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Tyrosine phosphorylation of Munc18-1 inhibits synaptic transmission by preventing SNARE assembly

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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 February 2017

Thank you for submitting your manuscript to The EMBO Journal. I am sorry for the slight delay in getting back to you with a decision, but I have now received input from the two referees.

As you can see from the comments below, the referees appreciate that the analysis adds new insight. While referee #1 is fairly supportive of publication here pending revisions, referee #2 is not convinced that the analysis goes far enough to consider publication here. One issue raised is that the physiological significance of the findings is unclear as we get limited support for a role of Src family kinases in this process.

I have discussed with the referees as well as with my colleagues about how much further the analysis should be extended for consideration here. In addition to addressing the specific concerns raised, we would need support for:

- That the phosphorylation happens in neurons like using mass spec data or phospho specific antibody

- Identification of the kinase involved and support for that this regulation is physiological significant

- Some insight into the dynamics of the phosphorylation event and when it happens.

Should you be able to extend the analysis along such lines then I am happy to consider a revised version. I can extend the revision deadline up to 6 months.

I also know that this would entail quite a bit of extra work and I have also taken the opportunity to discuss the manuscript further with my colleagues at EMBO Reports. EMBO Reports is interested in consider a revised version that addresses the specific comments raised including point 1 referee 1.

Let me know if you are interested in this option

REFEREE REPORTS

Referee #1:

The present paper by Meijer et al. reports on an interesting study concerned with the tyrosine phosphorylation of Munc18-1 (M18). M18 is an absolutely essential regulator of SNARE complexes and presynaptic transmitter release, and the activity of Src and related tyrosine kinases has profound (inhibitory) effects on transmitter release. The present paper demonstrates a functional convergence of these two important lines of research by providing data that indicate that tyrosine phosphorylation, probably by Src and related kinases, of M18 at a defined site strongly inhibits the release-promoting function of M18.

I think the study is in principle suitable for publication in The EMBO Journal. Still, I have a few issues that should be considered by the authors.

1. In their introduction, the authors make the valid point that the tyrosine phosphorylation of previously described substrate proteins cannot explain the robust effects of tyrosine kinases on presynaptic function, and a key conclusion of the present study is that the tyrosine phosphorylation of M18 can. I think this is a critically important issue. The consequent prediction would be that the presynaptic functionality of neurons expressing the M18-YA mutant, in which synaptic transmission is still robustly measurable, should be at least partly insensitive to the effects of Src activity. I think this could be tested, e.g. by expressing active Src or by inhibiting it. Can this be done?

2. Partly based on Figure 1G, the authors argue that M18-YD does not have the characteristic fusion-stimulating activity of M18 anymore. However, it seems to be only the maximal fusion and apparently not the (initial) rate of stimulated fusion that is affected. I realize that this is different in the -CpxII dataset shown in Figure 1F, but I am still confused. Do the authors mean to say that in the presence of Cpx, i.e. in the context of a more complete reconstitution of the release machinery, the effect of M18 is restricted to promoting some additional t-SNARE assembly? If so, how does this relate to the inhibitory effect of M18 shown in Figure 1I, which is equally affected by all M18 variants tested?

3. I am a bit confused by the quantifiation of the expression levels of the different M18 variants tested. Figure 1A (right panels) indicates very substantially reduced expression levels of M18-YD as compared to WT, while the difference reported in Figure 1B (left - synapses) is marginal. I think it is important to tell the reader how the total expression levels compare between M18-YD and WT M18. Otherwise it is difficult to judge how close the M18YD expression approaches a bona fide loss of function as seen in the KO. The same is true for the data on M18-YA.

4. A reliable demonstration of a combination of normal vesicle docking (Figure 3) and total loss of primed vesicles (Figure 2) would be interesting. As far as I see it, the evidence from work in worm NMJ and mouse hippocampus is still in favour of a close correlation between SNARE complex assembly, priming, and docking - unless the fusion process is halted at a stage prior to full SNARE complex zippering or the active zone is shot to pieces, which probably affects vesicle mobility and recruitment during hypertonic sucrose stimulation. In the present study, the authors assessed vesicle docking in ultrathin sections of aldehyde fixed material. I think this is not really sufficient to make a reliable call - and in fact, neurons expressing M18-YA do show a partial docking defect. I see no easy way out of this apart from either doing the EM analysis with HPF material and tomography or toning down or leaving out the docking data. A conceptually related issue concerns the degree and efficiency of SNARE complex assembly in the presence of M18-YD vs. WT M18. How can the authors deduce from the present data with confidence that SNARE complex assembly is indeed differentially affected by M18-YD vs. WT M18? After all, Figure 1I shows that the two protein

variants have the same inhibitory effect on SNARE assembly and fusion ...

Overall, I support the publication of the present work in EMBO Journal, but prior to a final decision I would like to see the authors' response to my comments above.

Referee #2:

Munc18-1 is required for neurotransmitter release, and Munc18-1 homologs (collectively SM proteins) may be universally required for SNARE-mediated fusion. How exactly SM proteins promote membrane fusion has been a long-standing mystery, but recent developments (Parisotto 2014 and Baker 2015) support a templating role in SNARE complex assembly. Several mutants in or near domain 3a have rather dramatic effects on Munc18-1 function, which together with structural data on another SM protein (Vps33) implicate this region in the templating role.

The authors find that Src family kinases phosphorylate Munc18-1 at Tyr 473, a residue that lies adjacent to, but not within, domain 3a. They then make three mutations, Y473F, Y473D, and Y473A, and test the effects of these mutations on an in vitro lipid mixing assay and also in vivo using munc18-1 null neurons rescued with lentivirus expressing wild-type or mutant Munc18-1s. Near the end of the manuscript, they also combine Y473D with a second mutation, P335A, this one inside domain 3a and previously shown to augment Munc18 function. From their results they conclude that Tyr 473 phosphorylation hinders Munc18-1 function, either by affecting the conformation of domain 3a or by blocking synaptobrevin binding (the latter assumed to be analogous to Vps33-Nyv1 binding as per Baker 2015). This is an interesting model, although of uncertain physiological significance absent evidence that Src family kinases play a role in regulating neurotransmitter release, or that eliminating the ability of Tyr to be phosphorylated (via the Y473F mutation) has an impact on synaptic transmission.

Main points:

1. Phe is a fine mimic for unphosphorylated Tyr and, indeed, is found in Munc18-3 according to Fig. 1C. Asp seems like a much poorer mimic for phospho-Tyr. The hydrophobic aromatic ring is gone, to be replaced by a highly polar - indeed fully charged - carboxylate. The position of the negative charge is completely wrong. This may well trigger structural changes at the domain 3a/3b interface, but it seems like a stretch to argue that these changes would resemble the impact of Tyr 473 phosphorylation.

2. I would argue that the field now demands content-mixing assays in addition to, or instead of, lipid-mixing assays. There is simply too much evidence from too many labs that lipid mixing is not necessarily reporting on membrane fusion.

3. I'm not sure why the authors used different experiments in Figs. 1 and 5. Fig. 5 needs to include the experiment shown in Fig. 1f (with a content mixing read-out, of course) to be convincing.

4. At several points in the manuscript (e.g., on p. 10 and pp. 14-15), the authors argue that Y473D likely inhibits fusion by inhibiting helix 12 extension. I do not find this convincing (and indeed the abstract and the final paragraph of the Discussion, for example, suggest an alternative explanation). Why am I unconvinced? Having shown that Y473D has reduced function, and knowing from published work that P335A has enhanced function, the authors now find that P335A partially rescues Y473D. I can't see why this suggests that P335A and Y473D impinge on the same aspect of Munc18-1 mechanism. Aren't the results consistent with the combined impact of independent negative (Y473D) and positive (P335A) effects?

Rather than inhibiting helix 12 extension, it seems more plausible that Y473D destabilizes synaptobrevin binding. First, this is consistent with data presented in Fig. 5b. Moreover, Y473 (and L348) appear to lie in the synaptobrevin binding groove. Finally, P335A does not rescue the synaptobrevin binding defect exhibited by Y473D (Fig. 5b). Thus, taken together, the evidence seems largely unsupportive of the authors' model that 473D acts at a distance to inhibit helix 12 extension.

Minor point: On p. 6, "occluded" should be "omitted".

1st Revision - authors' response 30 August 2017

Referee #1:
The reviewer 'supports publication of the work in EMBO Journal' and raises 4 main issues:

1. *The reviewer points out that a non-phosphorylatable Munc18-1 mutant "should be at least partially insensitive to the effects of Src activity", and asks whether this prediction can be tested by expressing active Src or inhibiting Src.*
We agree with this idea and in fact we had already looked into the suggested experiments earlier. We learned that SFK activation in the brain is not as straight forward as for other kinases. From the cancer field we know that several cellular mechanisms tonically inhibit SFKs and activation involves a multistep process (unlatch–unclamp–switch, (Roskoski, 2015)). It is known that SFKs are important for synaptic plasticity, for instance during LTP induction, but it is unknown how and where the kinases are activated and escape their tightly regulated inhibition. Consistent with this, we have not observed an effect of Src inhibitors on synaptic transmission (new Fig EV3a-b), confirming conclusions from previous studies that Src is largely inactive under basal conditions (Roskoski, 2005). Conversely, SFK over-expression does not lead to higher kinase activity (new Fig EV3c), also confirming previous studies (Kotani et al., 2007). Hence, it remains unclear how/when/where these kinases are activated. Unraveling physiological SFK activity regulation in the brain is a study on its own. Instead, we have now strengthened the evidence that native, brain Munc18-1 is indeed phosphorylated by SFKs and we show that when that happens, synaptic transmission is effectively shut down. We feel this is important and novel, especially with the new molecular insights into how Munc18-1 shuts down transmission. We have also added the experiments to modulate the kinase as a supplemental figure to the revised manuscript (Fig EV3 (p12,l16-30) and added more background information on the fact that physiological SFK activity regulation is complex and largely unknown, at least in brain (p15,l13).

2. *The reviewer points out that the interpretation of the phosphomimetic Munc18-1(Y473D) variant in the liposome fusion assays is confusing and request clarification of our interpretation in relation to the presence/absence of CpxII.*
The reviewer is correct in concluding that in the presence of CpxII, Fig. 1G, the effect of Y473D on the initial fusion rate is less obvious. In general, Complexin inhibits in particular the initial fusion rates in the presence or absence of Munc18-1 and calcium is required to trigger high fusion rates (consistent with our earlier findings (Parisotto et al., 2014)). We argue that also in the presence of CpxII, Munc18-1 stimulates the initial lipid-mixing rates before calcium is added, see the first 2 min in Fig 1G (compare the grey line (-) with the black one (+M18)). The reviewer is correct that Munc18-1 also increases the final fusion signal (this was also observed without CpxII). This is also in line with our previous data (Parisotto et al., 2014). We argue that Munc18-1 increases the number of functional v- /t- SNARE complexes, irrespective of the presence of CpxII. In addition, Munc18-1 also stabilizes functional t-SNARE conformations (Weninger et al., 2008). We have clarified this interpretation in the revised manuscript (p6, l31 to p7, l11). We realized that an inconsistent use of color in Fig 1F-I relative to later figures may have contributed to the confusion. We have now corrected the color use in Fig 1.

The reviewer also asks how the effects on the stimulatory function of Munc18-1 relate to "the inhibitory effect of M18 shown in Figure 1I, which is equally affected by all M18 variants tested".
First, we have to clarify that Fig 1I demonstrates that the inhibitory effect is equally retained in all Munc18-1 variants comparable to Munc18-1 (WT, black line), not equally affected. However, the reviewer is correct that the Y473D mutant is defective in its stimulatory function, while retaining its inhibitory function. The inhibitory and stimulatory role of Munc18-1 rely on distinct Munc18-1/SNARE protein interaction modes (Schollmeier et al., 2011). Experiments in Fig 1F-I were specifically designed to selectively investigate the stimulatory effect (by starting with assembled t-SNARE complexes), or the inhibitory function (by starting with Syntaxin1 and replacing VAMP2 with VAMP8 thereby removing the VAMP2-Munc18-1 interaction critical to the stimulatory function of Munc18-1). On Syntaxin1 GUVs, Syntaxin1 (in contrast to preassembled t-SNAREs) adopts a closed conformation, which is stabilized by Munc18-1 (Misura et al., 2000). In contrast to

VAMP2, VAMP8 cannot release the closed state (Schollmeier et al., 2011). Thus, Y473D affects only the stimulatory effect of Munc18-1 by inhibiting VAMP2 binding.

3. *The reviewer raises concerns about the quantification of Munc18-1 expression levels, and argues it is important to show "the total expression levels compared between M18-YD and WT M18" to be able to compare it to "a bona fide loss of function as seen in the KO".*
Since mutations can alter the localization or subcellular targeting of a protein, we have specifically quantified the fluorescent intensity of Munc18-1 signal within synapses to investigate how much Munc18-1 is available for synaptic transmission (using automated image analysis to detect the soma, dendrites and synapses (Schmitz et al., 2011)). We have now also added Munc18-1 staining intensity in the soma to Fig 2B (M18-YD) and Fig 8C (M18-YA).

4. *The reviewer is concerned that the current method used to address vesicle docking (aldehyde fixed material) is "not sufficient to make a reliable call" on vesicle docking.*
We agree. We have now repeated the vesicle docking experiments using cryofixation. The new data lead to the same conclusion, that vesicle docking is normal in neurons expressing the M18-YD mutant. These data are added to the revised manuscript (Fig. 3f-j), result section (p9, l2-6).

The reviewer also raises concerns on whether we can conclude "with confidence that SNARE complex assembly is indeed differentially affected by M18-YD vs. WT M18", especially since "the two protein variants have the same inhibitory effect on SNARE assembly and fusion".
We agree that liposome fusion assays have their limitations to draw conclusions on the function of Munc18-1. Depending on the experimental design, Munc18-1 can both facilitate and inhibit SNARE-assembly. In this study, we have specifically chosen the conditions such that we can investigate either the stimulatory role (by starting with assembled t-SNAREs) or inhibitory role (by replacing VAMP2 with VAMP8). Therefore, we believe we can deduce with confidence that Munc18-1(Y473D) cannot facilitate SNARE complex assembly. As further proof, we have now included a trans-SNARE formation assay in which t-SNARE SUVs were mixed with v-SNARE SUVs and incubated with or without Munc18-1. While Munc18-1(WT) promoted VAMP binding to t-SNAREs by 350% (immunoblotted VAMP2 precipitated using nickel beads that bind His6-SNAP-25), Munc18-1(Y473D) does not (Fig. 5E) , result section (p11,l10-22) and discussion (p18,l4-12).

Referee #2:

This reviewer states our model "is an interesting model" and raises 4 major and 1 minor point

1. *The reviewer argues that "it seems like a stretch to argue that these changes would resemble the impact of Tyr 473 phosphorylation".*
This is a valid point, but there is no amino-acid substitution that fully resembles phospho-tyrosine. We have tried to phosphorylate Munc18-1 to use in our liposome fusion assays, but the amount of phosphorylated Munc18-1 obtained (maximum of 8%) was insufficient to perform functional assays. Our AA substitution approach is the best available alternative and similar approaches have provided many valuable insights on the impact of phosphorylation events in the past.
Moreover, we feel that the observed effect of local charge change, mimicking the effect of phosphorylation as good as possible, produces a plausible and interesting effect on Munc18 function and synaptic transmission, which is important and novel, especially with the new molecular insights into how Munc18-1 shuts down transmission. In addition, adding a phosphate to Y473 is predicted to obstruct the proposed VAMP2/Synaptobrevin2 binding groove, which is exactly what we see in our VAMP2-binding assay using Y473D (Fig. 5b). We have added this to the argumentation of the amino acid replacement strategy (p17, l2-8). Nevertheless, we have now added a more explicit remark about the limitations of AA substitutions to mimic Tyrosine phosphorylation events (p16,l29).

2. *The reviewer states that "the field now demands content-mixing assays".*
We have performed content-mixing assays and added the data to the revised manuscript (Fig. EV2A-B), see result section (p7,l9). The results of the content-mixing assay are in line with the lipid-mixing assays.

3. *The reviewer points out discrepancies in the experimental design between Fig. 1 and Fig. 5 and states that a content mixing assay is required to be convincing.*
Indeed, the assay in Fig 5D uses GUVs containing monomer Syntaxin, instead of the assembled t-SNARE GUVs used in Fig 1. This was chosen to allow formation of inhibitory Syntaxin-Munc18-1 dimers. As we reported previously (Schollmeier et al., 2011), subsequent incubation with SNAP-25 and VAMP2 liposomes reverses the inhibitory effect of Munc18-1 and M18-WT stimulates fusion. On the other hand, Y473D retains its inhibitory function but does not stimulate fusion. To be consistent with Fig 1, we replaced the experiments in Fig 5D with a new dataset which also includes Y473F. In line with Fig 1, Y473F also acted like M18-WT in these new experiments. In addition, we have added data of a content-mixing assay to Fig 5D as requested (see Fig. EV2C) and result section (p11,l8).

4. *The reviewer is not convinced by our proposed model "that Y473D likely inhibits fusion by inhibiting helix 12 extension", and mentions several arguments.*
We agree. From the data presented in figure 5C it is impossible to tell whether Munc18-1(P335A/Y473D) reversed the inhibitory effect of M18-1-Y473D or (partly) restored the stimulatory effect of Munc18-1. We therefore performed a new assay to address trans-SNARE formation in Fig 5E using t-SNAREs and v-SNARE SUVs (p11,l10-22). Surprisingly, this new experiment showed that the stimulatory function of Munc18-1 is not restored by P335A/Y473D. We conclude that P335A either renders Y473D unable to inhibit fusion (likely because the P335A single mutant also stimulates fusion under conditions that normally inhibit fusion (Parisotto et al., 2014)) or was not able to reverse the inhibitory effect of Munc18-1. These interpretations were added to the discussion (p18, l4-12). We agree with the reviewer that there are alternative explanations for the reversal the Y473D phenotype by P335A. We have revised this by removing over-interpretation from the result section (p10, l27 and p12,l1), changed the order of argumentation to prioritize VAMP2 binding deficits (p2,l12-14; p4,l11) and modified the last paragraph of the discussion. We thank the reviewer for pointing out the over-interpretation.

Minor point: *On p. 6, "occluded" should be "omitted"*. This is corrected.

RESPONSE TO THE EDITOR'S COMMENTS

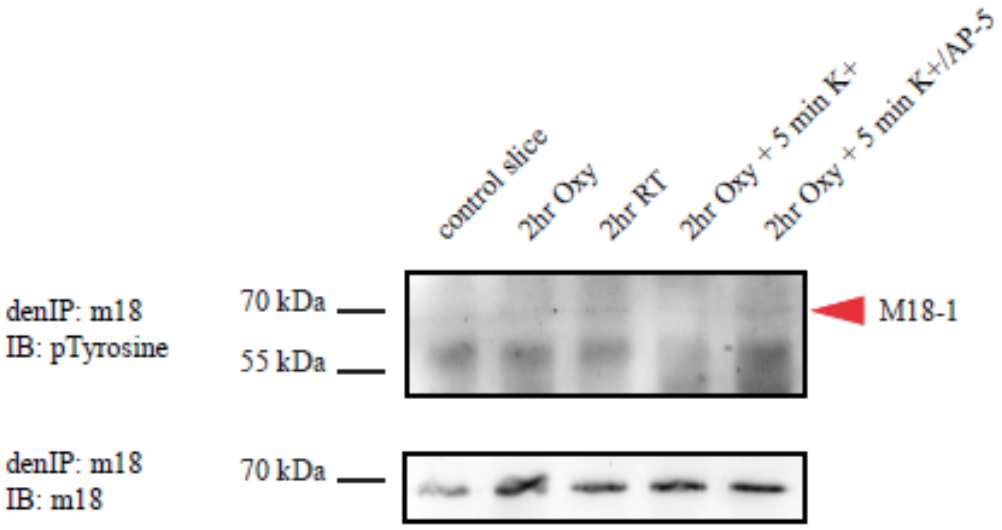
In the editor's letter, 3 points were listed on how much further the analysis should be extended, listed below.
1- That the phosphorylation happens in neurons like using mass spec data or phospho specific antibody
We agree that this is crucial. We have now added to the revised manuscript (i) information on mass spec data to confirm that Munc18-1 is indeed phosphorylated at Y473 (p5, l3) and (ii) because we have tried generating phospho-specific antibodies for Munc18-1 for years without success, we present an alternative that we feel is equally convincing, using a phospho-tyrosine antibody: we have immuno-precipitated Munc18-1 from brain lysates, brain slices and from cultured neurons under denaturing conditions, i.e., no interacting proteins are-co-precipitated, and subsequently stained blots using the phospho-tyrosine antibody (see Fig. EV1 and Fig. EV3, and figure below). The results are described in (p5, l17-25) and p12,l21-30. Using these additions we feel we have convincingly shown that SFK-dependent phosphorylation of native Munc18-1 happens in neurons.

2- Identification of the kinase involved and support for that this regulation is physiological significant
We have now added new experiments to identify the kinase: we have added native Src (expressed in mammalian cells) to brain lysates and show that the kinase indeed phosphorylates native, brain Munc18-1 (Fig EV1B). In addition, we already had HEK-cell co-expression data in the original manuscript. Together, we feel these two data sets show convincingly that Src (and related kinases) phosphorylate (endogenous) Munc18-1.
In response to reviewer 1 (point 1) we already indicated how the lack of tools and knowledge on Src-pathways in neurons limits our options. SFKs are tonically inhibited and the mechanism of disinhibition is complex and only characterized in any detail in cancer cells. Increasing Src activity by overexpression does not lead to higher kinase activity (new Fig EV3C), consistent with previous studies (Kotani et al., 2007). Unraveling physiological SFK activity regulation in the brain is a study on its own. We also wish to point out that our paper does not make any claims on the physiological regulation of phosphorylation. Our paper claims that native, brain Munc18-1 is phosphorylated by

SFK kinases and when that happens, synaptic transmission is effectively shut down. We feel this is important and novel, also with the new molecular insights into how Munc18-1 shuts down transmission.

3- Some insight into the dynamics of the phosphorylation event and when it happens.
Our new experiments show that phosphorylation of neuronal Munc18-1 happens within 10 minutes at room temperature (Fig EV1B). We investigated physiological triggers of the phosphorylation event in slices (see figure below) and cultured neurons, but with the current tools we do not have the sensitivity to detect phosphoylation of small fractions of Munc18-1. Analysis of the kinetics in neurons requires a phospho-specific antibody, which we failed to generate (see above). We also point out that this issue is actually not raised by the reviewers. As indicated under point 2, Src activity is tightly regulated and unraveling the physiological SFK activity regulation in the brain is a study on its own.

FIGURE: Munc18-1 phosphorylation in brain slices



Adult mouse brain was sliced in ASCF (250 nm) and either directly denatured (control slice) or first treated with the following conditions: 2 hours at room temperature (RT), oxygenized for 2 hours on ice (Oxy), Oxy with 5 minutes depolarization at end by adding 40mM potassium (Oxy + K+), Oxy with K+ and NMDAR blocker AP-5 (1 mM) during depolarization. Tyrosine phosphorylation was probed by denatured immunoprecipitation with Munc18-1 antibody and immunoblotting against anti-phosphotyrosine.

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2nd Editorial Decision 04 October 2017

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by the two referees and both referees appreciate the added data. I am therefore very pleased to accept the manuscript for publication here.

REFEREE REPORTS

Referee #1:

This is revised version of an earlier EMBO submission I reviewed.

I think the authors made a serious effort to respond to my comments, and I am satisfied with this.

Referee #2:

The authors have, as requested, included contents mixing assays. As well, they now mention the limitations of Asp as a mimetic for phospho-Tyr (although not before using the term 'phosphomimetic' ten times, including in several section headings). Based on new experiments, it seems fairly clear that Y473D acts by inhibiting VAMP binding, which is a satisfying outcome that is reasonably consistent with expectation doi: 10.1126/science.aac7906 and doi: 10.7554/eLife.24278 (the second of which was published in May and needs to be referenced).

It is less clear to me that the authors have fulfilled the task set by the other reviewers of establishing a clear role for Y473 phosphorylation by Src in the regulation of neurotransmitter release. If that demonstration is in their view conclusive, then the biochemical experiments provide a plausible mechanistic basis, and I would support publication in EMBO J.

2nd Revision - authors' response 12 October 2017

We have produced and added 10 new datasets to the manuscript: electron microscopy of cryofixed samples, new phosphorylation assays in cultured neurons and brain lysate, *in vitro* kinase assays, physiology on the effect of SFK inhibitor on synaptic transmission, a trans-SNARE formation assay, content-mixing assays and a new liposome fusion assay containing all mutants to be consistent.

We have fully addressed all comments by the reviewers except part of the 1st point of reviewer 1. We have also addressed your 3 points, except half of the second point, which overlaps with the remaining reviewer point. We feel that this remaining issue can only be addressed with a phospho-specific antibody, which we failed to generate.

After reading your message and the reviewers' reports, we realized something that has become clear to us along the way, that SFK neurobiology is largely uncharted territory, was not properly expressed in the manuscript (and triggered several justified questions). In the revised manuscript, we have now added some of the crucial facts, for instance that increased SFK levels do not necessarily lead to higher kinase activity (even in *in vivo* studies) and that pharmacological inhibition is poorly documented in brain except for some specific cases (during LTP or upon 40mM KCl in brain slices). Several mechanisms have been identified in the cancer field that tonically inhibit SFKs and activate the kinase in a multistep process (unlatch–unclamp–switch, Roskoski, 2015).

Still, SFKs are highly expressed in the brain and under specific conditions are crucial for synaptic plasticity, for instance during LTP. Hence, we can be sure that Src-dependent phosphorylation is relevant in the brain, but its experimental manipulation is not as straight forward as for other kinases. Unraveling physiological SFK activity regulation in the brain is a study on its own. Our paper claims that native brain Munc18-1 is phosphorylated by SFK kinases and when that happens, synaptic transmission is effectively shut down. We feel this is important and novel, especially with the new molecular insights into how Munc18-1 shuts down transmission.

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This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Inhibitory neurons were excluded based on decay of postsynaptic currents. Electrophysiological recordings were included if the following quality criteria were passed: access resistance < 12 Mohm and leak current < 300pA. These criteria were pre-established.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Drug treatments were blinded during data acquisition and analysis
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Data samples were tested for normality and heterogeneity of variance. If data allowed, unpaired two-way t-tests (with Welch correction in case of unequal variance) were performed. Otherwise, non-parametric tests were used. For data that were nested (e.g. assessing synapse ultrastructure with electron microscopy, in which multiple synapses origining from a single neuron or single culture), multilevel analysis was performed.
Is there an estimate of variation within each group of data?	Yes, tdata were tested for heterogeneity of variance
Is the variance similar between the groups that are being statistically compared?	Yes, unless stated in the legends

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Munc18-1 antibody (de Vries, 2000), anti-phosphotyrosine (clone 4G10). Anti-VAMP (SySy) and anti-MAP2 (Abcam) are widely used antibodies.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HEK293T cells

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Munc18-1 deficient E18 embryos were obtained by ceasarian section from pregnant females from timed matings of munc18-1 heterozygous mice. Newborn P0-P1 pups from pregnant female Wistar rats were used for glia preparations. Animals were housed and bred according to institutional, Dutch and U.S. governmental guidelines.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA