Supplemental information about the study "Arachidonic acid does not grease the exocytotic SNARE machinery"

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A brief description of my study: These were experiments that I had carried out because I considered the study by Darios and Davletov (Nature 2006: 440: 813-17), which had appeared shortly before, to be untrustworthy. I performed a series of experiments which showed that the effect observed by Darios and Davletov is not specifically caused by arachidonic acid, but can be attributed to the formation of micelles independent of their composition.

Nature refused to publish my data. In the following you can find the correspondence, what the authors had to say. At the end you will find my reply to their comments, which, on the advice of others, I never sent. I had learned a lesson.

I have now decided to make these old data accessible. A lot of time has passed. Nevertheless, negative findings should not simply disappear in the drawer.

My original letter to Nature

22nd May 2006

Dear Editor:

In a recent letter to Nature, Darios and Davletov (Nature 2006: 440: 813-17) have proposed to have found the molecular basis for a previously established activating effect of polyunsaturated fatty acids, in particular arachidonic acid, in neurite outgrowth. They claim that arachidonic acid directly acts on the secretory machinery of cargo vesicles, triggering SNARE complex formation between syntaxin 3, SNAP-25 and synaptobrevin. They state that syntaxin 3 is a single molecule effector of arachidonic acid, switching it into a so-called open conformation. Only in its open conformation syntaxin 3 can engange in SNARE interactions.

Interestingly, Davletov and colleagues had shown before that syntaxin 3 can also be activated for SNARE interaction ('opened') by detergents. They argue, however, that detergents in contrast to arachidonic acid need to be employed at much higher concentrations. Nevertheless, the sigmoidal concentration dependence of the accelerating effect corresponds to the critical micellar concentration of arachidonic acid. Therefore, it seems very likely that the observed effect on syntaxin 3 is simply due to a detergent/surfactant-like action of arachidonic acid micelles.

Due to this discrepancy in their claim, I have decided to repeat their experiments using existing assays in our laboratory. As outlined in detail in the enclosed text entitled 'Arachidonic acid does not grease the exocytotic SNARE machinery', my data reveal that arachidonic acid rather unspecifically affects SNARE assembly, and therefore are incompatible with the findings of Darios and Davletov. For the purpose of scholarly debate, I would like to publish my results as 'Communications Arising' in your journal.

Looking forward to hearing from you

Dirk Fasshauer

PS: Last Thursday, I have sent a copy of my manuscript to both authors, but so far did not receive any reply.

Answer from Nature

6th July 2006

Dear Dr Fasshauer

Thank you for your comment on the Nature paper by Davletov et al., which I am afraid we must decline to publish. As is our policy on these occasions, we showed your comment to those authors, and their response is enclosed.

In the light of this reply and of the competition in this section of the journal, we have regretfully decided that publication of this debate is not justified as it would not add to our understanding or otherwise clarify the issues for our readers.

Thank you again, however, for writing to us.

Yours sincerely

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Nature Author #1(Remarks to the Author):

The study by D. Fasshauer addresses the effect of arachidonic acid (AA) on syntaxin. The fact that the author does see arachidonic acid-stimulated SNARE interaction undermines the message of his title. We showed recently(Ref1)in vivo that native syntaxin 3:
1) is involved in neuronal growth
2) responds to the presence of a growth factor by enhanced interaction with SNAP-25
3) responds similarly to the presence of AA.
We also showed that recombinantly-produced syntaxin 3:
4) is activated by AA and several other polyunsaturated fatty acids in a direct manner.
Fasshauer questions this latter point and states that (i) syntaxin 3 readily forms SNARE complexes in the absence of fatty acids, and (ii) AA has a further non-specific micelle-related effect.
Analysis of the technical details reveals the likely causes of the discrepancies. The author routinely uses 10-20 µM protein, 10-fold higher than we used. Furthermore, it appears from his cited publication(Ref2) that some incubations were done overnight at 4{degree sign}C - our reactions were 30 min at 22{degree sign}C(Ref1). High protein concentrations, long incubation times and low temperature are known to favour protein interactions and can explain Fasshauer's in vitro observations. Our own results(Ref1) are in accord with the original study of syntaxin isoforms that highlighted a relative inability of syntaxin 3 to engage its SNARE partners(Ref3).

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Fasshauer observes AA-induced activation of the isolated SNARE motif of syntaxin 3 and therefore questions whether AA acts on the helical syntaxin fold. However, isolated SNARE motif oligomerises to form a helical bundle(Ref4) and AA may affect such a state, leading to release of the truncated motif for further interactions.

Fasshauer observes that AA can activate syntaxin 4 but this does not affect the conclusions of our paper. Indeed, we showed previously that Munc18-regulated syntaxin 1 is also sensitive to AA(Ref5) and yet syntaxin 1 is not involved in neurite outgrowth(Ref1). It is possible that the regulation of syntaxins by unsaturated fatty acids is a conserved mechanism. Fasshauer states that AA has a non-specific, micelle-induced effect, despite knowing that only 'certain detergents' (e.g. dodecylmaltoside) affect syntaxin 3. We addressed this issue previously (Ref5) showing that synthetic detergents with a certain carbon chain length can substitute for polyunsaturated fatty acids. Does this rule out a role of AA in syntaxin activation? No; for example, synthetic opiates can stimulate endorphin receptors - but this does not mean that endorphins act non-specifically. Furthermore, AA at a concentration of 50 uM, i.e. below the CMC value of 60 uM (Ref6), still activates syntaxins (Refs1,5)

Taken together, our published results show that micelle formation and surfactant properties per se can not account for syntaxin activation and that naturally occurring fatty acids act in a specific way. It is worth remembering that in vitro reactions can only attempt to mimic physiological conditions. Although Fasshauer questions the relevance of the AA effect, the author neither provides in vivo results nor finds an indication in literature for possible local and transient AA concentrations to support his arguments (for further discussion see Ref7).

References:

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My reply that I have never sent

Re: Decision on Nature Manuscript 2006-05-05495

Dear Dr. Nath,

Thank you very much for the response to my criticism on the Nature paper by Darios and Davletov entitled "Arachidonic acid does not grease the exocytotic SNARE machinery". In fact, after reading the recent feature article on replication of published results {Giles, 2006 #44}, I understand that editors are generally reluctant to deal with challenges of papers that have first been selected by the editors as interesting and then have passed peer review. However, the response of the authors does nothing to dispel my rather fundamental critique – rather the contrary is the case. In fact, in their answer the authors are already backtracking from one of their fundamental statements – that the effect of arachidonic acid is specific for syntaxin 3. To make it perfectly clear: I reiterate that the main conclusions of Darios and Davletov are erroneous because of fundamental flaws in the experimental design and basic ignorance about the biology and chemistry of fatty acids. As the authors are unable to counter my arguments, it is my opinion that the original study should be retracted. Apparently, no expert in amphiphilic molecules has seen my comments, because otherwise the smokescreen of vague and factually incorrect statements provided by the authors would not have been taken for granted. Apparently Nature accepts those statements as facts. I consider myself as an expert in conformational regulation of SNARE proteins who has contributed significantly to our current picture of how SNARE proteins work. Therefore, it is definitely unsatisfactory that my comments are so easily rejected at the editorial level.

In their response, Darios and Davletov have emphasized that I criticized only a minor point of their study, the *in-vitro* part. They aim to downplay this part by saying for example: *"It is worth remembering that in vitro reactions can only attempt to mimic physiological conditions."* This is true, but the in-vitro part is where the strength of the argument is coming from. While we have not tried to repeat the cell culture part of the work, one cannot fail to notice that this section is weak as well. The reason why the study is considered by the community as "news-worthy" is the authors claim to have identified a molecular target of arachidonic acid, and that this target is surprising and completely novel for a fatty acid (FA) or FA derivative, particularly for one that is in the frequently in the news.

My data that I have submitted show:

i) SNARE complex formation does not require the presence of arachidonic acid (AA). To explain the discrepancy between my and the published data, I assume that the recombinant proteins used by Darios & Davletov are inactive, probably due to aggregation.

ii) In my hands, the accelerating effect of arachidonic acid on complex formation is rather moderate. This effect correlates with the presence of a micellar phase that is characteristic for all surfactants. In fact, it can be reproduced by detergents that are structurally unrelated to AA

(DDM or Triton). Thus, syntaxin 3 is not activated by single molecules of AA but rather by micelles (i.e. by a surfactant action). Consequently, the acceleration cannot be due to a specific, i.e. receptor-ligand type of interaction between single molecules of AA and syntaxin 3.

iii) The accelerating effect of AA and other detergents at concentrations above their critical micellar concentrations (CMC) is also observable when the N-terminal domain of syntaxin 3 is truncated and only the SNARE motif is present. This finding excludes that acceleration is due to a transition of syntaxin 3 from the closed to the open conformation as suggested by the authors – the closed conformation is defined by an intramolecular complex between the N-terminal domain and the SNARE motif.

iv) AA and detergents also accelerate the formation of SNARE complexes when syntaxin 4 instead of syntaxin 3 is used, revealing that the effect is not specific for this particular syntaxin species.

v) Complex formation is also accelerated by oleic acid, a monounsaturated fatty acid, as long as it is used above CMC. Thus, the conclusion of Darios and Davletov that the effect is confined to omega-3 and omega-6 polyunsaturated fatty acids cannot be sustained.

vi) Darios and Davletov have used several saturated and monounsaturated fatty acids at concentrations that were several times above their solubility level (stearic acid ≈ 10.5 µM, palmitic acid ≈ 27 µM). Due to their poor solubility in water, these FA cannot reach free concentrations that would allow for micelle formation. Not surprisingly, the authors classified those fatty acids as 'non-activating'. At the concentrations employed by Darios and Davletov, these FA yield turbid solutions containing insoluble aggregates or even solids, compromising the interpretation of the experiments.

vii) AA and oleic acid cause precipitation of SNARE proteins when used at 100 µM.

viii) The authors report that AA causes a structural change (i.e. an increase in alpha-helical content) only in syntaxin 3, which constitutes the main argument of the authors in support of a specific interaction between AA and syntaxin 3. I have not been able to reproduce such a specific effect. Rather I have observed that all three SNARE proteins showed a structural change in the presence of arachidonic acid. In fact I observed a small reduction of alpha-helical content of syntaxin 3.

To conclude: The main conclusion of the paper is wrong. The effects of AA on SNARE assembly are caused by a detergent-like effect that is (i) not specific for AA because structurally unrelated detergent have similar effects, (ii) not specific for syntaxin 3 because syntaxin 4 (and probably many others) exhibit similar behavior, (iii) unrelated to a conformational change between closed and open conformations as the same effect is visible in the absence of the N-terminal domain of syntaxin, and (iv) thus caused by a surfactant effect that is extremely unlikely to be of any biological relevance whatsoever.

In conclusion, it is obviously your decision how to handle such a challenge to a published paper. If, as I expect, you will maintain your decision without involving outside experts, I will have learned a lesson about the editorial policy of Nature and will publish these data elsewhere.

Yours sincerely,
Dirk Fasshauer

Detailed response to the answer of Darius and Davletov:

They state that "Analysis of the technical details reveals the likely causes of the discrepancies. The author routinely uses 10-20 µM protein, 10-fold higher than we used."

First, the paper does not contain any information about the concentrations used by Darios and Davletov, so I needed to guess. Second, I have difficulties in accepting that the experiments of Darios and Davletov were carried out at 1-2µM. To be able to visualize proteins after SDS-PAGE by Coomassie-Blue staining (as done in the paper), at least 1-2 µg are required for each protein, corresponding to approximately 10 µM. Our data show that syntaxin 3 forms SDS-resistant complexes in the absence of arachidonic acid. The proteins concentrations were chosen solely to allow for visualizing the SDS-resistant complexes. Since the intensity of the bands of the individual proteins in our experiments is comparable to that shown in Figure 3 in Darios & Davletov, it appears that the concentrations are comparable. My CD and fluorescence measurements have been indeed carried out at 10 -20 µM concentrations of syntaxin 3 (labeled SNAP-25 was at $\approx$ 200 nM). However, this was only done to accelerate the reaction since syntaxin 3 is slower than syntaxin 1 in forming a SNARE complex. Nevertheless, I have observed effective complex formation at lower concentrations, and if required these data can be provided.

Thus, the inability of Darios and Davletov to observe formation of SNARE complexes cannot be explained by a concentration difference. Rather, I assume that the accelerating effect by detergents on complex formation is caused by a dissolving/reactivating effect on protein aggregates that may have formed due to inappropriate handling of the proteins. For example, I noticed that the buffers used by Darios and Davletov do not contain DTT which is needed to protect the protein from oxidation.

Darios and Davletov: *"Furthermore, it appears from his cited publication(Ref2) that some incubations were done overnight at 4°C - our reactions were 30 min at 22°C(Ref1)."*

As clearly stated, we observe an SDS-resistant SNARE complex band after 30 minutes of incubation at room temperature.

Darios and Davletov: *"High protein concentrations, long incubation times and low temperature are known to favour protein interactions and can explain Fasshauer's in vitro observations."*

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As outlined above, there has been no difference in our incubation conditions. Furthermore, it is well known that low temperatures decrease rather than increase the kinetics of protein-protein interactions.

Darios and Davletov: *Fasshauer observes AA-induced activation of the isolated SNARE motif of syntaxin 3 and therefore questions whether AA acts on the helical syntaxin fold. However, isolated SNARE motif oligomerises to form a helical bundle(Ref4) and AA may affect such a state, leading to release of the truncated motif for further interactions.*

In other words, the authors believe that the accelerating effect of AA is due to a different mechanism when either full-length or truncated syntaxin is used. While such a difference cannot be completely excluded, I prefer the easier explanation, i.e. that the accelerating effect is due to the same mechanism – after all, it is the SNARE motif that forms SNARE complexes. It is likely that micelles constrain the conformational space of this domain, allowing for more efficient complex formation – after all, the effect is moderate.

Darios and Davletov: *Fasshauer observes that AA can activate syntaxin 4 but this does not affect the conclusions of our paper. Indeed, we showed previously that Munc18-regulated syntaxin 1 is also sensitive to AA(Ref5) and yet syntaxin 1 is not involved in neurite outgrowth(Ref1). It is possible that the regulation of syntaxins by unsaturated fatty acids is a conserved mechanism.*

Remember: the major message was that syntaxin 3 serves as a specific receptor for AA in order to accelerate the formation of SNARE complexes and accelerate vesicle fusion. Do the authors believe, for instance, that AA regulates neurotransmitter release because – according to this statement – it also activates syntaxin 1? Would one thus expect that AA stimulates neurotransmitter release by changing SNARE conformations?

Darios and Davletov: *Fasshauer states that AA has a non-specific, micelle-induced effect, despite knowing that only 'certain detergents' (e.g. dodecylmaltoside) affect syntaxin 3. We addressed this issue previously (Ref5) showing that synthetic detergents with a certain carbon chain length can substitute for polyunsaturated fatty acids. Does this rule out a role of AA in syntaxin activation? No; for example, synthetic opiates can stimulate endorphin receptors - but this does not mean that endorphins act non-specifically. Furthermore, AA at a concentration of 50 uM, i.e. below the CMC value of 60 uM (Ref6), still activates syntaxins (Refs1,5)*

Synthetic opiates are specific high-affinity ligands that are fashioned to fit into the binding pocket of the endorphin receptor – they structurally fullfil the requirements for binding and activation. Receptors for eicosanoid hormones, for which AA is a precursor, were found among the large 7TM protein family. If indeed AA acts without interconversion into a hormone, it is probably more likely that one can find the putative AA-receptor in that protein family.

CMC: Did Darios and Davletov ensure that there are no micelles in the solution – even to a non-expert to claim a difference between 50 and 60µM may not be convincing. The transformation from a monomeric solution to a micellar solution does not occur as an abrupt transition but over a narrow concentration range. I suggest that the authors educate themselves about basic physico-chemical properties of detergents and fatty acids (see for example {Small, 1986 #42; Serth, 1991 #28}). It is well known that the CMC is variable and depends on the environmental conditions, i.e. buffer, salt, and temperature and the nature of the detergent. For instance, it may be lower in the presence of proteins (e.g. ≈ 10 µM for AA in{Necula, 2003

#23}). Considering these facts, the statement: *"Taken together, our published results show that micelle formation and surfactant properties per se can not account for syntaxin activation ..."*. cannot be sustained unless experimental evidence is provided that AA forms no micelles under the experimental conditions employed.

Furthermore, it is surprising that none of the reviewers challenged the authors' view that the detergents Triton X-100 and DDM resemble arachidonic acid. Except of being amphiphiles, these compounds are completely unrelated with each other. DDM consists of a sugar (polar headgroup) linked to a saturated C12 chain. Triton is a mixture of polyoxyethylene detergents, i.e. chemically completely unrelated! The property they share with AA is solely the fact that the CMC is comparatively low.

Darios and Davletov: *Although Fasshauer questions the relevance of the AA effect, the author neither provides in vivo results nor finds an indication in literature for possible local and transient AA concentrations to support his arguments (for further discussion see Ref7).*

Apparently there appears to be no evidence that such high concentrations of free AA ever exist inside cells. Therefore, I can only again reiterate my strong doubts that these concentrations can ever be reached – especially not in the cytosol as the concept of the authors would require. In every biochemistry textbook it can be read that fatty acids, as all lipophilic substances, are bound to carrier proteins.