

# Subsynaptic mobility of presynaptic mGluR types is differentially regulated by intra- and extracellular interactions

Anna Bodzeta, Florian Berger, and Harold MacGillavry

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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.)

RE: Manuscript #E21-10-0484

TITLE: Subsynaptic mobility of presynaptic mGluR types is differentially regulated by intra- and extracellular interactions

Dear Dr. MacGillavry:

Your manuscript, entitled "Subsynaptic mobility of presynaptic mGluR types is differentially regulated by intra- and extracellular interactions" has been seen by two referees whose verbatim comments are enclosed. Both referees felt that your findings, in principle, would be of interest to our MBoC readership, but also raised some important points that need to be addressed. The majority of these concerns can be addressed by additional text and clearly describing the statistical comparisons. However, it is important to address the concern about the low number of independent experiments. Also, please review the Author Submission Checklist to better align with the required information on experimental sample size and statistical analysis. We look forward to receiving your revised manuscript and a letter indicating your response to the referees in the near future.

Sincerely,

Diane Lidke  
Monitoring Editor  
Molecular Biology of the Cell

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Dear Dr. MacGillavry,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office ([mboc@ascb.org](mailto:mboc@ascb.org)).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

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In preparing your revised manuscript, please follow the instruction in the Information for Authors ([www.molbiolcell.org/info-for-authors](http://www.molbiolcell.org/info-for-authors)). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised manuscript, and figures, use this link: [Link Not Available](#)

Please contact us with any questions at [mboc@ascb.org](mailto:mboc@ascb.org).

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker  
Journal Production Manager  
MBoC Editorial Office  
[mbc@ascb.org](mailto:mbc@ascb.org)

Reviewer #1 (Remarks to the Author):

Review of Bodzeta, Berger and MacGillavry, MBoC 30326\_0; 2021

This is an interesting manuscript aiming to elucidate whether and why different metabotropic glutamate receptors (mGluR) might show differential subcellular localization. Specifically, the authors investigated the high affinity group II receptor mGluR2 and the low affinity group III receptor mGluR7, and they investigated presynaptic sites (but it takes until you come to methods to learn that these presynaptic sites were those found in hippocampal cultures from embryonic day 18 (E18) Wistar rats).

The key findings are that the two receptors show very different localization and mobility, plus that the different behavior is controlled by different regions of the receptor molecules: mGluR2 localizes diffusely axons and is highly mobile, whereas mGluR7 is stably anchored at the active zone. This different behavior appears to be dictated by the intracellular receptor domain in the case of the mGluR2, while the immobilization of mGluR7 is determined by its extracellular domain. Finally, the authors present a computational model to describe the consequences of their findings as a function of receptor localization (distance from transmitter release site).

The authors use a set of advanced microscopy methods, ranging from superresolution to single molecule tracking, and combine it with receptor tagging to study the localization as well as the dynamic behavior of the two different receptor subtypes. These methods appear to have been used with care and a sound technological knowledge. The authors take care not to overexpress their proteins of interest, which is a very important issue in the context of localization and mobility studies.

A problem in providing a "simple" explanation for the differences between the receptors is that many parameters were found to differ between them, i.e. often both the mobile fraction and the mobility of the moving species are both different, and these individual differences are often not very big. However, the resulting overall difference in the diffusion behavior is quite striking (compare, notably, the two panels in Fig. 2E).

Quite naturally, the authors have to fight with a large biological scatter; therefore, they generally investigate a large number of individual samples. Unfortunately, however, these are often from only a (very) low number of really independent experiments. In fact, often this number of independent experiments is only 2, which is really very low and would call for repetition at least of critical experiments.

I have a number of comments that the authors may want to consider:

p.6 - the use of the specific labeling procedure used should be explained. pHluorins are usually used to study protein cycling between compartments with distinct pH - why was it chosen here, particularly since its reliable detection appeared to require additional use of anti-GFP labeling. And finally, what does this labeling approach to receptor mobility (has this been assessed for example in a simple cellular expression system)? This issue comes up also on p.8, when they use Atto647N-labeled anti-GFP nanobodies.

p.9 - from their tracking analyses in different regions of the neuron the authors conclude "showed that at a subset of synapses the mobility of SEP-mGluR7 is considerably lower inside boutons". What does this "subset" mean and how is it defined? In Fig. 2M, I see some tracks having a higher  $D_{eff}$  in axonal vs. synaptic tracks, while in others it is the opposite! Even though the authors attach a p-value of  $<0.05$ , the mean and distribution look quite identical to me.

p.10ff - The experiments on domain swapping are impressively clean. In contrast, I was not much convinced by the effects of latrunculin B on mGluR2 mobility (p.11) - the effects are really very modest.

p.15ff - The experiments to study the effects of stimulation or inhibition of synaptic activity are quite drastic: potassium depolarization and TTX are really extremes, and the reader wonders whether their use really demonstrates an effect of synaptic function on receptor mobility or rather the contrary.

Fig.6 - I am not sure whether and how much the computational model provides new insights. The key point that I derive from the data shown in Fig. 6 is that under all conditions considered the activation of mGluR7 at any location is at best minimal. One wonders whether this is really a functionally relevant effect.

The discussion is very long and might benefit from focusing.

Finally, an editorial point that the authors may or may not want to consider: I found some passages of the text really difficult to read because the sentences were disrupted by long brackets giving individual values (which are the same as those given in the figures that the text describes). An example is p.12.

Reviewer #2 (Remarks to the Author):

The authors investigate the spatial distribution and diffusion properties of two metabotropic glutamate receptors, mGluR2 and mGluR7. They use several methods to characterize the diffusion properties of these neurons, including several imaging modalities, co-culture assays, and chimera analyses. Their results reveal interesting differences in the receptor mobility of these mGluRs and provide a potential mechanism: the intracellular mGluR2 domain interacts with the actin cytoskeleton whereas mGluR7 interacts transsynaptically with ELFN2, tethering it to synaptic sites. Their differential distribution and sensitivity could have a significant impact on the modulation of synaptic transmission at hippocampal synapses. They are able to put forward a model to confirm this impact, nicely conveying the consequences of heterogeneous distribution and sensitivity on receptor function. The manuscript is of sufficient interest and impact to warrant publication, but several discrepancies and inconsistencies in the presentation of the data should be clarified before publication, which are outlined below.

#### Major Points:

The authors make several statements about the distribution/diffusion of variants in the axon or boutons and refer to Figures 3A, 4A, and 5A but those figures do not directly make statistical comparisons and it is not clear if the subsequent analyses are broken down by the axonal/bouton spatial positioning of the variants

- page 10: "...all chimeric mGluR2 variants displayed rapid diffusion throughout the axon and presynaptic boutons, similar to wild-type mGluR2 (Fig. 3A)".
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- page 15: "We found that activation SEP-mGluR2 with the selective group II mGluR agonist LY379268 (LY) did not change the distribution of receptor trajectories (Fig. 5A)"

It is difficult for the reader to reconcile differences between conditions across multiple figures in the main text and supplement. The interpretation would be improved by side-by-side comparisons of mGluR2 and mGluR7 variants in the main Figure 3 & 4. The authors should clarify some specific points, outlined below:

- It is hard to tell for sure with staggered points, but it appears that data in Figure 2C and 2D are presented again in Figure S3D and E. If this is the case, it should be noted. The legend indicates that the GluR2 vs GluR7 comparison for tau and mobile fraction was not significant by the ANOVA + Dunnett's, but then a paired t-test was run on the (same ?) data and presented in Figure 2C as significant.
- The ECD of mGluR7 decreases the mobile fraction in the mGluR2-ECD7 chimera in Figure 3B, but not in Figure S3E.
- Mean mobile fractions appear significantly different between the same experimental paradigms in Figure 3C/4C and Figure S3E. The differences in the mean are around the same magnitude as changes quantified and the authors should either indicate how these experiments differ which might give rise to the different mean values:
  - Mobile fraction wildtype mGluR2 is ~0.39 (Fig3C) vs ~0.58 (FigS3E).
  - Mobile fraction mGluR2-ECD7 is ~0.35 (Fig3C) vs ~0.59 (FigS3E).
  - Mobile fraction mGluR7-ECD1/2 is ~0.37 (Fig4C) vs ~0.55 (FigS3E).
- The ECD of mGluR7 alone does not seem to change the mobile fraction of mGluR2 (Figure S3E) nor does its substitution with EDC 1 or 2 change the recovery kinetics. One might conclude from these experiments that the mGluR7-ECD is neither necessary (no change in tau of mGluR7-ECD1 variant) nor sufficient (no change in the mobile fraction in mGluR2-ECD7 variant), but the authors make the opposite conclusion: stating that mGluR7 is necessary (Change in Deff, Figure 4B) and sufficient (Synaptic tracks, Figure 4E). The authors should comment on how effects on tau and Deff may diverge in these populations and how the mobile fraction may be differentially affected by these chimeras.
- One explanation of the above could be a preferential effect on synaptic tracks. Interpretation of the effect of ECD7 on synaptic vs axonal diffusion would be improved by a comparison to the axonal vs synaptic tracks of wild type mGluR7 and mGluR2 (Figure 4 E-F)

#### Minor Points

1. "All SEP-tagged chimeric mGluR2 variants were targeted to the axon, similar to wildtype mGluR2, indicating that axonal targeting and surface expression were not altered by replacing these domains (Fig. S3A)." The authors should comment if dendritic targeting was altered.
2. It should be clearly noted in the figure legends when SEP knock-in neurons are used and when neurons are transfected.
3. Figure 1A&C and 2A. The scale bars indicate these images are taken at two different magnifications. Since a comparison is being made across mGluRs, these should be at the same magnification.
4. mGluR7 density appears very high on ELFN2-containing neurons (Figure 4G), compared with a randomly elected FOV, Figure 1C. The authors should comment on how FOVs are selected.
5. MBoC Author Submission Checklist, I. 4. c. Tests are identified as one-sided or two-sided.





## Reviewer #1:

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We thank the Reviewer for the detailed and careful analysis of our manuscript and appreciate the raised points. We have addressed these points in the revised manuscript as detailed below.

We regret it was unclear that rat hippocampal neurons are used. We had attempted to clarify this already in the abstract and introduction, but we now include more detailed information on the preparation used in this study in the Results section, see page 6.

The key findings are that the two receptors show very different localization and mobility, plus that the different behavior is controlled by different regions of the receptor molecules: mGluR2 localizes diffusely axons and is highly mobile, whereas mGluR7 is stably anchored at the active zone. This different behavior appears to be dictated by the intracellular receptor domain in the case of the mGluR2, while the immobilization of mGluR7 is determined by its extracellular domain. Finally, the authors present a computational model to describe the consequences of their findings as a function of receptor localization (distance from transmitter release site).

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Quite naturally, the authors have to fight with a large biological scatter; therefore, they generally investigate a large number of individual samples. Unfortunately, however, these are often from only a (very) low number of really independent experiments. In fact, often this number of independent experiments is only 2, which is really very low and would call for repetition at least of critical experiments.

Indeed, there is considerable variation in some experiments that stems from both biological and experimental factors. Because we find that most of the variation is at the level of individual neurons, we aimed to include a sufficient number of observations from neurons from each individual culture to reach average values that are representative of the population. Overall, our data is based on values from ~10-30 neurons per conditions from two or more individual cultures. In fact most experiments, and certainly the most critical experiments, are from 3 or more individual cultures, which we believe is up to the standard in the field. Also, we would like to point out that the uPAINT single-molecule tracking approach

that we took ensures high density of single-molecule trajectories, allowing estimations of  $D_{\text{eff}}$  to be based on the recording of hundreds to thousands of single-molecule trajectories. We also want to stretch that we have been very vigorous in selecting data to include for further analysis. If the number of observations from a single culture was not sufficient ( $< 3$ ) to reach a reliable, representative average value, we discarded the experiment. Moreover, the main conclusions on differences in the mobility of mGluR2 and mGluR7, and different chimera receptors are supported by an independent approach, i.e., fluorescence recovery after photobleaching (FRAP). For the most critical comparison (mGluR2 vs. mGluR7 mobility), we included an extra data set increasing the number of observations (Figure 2B-D). Thus, the conclusions presented in the manuscript are based on a sufficient number of individual cultures, with data collected from a large number of individual neurons, and further strengthened by independent experimental approaches.

I have a number of comments that the authors may want to consider:

p.6 - the use of the specific labeling procedure used should be explained. pHluorins are usually used to study protein cycling between compartments with distinct pH - why was it chosen here, particularly since its reliable detection appeared to require additional use of anti-GFP labeling. And finally, what does this labeling approach to receptor mobility (has this been assessed for example in a simple cellular expression system)? This issue comes up also on p.8, when they use Atto647N-labeled anti-GFP nanobodies.

We tagged presynaptic mGluRs with super-ecliptic pHluorin (SEP-tag) to visualize the surface pool of receptors (indicated on page 7), that allowed us to for instance perform FRAP measurements specifically on surface-expressed receptors (without further enhancement by anti-GFP labeling). For the uPAINT single-molecule tracking technique, it is critical to stochastically label individual receptors with a bright, photostable fluorophore, such as ATTO647N, allowing the reliable detection, localization and tracking of single molecules. Here, we thus chose to use ATTO647N-labeled anti-GFP nanobodies, that are small, monovalent binders, at very low concentration. This is a broadly used approach to achieve reliable, high-density single-molecule trajectory datasets in neurons (see e.g., Chama et al., Nat Comm 2016, Albrecht et al., JCB 2016). In case of CRISPR/Cas9 knock-in of mGluR2, enhancement of the signal with anti-GFP antibodies was necessary to detect endogenous expression of this receptor in hippocampal neurons. We have expanded our explanation of why specific labeling procedures are used in the main text.

The effect of the labeling procedures on receptor mobility is difficult to assess and we cannot exclude a small contribution of the tag to the mobility characteristics we observed. Nevertheless, the direct comparisons between receptor types and chimeras indicates that we can reliably detect changes in mobility properties across a wide range of diffusion coefficients. Also, the independent FRAP experiments, do not rely on the addition of the nanobody, and thus further strengthen the main conclusions. Altogether, we believe that we carefully selected the labeling procedures to minimize effects on experimental measurements of receptor mobility, and used independent, complementary methods to support the conclusions that we present.

p.9 - from their tracking analyses in different regions of the neuron the authors conclude "showed that at a subset of synapses the mobility of SEP-mGluR7 is considerably lower inside boutons". What does this "subset" mean and how is it defined? In Fig. 2M, I see some tracks having a higher  $D_{\text{eff}}$  in axonal vs. synaptic tracks, while in others it is the opposite! Even though the authors attach a p-value of  $< 0.05$ , the mean and distribution look quite identical to me.



In this experiment we detected a significant lower  $D_{\text{eff}}$  for mGluR7 tracks inside boutons compared to axonal tracks (mean  $D_{\text{eff}}$  axonal tracks:  $0.058 \pm 0.006 \mu\text{m}^2/\text{s}$ , mean  $D_{\text{eff}}$  synaptic tracks:  $0.045 \pm 0.006 \mu\text{m}^2/\text{s}$ ,  $P < 0.05$ , paired t-test), i.e., a ~20% lower diffusion coefficient in boutons compared to axons. Nevertheless, the data in Figure 2M indeed does not unequivocally indicate that at *all* boutons the diffusion coefficient of mGluR7 is lower than the diffusion coefficient in the axon. We interpreted this data to indicate that mGluR7 mobility is reduced at a subset of synapses. Consistent with our observations that ELFN2 is not expressed at all synapses, this could for instance suggest that not at all synapses anchoring mechanisms are available to effectively immobilize mGluR7. Nevertheless, we agree that we can not quantitatively discriminate this subset of boutons where mGluR7 is immobilized and we rephrased the conclusion to better reflect the presented results (see page 9).

p.10 - The experiments on domain swapping are impressively clean. In contrast, I was not much convinced by the effects of latrunculin B on mGluR2 mobility (p.11) - the effects are really very modest.

We agree with the Reviewer that the effects of actin disassembly by latrunculin B on mGluR2 mobility are modest ( $D_{\text{eff}}$  control:  $0.063 \pm 0.002 \mu\text{m}^2/\text{s}$  vs. Lat-B:  $0.069 \pm 0.002 \mu\text{m}^2/\text{s}$ ,  $P = 0.019$ , paired t-test). We were therefore motivated to substantiate this observation and tested the effect of latrunculin B on the mobility of an mGluR2 mutant lacking the C-terminal domain (mGluR2- $\Delta$ ICD). The diffusion coefficient of this mutant was not affected by latrunculin B. These data thus strengthen the conclusion that the actin cytoskeleton modulates the mobility of mGluR2 through its C-terminal domain. Further studies need to be conducted to elucidate in more detail how the ICD of mGluR2 regulates receptor mobility.

p.15- The experiments to study the effects of stimulation or inhibition of synaptic activity are quite drastic: potassium depolarization and TTX are really extremes, and the reader wonders whether their use really demonstrates an effect of synaptic function on receptor mobility or rather the contrary.

In this set of experiments, we investigated whether changes in activity induced alterations in mGluR mobility. We addressed this by pharmacological approaches that act specifically on mGluR2 or mGluR7, and treatments that are broadly used to globally increase or decrease neuronal network activity. To avoid long-term adaptations in synaptic functioning caused by prolonged changes in synaptic activity, we only studied acute effects (incubation times did not exceed 15 min). Surprisingly, we found that specific receptor agonists and antagonists do not influence receptor mobility. And, indeed, even strong chemical stimulation of neurons with 25 mM  $\text{K}^+$  did not change mobility of presynaptic mGluRs. So, even quite drastic changes in synaptic activity, or direct manipulation of receptor activity do not alter receptor mobility. Of interest however, acute *silencing* of spontaneous activity with TTX caused a decrease in the mobility of both receptors. This thus seems to be a relatively fast effect of synaptic *inactivity* on receptor mobility. Particularly since we found that direct activation or inactivation of mGluR2 or mGluR7 did not change receptor mobility, it is difficult to conceive how acute application of TTX would change synaptic function via a change in receptor mobility. We therefore concluded that acute synaptic silencing changes mobility of presynaptic mGluRs, for instance by rearrangements of scaffold proteins or other interaction partners.

Fig.6 - I am not sure whether and how much the computational model provides new insights. The key point that I derive from the data shown in Fig. 6 is that under all conditions considered the activation of mGluR7 at any location is at best minimal. One wonders whether this is really a functionally relevant effect.

The results from our computational model are fully consistent with the experimental data and our interpretation. We agree with the Reviewer that, therefore, they do not provide an unexpected, surprising behavior. However, we want to emphasize that our description includes the rather complex 'cubic' GPCR model, which makes the relationship between the time-dependent glutamate concentration and G protein activation non-trivial. Whether the low activation of mGluR7 is functionally relevant, as asked by the Reviewer, we cannot answer with the current model. The current model does not include a functional interpretation of mGluR activation and was not built to address such questions.

We strongly believe that our model makes a valuable contribution to our study by integrating previously published knowledge with our novel experimental results, as acknowledged by the other reviewer: 'They are able to put forward a model to confirm this impact, nicely conveying the consequences of heterogeneous distribution and sensitivity on receptor function.' Importantly, we intend to contribute our manuscript to MBoC's special issue on 'Quantitative Cell Biology', and therefore a quantitative model that nicely integrates our experimental results is appropriate and highly desired.

The discussion is very long and might benefit from focusing.

We reduced the length of the discussion and attempted to focus on the most critical conclusions.

Finally, an editorial point that the authors may or may not want to consider: I found some passages of the text really difficult to read because the sentences were disrupted by long brackets giving individual values (which are the same as those given in the figures that the text describes). An example is p.12.

We think that giving individual mean values of  $D_{\text{eff}}$  and mobile fraction in the text, in addition to the graphs in the figures, is beneficial and allows readers to directly evaluate the described results.

#### **Reviewer #2:**

The authors investigate the spatial distribution and diffusion properties of two metabotropic glutamate receptors, mGluR2 and mGluR7. They use several methods to characterize the diffusion properties of these neurons, including several imaging modalities, co-culture assays, and chimera analyses. Their results reveal interesting differences in the receptor mobility of these mGluRs and provide a potential mechanism: the intracellular mGluR2 domain interacts with the actin cytoskeleton whereas mGluR7 interacts transsynaptically with ELFN2, tethering it to synaptic sites. Their differential distribution and sensitivity could have a significant impact on the modulation of synaptic transmission at hippocampal synapses. They are able to put forward a model to confirm this impact, nicely conveying the consequences of heterogeneous distribution and sensitivity on receptor function. The manuscript is of sufficient interest and impact to warrant publication, but several discrepancies and inconsistencies in the presentation of the data should be clarified before publication, which are outlined below.

We thank the Reviewer for the careful assessment and positive notes on the manuscript. We took all the questions and concerns into account and revised the manuscript accordingly. Please find our detailed response below.

Major Points:

The authors make several statements about the distribution/diffusion of variants in the axon or boutons and refer to Figures 3A, 4A, and 5A but those figures do not directly make statistical comparisons and it is not clear if the subsequent analyses are broken down by the axonal/bouton spatial positioning of the variants

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- page 15: "We found that activation SEP-mGluR2 with the selective group II mGluR agonist LY379268 (LY) did not change the distribution of receptor trajectories (Fig. 5A)"

These statements were intended to convey a qualitative description of the distribution of the receptor trajectories we observed in our experiments comparing the mobility between wild-type receptors and chimeras. We regret if our wording implied that we addressed these specific statements quantitatively. We adjusted the sentences pointed out by the Reviewer to avoid this confusion.

Nevertheless, we would like to make clear that the graphs presented in Figures 3B and C, Figure 4B and C and Figure 5B respectively, are based on rigorous quantification of the receptor trajectories observed in many fields of view. In these data sets, average diffusion coefficients and mobile fractions were calculated from all (i.e., both axonal and synaptic) trajectories.

It is difficult for the reader to reconcile differences between conditions across multiple figures in the main text and supplement. The interpretation would be improved by side-by-side comparisons of mGluR2 and mGluR7 variants in the main Figure 3 & 4.

We have considered a side-by-side comparison of mobility of chimeric variants of mGluR2 and mGluR7 in earlier versions of the figures. However, we found that it made the description of the results in the text less clear. We compared multiple configurations of presenting these data sets, and we decided to keep the data about mGluR2 and mGluR7 variants in separate figures as the most clear way of describing our results and most effectively conveying the most important conclusions.

The authors should clarify some specific points, outlined below:

It is hard to tell for sure with staggered points, but it appears that data in Figure 2C and 2D are presented again in Figure S3D and E. If this is the case, it should be noted. The legend indicates that the GluR2 vs GluR7 comparison for tau and mobile fraction was not significant by the ANOVA + Dunnett's, but then a paired t-test was run on the (same ?) data and presented in Figure 2C as significant.

Indeed, the data in Figure 2C and D are also included in Figure S3D and E for comparison. We regret that we omitted this information from the figure legend and have now included this information in the legend of Figure S3.

We also adjusted the graphs in Figure S3D and E and now present the data from mGluR2 and its ECD chimera, and mGluR7 and its ECD chimeras in separate graphs. The previous version, as pointed out by

the Reviewer, only indicated significant differences between wild-type receptors and its chimeric variants but an indication of the significant difference between wild-type receptors was missing. We hope that the new version of this figure presents the FRAP data in a more clear way.

The ECD of mGluR7 decreases the mobile fraction in the mGluR2-ECD7 chimera in Figure 3B, but not in Figure S3E.

Mean mobile fractions appear significantly different between the same experimental paradigms in Figure 3C/4C and Figure S3E. The differences in the mean are around the same magnitude as changes quantified and the authors should either indicate how these experiments differ which might give rise to the different mean values:

- Mobile fraction wildtype mGluR2 is  $\sim 0.39$  (Fig3C) vs  $\sim 0.58$  (FigS3E).
- Mobile fraction mGluR2-ECD7 is  $\sim 0.35$  (Fig3C) vs  $\sim 0.59$  (FigS3E).
- Mobile fraction mGluR7-ECD1/2 is  $\sim 0.37$  (Fig4C) vs  $\sim 0.55$  (FigS3E).

To investigate mobility of presynaptic mGluRs, we used two complementary but explicitly different experimental approaches: high-resolution single-molecule tracking (SMT; as in Figures 3 and 4) and confocal-based (diffraction-limited) fluorescence recovery after photobleaching (FRAP; as in Figure S3). These two techniques differ in many aspects. Most notably the spatial resolution ( $\sim 20$  nm for SMT vs.  $\sim 200$  nm for FRAP) and the time scale of observation (few second long trajectories vs. minutes of recovery of fluorescence signal). Moreover, in single-molecule tracking experiments, the  $D_{\text{eff}}$  is estimated from many (hundreds to thousands) trajectories of individual molecules, while the kinetics ( $\tau$ ) obtained from FRAP experiments represent the average 'ensemble' behavior of many molecules. Importantly, in single-molecule tracking experiments, we classified tracks as mobile or immobile based on geometry-based thresholds of the tracks (Figure S2F-H). This parameter is thus independent from the MSD curve-based estimation of the diffusion coefficient. Population estimates of changes in  $D_{\text{eff}}$  are therefore often, but not always reflected in changes in the mobile fraction. In FRAP experiments, the mobile fraction was calculated as the average recovery of fluorescence in the bleached spot. In most single-molecule tracking experiments, we evaluated both axonal and synaptic trajectories, while in FRAP experiments only presynaptic boutons were bleached. Therefore, the absolute values of mobile fractions presented in Figure 3 and 4 cannot be directly compared with the absolute values in Figure S3. However, both approaches showed highly consistent results, indicating that mGluR2 is considerably more mobile than mGluR7 and that swapping the ECD of mGluR7 with the ECD of mGluR1 or mGluR2 increased mobility of chimeric receptors. Together, we thus believe that the results from these two complimentary experimental approaches together significantly strengthen our conclusions.

The ECD of mGluR7 alone does not seem to change the mobile fraction of mGluR2 (Figure S3E) nor does its substitution with EDC 1 or 2 change the recovery kinetics. One might conclude from these experiments that the mGluR7-ECD is neither necessary (no change in  $\tau$  of mGluR7-ECD1 variant) nor sufficient (no change in the mobile fraction in mGluR2-ECD7 variant), but the authors make the opposite conclusion: stating that mGluR7 is necessary (Change in  $D_{\text{eff}}$ , Figure 4B) and sufficient (Synaptic tracks, Figure 4E). The authors should comment on how effects on  $\tau$  and  $D_{\text{eff}}$  may diverge in these populations and how the mobile fraction may be differentially affected by these chimeras.

One explanation of the above could be a preferential effect on synaptic tracks. Interpretation of the effect of ECD7 on synaptic vs axonal diffusion would be improved by a comparison to the axonal vs synaptic tracks of wild type mGluR7 and mGluR2 (Figure 4 E-F)

Based on the presented data we indeed conclude that mGluR7 mobility is reduced at synaptic sites by extracellular interactions. First, we found that the diffusion coefficient of mGluR2 is similar in axons and presynaptic boutons, while synaptic tracks of mGluR7 display significantly lower diffusion coefficients than axonal trajectories (Figure 2J-M). Second, results of single-molecule tracking show that replacement of the mGluR7 ECD with the ECD of mGluR1 or mGluR2 increased the average diffusion coefficient as well as the mobile fraction of the chimeric variants (Figure 4B and C). Adding the ECD of mGluR7 to mGluR2 affected the mobile fraction (Figure 3C), but had no detectable effect on the average  $D_{\text{eff}}$  of the total population (Figure 3B). Together, we interpreted these data to be consistent with the model that the ECD of mGluR7 immobilized receptors specifically at presynaptic sites and would thus affect only a sub-population of the receptors.

As pointed out above, direct comparison of single-molecule tracking data with FRAP recovery curves can be complicated. Most importantly, since FRAP is a diffraction-limited technique that reports the average behaviour of a bulk population of receptors, subtle differences in diffusional behaviour of receptor sub-populations residing in different subsynaptic compartments cannot be resolved. Nevertheless, the FRAP experiments in Figure S3 confirm the general notion: the overall population of mGluR2-ECD7 seems unaffected, but the altered recovery kinetics ( $\tau$ ) are an independent suggestion that a sub-population of the receptors became hampered in their diffusion. These FRAP data motivated us to use high-resolution single-molecule tracking experiments to measure specifically the effect of the mGluR7 ECD on mGluR2 mobility in synapses as presented in Figure 4E. Swapping the ECD of mGluR7 into mGluR2 decreased the diffusion rate of the chimeric receptor inside presynaptic boutons similar to WT mGluR7. Altogether, we think these experiments provide strong evidence that the ECD of mGluR7 is involved in immobilizing receptors at the active zone.

#### Minor Points

1. "All SEP-tagged chimeric mGluR2 variants were targeted to the axon, similar to wildtype mGluR2, indicating that axonal targeting and surface expression were not altered by replacing these domains (Fig. S3A)." The authors should comment if dendritic targeting was altered.

We did not observe alterations in dendritic targeting of chimeric variants of mGluR2. We added this statement to the Results part (page 10).

2. It should be clearly noted in the figure legends when SEP knock-in neurons are used and when neurons are transfected.

Images of SEP-mGluR2 knock-in cells are labeled as "KI SEP-mGluR2" in the figures. In the figure legends, these images are described as SEP-mGluR2 CRISPR/Cas9 knock-in neurons.

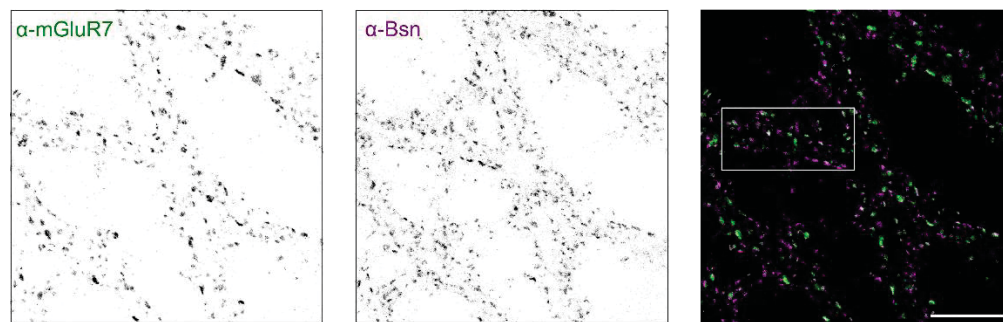
3. Figure 1A&C and 2A. The scale bars indicate these images are taken at two different magnifications. Since a comparison is being made across mGluRs, these should be at the same magnification.

We thank the Reviewer for pointing out these inconsistencies. We corrected the scale bar in Figure 1A (see new version of Figure 1). Images in Figure 1A and C were taken with the same magnification but during figure preparation, we erroneously mislabeled the scale bar. We also corrected this for the images in Figure 2A (see new version Figure 2). These changes do not have any influence on the data analysis or interpretation of the results.

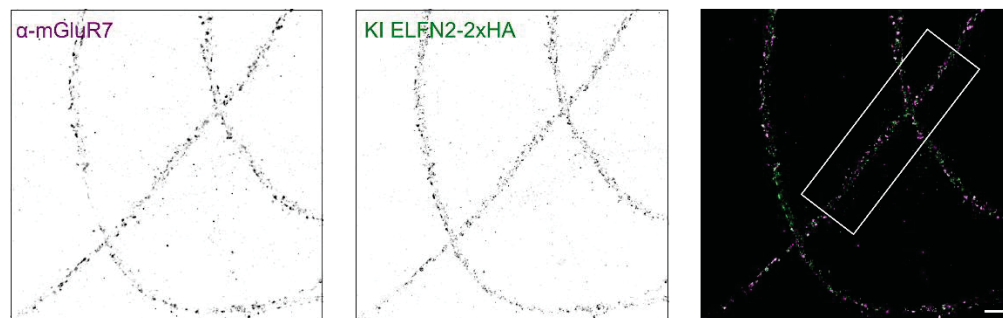
4. mGluR7 density appears very high on ELFN2-containing neurons (Figure 4G), compared with a randomly elected FOV, Figure 1C. The authors should comment on how FOVs are selected.

We indeed observed small differences in the density of mGluR7-positive puncta between images caused by variations in neuronal network architecture (number of synapses, neurons) observed between coverslips, but the impression that the density of mGluR7 spots in Figure 1C is different than in Figure 4G is most likely due to different magnifications of these images (see the Figure below showing the uncropped images). The image in Figure 1C is a STED image with  $\sim 20$  nm pixel size, while the image in Figure 4G is a confocal image with  $\sim 80$  nm pixel size yielding different effective magnifications. More example mGluR7 images of the uncropped fields of view are shown in Figure S1D.

**A. Full image from Figure 1C**



**B. Full image from Figure 4G**



**Figure.** (A) Full field of view of gSTED image of neurons co-stained with anti-mGluR7 and anti-Bsn. Scale bar, 5  $\mu$ m. (B) Full field of view of confocal image of ELFN2-2xHA CRISPR/Cas9 knock-in neuron co-stained with anti-mGluR7. Scale bar, 5  $\mu$ m. White boxes in the merge images indicate regions shown in Figures 1C and 4G in the manuscript.

5. MBoC Author Submission Checklist, I. 4. c. Tests are identified as one-sided or two-sided.

Information whether one-sided or two-sided test was used are added to the manuscript.

RE: Manuscript #E21-10-0484R

TITLE: "Subsynaptic mobility of presynaptic mGluR types is differentially regulated by intra- and extracellular interactions"

Dear Dr. MacGillavry:

Thank you for revising your manuscript in response to the reviewers' comment. I find that you have addressed their concerns. At this point, before acceptance, there is still one point of clarification needed. As you can see from Reviewer 2's comments, the statistical comparison for the data in Figure 3 is not clear. The appropriate comparison would be to compare data in Figures 2C&D with respect to all the FRAP conditions (found in the Figure S3), rather than pulling out the two conditions and performing an isolated t-test (as is currently indicated in the legend). Please confirm that the GluR2 and GluR7 differences retain statistical significance when compared in an ANOVA multiple comparisons test with the other corresponding FRAP measurements? Also note this outcome in the figure legend(s).

Sincerely,  
Diane Lidke  
Monitoring Editor  
Molecular Biology of the Cell

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Dear Dr. MacGillavry,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office ([mboc@ascb.org](mailto:mboc@ascb.org)).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us immediately at [mboc@ascb.org](mailto:mboc@ascb.org).

In preparing your revised manuscript, please follow the instruction in the Information for Authors ([www.molbiolcell.org/info-for-authors](http://www.molbiolcell.org/info-for-authors)). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL):  
Link Not Available

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Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker  
Journal Production Manager  
MBoC Editorial Office  
[mbc@ascb.org](mailto:mbc@ascb.org)



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Reviewer #2 (Remarks to the Author):

I thank the authors for their explanation and clarification of several of the issues I brought up, and for resolving those issues with edits to the text and figures. The only remaining issue I would like to address is one point on multiple comparisons. As this is specifically stated in the MBoC Author Submission Checklist that the authors must correct for multiple comparisons, I wanted to bring it to the attention of the journal. Please see below the thread related to the issue of multiple comparisons. I will defer to the journal if it feels the authors have met this criterion.

Reviewer Response: It is hard to tell for sure with staggered points, but it appears that data in Figure 2C and 2D are presented again in Figure S3D and E. If this is the case, it should be noted. The legend indicates that the GluR2 vs GluR7 comparison for tau and mobile fraction was not significant by the ANOVA + Dunnett's, but then a paired t-test was run on the (same ?) data and presented in Figure 2C as significant.

Author Rebuttal:

Indeed, the data in Figure 2C and D are also included in Figure S3D and E for comparison. We regret that we omitted this information from the figure legend and have now included this information in the legend of Figure S3.

We also adjusted the graphs in Figure S3D and E and now present the data from mGluR2 and its ECD chimera, and mGluR7 and its ECD chimeras in separate graphs. The previous version, as pointed out by

the Reviewer, only indicated significant differences between wild-type receptors and its chimeric variants but an indication of the significant difference between wild-type receptors was missing. We hope that the new version of this figure presents the FRAP data in a more clear way.

Reviewer Response to Rebuttal: I appreciated the addition of text indicating the data was the same as in the main figures. I apologize for some apparent lack of clarity in my previous comment. I was not concerned that the data for all mutants are displayed together in one figure. My concern was if the authors corrected for multiple comparisons. Extracting a subset of conditions and running a t-test on those conditions rather than an ANOVA w/ post hoc Tukey's Test circumvents the correction for multiple comparison. In this case, it seems like the effect would not have been statistically significant (at  $p < 0.05$ ) when corrected. In my view, if they were run as a one experiment, the convention would be ANOVA + multiple comparisons. If the mGluR2 and mGluR2-ECD7 were run as a separate experiment, I agree with the authors that t-test will suffice. I will defer to the journal regarding their best practices surrounding corrections for multiple comparisons.



## **Monitoring Editor's**

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We appreciate the thorough evaluation of the statistical procedures. We attempted to approach the statistical analyses correctly and would like to clarify our reasoning.

In initial experiments, we performed only FRAP experiments for wild-type mGluR2 and mGluR7 to directly compare the recovery kinetics for these receptors. In follow-up experiments we also included the chimera receptors. In the figures and accompanying statistical analyses we therefore compared what we think are the most meaningful comparisons:

- wild-type mGluR2 vs. mGluR7 in Figure 2B, C and D
- wild-type mGluR2 vs. mGluR2-ECD7 in Figure S3D
- wild-type mGluR7 vs. mGluR7-ECD1 and mGluR7-ECD2 in Figure S3E

Nevertheless, as the dataset could be considered as a single experiment, it would indeed be conventional to run an ANOVA + Tukey's multiple comparison on the complete dataset.

Please find below the table with results for the multiple-comparison test on (A) the half-time ( $\tau$ ) and (B) mobile fractions.

When corrected for multiple comparisons indeed the mGluR2 vs. mGluR7 comparison remains significant when comparing the mobile fractions, the  $\tau$  is not significant. We adjusted this in the main text as well as in the legends for Figure 2 and Figure S3 to indicate this. Thus, the overall conclusion that mGluR2 and mGluR7 display differences in mobility remains valid and further supported by the single-molecule tracking experiments.

## (A) Tau

Number of families 1  
 Number of comparisons per family 10  
 Alpha 0.05

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
mGluR2 vs. mGluR7	-7.139	-23.36 to 9.085	No	ns	0.7443 A-B
mGluR2 vs. mGluR2-ECD7	-27.67	-42.69 to -12.65	Yes	****	<0.0001 A-C
mGluR2 vs. mGluR7-ECD1	-5.964	-20.82 to 8.889	No	ns	0.8031 A-D
mGluR2 vs. mGluR7-ECD2	-11.73	-26.51 to 3.049	No	ns	0.1894 A-E
mGluR7 vs. mGluR2-ECD7	-20.53	-33.23 to -7.829	Yes	***	0.0001 B-C
mGluR7 vs. mGluR7-ECD1	1.175	-11.33 to 13.68	No	ns	0.999 B-D
mGluR7 vs. mGluR7-ECD2	-4.59	-17.00 to 7.821	No	ns	0.8464 B-E
mGluR2-ECD7 vs. mGluR7-ECD1	21.71	10.81 to 32.60	Yes	****	<0.0001 C-D
mGluR2-ECD7 vs. mGluR7-ECD2	15.94	5.148 to 26.73	Yes	***	0.0007 C-E
mGluR7-ECD1 vs. mGluR7-ECD2	-5.765	-16.32 to 4.790	No	ns	0.5608 D-E

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
mGluR2 vs. mGluR7	12.73	19.87	-7.139	5.889	17	28	1.714	185
mGluR2 vs. mGluR2-ECD7	12.73	40.4	-27.67	5.453	17	45	7.176	185
mGluR2 vs. mGluR7-ECD1	12.73	18.7	-5.964	5.392	17	49	1.564	185
mGluR2 vs. mGluR7-ECD2	12.73	24.46	-11.73	5.364	17	51	3.092	185
mGluR7 vs. mGluR2-ECD7	19.87	40.4	-20.53	4.611	28	45	6.297	185
mGluR7 vs. mGluR7-ECD1	19.87	18.7	1.175	4.538	28	49	0.3663	185
mGluR7 vs. mGluR7-ECD2	19.87	24.46	-4.59	4.505	28	51	1.441	185
mGluR2-ECD7 vs. mGluR7-ECD1	40.4	18.7	21.71	3.955	45	49	7.762	185
mGluR2-ECD7 vs. mGluR7-ECD2	40.4	24.46	15.94	3.918	45	51	5.754	185
mGluR7-ECD1 vs. mGluR7-ECD2	18.7	24.46	-5.765	3.832	49	51	2.128	185

## (B) Mobile fraction

Number of families 1  
 Number of comparisons per family 10  
 Alpha 0.05

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
mGluR2 vs. mGluR7	0.3617	0.2326 to 0.4907	Yes	****	<0.0001 A-B
mGluR2 vs. mGluR2-ECD7	-0.0006434	-0.1205 to 0.1193	No	ns	>0.9999 A-C
mGluR2 vs. mGluR7-ECD1	0.0579	-0.06236 to 0.1782	No	ns	0.675 A-D
mGluR2 vs. mGluR7-ECD2	-0.02999	-0.1486 to 0.08861	No	ns	0.957 A-E
mGluR7 vs. mGluR2-ECD7	-0.3623	-0.4625 to -0.2622	Yes	****	<0.0001 B-C
mGluR7 vs. mGluR7-ECD1	-0.3038	-0.4044 to -0.2032	Yes	****	<0.0001 B-D
mGluR7 vs. mGluR7-ECD2	-0.3917	-0.4903 to -0.2931	Yes	****	<0.0001 B-E
mGluR2-ECD7 vs. mGluR7-ECD1	0.05854	-0.03002 to 0.1471	No	ns	0.3645 C-D
mGluR2-ECD7 vs. mGluR7-ECD2	-0.02934	-0.1156 to 0.05696	No	ns	0.8822 C-E
mGluR7-ECD1 vs. mGluR7-ECD2	-0.08789	-0.1747 to -0.001088	Yes	*	0.0456 D-E

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
mGluR2 vs. mGluR7	0.6139	0.2523	0.3617	0.04683	17	29	10.92	182
mGluR2 vs. mGluR2-ECD7	0.6139	0.6146	-0.0006434	0.04351	17	46	0.02091	182
mGluR2 vs. mGluR7-ECD1	0.6139	0.556	0.0579	0.04364	17	45	1.876	182
mGluR2 vs. mGluR7-ECD2	0.6139	0.6439	-0.02999	0.04304	17	50	0.9852	182
mGluR7 vs. mGluR2-ECD7	0.2523	0.6146	-0.3623	0.03635	29	46	14.1	182
mGluR7 vs. mGluR7-ECD1	0.2523	0.556	-0.3038	0.03651	29	45	11.77	182
mGluR7 vs. mGluR7-ECD2	0.2523	0.6439	-0.3917	0.03578	29	50	15.48	182
mGluR2-ECD7 vs. mGluR7-ECD1	0.6146	0.556	0.05854	0.03214	46	45	2.576	182
mGluR2-ECD7 vs. mGluR7-ECD2	0.6146	0.6439	-0.02934	0.03132	46	50	1.325	182
mGluR7-ECD1 vs. mGluR7-ECD2	0.556	0.6439	-0.08789	0.0315	45	50	3.945	182

RE: Manuscript #E21-10-0484RR

TITLE: "Subsynaptic mobility of presynaptic mGluR types is differentially regulated by intra- and extracellular interactions"

Dear Dr. MacGillavry:

Thank you for clarifying the statistical analysis. I am pleased to now accept your paper for publication in Molecular Biology of the Cell. Congratulations to you and your colleagues.

Sincerely,  
Diane Lidke  
Monitoring Editor  
Molecular Biology of the Cell

-----  
Dear Dr. MacGillavry:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at [www.molbiolcell.org/toc/mboc/0/0](http://www.molbiolcell.org/toc/mboc/0/0) is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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