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Deorphanizing FAM19A Proteins as Pan-Neurexin Ligands with an Unusual Biosynthetic Binding Mechanism

Anna Khalaj, Fredrik Sterky, Alessandra Sclip, Jochen Schwenk, Axel Brunger, Bernd Fakler, and Thomas Südhof

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May 20, 2020

RE: JCB Manuscript #202004164

Dr. Anna J Khalaj
Stanford University
Department of Molecular and Cellular Physiology
265 Campus Dr
Stanford, CA 94305

Dear Dr. Khalaj:

Reviews of the manuscript "Deorphanizing FAM19A Proteins as Pan-Neurexin Ligands with an Unusual Biosynthetic Binding Mechanism" that you submitted recently to this journal are attached to this letter. The reviewers are both impressed with the quality and importance of the work and simply request a few clarifications and recommend some other largely minor revisions. A few new experiments are also suggested, but these do not seem essential to me.

I would like to ask you to consider these comments, make revisions that seem appropriate in response to them, and return the manuscript to the JCB office for a final decision. I look forward to receiving your revision.

I hope you, Dr. Sudhof and your other lab members are well isolated, well and in reasonably comfortable accommodations.

Sincerely yours,

Louis F. Reichardt For the JCB

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3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel

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4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (either in the figure legend itself or in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

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14) A separate author contribution section following the Acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

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Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Louis Reichardt, PhD
Monitoring Editor

Andrea L. Marat, PhD
Scientific Editor

Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

This manuscript by Khalaj et al. finds a novel ligand partner of Neurexin called FAM19A, previously known as a cytokine that binds to G-protein coupled receptors. FAM19A1-A4, but not FAM19A5, binds specifically to the cystein-loop domain of neurexin by disulfide bonding during the secretory pathway. Neurexin is required for the surface transport of FAM19A1, while FAM19A1 suppresses the post-translational modifications of neurexin (heparan sulfate and O-glycosylation).

Overall, I believe that this manuscript thoroughly reports a novel function of FAM19A with multiple lines of high-quality evidence through various experiments (co-IP, IHC, cell surface binding assay, and mass spectrometry). In addition, FAM19A is unique in that it binds to the cystein-loop of neurexins unlike other known neurexin partners binding to LNS domains. Given the increasing importance of post-translational modifications in synaptic cell adhesion molecules, in particular, those involving glycosylation and heparan sulfate, this study is a timely and important addition to the synaptic adhesion field. I have only the following minor comments.

Some minor comments are:

1. The results from the point mutation of the aa residues in the Cys-loop domain are quite surprising and interesting. I guess the authors have already performed sufficient levels of experiments, although I am curious to know about the effects of a 'single' cysteine mutation which would disrupt the loop structure but decrease the intermolecular disulfide bonds (between neurexin and FAM19) only by half. Some additional experiments or discussion would be nice.

2. It is interesting that FAM19A1 causes a decrease in mIPSC frequency. What may be the mechanisms underlying this inhibitory synapse-specific effects? A decrease in mIPSC frequency often involves a change in presynaptic release. Could it involve decreased presynaptic release at inhibitory synaptic sites? Here, the authors show changes in mIPSCs in the presence and absence of FAM19A1 in WT Nrxn123 cells (Figure 6). I am curious to know how mIPSCs might change in the absence of Nrxn123. Some additional experiments or discussion would be nice.

3. Nearly all neurexins bind to FAM19A1-A4, highlighting shared functions of FAM19A1-A4 in inhibiting neurexin modifications (by O-glycosylation and heparan sulfate). Notably, however, FAM19A1-A4-mutant mice show largely different behaviors. Although there are big gaps between

molecular interactions and behaviors, some discussions may be needed on potential differential functions of the neurexin- FAM19 interactions.

4. In figure S4B, FAM19A1 dimers are not really visible.

5. Figure 2 is not easy to read. Perhaps, color coding of the SS4+ and SS4- labels may help.

Reviewer #2 (Comments to the Authors (Required)):

Adhesion molecules organize the formation and stability of synaptic connections by linking core neurotransmission machinery to a vast array of extracellular ligands. Nearly all known neurological disorders are associated with alterations to synapse function, but efforts to understand and treat synaptic disease have been complicated by the sheer diversity of adhesion molecules, the abundance of binding partners, and their complex contribution to synapse formation and function. In order to begin to understand the synaptic basis of neurological diseases, a complete catalog of adhesion molecules and their binding partners is therefore required.

The study by Khalaj et al. has addressed this important issue with a comprehensive and clear approach. Focusing on neurexins, Khalaj et al. first use a rigorous and unbiased proteomic screen to identify that the secreted proteins, FAM19A1-4, are novel endogenous ligands of neurexins in mouse brain. They then fully characterize the properties and specificity of neurexin/FAM19A1-4 binding using in vitro assays and begin to assess the role of this binding in synapse function using dissociated cultures of primary neurons. Overall, the study by Khalaj et al. is thorough, the experiments are well-designed, and the data is clear and well-presented. This is a very strong first step towards a deeper understanding of how FAM19A may regulate synapse function via neurexins. Moreover, the biochemical interactions described for neurexin/FAM19A here may have implications beyond the synapse (Bottos et al 2009). I strongly recommend this work as suitable for publication in The Journal of Cell Biology.

It is possible that other reviewers may perceive limitations with the paper in two areas. The first is reliance of protein overexpression. The second revolves around the lack of antibodies to examine endogenous FAM19A. These should not preclude rapid publication of the existing data. The biochemical interactions necessitate sufficient protein to assess binding interactions and this is the major emphasis of the work. Again, I am strongly in support of publication of these data which represent a substantial advance in a fundamental area of neuroscience.

There are a few points that the authors may wish to consider - all of which are of minor importance.

Minor points:

Line 156 - Please identify the site of V5 tag insertion on the FAM19A peptide.

Line 157 - Please identify the site of Myc tag insertion on the neurexin peptide (related to point re: Line 149).

Line 184 - It is possible that fixation itself may permeabilize cell membranes and that the images shown in Figure 2 represent both surface and cytosolic complexes. A control might be warranted, in line with other studies using similar approaches.

Line 350 - Please provide a reference or information regarding the nature of the Cre mutation that results in an inactive isoform.

Line 352 - What is the efficiency of lentiviral infection in neuronal cultures?

Figure 6E&F, K&L Are these cumulative distributions obtained by pooling all individual PSC events across all recorded cells? Please clarify. If pooled, it would be appropriate to give the average of each sample and compare statistically.

Line 382 - Discussion of whether neurexin is a major/the primary binding partner to FAM19A might be better restricted to the discussion.

Figure 7A - Please clarify why some neurexin bands are considered 'non-specific' (especially β-Nrxns HS-; MW 63). The Cre-mediate knockout approach is not guaranteed to remove all neurexin protein (as indicated in Figure S7), so some residual protein is expected.

Figure 7B - The quantification does not seem to align well with a visual inspection of binds (FAM19A1-V5; MW11). Is it possible the input and flow-through lanes were flipped accidentally?

Figure 8 - The influence of overexpressed FAM19A on the migration of Nrxn bands is quite strong. How can this be interpreted in terms of the contribution of endogenous FAM19A to Nrxn modification? A short discussion of the relative influence of glycosidases/heparinases on Nrxn migration in the absence of FAM19A overexpression may help to clarify the function and the extent by which endogenous FAM19A modifies neurexins in cultured neurons.

In summary, the authors should be congratulated for an excellent study.

Graeme Davis.

Authors' Response to the Reviewers' Comments for Khalaj *et al.*, and Changes Introduced into the Revised Paper

Reviewers' comments are in black font and our responses are in blue font.

We thank the Reviewers for their very helpful and constructive comments. As recommended, in the revised paper we have introduced the corrections and changes described below in response to these comments. In addition, we have shortened the paper considerably to meet the character requirements of the *J. Cell Biol.* We have also moved Suppl. Figure 8 to the main figures and combined Suppl. Figures 3 and 4 as well as Suppl. Figures 5 and 6 to conform to the limit of five Suppl. Figures for the *J. Cell Biol.*

Reviewer #1 (Comments to the Authors (Required)):

This manuscript by Khalaj et al. finds a novel ligand partner of Neurexin called FAM19A, previously known as a cytokine that binds to G-protein coupled receptors. FAM19A1-A4, but not FAM19A5, binds specifically to the cystein-loop domain of neurexin by disulfide bonding during the secretory pathway. Neurexin is required for the surface transport of FAM19A1, while FAM19A1 suppresses the post-translational modifications of neurexin (heparan sulfate and O-glycosylation).

Overall, I believe that this manuscript thoroughly reports a novel function of FAM19A with multiple lines of high-quality evidence through various experiments (co-IP, IHC, cell surface binding assay, and mass spectrometry). In addition, FAM19A is unique in that it binds to the cystein-loop of neurexins unlike other known neurexin partners binding to LNS domains. Given the increasing importance of post-translational modifications in synaptic cell adhesion molecules, in particular, those involving glycosylation and heparan sulfate, this study is a timely and important addition to the synaptic adhesion field. I have only the following minor comments.

We thank the Reviewer for the positive and constructive comments.

Some minor comments are:

1. The results from the point mutation of the aa residues in the Cys-loop domain are quite surprising and interesting. I guess the authors have already performed sufficient levels of experiments, although I am curious to know about the effects of a 'single' cysteine mutation which would disrupt the loop structure but decrease the intermolecular disulfide bonds (between neurexin and FAM19) only by half. Some additional experiments or discussion would be nice.

We thank the Reviewer for this interesting suggestion. We have generated single cysteine mutants of the IgK signal peptide-Nrxn1β$^{SS4\text{-}SS5}$-ECD-Myc-6xHis construct and tested their secretion into the medium of HEK293T cells once by immunoblotting for Myc. We did not detect these constructs in the medium, but did not check whether we could detect them in the cell lysates, or via immunoprecipitation of the medium. This suggested to us that single cysteine mutant Nrxn1β-ECD does not fold properly and we did not pursue experiments using these constructs.

Given that our data point to the formation of a covalent neurexin/FAM19A complex within the secretory pathway, it is possible that the co-expression of FAM19A1 and a single cysteine mutant Nrxn1β-ECD would result in complex formation and secretion into the medium. In other words, it is conceivable that the formation of a single intermolecular disulfide will allow the single cysteine mutant Nrxn1β-ECD to fold properly. However, this would also result in FAM19A1 having a free

cysteine, which could negatively impact its folding, cause it to aggregate, and/or alter the stoichiometry of the Nrxn1β-ECD/FAM19A1 complex. While interesting, we believe that it would be difficult to interpret such results in a physiologically relevant manner.

Owing to the strict space limits for the *J. Cell Biol*., we have not added an extensive discussion of these possibilities to the text. We hope this is acceptable.

2. It is interesting that FAM19A1 causes a decrease in mIPSC frequency. What may be the mechanisms underlying this inhibitory synapse-specific effects? A decrease in mIPSC frequency often involves a change in presynaptic release. Could it involve decreased presynaptic release at inhibitory synaptic sites? Here, the authors show changes in mIPSCs in the presence and absence of FAM19A1 in WT Nrxn123 cells (Figure 6). I am curious to know how mIPSCs might change in the absence of Nrxn123. Some additional experiments or discussion would be nice.

We agree with the Reviewer that these are interesting questions, but addressing them, especially under the current conditions of the coronavirus crisis, is beyond the scope of the present paper. We concur with the Reviewer that a decrease in release probability is most likely the cause for the decrease in mIPSC frequency that we observed, given that the density of inhibitory synapses assessed by vGAT staining is unchanged by FAM19A1 overexpression. However, many potential mechanisms could be involved, and other causes could also account for this decrease. Studying the mechanisms of the FAM19A1 effect on mIPSCs and their relation to Nrxn123 triple deletions would require a major set of new experiments that would take months to complete.

3. Nearly all neurexins bind to FAM19A1-A4, highlighting shared functions of FAM19A1-A4 in inhibiting neurexin modifications (by O-glycosylation and heparan sulfate). Notably, however, FAM19A1-A4-mutant mice show largely different behaviors. Although there are big gaps between molecular interactions and behaviors, some discussions may be needed on potential differential functions of the neurexin- FAM19 interactions.

We agree and have now discussed this interesting question more explicitly in the paper (see Discussion, lines 491-496). As mentioned in the Introduction, *Fam19a1-a4* mRNAs are differentially expressed across brain regions and during development (Tom Tang et al., 2004; Yong et al., 2020; Figure S1A-C). Additionally, while *Fam19a1* mRNA is primarily synthesized in subsets of excitatory neurons, *Fam19a2* mRNA is preferentially synthesized in subsets of both excitatory and inhibitory neurons (Figure S1E-F). Therefore, differences in FAM19A1-A4 function and, putatively, neurexin/FAM19A complexes may reflect differences in developmental, regional, and/or cell subtype-specific expression profiles of FAM19A proteins.

4. In figure S4B, FAM19A1 dimers are not really visible.

We thank the Reviewer for noting this. The FAM19A1 dimer-indicating asterisks were included in former Figure S4B (now Figure S3I) by mistake and we have corrected this oversight.

5. Figure 2 is not easy to read. Perhaps, color coding of the SS4+ and SS4- labels may help.

We thank the Reviewer for letting us know this. We have modified the Figure 2 labels to make them easier to read.

Reviewer #2 (Comments to the Authors (Required)):

Adhesion molecules organize the formation and stability of synaptic connections by linking core neurotransmission machinery to a vast array of extracellular ligands. Nearly all known

neurological disorders are associated with alterations to synapse function, but efforts to understand and treat synaptic disease have been complicated by the sheer diversity of adhesion molecules, the abundance of binding partners, and their complex contribution to synapse formation and function. In order to begin to understand the synaptic basis of neurological diseases, a complete catalog of adhesion molecules and their binding partners is therefore required.

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It is possible that other reviewers may perceive limitations with the paper in two areas. The first is reliance of protein overexpression. The second revolves around the lack of antibodies to examine endogenous FAM19A. These should not preclude rapid publication of the existing data. The biochemical interactions necessitate sufficient protein to assess binding interactions and this is the major emphasis of the work. Again, I am strongly in support of publication of these data which represent a substantial advance in a fundamental area of neuroscience.

We also thank the Reviewer for his constructive and positive comments that have been most helpful.

There are a few points that the authors may wish to consider - all of which are of minor importance.

Minor points:

Line 156 - Please identify the site of V5 tag insertion on the FAM19A peptide.

Line 157 - Please identify the site of Myc tag insertion on the neurexin peptide (related to point re: Line 149).

In the revised manuscript, we have now described these constructs in more detail within the Results section (lines 133-134), in addition to the Materials and Methods section (under “Constructs”).

Line 184 - It is possible that fixation itself may permeabilize cell membranes and that the images shown in Figure 2 represent both surface and cytosolic complexes. A control might be warranted, in line with other studies using similar approaches.

We did not perform in parallel controls for the possible permeabilization of cell membranes due to fixation because in earlier experiments we observed no permeabilization of cells by fixation alone. Furthermore, the experiment shown in Figure 2 was performed at least once using live surface labeling (no fixation prior to staining) and yielded similar results. Moreover, in published

experiments we found fixation alone to be ineffective in permeabilizing cells (e.g., see Trotter et al., *J. Cell Biol.* 2019).

Line 350 - Please provide a reference or information regarding the nature of the Cre mutation that results in an inactive isoform.

Agreed - ΔCre is a truncated Cre recombinase that is recombination-deficient (Kaeser et al. 2009). We have widely used this construct, and added the relevant citation (line 292).

Line 352 - What is the efficiency of lentiviral infection in neuronal cultures?

The efficiency of lentiviral infection is nearly 100%, as documented extensively in previous papers (Kaeser et al., 2009; Chanda et al., 2017).

Figure 6E&F, K&L Are these cumulative distributions obtained by pooling all individual PSC events across all recorded cells? Please clarify. If pooled, it would be appropriate to give the average of each sample and compare statistically.

We are using standard procedures for the analyses of these data, as described in the Materials and Methods. Both cumulative distributions and averages are shown. Specifically, the graphs in the inset of Figure 6E-F, K-L show the average of the amplitude and frequency of each sample and have been statistically compared using two-tailed Student t-tests, as indicated in the figure legend. For mEPSC recordings, data in these graphs are means ± SEM of 33 and 37 cells recorded across 6 independent cultures; for mIPSC, data in these graphs are means ± SEM of 25 cells recorded across 5 independent cultures. For cumulative distributions, we plotted the amplitude and inter-event interval of single events across all recorded cells (180 events for all cells except for one cell, for which only 133 events could be scored). We used the standard approach of analyzing the same number of events for each cell in order to avoid biasing the analysis towards outliers. The cumulative distributions have been compared using the Kolmogorov-Smirnov (KS) test.

Line 382 - Discussion of whether neurexin is a major/the primary binding partner to FAM19A might be better restricted to the discussion.

We agree that most discussion of results should be reserved for the actual Discussion, but sometimes it is helpful to explain why certain experiments were done by discussing the data. In the revised manuscript, we have tried to avoid too much discussion in the Results section, but kept whatever was helpful for the reader in understanding the data.

Figure 7A - Please clarify why some neurexin bands are considered 'non-specific' (especially β-Nrxns HS-; MW 63). The Cre-mediate knockout approach is not guaranteed to remove all neurexin protein (as indicated in Figure S7), so some residual protein is expected.

We thank the Reviewer for bringing up this point. We agree that some residual protein may be expected when using the Cre-mediated knockout approach. We identified non-specific bands within the pan-neurexin immunoblots based on their persistence, without a qualitative change in band intensity, after Nrxn123 deletion (i.e., after Cre recombination). We observed a persistent (though lower abundance) diffuse band at ~63 kDa after Cre recombination in the biotinylated fraction. It is possible that this band corresponds to HS- β-neurexins and is not non-specific, so we have removed the asterisk corresponding to it in Figure 7A. However, this is extremely unlikely given the fact that other neurexin bands are quantitatively removed upon Cre recombination.

Figure 7B - The quantification does not seem to align well with a visual inspection of binds (FAM19A1-V5; MW11). Is it possible the input and flow-through lanes were flipped accidentally?

We checked this possibility, but did not detect a mistake. To our eyes, the immunoblots do look fairly representative. Please note that immunoblots, when visualized with the naked eye or by immunoperoxidase staining, are not quantitative but saturate very quickly. This is why we perform all quantitative analyses using fluorescently-labeled secondary antibodies, which are more quantitative because they have a large dynamic range.

Figure 8 - The influence of overexpressed FAM19A on the migration of Nrxn bands is quite strong. How can this be interpreted in terms of the contribution of endogenous FAM19A to Nrxn modification? A short discussion of the relative influence of glycosidases/heparinases on Nrxn migration in the absence of FAM19A overexpression may help to clarify the function and the extent by which endogenous FAM19A modifies neurexins in cultured neurons.

We thank the Reviewer for this suggestion. While the majority of neurons express exogenous FAM19A1-V5 after lentiviral infection, based on single-cell RNAseq data it is likely that only a subset of hippocampal neurons normally express significant levels of endogenous FAM19A1. Because *Fam19a* mRNAs are expressed in specific brain regions and in specific subsets of neurons (Figure S1), we were not surprised to observe that the endogenous contribution of FAM19A1 on neurexin modification is different from that of overexpressed FAM19A1. We would expect endogenous FAM19A-induced neurexin modifications to become diluted in bulk analyses of mixed hippocampal cultures.

In summary, the authors should be congratulated for an excellent study.

Graeme Davis.

We again thank the Reviewers for their efforts in assessing our paper – reviewing papers is a lot of work, and we are grateful for the Reviewers' time.