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Independent localization of MAP2, CaMKII and -actin RNAs in low copy numbers

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1st Editorial Decision 21 February 2011

Thank you for the submission of your manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, the referees agree that the study is potentially suitable for publication in EMBO reports. They do raise a few important concerns though, which need to be addressed before the manuscript can be considered for publication in our journal.

Most importantly, referee 3 points out that additional controls and quantification of single particle intensity are required to support the conclusion that only few mRNA copies are present in a given RNP. Referee 2 further remarks that the quality of the figures needs to be improved and that it should be investigated whether mRNA distribution to RNPs changes upon synapse formation. The referee also remarks that it needs to be addressed whether Staufen2 affects gene transcription and RNP assembly or transport. Mechanistic insight into how Staufen2 influences different types of RNPs is, however, not required for publication of the manuscript in EMBO reports.

Given these evaluations and the constructive referee comments, I would like to give you the opportunity to revise your manuscript, with the understanding that the referee concerns must be fully addressed and their suggestions (as detailed above and in their reports) taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that,

therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

I look forward to seeing a revised version of your manuscript when it is ready.

Yours sincerely

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

This paper addresses the question on whether dendritic RNAs are part of large ribonucleoprotein particles with many mRNAs attached, or part of small particles that can be individually transported and associated with ribosomes for translation. The authors provide evidence that the mRNAs for MAP2, CaMKIIalpha and beta-actin localize to distinct particles, and, most importantly, this paper is also the first to describe that theses particles contain only very few RNA molecules. These are new and important findings that deserve attention. The authors present additional evidence that Stau2 and synaptic activity are involved in assembly of these specific RNPs and/or transport to dendrites. These findings could be of broad interest to understand the contribution of dendritic mRNA transport and local translation at postsynaptic spines to dendritic development and synaptic activity in the nervous system.

Specific criticism:

1.The pictures shown in Fig. 1 and 2 are relatively low resolution. The quality should be improved. In particular I would like to see more details on the dendritic spots in which the mRNAs for MAP2, CaMKIIalpha and beta-actin are contained, and their spatial relation to postsynaptic densities. Moreover, size bars should be included in these figures.

2.The hippocampal neurons used for the colocalization experiment were kept in culture for 13 days for most of the experiments shown in this paper. This is a time point when synaptic differentiation just starts. One would like to know whether distribution of these mRNAs and colocalization changes from early time points (7 to 10 days DIV) to later time points when synapses, either in form autapses or as synaptic contacts between neurons in these cultures have formed. Supplementary Fig. 1b presents some data that go into this direction, but they are not sufficient to make a clear statement on whether synaptic differentiation enhances accumulation of this mRNAs, and whether the transport and/or colocalization of these mRNAs changes when synapses mature.

3.The authors raise the point that their data provide evidence that these different mRNAs are selectively delivered to individual synapses. To my mind, this is important and should further characterized and quantitated. Are there individual post-synaptic areas in which only one or two of these mRNAs accumulate, and is there any evidence that occupation of the spine by a pre-synaptic terminal alters the distribution of any of the three mRNAs under investigation?

4.The authors provide evidence in Fig. 4, that levels of MAP2 and beta-actin mRNA were lower in the cell bodies of Stau2 deficient neurons. It needs to be excluded that Stau2 has an effect on differentiation of these neurons that affects gene transcription, and it needs to be worked out whether this protein affects stability or increases the assembly of RNP particles for transport. At least, these different possibilities should be discussed, and previously published evidence on this theme included in this discussion.

5.It would be interesting to know whether Stau2 is involved in RNP particles assembly and to

distinguish this function from transport of RNP particles containing beta-actin and MAP2 mRNA.

6.It would be good to know whether the lack of effect in Stau2 deficient neurons on cell body levels of CaMKIIalpha and Septin7 is due to the fact that Stau2 is not contained in these particles. This part of the paper is a bit confusing, and mechanistic insights on how Stau2 influences these two types of RNP particles should be included.

Referee #2:

Mikl et al. try to answer several important questions regarding the composition of neuronal ribonucleorprotein particles (RNPs): do different RNAs localize to dendrites in distinct RNPs; how many RNA molecules do the particles contain; is organisation of the RNPs affected by synaptic activity. In a recent published study using microinjection of labelled RNAs and double in situ hybridization (ISH), the group have shown that MAP2 and CaMKIIa RNAs colocalized only at low levels in the dendrites of hippocampal neurons. Here, they repeated double ISH experiments with MAP2, CaMKIIa, b-actin and b-tubulin RNAs and found that all tested RNAs rarely localized to the same dendritic RNPs. The colocalisation was not affected by synaptic activity. Further experiments, using double ISH with probes competing for the same sequence within one RNA, showed that RNPs contained an average of one to two RNA molecules. Stimulation of neuronal synaptic activity decreased this number to one RNA molecule in MAP2 particles but had no effect on b-actin RNPs composition. Depletion of Stau2 by shRNA increased RNA content in MAP2 particles but had no effect on other tested RNAs.

In my view, this is interesting work that provides new and significant data about the number of mRNA molecules in neuronal RNPs and how Stau2 and synaptic activity can modulate it. This is important because others have argued that many mRNAs are co-transported and co-regulated in these RNPs without performing any careful quantification, and this study unambiguously refutes this view. However, it loses a bit of its novelty because the original observations on RNA colocalisation in neuronal RNPs have already been published by the Kiebler group, although this was only a single Figure in the paper.

Fig4A panel's organisation is confusing. It requires somewhat better arrangements. It is also sometimes hard to find nontransfected cells on EGFP panels, specifically on Septin7 one.

It is not clear why throughout the paper authors call in situ hybridisation ISH, but the chapter in Methods describing it is called FISH.

Page 10 line 5 Statements about Stau2 requires references.

Reference Tubling et al. needs to be updated.

Referee #3:

This manuscript aims at testing whether mRNAs targeted to specific sub-cellular compartments are transported and regulated independently, as single molecules, or whether they are co-assembled as multimolecular transport units. Specifically, the authors focus on three RNAs (MAP2, CAMKII and -actin) previously shown to be targeted to the dentritic compartment of neuronal cells, and carefully analyze the composition of their corresponding ribonucleoprotein particles (RNPs) in selected contexts. Using double fluorescent in situ hybridization techniques, they show that these RNAs are found in distinct dendritic RNPs in cultured hippocampal neurons. By performing competition with different concentrations of non-labelled probes, they then try to infer the number of RNA molecules present in single RNPs. Their conclusion is that this number would be lower than previously thought (around 2 RNA molecules/RNP). Interestingly, a differential response of MAP2 and -actin RNA is observed upon increased synaptic activity or inactivation of the RNA binding protein Staufen 2, suggesting that dendritic mRNAs localize independently from each other, and are regulated by

distinct machineries.

Overall, this manuscript is clear and well-written. It addresses an opened question in the field of intracellular mRNA targeting, and challenges the recent hypothesis according to which different RNA species targeted to the same destination would be co-transported in common high-order RNP complexes. As this model has been proposed based on imaging experiments where differentially tagged mRNAs were expressed at non-physiological levels, testing its validity on endogenously expressed molecules is of key importance. Furthermore, previous studies have mainly been limited to the analysis of single RNA species, rendering it hard to compare the regulatory properties of different RNA populations. This study, by simultaneously analyzing the behavior of two endogenously expressed mRNAs in wild-type and mutant contexts thus overcomes several limitations associated with previous work, and suggests a more complex and flexible spatio-temporal control of gene expression at synapses.
While the author's statement that dendritic mRNAs are transported independently and regulated by distinct machineries is supported by carefully controlled and quantitative experimental data, the claim that the number of RNA molecules per dendritic RNP is low requires further investigation (Fig 3, see comments below). The in situ hybridization technique described by the authors can be used to compare the relative composition of RNPs in different contexts (as done in Fig 3D and 4). However, important additional calibration controls are required before being able to interpret competition assays and conclude about the absolute number of RNA molecules present in single RNPs in vivo (Fig 3A-C).

Major concern:
The reasoning underlying the competition assays described in Figures 3B,C and their interpretation is valid, but implies that the following conditions are experimentally fulfilled:
1) hybridization efficiency of 100% (or close)
If a non-negligible proportion of endogenous molecules are not labelled, or if for some reason a maximum number of two molecules per RNP can be labelled, the interpretations of the experimental data are wrong.
2) detection method sensitive enough so that single RNA molecules can be detected
If the weakest signal detectable in the presented assay corresponds to the sum of the signals produced by several labelled molecules, then the quantification is not valid.
3) detection method linear over a wide range so that RNPs with 3 (or more) RNA molecules produce a signal 3 (or more) times brighter than RNPs containing a single RNA molecule
This point is particularly important as in the protocol used by the authors the in situ hybridization signal is amplified using an enzymatic HRP-mediated reaction. It is also critical for the interpretation of the "average intensity" values shown in Fig 3C.

Testing the above-mentioned conditions in vivo is not straightforward as the total number of RNA molecules accumulating in the dendritic compartment in vivo is unknown. However, the authors should provide appropriate controls showing the above-mentioned conclusions are likely to be fulfilled (calibration curves for example).

The best way to quantitatively measure RNP composition would be to perform calibration experiments combined to highly quantitative measurement of signal intensities. This should enable the authors to quantify single particle intensities (and not "average intensities"), and test if the distribution of signal intensities is unimodal (expected if RNPs contain a single RNA molecule) or bi-/multi-modal (expected if RNPs contain two/or more RNA molecules). To this end, intensities should be measured so that slightly out of focus signals are not taken into account (which is not the case in the conditions used by the authors), and in conditions where signal amplification is linear (to be tested).

Minor points:
- Page 6, line #1: "our data therefore provide a first hint that their independent distribution allows differential regulation and selective delivery to individual synapses"
This conclusion could be strengthened by triple-labelling experiments simultaneously showing the distribution of MAP2 mRNA and -actin mRNA concomitantly with a synaptic marker.

- page 7, line #13: "Normalization of the values ...."
This sentence could be deleted as this information is redundant with the following competition assays.

- Fig S3B: ** missing on the graph

1st Revision - authors' response 08 June 2011

DETAILED ANSWER TO THE REFEREES:

Reviewer 1

We are very grateful to the positive reception by this reviewer, when stating: *"these are new and important findings that deserve attention" and that: "these findings could be of broad interest to understand the contribution of dendritic mRNA transport and local translation at postsynaptic spines to dendritic development and synaptic activity in the nervous system."*
In the remainder, the following constructive criticisms were expressed:
1.The pictures shown in Fig. 1 and 2 are relatively low resolution. The quality should be improved. In particular I would like to see more details on the dendritic spots in which the mRNAs for MAP2, CaMKIIalpha and beta-actin are contained, and their spatial relation to postsynaptic densities. Moreover, size bars should be included in these figures.

We realized this ourselves when we uploaded the files into the submission system of *EMBO Reports*yielding a low resolution PDF file compared to our original TIFF files. As suggested to the editor, we will provide the reviewers with the original figures. Furthermore, we have added dendritic insets (see revised **Figure 1A**) and size bars.
To investigate the spatial relationship of these mRNAs to postsynaptic densities (this also addresses point 3 of this referee, see below), we performed the following experiments: 1) Double ISH against *MAP2* and *β-actin* on neurons transfected with PSD-95 (see revised **Figure 1B).** Please note that we tagged PSD-95 with GFP and then detected overexpressed PSD-95-GFP but not endogenous PSD-95 with anti-GFP, because during the stringent hybridization step, native epitopes often get damaged. 2) Double ISH against *MAP2* and *β-actin* combined with immunostaining against Synapsin I (see revised **Figure 1B**). As shown in **Supplementary Figure S1A**, PSD-95 and Synapsin I puncta are always found in close proximity. Therefore, we used Synapsin I as an additional marker to confirm our findings. Although not all of the RNA is found at synapses – some dendritic particles may actually represent transport intermediates, which have not yet reached their destination – we frequently observe *MAP2* and *β-actin* mRNAs near distinct postsynaptic densities or presynaptic sites.
You might notice that the RNA signal in **Figure 1B** is not as good as in the pictures shown in **Figure 1A**. This is due to the fact that double ISH, combined with immunostaining, especially on transfected neurons is challenging and less efficient than single or double stainings. See also our answer to point 3 of this referee.

2.The hippocampal neurons used for the colocalization experiment were kept in culture for 13 days for most of the experiments shown in this paper. This is a time point when synaptic differentiation just starts. One would like to know whether distribution of these mRNAs and colocalization changes from early time points (7 to 10 days DIV) to later time points when synapses, either in form autapses or as synaptic contacts between neurons in these cultures have formed. Supplementary Fig. 1b presents some data that go into this direction, but they are not sufficient to make a clear statement on whether synaptic differentiation enhances accumulation of this mRNAs, and whether the transport and/or colocalization of these mRNAs changes when synapses mature.

We now provide evidence that colocalization of *MAP2*, *β-actin*and *CaMKIIα* RNAs in younger neurons (see revised **Supplementary Figure S1B** and page 6 of the revised manuscript) does not change. This finding is not surprising, since Dotti and Banker have previously shown that

synaptogenesis starts from day 7 to 10 in dissociated primary neurons depending on the substrates used (Banker & Goslin, culturing nerve cells, 2nd edition, MIT press).
It is not straightforward to carefully quantify accumulation of RNA to dendrites in neurons of different age, due to the different expression levels of the RNA, as well as length and thickness of dendrites. We did not observe however any dramatic changes in the distribution of mRNAs with age.

3.The authors raise the point that their data provide evidence that these different mRNAs are selectively delivered to individual synapses. To my mind, this is important and should further characterized and quantitated. Are there individual post-synaptic areas in which only one or two of these mRNAs accumulate, and is there any evidence that occupation of the spine by a pre-synaptic terminal alters the distribution of any of the three mRNAs under investigation?

See our response to point 1 of this referee, referring to the data shown in **Figure 1B**. We frequently found *MAP2* and *β-actin* at or in close proximity to distinct synapses (see revised **Figure 1B** and page 6 of revised text), sometimes neighboring ones (for example, insets 1 and 2 of right panel), confirming our original suggestion that the localization of these RNAs in distinct RNPs mediates their delivery to individual synapses. This does not exclude that certain synapses might contain both mRNAs (since these RNAs colocalize to a low degree of 8%), which we indeed very occasionally observed.
We could not address whether occupation of the spine by a pre-synaptic terminal alters the RNA distribution because the vast majority (if not all) of the postsynaptic sites were in close contact with presynaptic terminals as shown in **Supplementary Figure S1A**.This is consistent with work of others (Kay et al, Nature Neuroscience 2011) and with the model that postsynaptic differentiation and maintenance depends on the presence of a presynaptic site (Friedman et al., 2000, *Neuron*; Okabe et al., 2001, *J.Neurosci*.). We got very similar results when we transfected younger neurons (8DIV) with PSD-95 and stained them for Synapsin I (see also Kay et al, Nature Neuroscience 2011).
However, we performed a triple detection of *CaMKIIα*, PSD-95 and SynapsinI by transfecting hippocampal neurons with PSD-95-RFP, *CaMKIIα* tagged with 24xMS2 sites and MCP-YFP followed by immunostaining for Synapsin I (please see **Figure for the reviewers only**). This experiment clearly shows that *CaMKIIα*localizes at synapses (like *MAP2* and *β-actin*), as it is found near postsynaptic densities, which are in close contact with presynaptic terminals. Since *CaMKIIα* has been reported recently to reside at the base of spines (Kao et al., 2010, *PNAS*) and due to space limitations, we have not included this data in the current manuscript.

4.The authors provide evidence in Fig. 4, that levels of MAP2 and beta-actin mRNA were lower in the cell bodies of Stau2 deficient neurons. It needs to be excluded that Stau2 has an effect on differentiation of these neurons that affects gene transcription, and it needs to be worked out whether this protein affects stability or increases the assembly of RNP particles for transport. At least, these different possibilities should be discussed, and previously published evidence on this theme included in this discussion.

Stau2 appears not to affect transcription globally since Stau2 downregulationdoes not alter*Septin7* or *CaMKIIα* mRNA levels (see **Figure 4**). Moreover, the enrichment of *β-actin*mRNAs in Stau2 particles isolated from brain or the colocalization of Stau2 with MAP2 in dendrites reported by others (Maher-Laporte et al, 2010, Lebeau et al, 2011) suggests that the effects we observe might be direct. Furthermore, although the RNA levels in the cytoplasm of Stau2 depleted neurons are dramatically reduced (see **Figure 4**), we can detect RNA signal (e.g. for *β-actin*, see revised **Supplementary Figure S3A**) in the nucleus of Stau2-depleted neurons – probably corresponding to newly transcribed RNAs – that does not appear to be lower compared to control.

We next tested whether Stau2 has an influence on the stability of the affected RNAs by blocking transcription using actinomycinD and determining RNA levels in neurons transfected with shStau2 or mismatch Stau2 plasmids. To restrict this analysis to hippocampal neurons, we used average cell body intensities as a measure for RNA levels, as this method indeed allows quantitative measurements (see revised **Supplementary Figure S2D**). Already after 4h of treatment, we observed a further decrease in *β-actin*and *MAP2* RNA levels in each of the three independent experiments, in addition to the ~50% reduction as a consequence of Stau2 depletion (see **Supplementary Figure S3B** and page 11 of the revised manuscript). These results point towards a

role of Stau2 in the stability of these transcripts in the cytoplasm. This is also consistent with our observation that *β-actin* and *MAP2* levels are reduced to a similar extent both in the cell body and in dendrites. This would not be expected ifStau2 would actually play a role in the assembly of transport RNPs.

5.It would be interesting to know whether Stau2 is involved in RNP particle assembly and to distinguish this function from transport of RNP particles containing beta-actin and MAP2 mRNA.

Actually, we are not suggesting that Stau2 is required for assembly of RNPs for transport or transport itself (at least not of those RNAs we investigated). Our data rather argues that it does not, as we see a similar effect in the cell body and dendrites of Stau2-deficient neurons, as well as an effect in the cell body levels of non-localizing mRNAs. We now clarify this in the text (see revised manuscript, page 10). We believe that Stau2 affects RNA stability in the cytoplasm (see also above). Furthermore, we now show that Stau2 does not change RNA colocalization in dendrites (see **Supplementary Figure S3D** and page 12 of revised manuscript), arguing against a role in the assembly of particles containing *MAP2* and *β-actin*.

6.It would be good to know whether the lack of effect in Stau2 deficient neurons on cell body levels of CaMKIIalpha and Septin7 is due to the fact that Stau2 is not contained in these particles. This part of the paper is a bit confusing, and mechanistic insights on how Stau2 influences these two types of RNP particles should be included.

It has been reported that *MAP2* colocalizes with Stau2 in dendrites of hippocampal neurons (Lebeau et al, 2011, published during the revision of our manuscript) and that *β-actin*, but not *CaMKIIα* or *Septin7* are enriched in Stau2 particles isolated from rat brain (Maher-Laporte et al, 2010). In the revised manuscript, we present data indicating that the reduction in cytoplasmic levels of a number of RNAs might be due to a role of Stau2 in RNA stability (see **Supplementary Figure S3B** and page 11 of revised manuscript). Furthermore, Stau2 depletion leads to an increase in the RNA content of dendritic *MAP2* RNPs (**Figure 4D** and **Supplementary Figure S3C**)_, arguing that it can affect packaging characteristics of certain RNAs. We feel, however, that further mechanistic insight into how Stau2 might influence different types of RNPs, is beyond the scope of this report and will be the focus of a follow-up study.

Reviewer 2

We appreciate the positive reception of our work by this referee, when stating: *"Mikl et al. try to answer several important questions regarding the composition of neuronal ribonucleoprotein particles (RNPs): do different RNAs localize to dendrites in distinct RNPs; how many RNA molecules do the particles contain; is organisation of the RNPs affected by synaptic activity. In my view, this is interesting work that provides new and significant data about the number of mRNA molecules in neuronal RNPs and how Stau2 and synaptic activity can modulate it. This is important because others have argued that many mRNAs are co-transported and co-regulated in these RNPs without performing any careful quantification, and this study unambiguously refutes this view."*

1. Fig4A panel's organisation is confusing. It requires somewhat better arrangements. It is also sometimes hard to find nontransfected cells on EGFP panels, specifically on Septin7 one.

We now added a smaller version of the ISH pictures, so that they are better related to the EGFP ones. We also increased the size of the asterisks, which mark the transfected cells in all ISH pictures (see revised **Figure 4**).

2. It is not clear why throughout the paper authors call in situ hybridisation ISH, but the chapter in Methods describing it is called FISH.

This is now corrected.

3. Page 10 line 5 Statements about Stau2 requires references.

This is now added, see page 10 of the revised manuscript

4. Reference Tübing et al. needs to be updated.

This is now corrected.

Reviewer 3

We are thankful for the positive reception of this referee by the following statements: *"Overall, this manuscript is clear and well-written. It addresses an opened question in the field of intracellular mRNA targeting, and challenges the recent hypothesis according to which different RNA species targeted to the same destination would be co-transported in common high-order RNP complexes. As this model has been proposed based on imaging experiments where differentially tagged mRNAs were expressed at non-physiological levels, testing its validity on endogenously expressed molecules is of key importance. Furthermore, previous studies have mainly been limited to the analysis of single RNA species, rendering it hard to compare the regulatory properties of different RNA populations. This study, by simultaneously analyzing the behavior of two endogenously expressed mRNAs in wild-type and mutant contexts thus overcomes several limitations associated with previous work, and suggests a more complex and flexible spatio-temporal control of gene expression at synapses. "*

We also appreciate the very constructive and insightful comments regarding our method and did our best to address them adequately.

While the author's statement that dendritic mRNAs are transported independently and regulated by distinct machineries is supported by carefully controlled and quantitative experimental data, the claim that the number of RNA molecules per dendritic RNP is low requires further investigation (Fig 3, see comments below). The in situ hybridization technique described by the authors can be used to compare the relative composition of RNPs in different contexts (as done in Fig 3D and 4). However, important additional calibration controls are required before being able to interpret competition assays and conclude about the absolute number of RNA molecules present in single RNPs in vivo (Fig 3A-C).

Major concern:

The reasoning underlying the competition assays described in Figures 3B,C and their interpretation is valid, but implies that the following conditions are experimentally fulfilled:

1) hybridization efficiency of 100% (or close)

If a non-negligible proportion of endogenous molecules are not labelled, or if for some reason a maximum number of two molecules per RNP can be labelled, the interpretations of the experimental data are wrong.

Hybridization efficiency: It is true that if only a maximum of 2 molecules per particle can be labeled and if the same two molecules are recognized by all differentially labeled probes (DIG, FL or cold), then our interpretations are wrong. But they would have to be the same two molecules that can be labeled by each probe, otherwise the competition experiments of Figures 2 and 3 would not have worked.

We do not know whether our hybridization efficiency is indeed 100%, but with our additional controls we know that it is very high. We tested in most cases 2-3 different probes per mRNA and we selected for the single ISH competition experiments the ones, which performed better (please see Material and Methods, page 28). Furthermore, in **Supplementary Figure S2A**, 1,2, 1' and 2), we have detected *MAP2* mRNA with one probe against the last third of its 3'-UTR) or with two probes targeting the first and last third of its 3'-UTR, respectively. Addition of the second probe did not significantly increase the number of detected particles arguing that the efficiency of the selected probe (used in all experiments of this manuscript) was already very high (see **Supplementary Figure S2A, 1,2, 1' and 2'**).

More importantly, the addition of the second probe increased significantly the intensity of the detected particles. In **Supplementary Figure S2C**, we have detected *MAP2* mRNA with the same probes as above, one DIG probe against the 3rd third of its 3'-UTR or with two DIG probes targeting the first and last third of its 3'-UTR, respectively. The last third contains 392 UTPs (including 11 from the sequence transcribed from the polylinker of the used vector) and the first third 323 UTPs. Therefore the addition of the latter is expected to increase the particle intensity by 82% if all molecules are bound by both probes. We observe a 75% increase of particle intensity, which argues that 91.5% (75/82) of RNA molecules are recognized by both probes. This suggests that the efficiency of hybridization in these experiments was indeed very high.

Accessibility of the target mRNAs: It is possible that dendritic particles contain many molecules of RNA, for example 20, but only 2 can be accessed by the non-overlapping probes (see above) or by different probes (see our competition experiments) but the remaining 18 are heavily decorated with proteins and not accessible at all, by any of the used probes. In this case, our interpretations are wrong. Unfortunately, we cannot think of a way to address this directly, but we do not think this to be very likely.
Furthermore, we can detect higher amounts of RNA when present. For example, particlesthat contain *CaMKIIα* mRNA tagged with YFP (MS2 system) at very high levels (as shown by their YFP content) after "deliberate" overexpression of the reporter, give a higher ISH signal than particles with lower amounts. This suggests that our observations in Figure 3are not very likely to simply result from low target accessibility. Please see **Figure for the reviewer only**, which we did not include in the manuscript due to space limitations.

We also employed an alternative ISH method (developed by the Singer lab), which utilizes a series of short 50nt probes. We used 6 singly labeled probes targeting *MAP2* and compared fluorescence intensity of puncta in dendrites to signal coming from individual probes sticking to the glass. We confirmed that the unspecific signal on the glass correspondsto single fluorophoresby bleaching experiments in which this signal showed decay to background levels in one step. In this case, we observed that the intensity of signal in dendrites typically corresponded to one or two molecules of RNA. Due to problems with finding adequate negative controls, the preliminary nature of these data and the lack of time to elaborate them, we prefer to not include this in the current manuscript.

2) detection method sensitive enough so that single RNA molecules can be detected
If the weakest signal detectable in the presented assay corresponds to the sum of the signals produced by several labelled molecules, then the quantification is not valid.

The probes that we used (standard reaction with 3.5mM DIG-UTP) contain 40-50 DIG molecules per kb, since DIG-UTP is incorporated at approximately once every 20-25nt (product information from the supplier). Each of these digoxigenin molecules is recognized by antibodies conjugated to peroxidase and each molecule of peroxidase catalyzes the activation of multiple molecules of tyramide, resulting in signal amplification. Therefore every probe can be labeled by many molecules of fluorophores. We believe that this detection method is sensitive enough to detect single molecules of RNA. To confirm that it is well above the detection limit, we reduced the number of labeled U's by decreasing the DIG-UTP concentration in the reaction mix from 3.5 mM to 1 mM. In this case, the fluorescence intensity of the detected particles and also the overall cell body levels were reduced accordingly, while the number of detected particles was unchanged (see revised **Supplementary Figure S2D** and revised **supplementary methods**, page 29). We therefore conclude that our detection method is sensitive enough to detect single RNA molecules, as a 3.5 fold reduction in labeling did not result in a loss in detected particles.

3) detection method linear over a wide range so that RNPs with 3 (or more) RNA molecules produce a signal 3 (or more) times brighter than RNPs containing a single RNA molecule
This point is particularly important as in the protocol used by the authors the in situ hybridization signal is amplified using an enzymatic HRP-mediated reaction. It is also critical for the interpretation of the "average intensity" values shown in Fig 3C.

We thought of a way to address this interesting question and to determine the linearity of our detection method and took two independent approaches. First, we performed ISH using two non-overlapping probes against the same mRNA (*MAP2*) that are both DIG labeled. Detection of the probes should result in a signal that is the sum of the two individual probes. We already showed previously, that this does not result in a significant increase in the number of detected particles in dendrites (**Supplementary Figure S2A**, 1,2, 1' and 2), arguing for a high efficiency of detection. In addition, the intensity of the detected particles was increased by 75% compared to one probe (**Supplementary Figure S2C** and page 29 of revised manuscript). This is very close to the maximum that can be achieved with the use of this particular additional probe, since its U content (and consequently the number of DIG molecules incorporated) is 82% of the first probe. Apart from confirming the high efficiency of our method, this result also indicates that our detection method is indeed quantitative, so that the binding of two probes results in a signal that is the sum of the individual probes.

In addition, we tested the linearity of detection over a broader range by performing ISH using probes that were synthesized in the presence of different DIG-UTP concentrations (1, 2, 3.5, 5 or 6.5 mM). This indeed resulted in different degrees of DIG incorporation in the RNA probe, observable by a shift in size on a gel. We measured particle intensities as well as average cell body intensities of the signal obtained with the different probes and observed in both cases that detection is very linear ($R > 0.99$) over a wide range. In **Supplementary Figure S2D**, the standard error of the mean of the 4 independent experiments is shown. In addition to the good fit of the combined data, also each individual repetition by itself confirmed the linearity of the method.
The overexpression experiment described above (see our response to your first criticism), also suggests that we can detect higher amounts of RNA when present.

Testing the above-mentioned conditions in vivo is not straightforward as the total number of RNA molecules accumulating in the dendritic compartment in vivo is unknown. However, the authors should provide appropriate controls showing the above-mentioned conclusions are likely to be fulfilled (calibration curves for example).
The best way to quantitatively measure RNP composition would be to perform calibration experiments combined to highly quantitative measurement of signal intensities. This should enable the authors to quantify single particle intensities (and not "average intensities"), and test if the distribution of signal intensities is unimodal (expected if RNPs contain a single RNA molecule) or bi-/multi-modal (expected if RNPs contain two/or more RNA molecules). To this end, intensities should be measured so that slightly out of focus signals are not taken into account (which is not the case in the conditions used by the authors), and in conditions where signal amplification is linear (to be tested).

We performed the suggested calibration experiments using two probes targeting the same RNA and probes with different DIG content (see above). Having confirmed that our method can indeed be used for quantitative intensity measurements, we analyzed the frequency distribution of the particle intensities and found that there was always one main peak that corresponded to half of the average intensity of all particles (see **Supplementary Figure S2C** and page 9 of revised manuscript). From our competition assay, we concluded that this average corresponds to two molecules per particle. Based on this result, we assigned this main peak to 1 molecule per particle. The frequency distribution also showed a fraction of particles with higher intensities, usually with peaks corresponding to multiples of the main peak (1 molecule). We assume that these represent 2, 3 or more detected molecules in one particle, and conclude that while most of the detected transcripts seem to be found in RNPs in single copies, some particles contain 2 or 3 and very few even more molecules of *MAP2* RNA, with decreasing likelihood/probability of occurrence, ultimately adding up to the observed average of 2 molecules per particle. Due to the amplification of the signal that makes our method sensitive enough to detect single molecules of RNA, we expect some variability in the signal intensity, even with very careful selection of analyzed particles taking into account only signal that does not seem to be out of focus. Nevertheless, we are confident that our results together with the control experiments (e.g. **Supplementary Figure S2C,D**) demonstrate that we are able to obtain quantitative information both on the level of "average intensities" as well as individual particles.

Minor points:
- Page 6, line #1: "our data therefore provide a first hint that their independent distribution allows differential regulation and selective delivery to individual synapses"
This conclusion could be strengthened by triple-labelling experiments simultaneously showing the distribution of MAP2 mRNA and β-actin mRNA concomitantly with a synaptic marker.

Please see revised **Figure 1B** and our response to points 1 and 3 of referee #1.

- page 7, line #13: "Normalization of the values ...."
This sentence could be deleted as this information is redundant with the following competition assays.

We agree that the conclusion of this sentence is redundant with the results of the competition assay, but we feel that the data of the double ISHs targeting the same RNA further corroborates our hypothesis and provides additional evidence. We would therefore prefer to keep this argument

mentioned in the text. (For more detailed discussion of the rational of the argument see **Supplementary Methods**, page 28).

*- Fig S3B: ** missing on the graph*

This is now corrected.

2nd Editorial Decision 22 June 2011

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

You can find referee's comments below. Both are very positive and have no further concerns, but referee #1 strongly recommends including in the report the figure that you submitted for referees only. Given the space limitations, you can include this figure as supplementary figure 4. If you agree, we can introduce this figure as it is now into the supplementary information, including its figure legend. Alternatively, you can provide us with a modified version of the figure or the legend. In case you need to modify the image, you can send the final version as an attachment in an e-mail, you do not need to use our submission system. Please, do not forget to indicate where in the main text you want this figure referenced.

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1 comments

The revised version of this paper is significantly improved and addresses all my original points of criticism.

The new Fig. 1A with the insets is much more convincing.

1. The authors have included additional data on double in situ-hybridization against MAP2 and -actin mRNAs, which makes this story more complete.

2. The original concerns raised in point 2 have also been adequately addressed.

I strongly recommend to include the data shown in the figure for the reviewers only, because these data provide good evidence that CaMKIIa mRNA localizes at postsynaptic densities. To my mind, these data make the story much better and should not be left out. In my opinion, these data go beyond to those published by Kao et al. last year because they provide the double labelling with -actin and the combination with immunohistochemistry with Synapsin-I, which has not been shown in the previous paper by Kao et al., 2010.

Referee #2 comments

All the concerns I had have been addressed with complementary

experiments displayed in supplementary figures.
To my opinion, this manuscript is of high interest and reports novel findings. I thus recommend it for publication in EMBO reports.

Correspondence – authors' response 24 June 2011

Many thanks for the kind email and the positive news. Of course, we are very excited about that!

We truly appreciate the positive and constructive comments of all referees and the strong support for our work. We have tried our best to perform as many experiments possible to provide experimental evidence to support our data. You might also notice that the key issues for the revision (see the decision letter) were to address the following essential points:

1. Provide additional controls and quantitation of single particle intensity to support our conclusion
2. Provide higher resolution files for some figures
3. Investigate whether synaptic stimulation changes the mRNA distribution to RNPs
4. Investigate whether Staufen2 might affect gene transcription and RNP assembly
5. Provide evidence whether mRNAs are delivered to synapses

In my opinion, we all successfully addressed these issues experimentally.

In addition, Referee #1 asked as one additional point related to the synaptic localization of localized mRNAs (this is shown in the revised version of Fig. 1B), whether there "is any evidence that occupation of the spine by a pre-synaptic terminal alters the distribution of any of the three mRNAs under investigation?"

As you see, this is a very special and detailed question that it not related to any of the above issues to be answered. We felt, however, that we did not want to ignore this aspect and decided to provide preliminary data for this interesting question. As outlined in detail in our rebuttal letter (page 2ff, point 3), this is a very difficult experiment. We wrote in the rebuttal letter:

Our response: "We could not address whether occupation of the spine by a pre-synaptic terminal alters the RNA distribution because the vast majority (if not all) of the postsynaptic sites were in close contact with presynaptic terminals as shown in Supplementary Figure S1A."

We therefore had to use a different technique to address this question. What we actually show in this Figure that we provided to the referees only, was that "CaMKIIalpha localizes at synapses (like MAP2 and beta-actin), as it is found near postsynaptic densities, which are in close contact with presynaptic terminals."

You can easily see that we already provide two examples ((like MAP2 and beta-actin) in Fig. 1B, whereas the Figure for the referee only provides a third example of the same kind. Most importantly for us, we decided to include this preliminary data for CaMKIIalpha only for the referees since we do not yet feel comfortable including it into the manuscript. As I made it clear, however, this does not add any novelty to any of the main points we were asked by the editor and the referees. Consequently, I would like to opt for not including this single experiment yet.

Correspondence – editor's response 24 June 2011

Thanks for your fast response and congratulations on the acceptance of your manuscript.
After reading your comments, if you consider that this supplementary figure is too preliminary, I agree in excluding it from the manuscript. However, I think that a sentence such as "preliminary results show a similar localization of CaMKIIalpha at synapses (data not shown)" should be included when discussing MAP-2 and beta-actin localization. The referee is quite positive about this piece of information.

Thanks again for your contribution to EMBO reports.

