Synaptic activity controls localization and function of CtBP1 via binding to Bassoon and Piccolo

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Review timeline:

Submission date: 25 April 2014
Editorial Decision: 10 June 2014
Revision received: 08 December 2014
Editorial Decision: 02 January 2015
Revision received: 07 January 2015
Accepted: 08 January 2015

Editor: Karin Dumstrei

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 10 June 2014

Thank you for sending me your response to the referees' comments. I have now had a chance to take a look at it and I appreciate the added work that you can do to address the raised concerns and in particular to address the issue of CtBP1intEGFP targeting. I would therefore like to invite you to submit a suitably revised version along the lines as indicated in your response. I should point out that it is EMBO Journal policy to allow one major revision only and that it is therefore important to address the concerns raised at this stage.

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:
http://emboj.embopress.org/about#Transparent_Process

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

The manuscript by Ivanova et al describes the role of the transcriptional co-repressor CtBP1 in activity-dependent regulation of gene expression in neurons. The authors show that CtBP1 is present
at synapses and in the nucleus and provide evidence that these pools are interconnected. Network activity regulates synaptic retention and shuttling of CtBP1 between synapses and the nucleus. CtBP1 binding to the active zone proteins Bassoon and Piccolo is essential for its synaptic localization and network activity and NAD/NADH levels regulate this interaction. The authors conclude that Bassoon and Piccolo regulate CtBP1 levels in the nucleus by trapping CtBP1 at the presynaptic terminal in an activity-dependent manner.

The manuscript reports on a novel role of Bassoon and Piccolo in the activity-dependent anchoring of a repressor of transcription, CtBP1, at presynaptic terminals. It also provides new insight into the molecular function of CtBP1 in neurons by convincingly showing that long-term changes in network activity lead to changes in nuclear levels of CtBP1 that inversely correlate with its synaptic expression levels. In addition the authors have identified several new neuronal genes controlled by CtBP1. The conclusions are reached using a variety of different approaches, usually the best possible. Together, this manuscript contains a very extensive and valuable data set which reveals a new mechanism to explain how synaptic activity is coupled to gene expression in the nucleus.

On the other hand, some of the data is not yet fully convincing, such as the functional validation of fusion proteins used in this study, some of the experiments leave room for alternative explanations and the statistical analyses in many of the data sets are incompletely described and might even be questionable.

Major points:

1- Validation of CtBP1(PA)EGFP constructs and potential off-target effects

Figures 2, 3 and 8 use overexpression of CtBP1(PA)EGFP constructs. Apart from co-localization with Bassoon there is no data to support functionality of these constructs. To prove functionality the authors should test whether these constructs rescue the effect of CtBP1 shRNA mediated knock-down on e.g. BDNF mRNA levels (Fig 4G). Rescue of the shRNA effect would also provide further support against off-target effects of the CtBP1 shRNAs.

2- Alternative explanation for role of Bassoon/Piccolo in synaptic localization of CtBP1

The total absence of synaptic CtBP1 in Bassoon/Piccolo DKO neurons (Fig 5D) is quite striking and an important finding. The authors conclude that Bassoon/Piccolo are required to anchor CtBP1 at synapses. However, from figure 5 one could also conclude that Bassoon/Piccolo are essential to transport CtBP1 to synapses (via PTVs, see Fig5C) where CtBP1 binds to other proteins for retainment. To prove that Bassoon/Piccolo function as synaptic anchors the authors should perform FRAP analysis of CtBP1 at synapses of DKO neurons or, alternatively, analyze CtBP1 localization in Bassoon KO neurons upon knock-down of Piccolo after synapse formation.

3- Alternative explanation for the effect of 2-DG on activity-dependent CtBP1 re-localization

An equally likely explanation for the effect of 2-DG on 4AP/Bic induced changes of CtBP1 localization is the fact that glucose deprivation prevents increased network activity upon 4AP/Bic treatment. The authors should show proof of increased network activity in 2DG/4AP/Bic treated cultures. The authors should also discuss why they did not use the NAD/NADH-binding mutant of CtBP1 in FRAP or re-localization studies to directly test that NAD/NADH binding to CtBP1 is essential for modulating synaptic retention and nuclear import.

4- Statistical analyses.

Throughout the manuscript, sufficient description of statistical procedures is lacking, observations that are not independent appear to be treated as independent and inappropriate tests appear to be performed. This may lead to inflation of the significance of certain effects and unjustified conclusions. For every test the number of independent observations should be clearly stated. Measurements on different neurons of the same trans/infection and/or the same coverslip are not independent observations (in this case capital N should be used for number of independent observations and
small case n for the total number of observations). Was the number of independent observations always used for statistical tests?

Figure 1G, H & I and Fig 3B, F: statistical comparisons should be between all groups (control and all experimental conditions), not between arbitrary pairs.

Minor:

- Figure 2D: please provide evidence that CtBP1 puncta co-localize with synapse markers.

- Control FRAP traces in Fig 3D and E clearly differ. Please comment on possible explanations.

- Figure 3A & B show the activity-dependent synaptic loss of PA-CtBP1 3hrs after photoactivation, do the authors observe a concomitant effect at the nucleus?

- Figure 6F: in line with major point 3, does 4AP/Bic increase network activity in DKO cultures?

- Figure 6G & H: over-expression of BassoonGFP could affect expression levels of CtBP1, which would also explain the lower nuclear levels, especially since the authors use untransfected cells as control. Please provide total cellular CtBP1 levels from the cells analyzed in 6H.

- Figure 7F: Bassoon is present in GFP pulldowns from cells expressing GFP only (lanes 3, 9). Please comment.

- Figure 4 legend mentions "Values in brackets in H, I are numbers of performed qPCRs on samples", but there are no such values in the figure.

Referee #2:

This study addresses how presynaptic activity is coupled to gene expression via the intracellular translocation of a signaling protein. Specifically, the authors report that CtBP1, a transcriptional co-repressor which is found at both the presynaptic terminals and the nucleus, directs gene expression according to the level of activity. The main findings show that (1) the presynaptic and nuclear CtBP1 pools are shared and their relative abundance is counter-regulated by activity; (2) CtBP1 is anchored to the presynaptic boutons by directly linking to bassoon and piccolo; and (3) activity modulates the interaction between CtBP1 and the active zone proteins by altered NAD/NADH levels. These findings featuring synapse to nucleus signalling along the axon, contrasts with the dendritic spine to nucleus signalling that has received much attention in recent years, and highlight the fact that presynaptic terminals are also equipped to communicate with the nucleus in regulating gene expression while providing clues about potential mechanisms. The topic is timely and the study should be of general interest to the broad readership of EMBO Journal. However, as the manuscript stands, some of the conclusions are not fully supported by the data shown, and the following points require careful consideration.

Why is the rate of nuclear accumulation of CtBP1 much slower than the rate of its nuclear loss with the contrasting activity manipulations?

Figure 2. The specificity of the signal appearing in the ROI following nuclear photoactivation (PA) of the exogenously expressed CtBP1 should be confirmed by including a control with Leomycin B. Conversely, is the nuclear accumulation of the fluorescence signal following PA in the processes blocked by preventing nuclear import? Note that in panel D, some of the neuronal processes look as though they might be dendrites. Also in panel F, the PA signals in the processes do not look like axons. A less saturated image and/or colabeling with an axonal marker should be included. Furthermore, the rate of fluorescence signal accumulation in synaptic areas following nuclear PA or vice versa would be expected to depend on distance if the change in fluorescence involves the transport of CtBP1, and should be tested.

Figure 3A. It is difficult to conclude for a redistribution of CtBP1 to neighboring synapses just based on the PA results. A quantification of the extent co-localization of CtBP1 PAEGFP with synaptic markers would help in interpreting the nature of PA-CtBP1 puncta changes. Moreover, either a
reference presynaptic fluorescent label of a different wavelength or the use of CtBP1 fused to a photoswitchable tag rather than to a photoactivation tag would be helpful.

Figure 3C-G. The size of the photobleached area as well as the relative size, intensity and the density of CtBP1 puncta in the target region appear to differ substantially between conditions. This could introduce some bias. For instance, in the 4AP group where the recovery is compromised, the photobleached area is broader than the control, whereas in the APV-CNQX group where the recovery is enhanced relative to the control, the photobleached CtBP1 puncta are immediately adjacent to large CtBP1 clusters.

Figure S4D. What is the basis by which CtBP1 levels in the brain lysates that is reduced in Bsn mutant neurons, recovers to the level found in wild type controls for the DKOs?

Figure 5F. Does co-expression of RP10 or RP16 fragment of piccolo along with GFP-bassoon prevent its ability to restore synaptic CtBP1? This should be tested in support of the specificity of synaptic accumulation of CtBP1 to its interaction with bassoon and piccolo in a competing manner.

Figure 8E,F. Given that 2DG treatment may have global metabolic consequences, it would be informative to perform additional experiments to determine the extent specificity of the observed effects of interfering with NAD/NADH levels on CtBP1 localization. For example, does 2DG occlude with APV-CNQX-induced reduction of synaptic CtBP1 and the corresponding increase in nuclear CtBP1? What about the effects of 2DG on CtBP1-dependent gene expression?

Referee #3:

In this manuscript, the authors provide evidence that CtBP1 shuttles between presynaptic sites and the neuronal nucleus to regulate the expression of BDNF, Arc and other targets. While much of the data presented is of good quality, this work represents two separate stories forced together by unconvincing and confusing data asserting a connection between nuclear and presynaptic pools of CtBP1. In one story, the authors elucidate the mechanisms underlying presynaptic targeting of CtBP1 and in a separate story show that activity-dependent nucleocytoplasmic shuttling of CtBP1 affects gene expression.

Major:

-The major concern regards the pre and/or postsynaptic localization of overexpressed GFP-tagged CtBP1, which affects the interpretation of several figures. The authors present high-quality STED evidence that endogenous CtBP1 is indeed presynaptic, yet overexpressed eGFP-CtBP-1 (Fig 2B) appears to be punctate throughout the dendrite. Shouldn’t it be present in axon terminals? If it is in fact presynaptic, and thus in a separate, different neuron, then the photoactivation studies are bizarre. For example, in Fig 2F how can photoactivation of dendritic branches (where CtBP1 is claimed to be presynaptic) result in its accumulation in the monitored cell body? Essentially, how does presynaptic CtBP1 reach the postsynaptic nucleus? Do the photoactivated patches contain axons of the monitored cell bodies? If so, how could so little CtBP1 (presynaptic CtBP1) contribute so much material to the cell soma? This is a recurring concern throughout the manuscript. In Figure 2D, photoactivation of CtBP1 in the nucleus results in puncta appearing in neurites over time. Are these postsynaptic, presynaptic? It seems like the authors used techniques to study dendritic intracellular trafficking, when they really needed evidence of retrograde transport along axons. If these experiments were simply used to show the potential for retrograde or anterograde transport of CtBP-1, then they do little to support the claims of the paper.

-A second concern is that given the kinetics of transport, which seems to be very different between endogenous and overexpressed protein, these results could simply reflect the diffusion of an overexpressed protein. If APV-CNQX can block the nuclear export of photoactivated CtBP-1, it would support their claims of a regulated process. The kinetics of transport (many hours) is also at odds with the regulation of transcription of immediate early genes such as Fos, Arc and BDNF. This should be discussed more thoroughly.

-While concerns regarding spontaneous photoactivation are addressed, the sensitivity is not. That is,
photoactivation of a patch could result in some photoactivation nearby. Indeed in Supplementary figure S2A, there is plenty of dendritic fluorescence at time 0 following nuclear photoactivation. A control should be developed to address this issue.

- Figure 4B-F represents weak and unconvincing correlation data. Promoter I is the most active promoter driving BDNF expression and can be regulated by CREB and REST. Moreover activity dependent regulation of BDNF expression has been extensively studied (4C-F). The results in 4H and 4I are important and interesting and do support their claim that CtBP-1 can regulate BDNF expression. It may be helpful to validate some of these findings by monitoring protein levels. However, using leptomycin to block all nuclear export is too broad and may lead to potentially damaging cellular effects that could greatly alter gene expression and thus confound their results. Ideally, the authors should employ a mutant CtBP-1 lacking a nuclear export sequence to identify CtBP-1 specific effects.

- The Western blots showing that activity/silencing do not alter global CtBP-1 levels do not convincingly support claims of transport as changes could be localized. Synaptic activity elevates protein ubiquitination and CtBP-1 has been shown to be ubiquitinated (Zhang et al 2005 PNAS). The authors must show that changes in the synaptic/nuclear abundance of CtBP-1 are not altered by proteosomal and/or translational inhibitors.

- The FRAP experiments are well done, but difficult to interpret. Again, is this overexpressed CtBP-1 presynaptic or postsynaptic? The dull fluorescence throughout the dendrites and the absolute lack of any axonal staining strongly suggests that CtBP-1 is dendritic and likely postsynaptic. Since it would not be associated with Bassoon/Piccolo, these experiments would have little impact on the assertions claimed in the manuscript.

-In Figure 5F, overexpressing GFP-Bassoon can rescue the synaptic expression of CtBP-1 in DKO cells. Again, wouldn't this Bassoon be dendritic/postsynaptic (i.e. in the cell overexpressing the construct?). Is the "rescued" synaptically localized CtBP-1 in the pre or postsynaptic cell?

Minor-
- Fig 1A requires a nuclear marker. Fig 1B requires a dendritic marker to better localize CtBP1.
- Figure 5C is confusing and needs a better explanation in the text.
- Wouldn't elevated NADH levels also increase CtBP-1 binding to transcription factors, thus retaining CtBP-1 in the nucleus?
- What is Supplementary Fig 4A?
- For Fig 7A- Why was a 30min activation tested and not the longer time points used for transport? It seems like the authors have antibodies, why wasn't endogenous CtBP-1 tested? Does activity alter Bassoon levels?
- Fig 8, Why were neurons treated with 2DG for 2 days (so long)? Any effects on viability?
- Wouldn't homeostatic effects induced by 48 hours of APV/CNQX result in opposite changes from those expected?

Point-by-point response to the referees' comments

First, we thank the reviewers for their overall very positive and encouraging assessment and their constructive comments, which helped us to significantly improve the manuscript.

General point:

An important general criticism of all three reviewers was a concern about 1) aberrant targeting of the over-expressed CtBP1int(PA)EGFP construct to other cellular compartments such as postsynapses and 2) problems that would result from photoactivation (PA) of the non-synaptic CtBP1int(PA)EGFP. This is of central importance and therefore we want to address it prior to answering concrete criticism of reviewers in point by point manner.
1) To demonstrate exclusive presynaptic localization of recombinant CtBP1intEGFP, we included images showing cells from cultures infected with lentivirus expressing CtBP1intEGFP (Fig 2B,C and in Fig 5D). In Fig 2B we show non-transduced cells with apparently many synaptic contacts on their dendrites containing CtBP1intEGFP. Here, CtBP1intEGFP can only originate from axons of other neurons, expressing CtBP1intEGFP and contacting dendrites of the non-transduced cell shown. This clearly demonstrates presynaptic targeting of CtBP1intEGFP. In Fig 2C we show a transduced cell expressing CtBP1intEGFP (Fig 2C). On dendrites of the transduced cells synapses are labeled with antibody against CtBP1, which labels virtually all synapses as seen in Fig 1B and 5D upper row. Only a fraction of these synapses contain also CtBP1int EGFP. These synapses might be autapses or synapses formed by axons of other transduced cells. If CtBP1int EGFP were targeted to postsynapses, it should be present in all postsynapses of transduced cell, which is evidently not the case. Dual color STED images show CtBP1intEGFP co-localized with presynaptic marker but only aligned with postsynaptic one, which further indicates its localization to presynapses (Fig. 2D).

2) We appreciate the comment about localization of overexpressed CtBP1intPAEGFP to cytoplasmic compartment. We agree that there is a considerable cytoplasmic pool of both endogenous CtBP1 and recombinant CtBP1intPAEGFP. To avoid PA of this extrasynaptic CtBP1intPAEGFP, we repeated all PA experiments with illumination spatially restricted to active synapses visualized by Syt1 Ab uptake prior to PA (Fig 3C). We believe that this strengthens our statement about the physical exchange of CtBP1 between its somatic and synaptic pools.

Referee #1:

The manuscript by Ivanova et al describes the role of the transcriptional co-repressor CtBP1 in activity-dependent regulation of gene expression in neurons. The authors show that CtBP1 is present at synapses and in the nucleus and provide evidence that these pools are interconnected. Network activity regulates synaptic retention and shuttling of CtBP1 between synapses and the nucleus. CtBP1 binding to the active zone proteins Bassoon and Piccolo is essential for its synaptic localization and network activity and NAD/NADH levels regulate this interaction. The authors conclude that Bassoon and Piccolo regulate CtBP1 levels in the nucleus by trapping CtBP1 at the presynaptic terminal in an activity-dependent manner.

The manuscript reports on a novel role of Bassoon and Piccolo in the activity-dependent anchoring of a repressor of transcription, CtBP1, at presynaptic terminals. It also provides new insight into the molecular function of CtBP1 in neurons by convincingly showing that long-term changes in network activity lead to changes in nuclear levels of CtBP1 that inversely correlate with its synaptic expression levels. In addition the authors have identified several new neuronal genes controlled by CtBP1. The conclusions are reached using a variety of different approaches, usually the best possible. Together, this manuscript contains a very extensive and valuable data set, which reveals a new mechanism to explain how synaptic activity is coupled to gene expression in the nucleus. On the other hand, some of the data is not yet fully convincing, such as the functional validation of fusion proteins used in this study, some of the experiments leave room for alternative explanations and the statistical analyses in many of the data sets are incompletely described and might even be questionable.

Major points:

1- Validation of CtBP1(PA)EGFP constructs and potential off-target effects

Figures 2, 3 and 8 use overexpression of CtBP1(PA)EGFP constructs. Apart from co-localization with Bassoon there is no data to support functionality of these constructs. To prove functionality the authors should test whether these constructs rescue the effect of CtBP1 shRNA mediated knockdown on e.g. BDNF mRNA levels (Fig 4G). Rescue of the shRNA effect would also provide further support against off-target effects of the CtBP1 shRNAs. We followed this recommendation and performed rescue experiments and included them in the...
revised manuscript – see Fig 5B,C,D and Results, section “Nucleo-cytoplasmic shuttling of CtBP1 underlies activity-driven transcriptional regulation in neurons”. In brief, expression of CtBP1intEGFP construct used for imaging and binding experiments throughout the study could revert the effect of knock-down of CtBP1 (CtBP1KD944) on expression of BDNF and Arc (Fig 5B,C) confirming its normal functionality. In contrast, expression of EGFP-CtBP1, which in previous studies was found to have aberrant nuclear localization (Fig 5D), did not rescue the CtBP1KD944-induced changes in gene expression of BDNF and Arc (Fig 5B,C).

2- Alternative explanation for role of Bassoon/Piccolo in synaptic localization of CtBP1

The total absence of synaptic CtBP1 in Bassoon/Piccolo DKO neurons (Fig 5D) is quite striking and an important finding. The authors conclude that Bassoon/Piccolo are required to anchor CtBP1 at synapses. However, from figure 5 one could also conclude that Bassoon/Piccolo are essential to transport CtBP1 to synapses (via PTVs?, see Fig5C) where CtBP1 binds to other proteins for retention. To prove that Bassoon/Piccolo function as synaptic anchors the authors should perform FRAP analysis of CtBP1 at synapses of DKO neurons or, alternatively, analyze CtBP1 localization in Bassoon KO neurons upon knock-down of Piccolo after synapse formation.

Unfortunately, the FRAP experiments of CtBP1 at synapses of DKO neurons are not possible as there is no detectable synaptic CtBP1 in these neurons. We followed the second recommendation and performed KD of Piccolo in Bsn-/- neurons. This experiment is shown in Fig S4H,I and in Results, section Bassoon and Piccolo regulate the synaptic vs. nuclear distribution and the corepressor activity of CtBP1. The synaptic levels of CtBP1 were strongly reduced upon shRNAPiclo28-induced depletion of Piccolo in Bsn-/- neurons. In these experiments the Piclo expression levels were reduced by 70-80%, which is in our opinion the reason for incomplete loss of CtBP1 from synapses in these experiments. Indeed, we cannot fully exclude the possibility that other proteins are involved in the synaptic retention of CtBP1. A strong argument against this alternative explanation is the lack of synaptic CtBP1 enrichment in DKO neurons despite of relatively high cytosolic levels of CtBP1. This cytoplasmic pool is most evident when looking at subcellular fractionation of brain tissue, showing high CtBP1 immunoreactivity in the cytosolic fraction S2 (please see Figure 1 in the attached file to reviewers). The cytosolic CtBP1 is very likely evenly distributed in neuronal processes and should be captured also in DKO neurons if other synaptic anchor than Bassoon and Piccolo existed. Overexpression of CtBP1-binding fragments of Bassoon in COS-7 cells, which sequester CtBP1 and retains from entering the nucleus, also argues for an anchoring function (Fig 7G).

At this point we are not able to distinguish definitelly whether Basson and Piccolo are impotant for trafficking or retention or both. Therefore we reworded the statement in abstract saying „we show that CtBP1 is targeted and/or anchored to presynapses via its direct interaction with the active zone scaffolding proteins Bassoon and Piccolo” instead of „we show that CtBP1 is anchored presynaptically via its direct interaction...”.

3- Alternative explanation for the effect of 2-DG on activity-dependent CtBP1 re-localization

An equally likely explanation for the effect of 2-DG on 4AP/Bic induced changes of CtBP1 localization is the fact that glucose deprivation prevents increased network activity upon 4AP/Bic treatment. The authors should show proof of increased network activity in 2DG/4AP/Bic treated cultures.
This is indeed an important point. We have tested the effect of 2-DG treatment on overall network neuronal activity in cultures by monitoring of endogenous activity-driven uptake of antibody against lumenal domain of integral synaptic vesicle protein synaptotagmin1 (Syt1 Ab uptake) in living neurons. The results are included in Fig. 9 G,H and Results, section Neuronal NAD/NADH ratio controls the molecular dynamics of CtBP1 at synapses and its subcellular localization via modulation of its affinity to Bassoon. In brief, inhibition of glycolysis slightly, but not significantly, reduced the synaptic activity monitored by Syt1 Ab uptake as compared to cultures not treated with 2-DG. However, 2DG treatment did not interfere with an enhancement of neuronal activity upon 4AP bicu application as the 4AP bicu-induced increase in Syt1 Ab uptake in 2-DG-treated cultures was indistinguishable from parallel cultures without the 2-DG treatment. In contrast, it fully interfered with the activity-induced changes in synapto-nuclear distribution of CtBP1.

The authors should also discuss why they did not use the NAD/NADH-binding mutant of CtBP1 in FRAP or re-localization studies to directly test that NAD/NADH binding to CtBP1 is essential for modulating synaptic retention and nuclear import.

As shown in Fig S6A the NAD/NADH binding mutant of CtBP1 does not localize to synapses, which makes the proposed experiment impossible.

4- Statistical analyses.

Throughout the manuscript, sufficient description of statistical procedures is lacking, observations that are not independent appear to be treated as independent and inappropriate tests appear to be performed. This may lead to inflation of the significance of certain effects and unjustified conclusions.

For every test the number of independent observations should be clearly stated. Measurements on different neurons of the same trans/infection and/or the same coverslip are not independent observations (in this case capital N should be used for number of independent observations and small case n for the total number of observations). Was the number of independent observations...
always used for statistical tests?
We added the statements about group character and size in the figure legend for each experiments. In general, minimum of 2 independent cultures (with comparable contribution to total n) were used for each measurement. It is important to state that for imaging experiments each cell comes from a different coverslip and for each IF quantification in each experiment images from at least 3 different coverslips were acquired and quantified to avoid effects given by experimental variance.

Figure 1G,H & I and Fig 3B,F: statistical comparisons should be between all groups (control and all experimental conditions), not between arbitrary pairs.
We did so, please see Fig 1E,F,J and Fig 4 B,F.

Minor:
- Figure 2D: please provide evidence that CtBP1 puncta co-localize with synapse markers.
In new experiments that are now included in the revised manuscript, we labeled active synapses with Syt1 Ab uptake in cells subjected to PA experiments. This allowed us not only to assess, whether the CtBP1intPAGFP photoactivated in soma translocates to synapses shown in Fig 3A, but also to photoactivate CtBP1intPAEGFP selectively in synaptic regions (shown in Fig 3C).
- Control FRAP traces in Fig 3D and E clearly differ. Please comment on possible explanations.
Yes, this is correct. We performed FRAP for 4AP/bicu and APV/CNQX treatments in different experiments, in which control and drug treatment were always applied to matched cultures. We think that the absolute recovery differs between individual experiments due to different basal activity state of the underlying neuronal network. Different expression levels leading to different abundances of CtBP1intEGFP fusion proteins in the cytoplasmic pool could also affect the recovery rates. This does not seem to be the case, as tested and shown in Fig 4G. The relative expression levels at synapses do not correlate with FRAP rate implying that the size of synaptic pool in individual synapses is not decisive for its dynamics.
- Figure 3A&B show the activity-dependent synaptic loss of PA-CtBP1 3hrs after photoactivation, do the authors observe a concomitant effect at the nucleus?
We could observe an opposing effect in cell bodies, which was, however, only a trend and did not pass the statistical significance check. Important to state here, we are not able to quantify exclusively nuclear staining by live-imaging, i.e. to discriminate between nuclear and somatic localization of the photoactivated construct.
- Figure 6F: in line with major point 3, does 4AP/Bic increase network activity in DKO cultures?
This is a good point. We tested this and included results in the revised manuscript (Fig S5 and Results, section Depletion of the synaptic pool of CtBP1 affects activity-dependent nucleo-cytoplasmic shuttling of CtBP1). In brief, we could show that in both wt and Bsn"/Pclo28KD cultures application of 4AP and bicu leads to significant increase of SV release/recycling using Syt1 Ab uptake.
- Figure 6G&H: over-expression of BassoonGFP could affect expression levels of CtBP1, which would also explain the lower nuclear levels, especially since the authors use untransfected cells as control. Please provide total cellular CtBP1 levels from the cells analyzed in 6H.
Our transfection efficiency with the Bassoon-GFP plasmid was quite low, likely due to the big size of this construct. Therefore, immuno-blotting based analysis of CtBP1 expression levels in lysates of transfected cells did not appear appropriate for us. To satisfy reviewer’s request for a suitable control, we expressed EGFP in a parallel experiment and measured effect of EGFP expression on CtBP1 nuclear levels. Results are in Fig. 7G,H and show clear decrease of nuclear CtBP1 in cells transfected with GFP-Bassoon but not with EGFP.
- Figure 7F: Bassoon is present in GFP pulldowns from cells expressing GFP only (lanes 3,9). Please comment.
This is correct and most likely due to the unspecific binding of RB29 to the magnetic beads. We quantified this unspecific signal; it was 10-fold lower than the signal of Bassoon fragment co-precipitated with CtBP1intEGFP. We added corresponding statement in legend of Fig 8F.

Figure 4 legend mentions "Values in brackets in H, I are numbers of performed qPCRs on samples", but there are no such values in the figure. This was mistake from our side. We corrected it; see Fig 5E,F of revised manuscript.

Referee #2:

This study addresses how presynaptic activity is coupled to gene expression via the intracellular translocation of a signaling protein. Specifically, the authors report that CtBP1, a transcriptional co-repressor which is found at both the presynaptic terminals and the nucleus, directs gene expression according to the level of activity. The main findings show that (1) the presynaptic and nuclear CtBP1 pools are shared and their relative abundance is counter-regulated by activity; (2) CtBP1 is anchored to the presynaptic boutons by directly linking to bassoon and piccolo; and (3) activity modulates the interaction between CtBP1 and the active zone proteins by altered NAD/NADH levels. These findings featuring synapse to nucleus signalling along the axon, contrasts with the dendritic spine to nucleus signalling that has received much attention in recent years, and highlight the fact that presynaptic terminals are also equipped to communicate with the nucleus in regulating gene expression while providing clues about potential mechanisms. The topic is timely and the study should be of general interest to the broad readership of EMBO Journal. However, as the manuscript stands, some of the conclusions are not fully supported by the data shown, and the following points require careful consideration.

Why is the rate of nuclear accumulation of CtBP1 much slower than the rate of its nuclear loss with the contrasting activity manipulations?

This is probably due to the particular activity modulations that we have used in this study (combined NMDA/AMPA receptor blockage). Currently, in a project that should follow up on this initial study we tested carefully various treatments for their impact on CtBP1 synapto-nuclear redistribution. We identified TTX treatment as very powerful to induce nuclear import and observed significant effects on nuclear levels of CtBP1 already 1 hr after treatment. However, we still do not know why the TTX effect is faster than the one of APV/CNQX and in fact this is topic of a follow up study we are pursuing. One possibility is that presynaptic Ca\textsuperscript{2+} influx is significantly lowered in TTX treated cells and in fact it appears that the function of Ca\textsuperscript{2+} channels is required to induce nuclear export of CtBP1 by 4AP/bicu treatment. We hope it will be acceptable for the editor and the reviewers to consider this information without including it to the manuscript, but we can make respective statement in discussion, if requested.

Figure 2. The specificity of the signal appearing in the ROI following nuclear photoactivation (PA) of the exogenously expressed CtBP1 should be confirmed by including a control with Leomycin B. Conversely, is the nuclear accumulation of the fluorescence signal following PA in the processes blocked by preventing nuclear import? Note that in panel D, some of the neuronal processes look as though they might be dendrites. Also in panel F, the PA signals in the processes do not look like axons. A less saturated image and/or colabeling with an axonal marker should be included.

As stated in general point 1 and 2 at the beginning of this document we included 1) careful characterization of localization of the CtBP1intEGFP construct showing its localization to presynapses and 2) repeated the PA experiments doing PA selectively in spots identified as active synapses by Syt1 Ab uptake beforehand. We provide completely new images and quantifications in Fig 3C,D and Fig 4A,B. We hope that this improvement dispels doubts of reviewer.

It is important to state at this point that with our current resolution (due to the usage of activation by single laser, wide-field fluorescence microscopy and CCD camera) we cannot image the nucleus without getting signal from cytoplasm above and underneath. Therefore we were in fact very careful about our statements and always put forward that CtBP1intEGFP can translocate from
axons/presynapses to soma/nucleus and from soma/nucleus to axons/presynapses. This technical restriction is also the reason why interference with nuclear import/export is not yet very meaningful at this point.

Furthermore, the rate of fluorescence signal accumulation in synaptic areas following nuclear PA or vice versa would be expected to depend on distance if the change in fluorescence involves the transport of CtBP1, and should be tested. This is a very interesting point and surely worth to follow. However, it is impossible to judge the real distance of presynapse from the cell bodies, as axons are not growing straight but highly irregularly in dissociated cultures. We plan to do experiments like this in hippocampal slices where axons grow regularly. This allows measurement of their distance e.g. between activated nuclei in CA3 and in presynapses of Schaffer collaterals along CA1. However, this needs to be established and is beyond what we could do for this study.

Figure 3A. It is difficult to conclude for a redistribution of CtBP1 to neighboring synapses just based on the PA results. A quantification of the extent co-localization of CtBP1intPAEGFP with synaptic markers would help in interpreting the nature of PA-CtBP1 puncta changes. Moreover, either a reference presynaptic fluorescent label of a different wavelength or the use of CtBP1 fused to a photoswitchable tag rather than to a photoactivation tag would be helpful.

This is a valuable comment and we tried to address it as follows. We labeled synapses by Syt1Ab uptake in new PA experiments included in revised manuscript in Fig 3A,B and 4A,B. This identifies active presynapses at which photoactivation was performed or where CtBP1intPAEGFP could be detected after PA in nucleus/soma. We hope that this sufficiently addresses the reviewer’s comment.

Figure 3C-G. The size of the photobleached area as well as the relative size, intensity and the density of CtBP1 puncta in the target region appear to differ substantially between conditions. This could introduce some bias. For instance, in the 4AP group where the recovery is compromised, the photobleached area is broader than the control, whereas in the APV-CNQX group where the recovery is enhanced relative the control, the photobleached CtBP1 puncta are immediately adjacent to large CtBP1 clusters. This would be indeed true if the size of bleached area would vary repetitively between different treatments. However, the size of bleached spot was set by laser control device to the constant pixel diameter for all imaging sessions in all experiments and a calibration of the laser was always performed when a new coverslip was inserted into the stage. The slight variations in effective bleaching between puncta might be due to small differences in refraction caused by particular morphology of the neuronal branch segments, where bleached synapse was located. Thus, the variability in the bleaching efficiency reflects the biological variability. The puncta set for bleaching were not preselected. The only criteria was not to hit puncta located close to each other at the same axon to prevent additive effects and guarantee constant recovery by exchange with fluorescent molecules. There are 50-100 puncta analyzed per condition, which should compensate for incidental higher or lower bleaching efficiency of some puncta given by biological variability of the samples. We agree that the example image in the original manuscript could be misleading. We exchanged the criticized example image in new Fig 4C to show initial situation comparable between all treatments.

Figure S4D. What is the basis by which CtBP1 levels in the brain lysates that is reduced in Bsn mutant neurons, recovers to the level found in wild type controls for the DKO's?

There was no statistically significant difference in the expression levels of CtBP1 in the brain lysates from mice of different genotypes (now in Fig S4F,G). The slightly lower relative amount in Bsn mutant did not pass significance test and is likely due to experimental variation.

Figure 5F. Does co-expression of RP10 or RP16 fragment of piccolo along with GFP-bassoon prevent its ability to restore synaptic CtBP1? This should be tested in support of the specificity of synaptic accumulation of CtBP1 to its interaction with bassoon and piccolo in a competing manner. Due to technical limitations, it was not possible to double transfect DKO neurons with GFP-Bassoon (sized about 500kDa) together with Piccolo fragments. Important to state here that DKO animals are
not viable and we can obtain them only from mating of double heterozygote animals as homozygotes do not breed. This mating yield in only 1/16 of animals with correct genotype, which makes this type of experiments extremely challenging.

So far, rescue experiments were the gold standard to verify a specific effect of genetic mutation and therefore we did the rescue with GFP-Bassoon. Now we are requested to demonstrate the specificity of the rescue. We agree that additional proteins might be involved in synaptic retention of CtBP1 as we discussed above in the answer to point 2 of reviewer 1 and we reworded our statement in the abstract accordingly.

Figure 8E,F. Given that 2DG treatment may have global metabolic consequences, it would be informative to perform additional experiments to determine the extent specificity of the observed effects of interfering with NAD/NADH levels on CtBP1 localization. For example, does 2DG occlude with APV-CNQX-induced reduction of synaptic CtBP1 and the corresponding increase in nuclear CtBP1?

We appreciate reviewer’s request for controls in experiments with 2DG-treatments as they might have global effects. We tested the effect of 2DG treatment on neuronal survival (Fig S6G,H) and on synaptic activity and demonstrated that treated cells show same survival as non-treated controls and that they still respond to 4-AP bicu treatment with increase of synaptic vesicle recycling (Fig 9G,H).

As suggested by the reviewer we also tested the effect of 2DG treatment on APV CNQX-induced changes in CtBP1 synaptic and nuclear abundance (Fig S6C-F). The outcome of this experiment is not black and white. 2DG induced a decrease (which did not pass significance test) in synaptic CtBP1 levels, which is compatible with the requirement of NADH for efficient CtBP1 interaction with Bassoon and Piccolo. APV CNQX treatment induced a further decrease, which again did not pass significance test in comparison to cultures with basal activity levels treated with 2DG. Chronic (48h) APV CNQX treatment induced homeostatic presynaptic scaling, which is accompanied with extensive remodeling of presynaptic scaffolds including Bassoon and Piccolo (Lazarevic et al., 2011). Thus the decrease in CtBP1 abundance in 2DG treated cells might contribute to decreased abundance of Bassoon and Piccolo, which we propose to function as its synaptic anchors. Important to state here, the decrease of synaptic CtBP1 upon inactivity cannot be exclusively attributed to the regulation of abundance of Bassoon and Piccolo. FRAP experiments showed clearly that also synaptic dynamics of CtBP1 are acutely regulated by activity, what is not the case for Bassoon and Piccolo (Tsuriel et al, 2009; Lazarevic et al., 2011). Therefore it is most likely that activity-induced changes in CtBP1 synaptic dynamics are a consequence of activity-dependent regulation of its association to its synaptic anchors, Bassoon and Piccolo. 2DG treatment did not affect silencing-induced changes in nuclear CtBP1. This is in agreement with our conception that while Bassoon and Piccolo control the availability of CtBP1 for nuclear import, additional Bassoon/Piccolo independent molecular mechanisms control nuclear import and export of CtBP1. After all, CtBP1 is ubiquitously expressed and shuttles between the cytosol and the nucleus in response to extracellular stimuli also in non-neuronal cells that do not express Bassoon or Piccolo.

What about the effects of 2DG on CtBP1-dependent gene expression?

Effect of 2DG on CtBP1-mediated transcription in neurons has been reported in the study by Garriga-Canut and coauthors (2006). There it was demonstrated that 2DG inhibits the seizure-induced increase in the expression of BDNF and TrkB via CtBP1-dependent mechanism. This is well in agreement with our results showing that nuclear export of CtBP1 induced by 4AP bicu is affected by 2DG. However, it is likely that this is not exclusively due to interference with the Bassoon-CtBP1 interaction, but we would expect that 2DG also acts on Bassoon/Piccolo-independent mechanisms controlling nuclear import and export of CtBP1 as stated in previous paragraph.

Referee #3:
In this manuscript, the authors provide evidence that CtBP1 shuttles between presynaptic sites and the neuronal nucleus to regulate the expression of BDNF, Arc and other targets. While much of the data presented is of good quality, this work represents two separate stories forced together by unconvincing and confusing data asserting a connection between nuclear and presynaptic pools of CtBP-1. In one story, the authors elucidate the mechanisms underlying presynaptic targeting of CtBP-1 and in a separate story show that activity-dependent nucleocytoplasmic shuttling of CtBP-1 affects gene expression.

We apologize, but we cannot agree with reviewer in this point. CtBP1 is expressed in multiple cellular compartments of the same neuron. Therefore it was natural for us to investigate the effect of activity modulation of both prominent neuronal CtBP1 pools, namely the one at synapses and in the nucleus. The facts that 1) these pools are regulated in opposite manner upon bidirectional modulation of neuronal activity and metabolic status and 2) the interference with the synaptic pool has an effect on the nuclear one prompted us to formulate the hypothesis that recruitment of the CtBP1 to the synaptic pool controls its availability for nuclear import. The logical continuation of this thinking was to explore possible physiological consequences of the increased or decreased nuclear abundance of CtBP1 in neurons. We addressed this by studying the role of CtBP1 in the activity-dependent regulation of expression of activity-regulated neuronal genes. Finally, our imaging experiments provide first hint that synaptic and nuclear pools might communicate via the cytoplasmic pool, which connects them and can serve as a sink and supply for synaptic retention/liberation and nuclear import and export. We are excited about these data as they might provide new evidence how presynaptic activity can be translated into the regulation of neuronal gene expression. Taken together we provide here the principal idea that synaptic/axonal and somatic/nuclear pools of CtBP1 do communicate. That in addition multiple regulatory steps can influence this distribution is not doubted. I am afraid, we cannot answer all questions concerning this pathway in one paper. Rather we want to stimulate the field to dig deeper into this, as we feel, interesting and novel aspect of neuronal activity-induced signaling.

Major:
- The major concern regards the pre and/or postsynaptic localization of overexpressed GFP-tagged CtBP-1, which affects the interpretation of several figures. The authors present high-quality STED evidence that endogenous CtBP-1 is indeed presynaptic, yet overexpressed eGFP-CtBP-1 (Fig 2B) appears to be punctate throughout the dendrite. Shouldn't it be present in axon terminals? If it is in fact presynaptic, and thus in a separate, different neuron, then the photoactivation studies are bizarre. For example, in Fig 2F how can photoactivation of dendritic branches (where CtBP-1 is claimed to be presynaptic) result in its accumulation in the monitored cell body? Essentially, how does presynaptic CtBP-1 reach the postsynaptic nucleus? Do the photoactivated patches contain axons of the monitored cell bodies? If so, how could so little CtBP-1 (presynaptic CtBP1) contribute so much material to the cell soma? This is a recurring concern throughout the manuscript.

In Figure 2D, photoactivation of CtBP1 in the nucleus results in puncta appearing in neurites over time. Are these postsynaptic, presynaptic? It seems like the authors used techniques to study dendritic intracellular trafficking, when they really needed evidence of retrograde transport along axons. If these experiments were simply used to show the potential for retrograde or anterograde transport of CtBP-1, then they do little to support the claims of the paper.

This is indeed very important point and it is crucial to clarify this issue. As described in general points at the beginning of this document, we provide new images (Fig 2B,C; 5D) in the revised manuscript that support presynaptic localization of expressed CtBP1intEGFP and clarify all concerns of the reviewer in this point. Moreover, we redid all PA experiments, where we labeled active synapses before PA and did restricted PA only to small regions containing these synapses (Fig 3A-D, 4A,B). We hope that these considerable improvements are satisfactory and convincing.

Regarding the question about how can small synaptic amount contribute to somatic function? In Hüblier et al., 2012 we showed that there is considerable amount of CtBP1 located in synapses. In this manuscript we photoactivated 50-100 synapses and used extremely sensitive detection to monitor the increase in nucleus (Fig 3C).
More generally, the question about how a small amount of a presynaptic signaling molecule can account for changes in gene expression in nucleus is very interesting and it is actually a hot topic of discussion also in the field of spine/dendrite to nucleus signaling. We currently know too little about the turnover of CtBP1-based molecular complexes controlling gene expression in neurons. In principle and assuming the minimal requirement, the presence of one active complex is enough to associate with respective binding motif of one promoter region (which in many genes is present only once per promoter sequence) and repress its expression. Thus, small differences in the molecular abundance on top of a balanced steady state might have a significant effect on gene expression. Understanding of these mechanisms is of crucial importance and certainly on our future research, but goes far beyond the scope of this study.

A second concern is that given the kinetics of transport, which seems to be very different between endogenous and overexpressed protein, these results could simply reflect the diffusion of an overexpressed protein. If APV-CNQX can block the nuclear export of photoactivated CtBP-1, it would support their claims of a regulated process.

The activity dependent changes in nuclear pool of the endogenous proteins are significant as soon as 2 hrs after 4AP/bicu treatment (Fig S1A,D). This is in line with the appearance of fusion protein photoactivated in the distant neurite meshwork after 3 hrs, so we do not see any conflict in this point.

As stated above in the answer to the second question of reviewer 2; the imaging experiments that we performed have the clear limitation that we cannot activate the nuclear CtBP1 without activating the cytoplasmic pool above and underneath the nucleus, (which would be possible only doing 2-photon activation) and, similarly, as our detection is done using wide-field imaging we can not quantify nuclear expression without contribution of the cytoplasmic one. We could not overcome these technical limitations and therefore we limited the interpretations of these experiments as a principal prove of retrograde and anterograde flow of CtBP1 in neurons. Moreover, we avoided an interpretation of these results as de facto presynapse to nucleus shuttling, but claimed that the results support the idea about communication of the synaptic/axonal and the somatic (cytoplasmic/nuclear) pool. This is what our data clearly demonstrate.

We agree completely that it is necessary to test experimentally whether synaptic CtBP1 molecules once released from its synaptic anchor in response to activity block can translocate, enter nucleus and participate in the regulation of gene expression. This is technically very challenging, as minimal amounts of synaptic CtBP1 need to be detected in the nucleus in these experiments. We plan to address this issue in our forthcoming studies designed to investigate the mechanism of activity-regulated nuclear import/export of CtBP1 using 2-photon confocal imaging in brain slices. Here we will take advantage from clear circuitry allowing regional activation and detection.

The kinetics of transport (many hours) is also at odds with the regulation of transcription of immediate early genes such as Fos, Arc and BDNF. This should be discussed more thoroughly. This is indeed important point and one clearly needs to distinguish between regulation of gene expression occurring immediately after stimulus or in delayed time-frame. The immediate early genes are well known for their fast expression following transient neuronal activation, but they are also regulated during chronic changes in activity levels as e.g. epileptic seizures, chronic activity deprivation etc. We selected the 4AP/bicu treatment for 8 hrs, as it resembled well the condition of epilepsy, which was already previously connected to CtBP1 repressor function (Garriga-Canut et al, Cell, 2006). The robust regulation of c-fos, Arc and BDNF and many other genes classified as “immediate early” upon 8hrs treatment with 4AP/bicuculline was published previously by Xiang and colleagues (J. Cell. Physiol. 212: 126–136, 2007).

We included respective statement in Discussion, section “Nucleo-cytoplasmic shuttling of CtBP1 controls expression of activity-regulated genes”:

„While induction of immediate early genes takes place within minutes upon stimulus and is dependent on back-propagating action potentials or somatic/nuclear Ca²⁺ waves, expressional
control mediated by CtBP1 (and other activity-regulated synapse-to-nucleus messengers) occurs at longer time-scale (within hours) and might therefore play an important role in long-term modifications of gene expression during memory formation, homeostatic plasticity or adaptive plasticity."

We hope that this sufficiently address this reviewer’s point.

-While concerns regarding spontaneous photoactivation are addressed, the sensitivity is not. That is, photoactivation of a patch could result in some photoactivation nearby. Indeed in Supplementary figure S2A, there is plenty of dendritic fluorescence at time 0 following nuclear photoactivation. A control should be developed to address this issue.

We are aware that photoactivation experiments need to be performed in a highly controlled manner and we did our best to achieve this. To control for nonspecific photoactivation outside of the intended area we always measured fluorescence at time point 0 (immediately after photoactivation) in regions showing fluorescence at the final time point and normalized to the t=0 value as shown in plots 3B,D. Looking at images in Fig 3A,C it is evident that the fluorescence at time point 3h is much higher than the fluorescence at the time of photoactivation. We hope that this explanation is satisfactory.

- Figure 4B-F represents weak and unconvincing correlation data. Promoter I is the most active promoter driving BDNF expression and can be regulated by CREB and REST. Moreover activity dependent regulation of BDNF expression has been extensively studied (4C-F). The results in 4H and 4I are important and interesting and do support their claim that CtBP-1 can regulate BDNF expression. It may be helpful to validate some of these findings by monitoring protein levels.

We agree that data in formerly shown in Fig 4B-F are correlative, but nevertheless we find the results using promoter assay supportive for the role of activity-induced CtBP1 nuclear shuttling in the transcriptional regulation. Therefore, we have moved them to supplements (Fig S3A-F).

Regarding monitoring of protein levels: We did not go for quantification of protein levels of identified gene products as it is shown for many cases that changes at the transcriptional level are not mirrored linearly in the changes of protein levels. In fact looking at levels of mRNA appeared to us to be a more direct readout of CtBP1 function in transcriptional regulation.

However, using leptomycin to block all nuclear export is too broad and may lead to potentially damaging cellular effects that could greatly alter gene expression and thus confound their results. Ideally, the authors should employ a mutant CtBP-1 lacking a nuclear export sequence to identify CtBP-1 specific effects.

We are aware of the fact that leptomycin B affects all nuclear regulators undergoing exportin1-dependent export. To judge specific effects of leptomycin B on export of CtBP1 we compared the effect of leptomycin B in control cells with cells depleted from CtBP1 using CtBP1 KD (see figure 4I). The block of activity-induced expression by leptomycin B was clearly attenuated in cells depleted from CtBP1 suggesting that significant part of leptomycin B effect is CtBP1 dependent.

It is indeed a great idea to employ CtBP1 mutant lacking nuclear export signal in order to study the mechanisms of its nuclear import/export. However, although a motif similar to nuclear export consensus sequence was identified in CtBP1, a respective functional mutant was not cloned and tested yet. We will surely attempt to generate and test such mutant and employ it in future studies. However, the motif is highly degenerate and identification of the full export signal may take some time.

-The Western blots showing that activity/silencing do not alter global CtBP-1 levels do not convincingly support claims of transport as changes could be localized. Synaptic activity elevates protein ubiquitination and CtBP-1 has been shown to be ubiquitinated (Zhang et al 2005 PNAS). The authors must show that changes in the synaptic/nuclear abundance of CtBP-1 are not altered by proteosomal and/or translational inhibitors.
This is very good and important point. We followed reviewers recommendation and performed experiments as reviewer suggested and included them in Fig 1G,H. Neither interference with UPS-dependent protein degradation nor block of transcription affected activity-induced regulation of CtBP1 abundance in nucleus and synapses. We thank the reviewer for this valuable comment that helped us to support our claim.

- The FRAP experiments are well done, but difficult to interpret. Again, is this overexpressed CtBP-1 presynaptic or postsynaptic? The dull fluorescence throughout the dendrites and the absolute lack of any axonal staining strongly suggests that CtBP-1 is dendritic and likely postsynaptic. Since it would not be associated with Bassoon/Piccolo, these experiments would have little impact on the assertions claimed in the manuscript.

We invested great effort to clearly demonstrate a correct targeting of CtBP1intEGFP (Fig 2B,C and 5D; see also general points at the beginning of this point by point answer). Taking this in account we don’t see any problems with interpretation of the FRAP assay. In Fig 4C we interpret the CtBP1intEGFP signal as axonal/synaptic. We are not sure what the reviewer describes as dull fluorescence throughout the dendrites; we can state that dendritic signal intensity was similar to autofluorescence and by orders of magnitude lower than synaptic one. In fact, some of the FRAP experiments were done on synapses formed by transfected axons contacting dendrites of non-transfected neurons, which strongly argues against postsynaptic origin of recovered fluorescence.

-In Figure 5F, overexpressing GFP-Bassoon can rescue the synaptic expression of CtBP-1 in DKO cells. Again, wouldn’t this Bassoon be dendritic/postsynaptic (i.e. in the cell overexpressing the construct?). Is the “rescued” synaptically localized CtBP-1 in the pre or postsynaptic cell?

In the Fig 5F we are showing a neuron where axons are growing along the own dendrites and also over the own cell body. The two labeled fibers crossing diagonally (from lower right to upper left direction) over the cell body are clearly axons in our eyes.

Minor-
- Fig 1A requires a nuclear marker. Fig 1B requires a dendritic marker to better localize CtBP1. We provide requested co-staining in Fig S1A (with DAPI) and Fig 2C (with MAP2). We hope that this is satisfying; expanding of Fig 1 was from space reasons difficult.

-Figure 5C is confusing and needs a better explanation in the text. We apologize for lack of clarity here. We expanded our explanation in the figure legend (now Fig 6C).

-Wouldn’t elevated NADH levels also increase CtBP-1 binding to transcription factors, thus retaining CtBP-1 in the nucleus?

In our opinion it wouldn’t. The association of CtBP1 with NAD or NADH differentially influences affinity of CtBP1 to its interaction partners. Increasing NADH concentrations promote binding of Bassoon to CtBP1, while binding of CtBP1 to the nuclear repressors NRSF or p300 is reduced (Garriga-Canut et al., 2006, Kim et al., 2005). This fits well to our model how activity status controls synapo/nuclear targeting of CtBP1 via modulation of cellular NAD/NADH balance.

Accordingly we state in Discussion, chapter Activity regulates CtBP1 in nucleus and synapses: communication between the two cellular pools of CtBP1 „increasing NADH concentrations (occurring upon elevation of neuronal activity) promote binding of Bassoon to CtBP1, which mediates synaptic targeting/retention of CtBP1, while binding of CtBP1 to NRSF or p300, the nuclear regulators functionally associated with CtBP1, is reduced (Garriga-Canut et al, 2006; Kim et al, 2005).”

-What is Supplementary Fig 4A?
We apologize, but we do not understand this point.
This figure is now expanded and described as follows:

**Figure S4: CtBP1 is slightly reduced at synapses from Bsn-/− mice and is further depleted in Bsn-/−/Pclo KD neurons.**

A, B, C. Synaptic CtBP1 was stained in cultures in which equal numbers of neurons from WT and Bsn-/− neurons were mixed and plated (A) or in cultures infected with lentivirus expressing shRNA Pclo28 (B) or scrambled shRNA (C) together with EGFP-synapsin. Synapses were marked with staining for synaptophysin (A) or Homer (B,C). In A, synapses from Bsn-/− neurons lack Bassoon specific staining (arrowhead), which is clearly visible in synapses of WT neurons (arrows). In B and C synapses of transduced neurons express EGFP-synapsin (arrowheads). Synapses of neurons expressing Pclo28 shRNA show clear reduction in IF for CtBP1 (arrowhead in B) in comparison to synapses of non-transduced neurons (arrows in B). Synapses of neurons expressing scrambled shRNA (arrowheads in C) show no difference in the IF for CtBP1 when compared to synapses of non-transduced neurons (arrows in C).

We will change the figure legend adequately if reviewer could, please, specify what is the unclear point here.

- For Fig 7A: Why was a 30min activation tested and not the longer time points used for transport?
  We see clear differences in molecular dynamics of synaptic CtBP1 at time point 20 min after 4AP/Bicu application. As we interpret it, the synaptic recovery of CtBP1 depends on its association with its synaptic anchor, Bassoon/Piccolo. Therefore, stimulation 30 min before IP was done to test activity-dependent association of Bassoon with CtBP1.

  *It seems like the authors have antibodies, why wasn't endogenous CtBP1 tested?* We used overexpressed CtBP1 due to problems with low detection of the endogenous one.

  *Does activity alter Bassoon levels?* The FRAP experiments performed in Ziv lab (Tsuriel et al., 2009) do not suggest any effect of elevated neuronal activity on molecular dynamics of Bassoon in the investigated timeframe.

- Fig 8, Why were neurons treated with 2DG for 2 days (so long)? Any effects on viability?
  We performed this treatment as described previously. 2DG does not affect survival of cultured hippocampal cells during this period. In fact, it was reported that application of 2DG has a survival promoting effect, as it protects form oxidative injury and excitotoxicity (Lee et al., 1999, J of Neuroscience Research). In our hands 2DG treatment does not affect cell survival (Fig S6G,H).

- Wouldn’t homeostatic effects induced by 48 hours of APV/CNQX result in opposite changes from those expected?
  We don’t think it would. We have shown that the silencing of network activity for 48 hrs leads to a decrease in Bassoon and Piccolo immunoreactivity at synapses (Lazarevic et al., 2011), which is in good agreement with the observed decline in synaptic levels of CtBP1.

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been re-reviewed by the referees. As you can see below, the referees appreciate the introduced changes and support publication here. Referee #3 has just a few minor suggestions that I would like you to take into consideration. Once we get these last issues sorted out then we will proceed with the acceptance of the paper for publication here.

I look forward to seeing the final version.
REFEREE REPORT

Referee #1:

The authors provide a detailed and extensive rebuttal addressing all my main concerns. The additional data and analysis have in my opinion considerably strengthened the paper and I would recommend publication as is.

#1- concerning construct validation: The authors now convincingly show by rescue experiments that the CtBP1intEGFP is functional protein.

#2 - concerning an alternative explanation for role of Bassoon/Piccolo in synaptic localization of CtBP1: I acknowledge the authors' efforts to rule out alternative explanations and agree with the changes in the abstract. It would be interesting for future studies to revisit this issue.

#3- Alternative explanation for the effect of 2-DG on activity-dependent CtBP1 re-localization: The authors added the important control experiment and I fully agree with their conclusion.

#4- On statistical testing: the authors added the requested details.

All minor comments were successfully addressed with additional experiments.

Referee #2:

The revised manuscript has been significantly improved, and the authors have addressed my main concerns.

Referee #3:

The authors have addressed most of my concerns. However pre/postsynaptic localization concerns remain, but these can be addressed within the text. From Fig 2B it is clear that overexpressed CtBP-1 CAN be presynaptic. However Fig 2C is not a serious attempt at assessing postsynaptic localization (why didn't they use spine or postsynaptic markers?). Assumptions of autapses are unverified and arguments based on transduced/ untransduced neurons (Fig 2B/C) are contradicted by their claims of 100% transduction in the methods. Moreover, I am not convinced that in Fig 3A, regions 2,3,4 represent axon terminals of the neuron in region 1, nor am I convinced that the 50-100 puncta activated (should be stated in methods) in Fig 3C represent axon terminals of the cell bodies outlined by circles 3 and 4. However I am satisfied with the toned down language suggesting Fig 3 merely shows the retrograde and anterograde trafficking potential of CtBP-1. In addition, the authors should tone down claims like "The imaging of photoactivatable CtBP1 in living neurons supports this view and revealed physical retrograde translocation of CtBP1 from presynapses to the soma" and they should discuss the caveat that despite their evidence, nuclear CtBP-1 may be coming from other regions.

Regarding the Leptomycin block (there is no Fig 4I- I assume they meant 5F?), the argument "everything is messed up with leptomycin B, but slightly less if you remove CtBP-1" (essentially Fig 5F) is not particularly convincing or compelling. I suggest removing 5F.

Other than that, this work represents a high-quality and provocative paper that increases our understanding of CtBP-1 biology and should significantly contribute to our understanding of how nuclei and synapses communicate.

Point by point answer to Referee #3:

The authors have addressed most of my concerns. However pre/postsynaptic localization concerns remain, but these can be addressed within the text. From Fig 2B it is clear that overexpressed CtBP-
I CAN be presynaptic. However Fig 2C is not a serious attempt at assessing postsynaptic localization (why didn't they use spine or postsynaptic markers?). We actually present co-staining with postsynaptic marker homer in dual-color STED images in Fig 2D showing clear alignment without overlap suggesting pre- and not postsynaptic localization.

Assumptions of autapses are unverified and arguments based on transduced/untransduced neurons (Fig 2B/C) are contradicted by their claims of 100% transduction in the methods. We apologize for not being specific in this point. We added corresponding statement to the methods section (p.24).

Moreover, I am not convinced that in Fig 3A, regions 2,3,4 represent axon terminals of the neuron in region 1, nor am I convinced that the 50-100 puncta activated (should be stated in methods) in Fig 3C represent axon terminals of the cell bodies outlined by circles 3 and 4. However I am satisfied with the toned down language suggesting Fig 3 merely shows the retrograde and anterograde trafficking potential of CtBP-1. In addition, the authors should tone down claims like "The imaging of photoactivatable CtBP1 in living neurons supports this view and revealed physical retrograde translocation of CtBP1 from presynapses to the soma" and they should discuss the caveat that despite their evidence, nuclear CtBP-1 may be coming from other regions. We changed this sentence. Now it is:

... and revealed physical retrograde translocation of CtBP1 from distal neurites (presumably presynapses) to the soma. (Discussion p.16)

Regarding the Leptomycin block (there is no Fig 4I- I assume they meant 5F?), the argument "everything is messed up with leptomycin B, but slightly less if you remove CtBP-1" (essentially Fig 5F) is not particularly convincing or compelling. I suggest removing 5F.

We regret but we could not find citation of Fig 4I in the revised manuscript. We think, this misunderstanding was due to changes of figure numbering in the revised version in comparison with the initial submission.

Other than that, this work represents a high-quality and provocative paper that increases our understanding of CtBP-1 biology and should significantly contribute to our understanding of how nuclei and synapses communicate.