term, the sentence on page 4 might be easier to read when 'corticotropic cells' is used.

- It has been changed to ‘corticotropic cells’ (page 4, line 7 and 13, highlighted).

(2) It would help to explain the broken cell assay mentioned on page 9.

- We have added a summary table of γ-secretase activity assays in Figure 4 and 5 at supplementary data. The experiment for Figure 4A was using stably overexpressing C99 in H4 cells and we have corrected the sentence and added an explanation (page 10, line 11, highlighted).

(3) Page 11, third line (C99 stable lines): mention cell-type

- It is C99 stably expressing H4 cells lines. We have added cell type information (page 11, line 19, highlighted).

(4) Page 13, line 4: 'the system' and 'UCN ligands' need to be defined.

- ‘The system’ is the cell signaling system which is mediated by CRFR1 receptor and its ligands interaction. ‘UCN ligands’ is urocortin 1, 2, and 3 which are known to bind to CRFR and also mediate the CRFR cell signaling activation. We have changed the sentence at page 13 and 14, highlighted.

(5) Discussion page 11, second-last line; Typo: 'in some studies, we preferential...'

- ‘we’ has been deleted (page12, line 14, highlighted).

(6) Figure 2: What do the arrowheads show?

- Our IFA result in Figure 2B showed that the internalized CRFR1 is partially co-localized with EEA1, the early endosome markers. Internalized GPCRs have a punctate appearance when they are localized within early endosome or lysosome. The merging of the green (GFP-tagged CRFR1) and red (NCT) colors indicate the CRFR1-
NCT complex has internalized together into intracellular vesicles, probably endosomes or lysosomes. We have specified what the arrowheads shows in the script (page 8, line 1, highlighted).

(7) Excellent idea to not only list the antibodies but also the antibody epitopes.

REFERENCES


Thank you for submitting your revised manuscript to The EMBO Journal. Your manuscript has now been re-reviewed by the original referees.

While referee #2 is satisfied with revised version, referee #1 has some remaining concerns. I have discussed the issues further with referee #2 and while all the points raised by referee #1 are valid ones, we also find that not all of them have to be addressed for publication here. Please see below for my specific comments on what has to be resolved in a final revision - I have just paraphrased the concerns please see full referee comments below. Of course if you already have data on hand to address some of the points raised then please do include that in the revised version.

Referee #1 point 1A => provide more direct data to support a role for G protein signaling:
This is a good point and would be nice to have, but it is not essential to address this issue at this stage.

Referee #1 point 1B => Why does a reduction in the PS1 interaction not affect Ab42 generation with this truncation mutant:
Please clarify this point.

Referee #1 point 1C => Deals with the issue that your findings are not easy to reconcile with previous work and the referee suggests to look if cAMP and PKA levels are affected by treatment with CRFR1:
This is an important issue and I would like to ask you to address this point with additional experiments.

Referee #1 point 1D => gets back to mechanism of the mode of CRF action on Ab42 levels:
As above for point 1A valid and good point, but not essential to address at this stage.

Referee #1 point 2 => to provide graphic model:
I will leave that up to you.

Once you have resolve these last issues then please submit the revised manuscript using the link below.

REFEREE REPORTS

Referee #1:

The current manuscript does not succeed in providing a clear mechanism or proposed mechanism of action of CRFR1 agonism/antagonism on direct and/or indirect modulation of the γ-secretase function and Aβ generation. In addition, it is difficult to reconcile previously published studies with the current studies into a coherent "working model" for the involvement of CRFR1 in AD - would it be better to inhibit or stimulate CRFR1 function? The studies in the current manuscript suggest that neither approach would be beneficial. This referee wonders whether the progress made is sufficient for a publication in EMBO. In the revised manuscript, the authors have addressed many of the issues raised by this reviewer from the original version; however there are still critical concerns that have not adequately addressed.

Specific concerns:

1. The mechanism of action has not been thoroughly explored as requested in specific point 8. In the current study, Park and colleagues demonstrate that the interaction between the PS1-NTF and CRFR1 is reduced by ~45% with the Δ386 C-terminal truncation mutation and to a much greater extent with the Δ386/IC3 mutation. Furthermore, they demonstrate that these regions are necessary for the increased localization of the γ-secretase in lipid raft domains. Based on these studies and studies performed in β-arrestin 2 and β-arrestin 1/2 KO MEFs, the authors conclude that "a G protein-biased CRFR1 agonist that does not result in β-arrestin recruitment to CRFR1 might be
necessary to effectively target this pathway for therapeutic benefit in AD."

Several aspects remain unclear:

A. Based on the studies presented in the manuscript, the authors have not provided any direct evidence that G protein signaling is involved in the observed effect. This hypothesis is testable.

B. The schematic in Fig. 3B indicates that Δ386 deletion mutant drastically affects PS1 interaction but has no/minimal effect on Aβ40 generation and no effect on Aβ42. Why does a reduction in the PS1 interaction not affect Aβ generation with this truncation mutant?

C. The current observations and conclusions are difficult to reconcile with previous publications. For example, Dong et al. (2014) present evidence in primary hippocampal neuronal cultures that CRF stimulates Aβ accumulation through a G protein signaling mechanism based on an observed elevation in Aβ and PKA levels following CRF stimulation and the ability to inhibit the CRF-stimulated effect on Aβ levels with a PKA inhibitor. Furthermore, they demonstrate that co-treatment with CRF and Antalarmin attenuated the CRF-mediated effect on Aβ generation and the elevation in PKA. The authors also demonstrate that chronic treatment with Antalarmin leads to a reduction in the amyloid plaque burden in Tg2576 mice. Carroll et al. (2011) also demonstrate that chronic stress, but not variable stress, exacerbates Aβ levels in Tg2576. They go on to demonstrate that pre-stress treatment with the CRFR1 antagonist NB27914 reduces hippocampal neurodegeneration, stress-induced impairment in fear-associated memory, AT8-IR tau inclusions, and phospho-tau levels. They also determined that 4-month-old, WT, naive, mice display a transient increase in corticosterone levels after a 15 min acute restraint stress that returns to baseline within 90 minutes.

As the authors state "These data (Dong et al.) are difficult, at present, to reconcile with our results showing that 20 mg/kg Antalarmin did not block the rise in brain Aβ upon acute stress. However, the discrepant results could reflect differences in acute versus subacute and chronic dosing paradigms or alternatively the use of transgenic mice as opposed to non-transgenics." Indeed, these seemingly inconsistent results may represent a very different environment than a long-term, chronic stress paradigm. Given that CRFR1 stimulation primarily activates the cAMP/PKA-signaling cascade in neuronal cells and stress can impact PFC function and can lead to increases in cAMP and PKC intracellular signaling, it would be important to determine whether cAMP and PKA levels are affected by treatment with CRFR1 agonists/antagonists in a neuronal context. These studies are important if the authors propose that CRFR1 agonists/antagonists directly modulate the γ-secretase by functioning as iGSMs and that CRFR1 G protein-biased signaling is a therapeutic avenue for AD.

D. In specific point 10, the authors suggest "Based on our study (Figure 4 an 5), the increase of Aβ42 levels in CRFR1 Δ386/IC3 expressing cells (Figure 3F), could be a direct effect of CRF on γ-secretase, similar to the effect of inverse γ-Secretase modulators (iGSM)." Could G protein signaling be involved rather than direct modulation of γ-secretase function? In the in vitro cleavage assays with H4, these cells express endogenous levels of the CRFR1 receptor (Fig. S1). If membranes were prepared from these cells, could this explain the observed effect rather than direct modulation of the γ-secretase?

2. Given that some of results from the current studies are unexpected and difficult to interpret in the context of previously published literature, it is important that the authors provide a model diagram at the end of the manuscript to explain how their studies would fit into a physiologically relevant context. How would stress affect the constitutive interaction and localization between CRFR1 and the γ-secretase? At what stage would β-arrestin 2 enter into this model?

Referee #2:

The authors have considered my suggestions and comments satisfactorily.
We thank the reviewer for constructive criticisms.

Remaining concerns from referee #1 centered on the detailed role of GPCR signaling on the CRF-induced Aβ productions. Referee #1 also suggested identifying the detailed mechanism of CRFR1-independent γ-secretase activation by CRF and CRFR antagonists. As the editor suggested, we agree that those are good points for future studies and they will bring us one step closer to therapeutic approach. Since we have not shown if CRF or CRFR antagonist affects the intracellular signal transduction induced by the receptor activation, we have added the cAMP measurement result from CRF and/or CRFR antagonists treated SH-SY5Y cells. As shown in supplementary Figure 6, we observed significant increases in cAMP levels upon CRF treatment and CRFR antagonists efficiently blocked the CRF-induced cAMP activation.

A point by point response is provided below.

**Point by point response to editor’s suggestions**

While referee #2 is satisfied with revised version, referee #1 has some remaining concerns. I have discussed the issues further with referee #2 and while all the points raised by referee #1 are valid ones, we also find that not all of them have to be addressed for publication here. Please see below for my specific comments on what has to be resolved in a final revision - I have just paraphrased the concerns please see full referee comments below. Of course if you already have data on hand to address some of the points raised then please do include that in the revised version.

**Specific comments:**

1. **Referee #1 point 1A => provide more direct data to support a role for G protein signaling: This is a good point and would be nice to have, but it is not essential to address this issue at this stage.**
   - We also agree that it is a good point for future studies to identify details of stress-mediated Alzheimer’s disease pathogenesis.

2. **Referee #1 point 1B => Why does a reduction in the PS1 interaction not affect Ab42 generation with this truncation mutant: Please clarify this point.**
   - In this study, we have shown that two different mechanisms by which CRF/CRFR antagonists activate γ-secretase and increase Aβ productions. In the CRFR1-dependent γ-secretase activation mechanism, CRFR1 and γ-secretase interaction is required for enhancing γ-secretase activity and Aβ productions. It looks like that the levels of Aβ40 as well as Aβ42 increase by this mechanism. However, in series of our *in vitro* γ-secretase activity assays, we observed preferential increases in Aβ42 especially with high concentration of CRF and CRFR antagonists (Figure 4 and 5). In the Aβ assay using the CRFR1 Δ386/IC3 mutant, the interaction between CRFR1 and γ-secretase is reduced (Figure 3C and 3D) and the γ-secretase activation is mostly through the CRFR1-independent mechanism. As a result, total Aβ and Aβ40 significantly decreases but Aβ42 levels still remains high (Figure 3F).
(3) Referee #1 point 1C => Deals with the issue that your findings are not easy to reconcile with previous work and the referee suggests to look if cAMP and PKA levels are affected by treatment with CRFR1: This is an important issue and I would like to ask you to address this point with additional experiments.

- As we mentioned in discussion (page 14), Dong et al (2014) study showed the difference in only PBS-soluble Aβ fraction, which does not reflect total brain Aβ levels (Dong et al, 2014). In contrast, we have been using the highly sensitive rodent Aβ ELISA system which allows measuring endogenous Aβ levels in DEA-soluble fraction in vitro and in vivo. Carroll et al (2011) study, the CRFR1 antagonist efficiently blocked tau pathologies but they did not show the effect of antagonist treatment on Aβ production (Carroll et al, 2011). Surprisingly, CRFR1 antagonists not only failed to block the effects of CRF on Aβ levels but also increased Aβ42 levels by themselves in our study, whereas tau pathology is blocked by the CRFR1 antagonist treatment upon stress induction as shown in Carroll et al (2011) study.

- These results suggest that the molecular mechanism of stress-induced Aβ accumulation might be separated from that of tau phosphorylation. The antagonists could be used to treat tau pathology; nevertheless there is a riskiness to accumulate Aβ, considering that CRHR1 antagonists increase Aβ42 levels as we found in this study. Since Aβ is thought to have physiological roles to increase tau phosphorylation and inhibit tau degradation thereby result in tau pathology after all, elucidating precise mechanisms by which stress induces AD pathology and targeting both Aβ and tau pathology will be critical for AD therapy (See our graphic model of stress-induced AD pathogenesis above). In supplementary Figure 6, we have shown cAMP levels upon CRF and CRFR antagonist treatment to test the functionality of the CRFR antagonists in our system. Upon CRF treatment, cAMP production was stimulated approximately 6-fold over basal levels. Both peptide and small molecule antagonists significantly blocked CRF-induced cAMP production. We have chosen 15 min of CRF incubation for this experiment since it has been reported that Gs-coupled CRFR1 signaling via the cAMP-PKA pathway can be desensitized during longer incubation of CRF (3 hours or 24 hours)(Dautzenberg et al, 2001; Hauger et al, 1997). In our experimental condition for Aβ assays, we have incubated the assay system with CRF for 16 hours. Consequently, CRFR1 on cells would likely have been uncoupled from the Gs protein resulting in little or no cAMP generation due to homologous desensitization. In addition, the antagonists also increased Aβ productions even though it significantly decreased cAMP levels induced by CRF, suggesting that the stress-induced Aβ production is not dependent on the intracellular cAMP signaling activation. Astressin showed a little less efficient blocking in cAMP levels than Antalarmin, probably due to lower levels of concentration and less specificity to CRFR1.

(4) Referee #1 point 1D => gets back to mechanism of the mode of CRF action on Ab42 levels: As above for point 1A valid and good point, but not essential to address at this stage.
Based on the nature of many γ-secretase binding molecules which lower Aβ40 and raise Aβ42, we hypothesize that CRF and CRFR antagonists directly bind to γ-secretase. We agree that it is a good point for future studies.

(5) Referee #1 point 2 => to provide graphic model: I will leave that up to you.

- We have added a schematic diagram of CRF and CRF antagonist effects on γ-secretase activation in Figure 6.

References


Original comments from referees

Referee #1:

The current manuscript does not succeed in providing a clear mechanism or proposed mechanism of action of CRFR1 agonism/antagonism on direct and/or indirect modulation of the γ-secretase function and Aβ generation. In addition, it is difficult to reconcile previously published studies with the current studies into a coherent "working model" for the involvement of CRFR1 in AD - would it be better to inhibit or stimulate CRFR1 function? The studies in the current manuscript suggest that neither approach would be beneficial. This referee wonders whether the progress made is sufficient for a publication in EMBO. In the revised manuscript, the authors have addressed many of the issues raised by this reviewer from the original version; however there are still critical concerns that have not adequately addressed.

Specific concerns:

1. The mechanism of action has not been thoroughly explored as requested in specific point 8. In the current study, Park and colleagues demonstrate that the interaction between the PS1-NTF and CRFR1 is reduced by ~45% with the Δ386 C-terminal truncation mutation and to a much greater extent with the Δ386/IC3 mutation. Furthermore, they demonstrate that these regions are necessary for the increased localization of the γ-secretase in lipid raft domains. Based on these studies and studies performed in β-arrestin 2 and β-arrestin 1/2 KO MEFs, the authors conclude that "a G protein-biased
CRFR1 agonist that does not result in β-arrestin recruitment to CRFR1 might be necessary to effectively target this pathway for therapeutic benefit in AD."

**Several aspects remain unclear:**

A. Based on the studies presented in the manuscript, the authors have not provided any direct evidence that G protein signaling is involved in the observed effect. This hypothesis is testable.

B. The schematic in Fig. 3B indicates that Δ386 deletion mutant drastically affects PS1 interaction but has no/minimal effect on Aβ40 generation and no effect on Aβ42. Why does a reduction in the PS1 interaction not affect Aβ generation with this truncation mutant?

C. The current observations and conclusions are difficult to reconcile with previous publications. For example, Dong et al. (2014) present evidence in primary hippocampal neuronal cultures that CRF stimulates Aβ accumulation through a G protein signaling mechanism based on an observed elevation in Aβ and PKA levels following CRF stimulation and the ability to inhibit the CRF-stimulated effect on Aβ levels with a PKA inhibitor. Furthermore, they demonstrate that cotreatment with CRF and Antalarmin attenuated the CRF-mediated effect on Aβ generation and the elevation in PKA. The also demonstrate that chronic treatment with Antalarmin leads to a reduction in the amyloid plaque burden in Tg2576 mice. Carroll et al. (2011) also demonstrate that chronic stress, but not variable stress, exacerbates Aβ levels in Tg2576. They go on to demonstrate that pre-stress treatment with the CRFR1 antagonist NB27914 reduces hippocampal neurodegeneration, stress-induced impairment in fear-associated memory, AT8-IR tau inclusions, and phospho-tau levels. They also determined that 4-month-old, WT, naive, mice display a transient increase in corticosterone levels after a 15 min acute restraint stress that returns to baseline within 90 minutes.

As the authors state "These data (Dong et al.) are difficult, at present, to reconcile with our results showing that 20 mg/kg Antalarmin did not block the rise in brain Aβ upon acute stress. However, the discrepant results could reflect differences in acute versus subacute and chronic dosing paradigms or alternatively the use of transgenic mice as opposed to non-transgenics."

Indeed, these seemingly inconsistent results may represent a very different environment than a long-term, chronic stress paradigm. Given that CRFR1 stimulation primarily activates the cAMP/PKA-signaling cascade in neuronal cells and stress can impact PFC function and can lead to increases in cAMP and PKC intracellular signaling, it would be important to determine whether cAMP and PKA levels are affected by treatment with CRFR1 agonists/antagonists in a neuronal context. These studies are important if the authors propose that CRFR1 agonists/antagonists directly modulate the γ-secretase by functioning as iGSMs and that CRFR1 G protein-biased signaling is a therapeutic avenue for AD.

D. In specific point 10, the authors suggest "Based on our study (Figure 4 an 5), the increase of Aβ42 levels in CRFR1 Δ386/IC3 expressing cells (Figure 3F), could be a direct effect of CRF on γ-secretase, similar to the effect of inverse γ-Secretase modulators (iGSM)." Could G protein signaling be involved rather than direct modulation of γ-secretase function? In the in vitro cleavage assays with H4, these cells express endogenous levels of the CRFR1 receptor (Fig. S1). If membranes were prepared from these cells, could this explain the observed effect rather than direct modulation of the γ-secretase?

2. Given that some of results from the current studies are unexpected and difficult to interpret in the context of previously published literature, it is important that the authors provide a model diagram at the end of the manuscript to explain how their studies would fit into a physiologically relevant context. How would stress affect the constitutive interaction and localization between CRFR1 and the γ-secretase? At what stage would β-arrestin 2 enter into this model?

**Referee #2:**

The authors have considered my suggestions and comments satisfactorily.