

Stimulus-specific remodeling of the neuronal transcriptome through nuclear intron-retaining mRNAs

Maxime Mazille, Katarzyna Buczak, Peter Scheiffele, and Oriane Mauger

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Dear Oriane and Peter,

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and their comments are included below.

As you can see the referees find the analysis interesting and insightful. Should you be able to extend the findings along the lines as indicated then I would be interested in considering a revised version.

I think it would be helpful to discuss the raised points further and I am available to do so via email or video.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: <https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess>

I thank you for the opportunity to consider your work for publication. I look forward to discussing your revisions further with you.

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

I have attached a guide with helpful tips on how to prepare the revised manuscript

Guide For Authors: <https://www.embopress.org/page/journal/14602075/authorguide>

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (16th Mar 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

<https://emboj.msubmit.net/cgi-bin/main.plex>

Referee #1:

This is a very interesting and timely study based on a landmark paper by Mauger & Scheiffele 2016 in Neuron. Here, the authors systematically assess intron retention (IR) from fractionated mouse cortical neurons. In the first figure, the authors then convincingly show that their applied protocol including IR analysis nicely works. In the subsequent figures, they show that IR transcripts are kept in the nucleus and fully spliced transcripts are enriched in the cytoplasm. Next, they tested how different stimulation paradigms (Bic vs BDNF) may (differentially) regulate IR in their system. Interestingly, the majority of regulated transcripts occur in the nucleus. The authors also observe an increase of spliced isoforms upon stimulation, but also in some cases that intron excision can be slowed down under these conditions and that there is transcript-specific degradation. Furthermore, they investigated the localization of transcripts in the nucleus versus the cytosol after 1h of Bic treatment and confirmed that spliced transcripts were also enriched in the cytoplasm (not only in the nucleus) in contrast to transcripts with retained introns (Fig. 4). Particularly attractive is the stimulus-dependent regulation of transcripts, as this reveals that Bic and BDNF appear to specifically activate different pools of IR transcripts. Even more impressive is their findings that both stimulation paradigms act on common transcripts but with opposite outcome, as shown for example for the splicing factor SRSF2. In the last set of experiments, the authors investigated the underlying signal pathways, e.g. calcium signaling (via CaMK) vs MAPK and showed that Bic preferentially impacts NMDARs in contrast to BDNF that affects BDNF action in neurons.

Investigating systematically IR in neurons under physiologically relevant conditions is very interesting, adds important new insight to the field and will certainly yield as important source for future investigations. Overall, the manuscript is well written, concise and easy to follow. Experiments are well designed and executed, nicely controlled and clearly presented. The paper's conclusions are overall properly supported by the data. I have a few comments to be addressed before recommending the manuscript for publication:

1. I am not sure that the visualization of the data in Fig. 4 is optimal and does full justice to the current data set. It is the second part, in which the authors state that spliced transcripts "were also enriched in the cytoplasm, not only in the nucleus". If I got it right, there is a decrease (from grey to dark blue) in the first panel on the left but later in both cases (middle and right panel) there is an increase. This can certainly be emphasized more and additionally visualized in a more intuitive manner. Second, can the authors check, which transcripts shift from nucleus to cytosol and which ones stay. This would certainly be of utmost interest and very informative. Along those lines, do the authors have experimental evidence that this shift in the ratio is based on actual export and sequential localization? I would find this additional information of high importance and in my opinion represent a significant boost of the novelty and originality. In conclusion, this figure could certainly be improved by the addition of such new data.

2. The authors' findings that different stimulation paradigms yield different outcomes in IR is truly impressive. As they started with Bic vs BDNF stimulation, I would suggest to explain in more detail that Bic is not primarily used to interfere with GABA signaling, but to affect network stimulation. Also, the authors could then incorporate this aspect into their model (Fig. 6) and include GABAergic synapses. In this respect, I would suggest discussing their findings reg. NMDAR- vs BDNF-dependent synapses, and in what aspect the blocking of GABAA synapses might affect IR. Also, in their model, they show CaMK signaling back to the nucleus. Here, it might be worth mentioning that the CaMKII alpha transcript contains an intron, which itself undergoes regulation (Ortiz-R et al., 2017, Cell Rep, PMID: 28683307). Along those lines, this would allow the authors to improve their simplistic model by adding another layer of regulation: a certain set of intron-containing transcripts might not only be found in the cytosol, but be transported to synapses, as shown for CaMKII and Calm3 (Sharangdhar et al., 2017, EMBO Rep, PMID 28765142).

3. In my opinion, the discussion is not yet in the best possible shape. There are several key findings of the paper that are not discussed, e.g. why this paper represents indeed a significant step forward in our understanding of IR and why it actually provides new mechanistic insight. Second, to discuss why it makes sense that different signaling pathways affect distinct transcripts (and give a couple of examples). Finally, the authors could consider including the just mentioned departmental specific localization of transcripts, e.g. to excitatory spine synapses or inhibitory shaft synapses, just to name a few options.

Minor points

- Actual subheading is not ideal (p14): Targeting intron retentions is... I would rephrase
- Incomplete references, e.g. Suzuki et al.,

Referee #2:

In this paper Mauger and colleagues extend their work on intron retention in neuronal cells. Specifically, they define the changes in IRs across the transcriptome in response to two different neuronal stimuli. Interestingly, they observe and characterize distinct patterns of changes in IR with the two different stimuli clearly delineating a regulatory role for IR. Moreover, they show that unique cell signaling pathways (MAPK and CamK) govern this specificity. Overall, this is a very well written paper with interesting and important data for the field. The interpretations are consistent with the data and the experiments are rigorous with multiple validation approaches. I suggest only a few minor changes:

1. Please define what "density" is in Fig 1B. Do these data mean that nearly all nuclear transcripts have a PIR value of >20?
2. It may be nice to show a time course diagram for the experiments with transcription inhibition and neuronal stimuli in the figure. This way readers don't have to hunt for this information in the legends/methods. (Or, at least, state the timing more explicitly in the Results section).
3. Please include representative genome browser screenshots showing the effects of neuronal stimulation on specific transcripts. This can be incorporated into the main figures or in supplemental data but having visuals for these events on specific genes is quite helpful in illustrating the effects of the stimuli.
4. Literature on the role of spliceosome association on nuclear retention should be included. As early as the late 80's, two groups suggested that spliceosome assembly promotes nuclear retention (Legrain and Rosbash, Cell, 1989) (Chang and Sharp, Cell 1989). Therefore, the idea that IR promotes nuclear retention is not surprising. However, the observations here do imply at least partial spliceosome formation on these IR transcripts.
5. KN-62 activity should be stated in the Results, not left to the legend.

Referee #3:

This is a very interesting paper describing the presence and regulation of intron-retention (IR) in neurons. The work is nicely done, although I have some reservations especially on the statistical analysis (see below), but the validations suggest the findings to be robust. Most of the results reported in the paper were also reported in previous work

from the same authors (Mauger et al., Neuron 2016), including the characterization of features of retained introns and their activity-dependent regulation (although with just one paradigm). The main difference in this manuscript is the use of RNAseq from nuclei vs cytosol, whereas this was not done in a genome-wide fashion in the previous work. Nevertheless, I believe this data and their description could represent a meaningful contribution to EMBO Journal. However, as detailed below, a number of issues need to be addressed before this manuscript can be considered for publication.

MAJOR COMMENTS:

1) A big missing piece of information in the manuscript is whether IR affects all introns of a transcript more or less equally, or specific introns e.g. Does each transcript only contain a single retained intron? Is there a bias in which intron is retained (e.g. last intron)? What is the percentage of IR vs. fully-spliced transcripts for individual genes (e.g., how frequent is IR for individual genes)? This should be described. Moreover, this could act as an extra control given that it cannot be assumed that transcription is completely stopped and that unprocessed transcripts have been either spliced or degraded completely after 3h; in particular, in the stimulation experiment it is clear that it is only partially inhibited (Suppl Figure 2A). In the null hypothesis of retained introns being due to remaining unprocessed transcripts, we would expect a proportion of all introns of a transcript to be retained, plausibly in an increasing fashion towards the 3' end. Instead, if intron retention is due to specific regulation, it should depart from this pattern. This could easily be visualized and tested, and would provide an important addition to the paper.

2) The IR metric used is appropriate, but I find the statistical analysis lacking. In particular, I do not understand the use of z-scores which are known to be inadequate for that kind of data, when more robust statistical tools are readily available. This is bound to have both a lower power and a higher FDR than using established differential expression methods. The authors could easily harness GLMs in existing packages (e.g. edgeR/DESeq2) in the following way: build a matrix (features per samples) of exon-exon counts, join it to a matrix of exon-intron counts, fit a model of the form $\sim \text{countType} * \text{condition}$ (which amounts to $\sim \text{countType} + \text{condition} + \text{countType}:\text{condition}$), where countType indicates either the column gives exon-exon or exon-intron counts, and test for the interaction coefficient (countType:condition). (You can also add a sample covariate if you want). This is the standard way of addressing statistically similar problems (e.g. differential translation efficiency in ribosome footprinting), using all the benefits of discrete statistics and information sharing across genes. Moreover, an advantage of this approach is that the obtained logFC could be directly interpreted as PIR changes, rather than IR-transcript expression changes, therefore directly circumventing the question of changes that are truly transcription-independent.

3) The authors describe IR as "a widespread mechanism to acutely regulate subcellular compartmentalization of transcripts upon cellular signals", however the evidence presented is purely correlative, i.e. there is not experimental proof that IR/splicing is causative of localization, rather than merely associated with it. At the very least, the authors should assess whether the mobilization of specific sets of IR transcripts upon stimulation has an impact on their protein output.

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or a histogram on each axis), to know if the grey cloud in the middle hides a trend. In addition, it would be relevant to test whether the induced splicing is over-represented WRT to other quadrants.

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- What might be the physiological role of intron retention and stimulus-specific mobilization in non-coding transcripts (e.g. Mirg)? This should be discussed.

Please find below a detailed response to all points raised by the three referees. We have now extended the study and improved the clarity. We are confident this has strengthened our work and we hope the reviewers will find this manuscript suitable for publication.

Referee #1:

This is a very interesting and timely study based on a landmark paper by Mauger & Scheiffele 2016 in *Neuron*. Here, the authors systematically assess intron retention (IR) from fractionated mouse cortical neurons. In the first figure, the authors then convincingly show that their applied protocol including IR analysis nicely works. In the subsequent figures, they show that IR transcripts are kept in the nucleus and fully spliced transcripts are enriched in the cytoplasm. Next, they tested how different stimulation paradigms (Bic vs BDNF) may (differentially) regulate IR in their system. Interestingly, the majority of regulated transcripts occur in the nucleus. The authors also observe an increase of spliced isoforms upon stimulation, but also in some cases that intron excision can be slowed down under these conditions and that there is transcript-specific degradation. Furthermore, they investigated the localization of transcripts in the nucleus versus the cytosol after 1h of Bic treatment and confirmed that spliced transcripts were also enriched in the cytoplasm (not only in the nucleus) in contrast to transcripts with retained introns (Fig. 4). Particularly attractive is the stimulus-dependent regulation of transcripts, as this reveals that Bic and BDNF appear to specifically activate different pools of IR transcripts. Even more impressive is their findings that both stimulation paradigms act on common transcripts but with opposite outcome, as shown for example for the splicing factor SRSF2. In the last set of experiments, the authors investigated the underlying signal pathways, e.g. calcium signaling (via CaMK) vs MAPK and showed that Bic preferentially impacts NMDARs in contrast to BDNF that affects BDNF action in neurons.

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We thank the reviewer for this positive feedback and the constructive comments that we carefully implemented in the revised manuscript.

1. I am not sure that the visualization of the data in Fig. 4 is optimal and does full justice to the current data set. It is the second part, in which the authors state that spliced transcripts "were also enriched in the cytoplasm, not only in the nucleus". If I got it right, there is a decrease (from grey to dark blue) in the first panel on the left but later in both cases (middle and right panel) there is an increase. This can certainly be emphasized more and additionally visualized in a more intuitive manner. Second, can the authors check, which transcripts shift from nucleus to cytosol and which ones stay. This would certainly be of utmost interest and very informative. Along those lines, do the authors have experimental evidence that this shift in the ratio is based on actual export and sequential localization? I would find this additional information of high importance and in my opinion represent a significant boost of the novelty and originality. In conclusion, this figure could certainly be improved by the addition of such new data.

We have now modified the representation of the Figure 4 to better illustrate our model of the export of spliced mRNAs after activity-dependent intron removal.

In the main figure, we added a scheme describing the expected regulation (up- or down-) of the intron-retaining transcripts and the associated spliced isoforms in the cytosolic and nuclear compartments (new scheme in Figure 4A). In addition, in a histogram plot, we displayed the proportion of IR- and spliced isoforms up- versus down- regulated in the nucleus and the cytosol in response to stimulation (new data in Figure 4B). As expected for the splicing events, the vast majority (>90%) of IR-transcripts are decreased in the nucleus and the spliced isoforms are increased in the nucleus and also in the cytosol. Importantly, as displayed in the scatter plot (new data in Figure supplement/EV 4B), the IR-isoforms and the spliced isoforms are concomitantly down-regulated in the nucleus and up-regulated in the cytosol supporting that the increase of the spliced-isoforms in the cytosol arise from the splicing and the subsequent export of the newly spliced isoforms. Finally, we are providing a table (new Table 2) containing the list of IR events and their regulation in the different subcellular compartments. We hope this new representation is more intuitive and informative.

2. The authors' findings that different stimulation paradigms yield different outcomes in IR is truly impressive. As they started with Bic vs BDNF stimulation, I would suggest to explain in more detail that Bic is not primarily used to interfere with GABA signaling, but to affect network stimulation. Also, the authors could then incorporate this aspect into their model (Fig. 6) and include GABAergic synapses. In this respect, I would suggest discussing their findings reg. NMDAR- vs BDNF-dependent synapses, and in what aspect the blocking of GABA synapses might affect IR. Also, in their model, they show CaMK signaling back to the nucleus. Here, it might be worth mentioning that the CaMKII alpha transcript contains an intron, which itself undergoes regulation (Ortiz-R et al., 2017, Cell Rep, PMID: 28683307). Along those lines, this would allow the authors to improve their simplistic model by adding another layer of regulation: a certain set of intron-containing transcripts might not only be found in the cytosol, but be transported to synapses, as shown for CaMKII and Calm3 (Sharangdhar et al., 2017, EMBO Rep, PMID 28765142).

We have now better explained the rationale behind the use of bicuculline (text, page 7 and legend of Figure 2).

Moreover, we added a section in the discussion to elaborate the case of CaMKII alpha and Calm3 intron-retaining transcripts to illustrate the diversity of IRs and their widespread fates (text, page 15).

3. In my opinion, the discussion is not yet in the best possible shape. Here are several key findings of the paper that are not discussed, e.g. why this paper represents indeed a significant step forward in our understanding of IR and why it actually provides new mechanistic insight. Second, to discuss why it makes sense that different signaling pathways affect distinct transcripts (and give a couple of examples). Finally, the authors could consider including the just mentioned compartmental specific localization of transcripts, e.g. to excitatory spine synapses or inhibitory shaft synapses, just to name a few options.

We have now enriched the discussion and enlarged the section on the biological relevance of cue-specific regulation of IRs for plasticity (text, page 17). We also discussed in depth the heterogeneity of IR classes and their different fates in neurons (text, page 14-15).

Minor points

- Actual subheading is not ideal (p14): Targeting intron retentions is... I would rephrase

We have rephrased the title to: *"Targeting intron retentions is associated with an acute mobilization of mRNAs in the cytosol and an increase of protein levels"* (page 15).

- Incomplete references, e.g. Suzuki et al.,

We have now completed the references.

Referee #2:

In this paper Mauger and colleagues extend their work on intron retention in neuronal cells. Specifically, they define the changes in IRs across the transcriptome in response to two different neuronal stimuli. Interestingly, they observe and characterize distinct patterns of changes in IR with the two different stimuli clearly delineating a regulatory role for IR. Moreover, they show that unique cell signaling pathways (MAPK and CamK) govern this specificity. Overall, this is a very well written paper with interesting and important data for the field. The interpretations are consistent with the data and the experiments are rigorous with multiple validation approaches. I suggest only a few minor changes:

We thank the reviewer for this positive feedback and the constructive comments that we implemented in the revised manuscript.

1. Please define what "density" is in Fig 1B. Do these data mean that nearly all nuclear transcripts have a PIR value of >20?

The density plot used for the initial Figure 1B showed the distribution of the PIR values with a function based on a kernel density estimation. However, we realized the density plot is not the most intuitive way to represent the distribution of PIR values of retained introns. Therefore, we adopted a violin plot representation (new Figure 1B). Furthermore, to avoid any misleading information with regards to the category of plotted introns (only the retained introns were plotted in the initial version of Figure 1B), we have now displayed the distribution of PIR values for i) all introns and ii) retained introns. As expected, it indicates that most introns are constitutively removed through splicing.

2. It may be nice to show a time course diagram for the experiments with transcription inhibition and neuronal stimuli in the figure. This way readers don't have to hunt for this information in the legends/methods. (Or, at least, state the timing more explicitly in the Results section).

We have now added a time course diagram for each figure (Figures 1A, 2C-D, 3A-B, 4B-C, 5A, 6A-B).

3. Please include representative genome browser screenshots showing the effects of neuronal stimulation on specific transcripts. This can be incorporated into the main figures or in supplemental data but having visuals for these events on specific genes is quite helpful in illustrating the effects of the stimuli.

We have now displayed examples of genome browser tracks (new data in Figure supplement/EV 3C and 3D).

4. Literature on the role of spliceosome association on nuclear retention should be included. As early as the late 80's, two groups suggested that spliceosome assembly promotes nuclear retention

(Legrain and Rosbash, Cell, 1989) (Chang and Sharp, Cell 1989). Therefore, the idea that IR promotes nuclear retention is not surprising. However, the observations here do imply at least partial spliceosome formation on these IR transcripts.

Thank you for the suggestion, we have now included and elaborated on these references in the discussion (text, page 14).

5. KN-62 activity should be stated in the Results, not left to the legend.

We have now stated KN-62 in the Results (text, page 13).

Referee #3:

This is a very interesting paper describing the presence and regulation of intron-retention (IR) in neurons. The work is nicely done, although I have some reservations especially on the statistical analysis (see below), but the validations suggest the findings to be robust. Most of the results reported in the paper were also reported in previous work from the same authors (Mauger et al., Neuron 2016), including the characterization of features of retained introns and their activity-dependent regulation (although with just one paradigm). The main difference in this manuscript is the use of RNAseq from nuclei vs cytosol, whereas this was not done in a genome-wide fashion in the previous work. Nevertheless, I believe this data and their description could represent a meaningful contribution to EMBO Journal. However, as detailed below, a number of issues need to be addressed before this manuscript can be considered for publication.

We thank the reviewer for the valuable comments. We have addressed the different points and we are confident the new additions strengthen our conclusions.

MAJOR COMMENTS:

1) A big missing piece of information in the manuscript is whether IR affects all introns of a transcript more or less equally, or specific introns e.g. Does each transcript only contain a single retained intron? Is there a bias in which intron is retained (e.g. last intron)? What is the percentage of IR vs. fully-spliced transcripts for individual genes (e.g., how frequent is IR for individual genes)? This should be described. Moreover, this could act as an extra control given that it cannot be assumed that transcription is completely stopped and that unprocessed transcripts have been either spliced or degraded completely after 3h; in particular, in the stimulation experiment it is clear that it is only partially inhibited (Suppl Figure 2A). In the null hypothesis of retained introns being due to remaining unprocessed transcripts, we would expect a proportion of all introns of a transcript to be retained, plausibly in an increasing fashion towards the 3' end. Instead, if intron retention is due to specific regulation, it should depart from this pattern. This could easily be visualized and tested, and would provide an important addition to the paper.

We have now quantified the proportion of genes exhibiting intron retentions (new data in Figure supplement/EV 1C and D). Based on our filters (minPIR>20%), we found 8% of expressed genes containing at least one IR. The vast majority (88%) of these genes contains only 1 or 2 IRs (63% contains one IR and 25% contains 2 IRs). This supports that there are mechanisms to selectively prevent co-transcriptional excision of specific introns.

We investigated the relative localization of the IRs within transcripts (new data in Figure supplement/EV 2F). When examining all detected IR events there was a 2-fold enrichment at the 3'end. This is consistent with delayed processing of some introns towards the 3'end of the transcribed RNAs.

By contrast, the signaling-regulated events do not exhibit 3'end enrichment but are spread all along the transcripts. This supports the hypothesis that the regulated introns belong to a category of stable IR events.

2) The IR metric used is appropriate, but I find the statistical analysis lacking. In particular, I do not understand the use of z-scores which are known to be inadequate for that kind of data, when more robust statistical tools are readily available. This is bound to have both a lower power and a higher FDR than using established differential expression methods. The authors could easily harness GLMs in existing packages (e.g. edgeR/DESeq2) in the following way: build a matrix (features per samples) of exon-exon counts, join it to a matrix of exon-intro counts, fit a model of the form $\sim \text{countType} * \text{condition}$ (which amounts to $\sim \text{countType} + \text{condition} + \text{countType}:\text{condition}$), where countType indicates either the column gives exon-exon or exon-intro counts, and test for the interaction coefficient (countType:condition). (You can also add a sample covariate if you want). This is the standard way of addressing statistically similar problems (e.g. differential translation efficiency in ribosome footprinting), using all the benefits of discrete statistics and information sharing across genes. Moreover, an advantage of this approach is that the obtained logFC could be directly interpreted as PIR changes, rather than IR-transcript expression changes, therefore directly circumventing the question of changes that are truly transcription-independent.

We appreciate the comment of the referee. We have carefully investigated how to robustly identified IRs and their regulation. Using a previous dataset (Mauger et al., 2016, Neuron), we performed extensive PCR validation assays on IR identified as regulated or not (35 IR events were tested) (see appendix "IR pipeline validation"). This work highlights the high predictive power of our pipeline: 80% true positive and 86% true negative. We also tested the use of other packages to identified regulated IRs and found them much less reliable. A more thorough analysis and optimization of parameters used in other approaches would be required for more precise comparisons. We hope to perform such comparisons that might add statistical power to the pipeline in future studies.

3) The authors describe IR as "a widespread mechanism to acutely regulate subcellular compartmentalization of transcripts upon cellular signals", however the evidence presented is purely correlative, i.e. there is not experimental proof that IR/splicing is causative of localization, rather than merely associated with it.

The evidence linking activity-dependent intron excision and export of the arising spliced mRNAs are indeed correlative. We have now toned down this statement in the text. Nonetheless, we have extended Figure 4 to better support this hypothesis. We displayed a scatter plot exhibiting the fold change regulation of intron-retaining transcripts in the nucleus in function to the regulation of their spliced counterpart in the cytosol (new data in figure supplement/EV 4B and 4C). This showed that almost all (>90%) IRs regulated by splicing are associated with a concomitant decrease of the IR-isoform in the nucleus and an increase of the spliced isoform in the cytosol. This strongly supports our hypothesis that intron retention and excision are correlated with nuclear retention and export to the cytosol.

At the very least, the authors should assess whether the mobilization of specific sets of IR transcripts upon stimulation has an impact on their protein output.

To assess the impact of IR regulation on protein expression, we performed targeted mass spectrometry assays (parallel reaction monitoring, PRM). Consistent with our hypothesis that targeting IR represents a way to regulate functional gene expression, we found that IR-transcripts undergoing splicing completion in response to neuronal stimulation are associated with a significant increase in the level of the associated proteins (New data in Figure 4C). Importantly, this protein increase is sustained in the presence of a transcription inhibitor. This supports our model of a rapid post-transcriptional mechanism for gene regulation.

MINOR COMMENTS:

- According to their model, bicuculline is suggested to signal via calcium-dependent kinases. Thus, in Fig. 2A, activation of components of this signaling pathway rather than p-Erk should be tested.

We have now added further control conditions for the efficiency of stimulation paradigms.

In Figure supplement/EV 2B, we confirmed that bicuculline induces a substantial phosphorylation of CREB transcription factor as expected after an increase of intracellular calcium levels and the activation of the downstream CaMK pathways (Ca²⁺/Calmodulin dependent kinase pathways; CREB is a direct substrate of CaMKs) (Lee et al. 2005 DOI:10.1523/JNEUROSCI.4288-04.2005).

Neuronal stimulation with BDNF induces a robust activation of the AKT pathway as depicted by the increase of the phosphorylated state of AKT protein (Gottschalk et al. 1999 PMID#10492006; Guo et al. 2014 doi:10.1242/jcs.139964).

Finally, we also observed by western blot a substantial increase of the phosphorylation state of ERK (Gallo et al. 2011 DOI:10.1523/JNEUROSCI.0374-11.2011; Xu et al. 2017 doi: 10.1007/s12035-017-0555-x; Huang et al. 2003 doi: 10.1146/annurev.biochem.72.121801.161629).

- In Figure 3A/B, it would be useful to show some density information (e.g. coloring the points by density, showing 2D kernel density curves, or a histogram on each axis), to know if the grey cloud in the middle hides a trend. In addition, it would be relevant to test whether the induced splicing is over-represented WRT to other quadrants.

We have now displayed the density information on Figures 3A and 3B.

- A statistical assessment of the qPCR data is missing (Fig. 4-6). SD instead of SEM should be plotted.

We have now added the statistical assessment of the qPCR data and plotted on the graphs the SD instead of the SEM in all figures (Figures 3, 4, 5 and 6).

- Supplementary Figure 6A is unconvincing due to the unequal loading.

We have re-done our western blot with equivalent sample loading (Figure supplement/EV 6A).

- What are the factors on the IR transcripts (e.g. RNA-binding proteins) which confer specificity to different signaling cues (e.g. MAPK vs. bicuculline)? It would be worth running an enrichment analysis for RNA-binding protein motifs in and around the retained introns

We ran an enrichment analysis for RNA-binding protein (RBP) motifs using the DREME package. However, with this approach, we did not find any enriched motifs in regulated IRs (-/+300bp around the splice sites) (see text, page 17). Noteworthy, RBP motifs are short and degenerated, making their identification challenging. To tackle this hurdle, in the near future, we aim to combine computational analysis (motif enrichment, analysis of publicly available CLIP data, etc) as well as experimental approaches (minigenes with sequential intronic deletion, RNA-immunoprecipitation, etc) to identify RBPs controlling the cue-specific IR regulation.

- What might be the physiological role of intron retention and stimulus-specific mobilization in non-coding transcripts (e.g. Mirg)? This should be discussed.

We have now commented on the potential role of IR regulation in non-coding transcripts in the discussion section (text, page 16).

Dear Oriane,

Thank you for submitting your revised manuscript to The EMBO Journal. Your manuscript has now been seen by the original referees and their comments are provided below.

As you can see, the referees appreciate the introduced changes and support publication here. They have a few minor issues to resolve.

When you submit the revised version will you also take care of the following editorial points:

- you have at the moment 7 keywords, but can only have 5
- COI needs to be updated Disclosure Statement & Competing Interests
- The appendix needs a ToC, with page numbers. I see that the appendix has the pipe validation. Can you label them as figure(s)/table(s) number with title(s) and legend(s). The figures also need to be called out in the MS text. Please see nomenclature in our guide to authors.
- For the tables, please add a legend for each table in a separate tab in the excel file
- For the funding please add grant number 174153 to the MS.
- Please provide the accession numbers for the deposited dataset(s) in the data availability section.
- We don't encourage statistics when N=2 (4D and 6D). Can you simply show both data points?
- Our publisher has done their pre-publication checks on the MS. Please look at the word file called "data ed ms file" and the comments provided regarding the figure legends and incorporate their suggestions.

That should be all. Let me know if you have any further questions

Best Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Use the link below to submit your revision:

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Referee #1:

This is a thoroughly revised version of the original study, in which the authors systematically investigated intron retention (IR) from fractionated mouse cortical neurons under physiological conditions. Of particular interest for a wide audience is their findings that distinct pools of nuclear intron-containing RNAs are indeed released in the cytosol upon splicing to be used for protein synthesis.

Overall, three important new additions have been added to this already impressive manuscript that substantially improve the reported story. First, Fig. 4 was clearly improved to demonstrate the reported effect, e.g. by adding new data in Fig. 4B and EV 4B together with new Table 2. Second, explaining the rationale behind the bicuculine experiments clearly improves the logic of the stimulation experiments. Together with the new part in the discussion on the diversity of IRs and their widespread fates does better and more convincing justice to the authors' important findings. Third, the discussion improved significantly by talking about the biological significance of cue-specific regulation of IRs for plasticity.

Congratulations to this very fine and thorough study and the very constructive and extensive revision the authors performed. I strongly suggest publishing this important, timely and original study.

Referee #2:

The authors have addressed all of my concerns. This is a very nicely performed and impactful work on the role of intron retention in neuronal cells.

Referee #3:

The few key additions have brought important improvements to this already nice paper, and I fully support its publication. I would however have two small remaining suggestions:

1) EVFigure 2F is an important addition, and it strongly supports the claim that regulated IR cannot be the result of unprocessed transcripts. However, IR at baseline shows a strong 3' bias, which ought to be mentioned in the main text. It also prompts the question of whether there could be an enriched of baseline retained introns in the UTRs (like Calm3).

2) The authors write in the Results section that "compartmentalization of mRNAs through IR is a general mechanism in neurons". This is very similar to the other passage discussed in the previous round of reviews: while this interpretation is likely, the evidence is associative, i.e. it was not shown that intron retention/excision drives the localization (rather than merely being concomitant with it). While the paper as a whole does point in this direction, I would suggesting keeping the description close to the data, especially in the results section.

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We thank the reviewer for its great esteem about the revised manuscript and its support for the publication.

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We thank the reviewer for its appreciation of the data added in the revised version of the manuscript.

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We have now included a section about this finding in the discussion (text in green color, page 15).

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We have now modified the text and toned down this statement (text in green color, page 6).

Dear Oriane,

Thank you for submitting your revised manuscript to The EMBO Journal. I have now had a chance to take a look at the revisions and all looks good.

I am therefore very pleased to accept the manuscript for publication here.

Congratulations on a very interesting study

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Corresponding Author Name: Oriane Mauger & Peter Scheiffele
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This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: [10.31222/osf.io/9sm4x](https://doi.org/10.31222/osf.io/9sm4x)). Please follow the journal's guidelines in preparing your manuscript. **Please note that a copy of this checklist will be published alongside your article.**

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The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if $n < 5$, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/changed/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
 - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
 - are tests one-sided or two-sided?
 - are there adjustments for multiple comparisons?
 - exact statistical test results, e.g., P values = x but not P values < x;
 - definition of 'center values' as median or average;
 - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.

Select "Not Applicable" only when the requested information is not relevant for your study.

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Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	
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For antibodies provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and/or clone number - Non-commercial: RRID or citation	Yes	Materials and Methods
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Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Table 5
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Report if the cell lines were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	
Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	
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Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgments

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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Provide DOI OR other citation details if external detailed step-by-step protocols are available.	Not Applicable	

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Include a statement about sample size estimate even if no statistical methods were used.	Yes	Figures
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, have they been described?	Not Applicable	
Include a statement about blinding even if no blinding was done.	Not Applicable	
Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Not Applicable	
If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are statistical tests justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Figures

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was replicated in laboratory.	Yes	Figures
In the figure legends: define whether data describe technical or biological replicates .	Yes	Figures

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Studies involving human participants : State details of authority granting ethics approval (IRB or equivalent committee(s), provide reference number for approval).	Not Applicable	
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Studies involving human participants : For publication of patient photos , include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental animals : State details of authority granting ethics approval (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Materials and Methods
Studies involving specimen and field samples : State if relevant permits obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

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Could your study fall under dual use research restrictions? Please check biosecurity documents and list of select agents and toxins (CDC): https://www.selectagents.gov/sat/list.htm .	Not Applicable	
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Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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Are computational models that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective data citations in the reference list .	Not Applicable	