THE EMBO JOURNAL

Manuscript EMBO-2009-72338

Learning, AMPA receptor mobility and synaptic plasticity depend on n-cofilin mediated actin dynamics

Marco Rust, Christine Gurniak, Marianne Renner, Hugo Vara, Laura Morando, Marco Sassoe-Pogneto, Mummna Al Banchaabouchi, Maurizio Giustetto, Antoine Triller, Daniel Choquet, Andreas Görlich, Walter Witke

Corresponding author: Walter Witke, University Bonn

Review timeline:

Submission date:	26 August 2009
Editorial Decision:	08 October 2009
Revision received:	25 February 2010
Editorial Decision:	18 March 2010
Revision received:	25 March 2010
Accepted:	26 March 2010

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 08 October 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal, and please excuse the longer-than-usual delay in getting back to you with a decision. We have now received the reports and recommendations of three expert referees who had agreed to take a detailed look at your study. As you will see, all referees find your results of synaptic plasticity and behavioral effects upon neuron-specific cofilin deletion potentially important and interesting. However, especially referees 1 and 2 also raise a number of substantive issues that would need to be satisfactorily addressed in order to corroborate the main conclusions of the study. At the current stage, I think it is fair to summarize that although the reviewers consider the findings and conclusions interesting in principle, they are not yet convinced that the presented experimental evidence is sufficient to fully support these conclusions and interpretations. Main concerns in this respect are especially the lack of data on the extent and efficiency of cofilin gene deletion, the molecular, cellular and behavioral paradigms employed to derive conclusions on cofilin knock-out effects (combined with apparently only minor effects in some of these assays), as well as some inconsistencies (both internal and in comparison to previously reported findings).

Given the potential importance of your results and the overall interest nevertheless expressed by all referees, I am inclined to give you the opportunity to respond to the their criticisms through a revised version of the manuscript. However, I hope you understand that we are currently not in a position to make strong commitments with regard to publication of a revised manuscript, as it is presently not clear if the requested further work will be able to clarify the inconsistencies and

validate the main conclusions, or rather confound them. Still, should you feel confident that you might be able to address the key criticisms and to convince the critical referees, we should be happy to consider a revised manuscript further for publication. In this respect, please bear in mind that it is EMBO Journal policy to allow a single round of major revision only, and that it is therefore essential that you diligently answer to all the points raised at this stage if you wish the manuscript ultimately to be accepted. If required, we could in this case also discuss an extension of the revision duration (normally limited to three months). In any case, please do not hesitate to get back to us should you need feedback on any issue regarding your revision.

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

Yours sincerely,

Editor
The EMBO Journal

_____

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The paper of Rust et al. 'Learning, AMPA receptor mobility and synaptic plasticity depend on n-cofilin mediated actin dynamics' gives a detailed molecular, histological, physiological as well as behavioural analysis of a forebrain specific n-cofilin knock out mouse. N-cofilin is an actin filament depolymerizing protein and therefore potentially involved in synaptic remodel ling. The authors suggest a role of actin dynamics in associated learning, and an actin dependent mechanism of AMPA receptor availability. Furthermore the propose a model that n-cofilin can affect synaptic plasticity through the control of extrasynaptic excitatory AMPA receptor diffusion.

The manuscript presents a comprehensive description of the phenotype detected in mice with a specific n-cofilin gene inactivation in the forebrain of adult mice.
Rust et al. used a wide spectrum of methods with the primary goal to investigate the plasticity of spines and neuronal connectivity. Primary cultures and young and adult mice were recruited.
Most of the presented experiments were performed very professionally and are presented in an adequate format. The manuscript is of particular interest since deficiencies in actin mobility might underlie some forms of mental retardation. However, there are several major conceptual issues concerning several methods and the interpretation of the results. Al these issues need to be addressed seriously before the manuscript of Rust et al. can be accepted for publication.

1) The authors have to document the efficiency of the n-cofilin gene inactivation at E19 and new born pubs. Alpha CaMKII promoter controlled, forebrain specific Cre mice of Minichiello et al. and others were used in many experiments for gene deletions or gene expression in adult animals since it is known that the alpha CaMKII promoter dramatically increases its activity within the first two postnatal weeks of newborn mice. Using sensitive reporters CaMKII promoter embryonic activity could be detected in CaMKII promoter based transgenic animals, however it is not clear to which extent Cre/lox mediated gene deletion occurs in the different brain regions of a mouse embryo. The most central experiment - AMPA receptor mobility studies in absence of n-cofilin - of Rust et al., were done in cultured neurons. Therefore, it is absolutely necessary that the authors document the preparation of the culture in detail including the genotyping procedure which becomes necessary when selecting the embryos for the preparation (The current version of the manuscript doesn't contain a detailed description of the cultured neuron). But even more importantly it is obligatory to document the efficiency of the Cre induced gene deletion on the cellular level in hippocampal and cortical cultures.

2) The age of the mice used in all experiments needs to be given.

3) For the behavioural experiments Cre expressing mice without floxed alleles have to be included

as controls.

4) For the analysis of the dendritic tree a comparison of layer 5 cells between 'flox/flox' and 'flox/flox/Cre' mice makes absolutely no sense. It was documented by Minichiello et al. that the used Cre mouse doesn't induce recombination in layer V in a very sensitive Cre reporter mouse line.

5) Akashi et al. reported recently in J. Neuroscience 29, p. 10869 that the NMDA receptors (function) and the postsynaptic molecular organization is linked closely. One indication was the decreased F-actin/G-actin ratio on CA3 observed in CA3 specific epsilon2 knock out mice. Therefore a detailed investigation of synaptic NMDA receptor and AMPA receptor function has to be implemented. The presented ligand intensity blots (bar graphy) are not sufficient for the documentation of NMDA and AMPA receptor functions in adult mutant mice. There are plenty of molecular and physiological tools available that can be used to address this question.

6) For the LTP measurements the authors used a 4 x 100 Hz stimulation procedure. Using this strong stimulation a homeostatic potentiation is often observed in the tetanized CA1 cells. An unstimulated control pathway has to be included in the analysis. The stimulation strength (relative to the intensity needed for the induction of a population spike should be given). Given that the authors describe changes in LTP and LTD the response curves after 1 x 100 Hz tetanic stimulation (golden standard for LTP) should be included.

7) The mouse behaviour is an additional issue. The molecular and physiological studies of Rust et al. are focused on hippocampal function in n-cofilin mutant mice. Rust et al present data from different learning paradigms. But only two of the tests are well accepted tools for the analysis of hippocampal function. Presenting cued fear conditioning and drug induced place preference leaves the impression that the lack of hippocampal LTP and LTD is directly linked to learning impairments in fear and drug induced learning. It is interesting enough that the reference memory shows no improvement in the Morris water maze while the working memory is still perfect in-cofilin mutants. To substantiate this finding, a radial maze or Y-maze maze task should be included.

8) According to the authors the inhibition of the lateral movement of AMPA receptors is the dominant reason for the lack of plasticity in n-cofilin mutant mice. Indeed, it is well documented in cell culture and the GluA1 knockout mouse that the fast AMPA receptor trafficking and the huge extrasynaptic AMPA receptor pool (lateral movement) is directly linked to the expression of tetanus induced LTP in CA1 pyramidal cells. However, the 4 x 100 Hz induced LTP is not impaired in GluR-A knockout mice (Hoffman et al.2002 and Phillips et al. 2008) and the spatial reference memory is unchanged in absence of GluA1 (Zamanillio et al.1999;). Thus, all those learning and synaptic plasticity phenotypes described when the free diffusible, extrasynaptic AMPA receptor pool is missing (lack of working memory and operative 4x100Hz induced LTP) are normal in n-cofilin mutant mice. Interestingly, the GluA1 independent spatial memory and plasticity is missing in n-cofilin mutant mice. This contradiction needs to be addressed and needs an explanation.

Minor comments:

The introduction should explain how the forebrain specific of n-cofilin can be obtained.
Why the LIM kinase I pointed to a potential role of n-cofilin in spine morophology an synaptic plasticity needs to be explained already in the introduction.

Referee #2 (Remarks to the Author):

This is an important study describing the synaptic and behavioral phenotype of a genetically altered mouse strain with a region (forebrain)-specific ablation of n-cofilin. The authors showed that these mice are developmentally normal (including apparently normal brain anatomy and neuronal morphology--dendritic arborization) but with alterations in the dendritic spine and synapse, including increased spine density (specifically in the number of mushroom looking spines), enlarged spine heads and increased synaptic size. Electrophysiologically, these mice are impaired in both hippocampal long-term potentiation (LTP) and depression (LTD), but basal synaptic strength and presynaptic function remain intact. Behaviorally, these mice display defects in a number of associative learning/memory tests (e.g. water maze and fear conditioning), but the working memory

appears to be unaffected. In general, all the experiments are straightforward in terms of design and appear to be carefully done with significant findings.

General comments:

(1) It is not clear to me how much cofilin remains in the n-cof flx/flx, CaMKII-cre mice. Based on Figure 1D, n-cofilin signal can still be seen in these mice, in both the cortex and hippocampus. In addition, the actual activity of the remaining cofilin needs to be analyzed in order to assess the specificity and degree of n-cofilin knockout/knockdown. Without these data, it would be hard to evaluate the consequence of this particular genetic manipulation.
(2) In n-cof flx/flx, CaMKII-cre mice, both spine and synaptic (PSD and presynaptic terminal) sizes are enlarged. Accordingly, the AMPAR surface expression is also increased. However, basal synaptic strength is not altered. How these data are to be reconciled?

Specific comments:

(1) Fig. 1A: analysis of the activity of cofilin (possibly by looking at the amount of phosphorylated cofilin) over this period of time would be helpful.
(2) Fig. 1B,C: the amount of n-cofilin reduction in these mice should be quantified. Are there alterations in other forms of cofilin and their activity due to n-cofilin ablation.
(3) Fig. 2A,B: these images can not reveal any features of the dendritic spine. At least some large magnification images should be presented to illustrate spine morphology?
(4) Fig.2F: representative images are needed here too.
(5) Fig. 3E: how was the presynaptic bouton size defined/measured? I could not seem to find the description in the paper.
(6) Fig. 4E: the deficit in LTP is very mild. Therefore, I would not spend that much time on this. Also, it is not clear to me why such an inducing protocol was used (4X100Hz)?
(7) Fig. 4F: again, it is not clear to me why the paired low frequency protocol was used to induce LTD, which is know to elicit a complex form of LTD in terms of both the induction and expression mechanisms. Is this LTD NMDAR-dependent?
(8) Fig. 4E,F: given that the LTP/LTD changes, one can imagine that it may be possible to induce LTD in these mice if different protocols (i.e. different frequencies) were used.
(9) What about NMDAR functionality in these mice, although their surface expression is not altered?
(10) Changes in the lateral movement in AMPARS are important and interesting, but ultimately, receptor internalization may be needed to have sustained LTD. What about AMPAR insertion/internalization properties following LTD induction in these mice?

Referee #3 (Remarks to the Author):

Rust et al examined roles played by actin depolymerizing protein n-cofilin in neuronal plasticity as well as learning and memory. They generated conditional KO mice in which n-cofilin is selectively deleted from neurons of the postnatal forebrain. Using this mouse model, they showed that deletion of n-cofilin down-regulates actin dynamics and AMPA receptor mobility in the hippocampal neurons, which accompany impairments in synaptic plasticity and learning and memory. The authors concluded that n-cofilin-mediated actin dynamics is important for neural plasticity and learning through regulating the AMPA receptor mobility.

This is an interesting paper and provides an important insight how actin dynamics in neurons modulate synaptic plasticity and learning. I only have a few minor comments/questions.

1. The authors demonstrate a causal relationship between n-cofilin-mediated AMPA receptor movement and plasticity/learning (page 2, line 10; page 4, line 5). However, what they showed is a correlation. These statements should be re-written.

2. Discussion section is too long and should be shortened.

3. In Fig. 1D, what is the age of mice? It is helpful to add data showing developmental changes in n-cofilin level in flx/flx, CaMKII-cre mice.

4. In page 16, the authors demonstrate that LTP was impaired in flx/flx, CaMKII-cre mice. But n-cof deletion had small effect on LTP.

1st Revision - authors' response 25 February 2010

Reply to comments by Referee #1:

The authors have to document the efficiency of the n-cofilin gene inactivation at E19 and new born pubs ... the alpha CaMKII promoter dramatically increases its activity within the first two postnatal weeks of newborn mice ... however it is not clear to which extent Cre/lox mediated gene deletion occurs in the different brain regions of a mouse embryo.

We devote most of Fig. 1 to this question and provide data in 6 panels (Fig. 1A-F) showing that in the cortex and in the hippocampus, n-cofilin levels decrease postnatally. At P50 (after which the behavior experiments were performed) n-cofilin levels in n-cofflx/flx,CaMKII-cre mutants were reduced by more than 90% (see Fig. 1C, D of the revised manuscript). As an internal control we also show the cerebellum were CamKII-cre is not active and n-cofilin is not changed (Fig. 1E). The residual n-cofilin expression is due to interneuron and glia cell contribution in the lysates (CamKII-cre does not delete these cells) and to illustrate this better we now include another Western Blot of isolated synaptosomes (further enriched for neuronal cells) showing a more than 98% of reduction in n-cofilin. We suppose that one concern of the referee (see below) was the deletion of n-cofilin in the hippocampal cultures used for the receptor mobility experiments. We therefore also included Western Blots of these hippocampal cultures showing a significant decrease (>90%) in n-cofilin levels (Fig. 2F of the revised manuscript). In Fig. 1B we also show the deletion on the mRNA level by in situ hybridization. By all reasonable means employed we can conclude from these data that n-cofilin is efficiently removed from neurons in the forebrain at the times we performed the experiments.

The most central experiment - AMPA receptor mobility studies in absence of n-cofilin - of Rust et al., were done in cultured neurons…it is absolutely necessary that the authors document the preparation of the culture in detail including the genotyping procedure ... it is obligatory to document the efficiency of the Cre induced gene deletion.
We now provide immunoblot data demonstrating effective deletion of n-cofilin in hippocampal cultures at 21DIV (see above, and Fig. 2F of the revised manuscript). We now provide full information regarding generation of hippocampal cultures. Briefly, cultures were generated from individual n-cofflx/flx controls and n-cofflx/flx,CaMKII-cre mutants; genotypes of dissected embryos were determined by PCR using specific primers listed in the section "Material and Methods".

The age of the mice used in all experiments needs to be given.

We now specify the ages of all mice used in the experiments in the section "Material and Methods" (e. g. electrophysiological experiments, electron microscopy), age of mice used for experiments was between two and four month.

For the behavioural experiments Cre expressing mice without floxed alleles have to be included as controls.

We can see the point of referee 1, but would like to explain why we used the flox-allele as a control. In our study, age- and sex-matched n-cofflx/flx mice were used as controls. The CaMKII-cre line we used in our study to delete n-cofilin was generated by Minichiello and colleagues in 1999

(Minichiello et al., 1999). Until today, this paper was cited 290 times (Scopus) and numerous studies used the CaMKII-cre line to specifically delete a gene of interest in principal neurons of the forebrain. To our knowledge, in these studies (including behavioral analyses), the floxed-allele was used as a control since the CaMKII-cre mice themselves did not show any behavioral abnormalities (again confirmed by a personal communication by Liliana Minichiello, first author of the original CaMKII-cre description). Also in our hands we did not see any effect of the CaMKII-cre allele in behavior studies when we performed preliminary studies for the data presented here. For example, we used the same CaMKII-cre for our studies on ëanother gene of interestí in Y-maze, Morris water maze and the contextual fear conditioning in the same way as in the current study and never saw any effect of the CaMKII-cre. As these mutant mice do not show any deficits in these experiments (see attached file just for the eyes of the referee), we decided to exclude the CaMKII-cre transgenics and rather included the n-cofflx/flx to account for any possible disturbance of the n-cofilin locus by the presence of lox-site. For consistency ñ all the other experiments in our manuscript were performed with n-cofflx/flx genotypes. A critical referee might otherwise point out that despite the minimal changes by introducing the lox-sites this could affect the expression levels and therefore the outcome in a potential ëhypomorphí.

For the analysis of the dendritic tree a comparison of layer 5 cells between 'flox/flox' and 'flox/flox/Cre' mice makes absolutely no sense. It was documented by Minichiello et al. that the used Cre mouse doesn't induce recombination in layer V in a very sensitive Cre reporter mouse line.

We deleted Fig. 2B and the respective part of the manuscript

Akashi et al. reported recently in J. Neuroscience 29, p. 10869 that the NMDA receptors (function) and the postsynaptic molecular organization are linked closely. One indication was the decreased F-actin/G-actin ratio on CA3 observed in CA3 specific epsilon2 knock out mice. Therefore a detailed investigation of synaptic NMDA receptor and AMPA receptor function has to be implemented. The presented ligand intensity blots are not sufficient for the documentation of NMDA and AMPA receptor functions in adult mutant mice. There are plenty of molecular and physiological tools available that can be used to address this question.

In the study mentioned, Akashi et al. reported several alterations in postsynaptic structure (altered F-/G-actin ratio and dendritic spine density) upon deletion of the NMDA receptor subunit GluN2B (NR2B) in hippocampal CA3 neurons (Akashi et al., 2009). The data indicate that NMDA receptor mediated synaptic currents that were almost completely absent in GluN2B-/- mice are crucial for the induction of downstream effects such as rearrangements of actin in dendritic spines. The study is consistent with previous observations demonstrating that under resting condition 85% of the actin cytoskeleton turns over rapidly (Star et al., 2002). Influx of Ca2+ via NMDA receptors can block dendritic spine motility (Ackermann and Matus, 2003) (Ackermann and Matus, 2003) which is probably mediated by calmodulin (Fifkova and Van Harreveld, 1977). An altered F-/G-actin ratio as described by Akashi et al. is therefore probably the result of an reduced Ca2+ influx through NMDARs (also reviewed in Oertner and Matus, 2005 (Oertner and Matus, 2005)).
All effects described in Akashi et al. on the actin cytoskeleton are downstream of the NMDA receptor activity and no upstream feedback coming from altered F-/G-actin ratios was described. Based on the data by Akashi et al. one cannot necessarily conclude that vice versa our change in F-/G-actin ratio should have an effect on NMDA receptors function.
However, we took the advice of referee 1 and we have now performed and included additional electrophysiology experiments in which we recorded AMPAR- and NMDAR-mediated currents of single CA1 pyramidal cells. The new data show that the relation between AMPAR- and NMDAR-mediated currents is unchanged in n-cofflx/flx,CaMKII-cre mice (Fig. 5D of the revised mansucript).

For the LTP measurements the authors used a 4 x 100 Hz stimulation procedure. Using this strong stimulation a homeostatic potentiation is often observed in the tetanized CA1 cells. An unstimulated control pathway has to be included in the analysis.

This is an interesting comment, but we believe that there are strong evidences arguing against a possible confounding role of homeostatic potentiation in our 4x100Hz LTP experiments. As an activity-dependent mechanism, homeostatic plasticity requires a period of strong increase in

synaptic activity or a block of such activity as triggering event (see review by Turrigiano, 2007 (Turrigiano, 2007)). How long must this period be? In Drosophila neuromuscular junction (NMJ), homeostatic plasticity has been demonstrated to be triggered as soon as in 2-3 min, with full expression at 10 min. In this case, blocking postsynaptic glutamate receptors causes a homeostatic increase in the quantal content of presynaptically released neurotransmitter (Frank et al., 2006). However, other works in Drosophila NMJ had shown more slow homeostatic mechanisms, requiring chronic hyperpolarization of the muscle to induce a detectable gain in presynaptic function in the form of an increase in neurotransmitter release (Paradis et al., 2001). In a similar way, homeostatic forms of synaptic plasticity in NMJ and central nervous system (CNS) of vertebrates need long periods of time to develop. Works on cultures from rat visual cortex showed a homeostatic increase in pyramidal neuron firing rates as well as in intrinsic excitability of cortical neurons (pyramidal and interneurons) after 48 h of neuronal activity blockade with tetrodotoxin (Desai et al., 1999; Rutherford et al., 1997). More recently, results obtained by Rial Verde et al. in organotypic hippocampal cultures have revealed that reductions in synaptic AMPA, but not NMDA receptor-mediated currents can be detected after 10-20 hours of increasing the expression of Arc/Arg3.1 gene (which mimics the levels and time window of activity-dependent endogenous Arc/Arg3.1 upregulation) (Rial Verde et al., 2006). It has been proposed that the synaptic scaling, like that of AMPA receptors caused by Arc/Arg3.1 (see also Shepherd et al, 2006 (Shepherd et al., 2006)) may compensate for a form of synaptic plasticity like LTP of LTD. However, up to now all these forms of activity-dependent synaptic scaling in vertebrate CNS require a chronic period of neural activation or blocking of activity that ranges from several hours to few days. In the case of the long-term forms of synaptic plasticity -LTP and LTD - that we present in this work, a maximum of 60 minutes pass from the onset of any of the tetanization protocols applied to the hippocampal slices to the end of the experiment. This time window is much shorter than what has been described to be needed for homeostatic plasticity.
How strong does an increase in activity need to be? Recently, Roth-Alpermann et al. have addressed this question by analyzing whether induction of LTP in a (control) synaptic pathway is able to induce, by itself, homeostatic plasticity in a different (test) pathway on the same neuron (Roth-Alpermann et al., 2006). They have shown that a widespread strengthening of synapses like that evoked by chemical induction of LTP is required to produce homeostatic potentiation. In contrast, electrical protocols of repetitive high-frequency (100-Hz) trains applied to the point of inducing saturated LTP were not sufficient to evoke such homeostatic plasticity. Actually, impairment of LTP in a test pathway by saturation of LTP in a control one has been demonstrated to occur only in highly specialized experimental conditions like those reported by Moser et al. (Moser et al., 1998). In this work, the efficiency of saturation of LTP in the perforant path in vivo was maximized using lesions to reduce the volume of hippocampal tissue and employing a specially designed array of stimulating electrodes for LTP induction; even in this specific situation, impairing of LTP was not always achieved in the test pathway. These data supports the notion that strong and spatially diffused synaptic stimulation is required for homeostatic plasticity generation. This requirement is hardly fulfilled with the 4 x 100 Hz electrical protocol of LTP induction that we use in our hippocampal slices.
In light of all this information, we find it highly unlikely, both from a temporal and mechanistic point of view that homeostatic mechanisms of potentiation interfere with the molecular machinery responsible for long-term potentiation induced in our hippocampal slices.

The stimulation strength (relative to the intensity needed for the induction of a population spike should be given).

We consider "maximum" amplitude of the synaptic response when recognizable population spikes start to overlap with the recorded fEPSP. We agree that this was not clearly stated in the methods section, and in the revised version we have include this information.

Given that the authors describe changes in LTP and LTD the response curves after 1 x 100 Hz tetanic stimulation (golden standard for LTP) should be included.

As suggested by referee 1, we performed additional LTP experiments (1x100 Hz stimulation) and now show that in contrast to the 4x100 Hz stimulation LTP evoked by a single 100 Hz tetanus is not depending on n-cofilin (Fig. 4E of the revised manuscript). Previous studies demonstrated that different forms of LTP can be evoked with different stimulation procedures and that strong

stimulation (e.g. 4x100 Hz) is effective at producing so-called late LTP (L-LTP), whereas weaker stimulation (1x100 Hz) rather induces early LTP (E-LTP) (Albensi et al., 2007). Therefore, our data indicate that specifically L-LTP is affected by the inactivation of n-cofilin and that n-cofilin is not critical for E-LTP ñ an interesting finding that will be addressed in future work. In the revised manuscript we corrected the corresponding part accordingly and discuss the conclusions from the LTP experiments.
We should also note that for reasons of consistency (conditions, changing setups, etc.) we have not only performed the 1x100 Hz experiment, but also repeated the 4x100 Hz again. The old 4x100Hz experiment was therefore replaced by the new data (see Fig. 4F of the revised manuscript).

The mouse behaviour is an additional issue. The molecular and physiological studies of Rust et al. are focused on hippocampal function in n-cofilin mutant mice. Rust et al present data from different learning paradigms. But only two of the tests are well accepted tools for the analysis of hippocampal function. Presenting cued fear conditioning and drug induced place preference leaves the impression that the lack of hippocampal LTP and LTD is directly linked to learning impairments in fear and drug induced learning. It is interesting enough that the reference memory shows no improvement in the Morris water maze while the working memory is still perfect in-cofilin mutants. To substantiate this finding, a radial maze or Y-maze maze task should be included.

Our statement of intact working memory in n-cofflx/flx,CaMKII-cre mutants is based on normal performance in the hole board paradigm (e.g. working memory errors) and in the Y-maze (no changes in spontaneous alternation performance, alternate arm returns and same arm returns). Both experiments are established paradigms for analyzing working memory in mice. To again confirm the absence of working memory defects in mutant mice, we performed an additional modified Y-maze described to assess working memory and included the new data (Fig. 8D of the revised manuscript) (Fuchs, 2007). Also in this experiment no differences between n-cofflx/flx,CaMKII-cre mutants and controls were found. Together with the already presented data the new data bolsters the hypothesis, that working memory is not dependent on n-cofilin.

According to the authors the inhibition of the lateral movement of AMPA receptors is the dominant reason for the lack of plasticity in n-cofilin mutant mice. Indeed, it is well documented in cell culture and the GluA1 knockout mouse that the fast AMPA receptor trafficking and the huge extrasynaptic AMPA receptor pool (lateral movement) is directly linked to the expression of tetanus induced LTP in CA1 pyramidal cells. However, the 4 x 100 Hz induced LTP is not impaired in GluR-A knockout mice (Hoffman et al.2002 and Phillips et al. 2008) and the spatial reference memory is unchanged in absence of GluA1 (Zamanillio et al.1999). Thus, all those learning and synaptic plasticity phenotypes described when the free diffusible, extrasynaptic AMPA receptor pool is missing (lack of working memory and operative 4x100Hz induced LTP) are normal in n-cofilin mutant mice. Interestingly, the GluA1 independent spatial memory and plasticity is missing in n-cofilin mutant mice. This contradiction needs to be addressed and needs an explanation.

First, we would like to point out that we are not trying to give the impression that receptor diffusion alone is responsible for the observed defects in plasticity. We believe that the alteration in receptor diffusion contributes to the plasticity defects, as the stabilization of spines and the changes in morphology certainly does. The paper by Phillips et al. (2008) gives the answer to the refereeís concerns: "The first reports of GluR1 (GluRA, GluA1)-dependent LTP in the hippocampus indicated that LTP was completely absent in GluR1 knockouts (Zamanillo, 1999), but it was later discovered that GluR1 dependent LTP was present in younger animals and required a spike-timing protocol to induce it (Hoffman, 2002; Jensen, 2003)". Hoffman et al. showed that although tetanic LTP is largely impaired in GluR-A deficient mice, it could still be elicited by a theta burst LTP protocol. Therefore they stated that there are different forms of LTP even in one snyapse. We therefore find it difficult to follow the refereeís argument in saying that 4x100 Hz LTP is not impaired in GluR-A knock-outs. Hence we do not see a contradiction in our manuscript to the cited papers. In addition we would like to point out again that in our receptor diffusion experiments we looked at GluR2 and not GluR1.

Minor comments:
The introduction should explain how the forebrain specific of n-cofilin can be obtained.
We changed this part in the introduction accordingly
Why the LIM kinase I pointed to a potential role of n-cofilin in spine morophology an synaptic

plasticity needs to be explained already in the introduction.
We changed the introduction accordingly

Referee #2 (Remarks to the Author):

It is not clear to me how much cofilin remains in the n-cof flx/flx, CaMKII-cre mice. Based on Figure 1D, n-cofilin signal can still be seen in these mice, in both the cortex and hippocampus. In addition, the actual activity of the remaining cofilin needs to be analyzed in order to assess the specificity and degree of n-cofilin knockout/knockdown. Without these data, it would be hard to evaluate the consequence of this particular genetic manipulation.

We now include a variety of immunoblot data using cortical and hippocampal lysates demonstrating that n-cofilin levels were unchanged at P1 and strongly declined during postnatal development. We quantitated n-cofilin levels at P50 and found them down-regulated to ~9% and ~7% in cortical and hippocampal lysates, respectively (Fig. 1C, D of the revised manuscript). Moreover, we found n-cofilin down-regulated to ~2% in synaptic structures (Fig. 1F of the revised manuscript). By using an antibody directed against phosphorylated (inactivated) cofilin (that recognizes both isoforms, n-cofilin and ADF) we show that in n-cofflx/flx,CaMKII-cre mutants inactivated cofilin is strongly reduced at P21 and P50 (Fig. 1D of the revised manuscript). This was a surprising in vivo finding that to our knowledge has not been documented elsewhere and which we would like to follow up on. For the interpretation of the phenotype described here the phosphorylation has no significance.

In n-cof flx/flx, CaMKII-cre mice, both spine and synaptic (PSD and presynaptic terminal) sizes are enlarged. Accordingly, the AMPAR surface expression is also increased. However, basal synaptic strength is not altered. How these data are to be reconciled?

It is true that under "normal" conditions AMPAR surface expression correlates with dendritic spine size and PSD length. This does not mean that under pathological conditions as upon deletion of n-cofilin this correlation still persists. Indeed, we found in n-Cofflx,flx,CaMKII-cre mice by ligand binding assays a 13% increase in AMPAR surface expression in more rostral parts of the hippocampus but no changes in caudal parts. In contrast, changes observed in spine area (36% increase in EM) or PSD length (20%) were more pronounced. Therefore, we find it unlikely that the slight alteration in rostral AMPAR surface expression were sufficient to induce changes in basal synaptic strength. Also see Fig. 5D were normal AMPA/NMDA ratios were observed in mutant mice.

Other comments:
Fig. 1A: analysis of the activity of cofilin (possibly by looking at the amount of phosphorylated cofilin) over this period of time would be helpful.
By using an antibody that specifically recognizes phosphorylated cofilin (both, n-cofilin and ADF) we now provide data showing that in the cortex, the amount of phosphorylated cofilin became strongly reduced during early postnatal development and further declined at P21 and P50 (Fig. 1D of the revised manuscript). At P50, only a small fraction of the remaining cofilin, mainly ADF as n-cofilin levels were strongly reduced at that stage, was seen in the phosphorylated state (also see comments above).

Fig. 1B,C: the amount of n-cofilin reduction in these mice should be quantified. Are there alterations in other forms of cofilin and their activity due to n-cofilin ablation.
In the revised version of the manuscript we now provide quantitative data, indicating that n-cofilin levels were not changed in the cortex and the hippocampus of n-cofflx/flx,CaMKII-cre mice at postnatal day 1 (P1) (Fig. 1C+D of the revised manuscript). At P21, an age at which youngest mutant mice were used for experiments (LTD), n-cofilin was substantially down-regulated. Accordingly, also in hippocampal cultures generated at E16-17 and kept in culture for 21 days n-cofilin levels were strongly reduced (Fig. 2F of the revised manuscript). All other experiments were performed with older mice and we now show that at P50, n-cofilin levels decreased to ~7% in the cortex and ~9% in the hippocampus, respectively (Fig. 1C+D of the revised manuscript). No changes in n-cofilin expression were observed in the cerebellum, a brain region where CaMKII-cre

is not expressed (Fig. 1E of the revised manuscript). We completed our n-cofilin deletion studies by demonstrating that n-cofilin levels were reduced to 2% in synaptosomes of mutant mice (Fig. 1F of the revised manuscript).
Two members of the ADF/Cofilin protein family were shown to be present in the mouse brain. Therefore, functional redundancy of both isoforms is possible and investigating the role of ADF in n-cofilin deficient mice is a consequent continuation of the project. Functional redundancy of both isoforms in synaptic structures requires synaptic localization of ADF. By investigating protein lysates of synaptosomal preparations we show for the first time a synaptic localization of ADF (Fig. 1A of the revised manuscript). The revised manuscript now includes data demonstrating that ADF is up-regulated in the cortex and the hippocampus but not in the cerebellum of n-cofflx/flx,CaMKII-cre mutant mice (Fig. 1C-E of the revised manuscript). By using hippocampal synaptosomes we furthermore demonstrate an elevation of ADF levels also in synaptic structures (Fig. 1F of the revised manuscript).

Fig. 2A,B: these images can not reveal any features of the dendritic spine. At least some large magnification images should be presented to illustrate spine morphology?
We now provide images showing dendritic spines of 2nd order dendritic branches in the hippocampal stratum radiatum region that were used for evaluation of spine density, spine area and spine head/neck ratio (Fig. 2A of the revised manuscript).

Fig.2F: representative images are needed here too.
We now provide images showing dendritic spines of GFP-expressing hippocampal pyramidal cells kept in culture for 21 days that were used to calculate spine width and length (Fig. 2G of the revised manuscript).

Fig. 3E: how was the presynaptic bouton size defined/measured? I could not seem to find the description in the paper.
Measurement of presynaptic bouton size is now included in the respective part of material and methods.

Fig. 4E: the deficit in LTP is very mild. Therefore, I would not spend that much time on this. Also, it is not clear to me why such an inducing protocol was used (4X100Hz)?
As suggested by referee 1 we performed additional LTP experiments inducing LTP by weaker stimulation (1x100 Hz). We now show data demonstrating that in contrast to LTP evoked by strong stimulation (4x100 Hz), LTP that is evoked by weak stimulation (1x100 Hz) is not depending on n-cofilin. Previous studies showed that different forms of LTP can be evoked with different stimulation protocols and that 1x100 Hz stimulation rather evokes early LTP (E-LTP), whereas strong stimulation such as 4x100 Hz induces late LTP (L-LTP). Therefore, n-cofilin seems to be critical for L-LTP but not for E-LTP ñ a surprising and interesting finding that needs to be followed up by further investigations that go beyond the scope of the current study. As suggested by the referee, we downplay the LTP effects in the revised version.

Fig. 4F: again, it is not clear to me why the paired low frequency protocol was used to induce LTD, which is know to elicit a complex form of LTD in terms of both the induction and expression mechanisms. Is this LTD NMDAR-dependent?
The paired-pulse LFS (PPLFS) protocol is a stronger protocol compared to the classical LFS one. It can induce LTD even in hippocampal slices from adult animals, where LFS alone is unlikely to be as effective. In our experimental conditions, little or no LTD could be obtained with a classical non-paired LFS protocol in hippocampal slices from WT mice, while the paired protocol was able to consistently induce LTD in most slices. In rats, requirement for NMDA receptor activation in PPLFS-induced LTD is developmentally regulated. Although PPLFS is effective in inducing LTD both in young and in adult animals, PPLFS-induced LTD is NMDA receptor-dependent in young animals (<50 days) but NMDA receptor-independent in adults (12-15 weeks). (Kemp, 2000). In mice, however, NMDA receptor-dependency of PPLFS was not fully addressed yet. Some authors added NMDA receptor blocker D-AP5 trying to exclude a possible NMDA receptor-dependent component of the LTD induced by this protocol (Alarcon, 2004; Li, 2007; Kleppisch, 2001). In our study, we decided not to use D-AP5 in order to avoid any a priori assumption on the possible source of differences in the responses obtained in KO mice with respect to WT animals. Indeed, further electrophysiological investigations will be the target of future studies but it is not in the scope of the current manuscript.

Fig. 4E,F: given that the LTP/LTD changes, one can imagine that it may be possible to induce LTD in these mice if different protocols (i.e. different frequencies) were used.
We agree with the referee that LTD deficits in n-cofilin KO mice is an important issue. Indeed, it has been shown that low-frequency protocols classically used for inducing LTD can evoke LTP under certain circumstances (see review by Habib and Dringenberg, 2010). As the molecular mechanisms responsible for LFS-LTP seem to be different to those of "classic" LTP or LTD, it canít be assured that the molecular requirements for LTD induction under any protocol are correctly maintained in n-cofilin KO mice. Actually, n-cofilin could be one of the molecular determinants for the switch between "classic" LTD and LFS-LTP. In this line of evidences, Kleppisch et al. (2001) discovered that, in the absence of the protein G q, the NMDA receptor-independent LTD induced by a paired-pulse LFS protocol changes into an LTP, in a way very similar to what we observed in our n-cofilin ko mice (Kleppisch, 2001). We are aware that dissecting out such issues will be of interest, however, while this KO model will be extensively analyzed in future studies, this is beyond the scope of this first description.
What about NMDAR functionality in these mice, although their surface expression is not altered?
See response above to referee 1. Second the data shown in Fig. 5D suggest that overall functionality of NMDAR is preserved.

Changes in the lateral movement in AMPARS are important and interesting, but ultimately, receptor internalization may be needed to have sustained LTD. What about AMPAR insertion/internalization properties following LTD induction in these mice?

This is an interesting point made by the referee. At this juncture we honestly cannot exclude that internalization is affected by n-cofilin. To show this is technically non trivial and would have been beyond the time frame of the revision. We are aiming to perform such studies in the follow up work on n-cofilin in brain. In the revised version we have mentioned in the discussion that receptor trafficking might play a role as well and that we have not yet addressed this issue.

Referee #3 (Remarks to the Author):

I only have a few minor comments/questions.
The authors demonstrate a causal relationship between n-cofilin-mediated AMPA receptor movement and plasticity/learning (page 2, line 10; page 4, line 5). However, what they showed is a correlation. These statements should be re-written.

We revised the manuscript accordingly

Discussion section is too long and should be shortened.
We revised and shortened the discussion.

In Fig. 1D, what is the age of mice? It is helpful to add data showing developmental changes in n-cofilin level in flx/flx, CaMKII-cre mice.
The age of the animals used for investigating deletion of n-cofilin in various brain regions was P70 (Fig. 1D of the previous manuscript). We now include analyses demonstrating the deletion of n-cofilin at various postnatal stages (P1, P21, P50) in the cortex, hippocampus and cerebellum (Fig. 1C-E of the revised manuscript)

In page 16, the authors demonstrate that LTP was impaired in flx/flx, CaMKII-cre mice. But n-cof deletion had small effect on LTP.

We performed different LTP paradigms (see above) to address this issue and we found a more complex dependence of LTP from n-cofilin. With mild stimulation LTP was not altered in n-cofilin mutant mice. We have changed the text and discussion accordingly.

References

Ackermann, M. and Matus, A. (2003) Activity-induced targeting of profilin and stabilization of dendritic spine morphology. Nat Neurosci, 6, 1194-1200.
Akashi, K., Kakizaki, T., Kamiya, H., Fukaya, M., Yamasaki, M., Abe, M., Natsume, R., Watanabe, M. and Sakimura, K. (2009) NMDA receptor GluN2B (GluR epsilon 2/NR2B) subunit is crucial for channel function, postsynaptic macromolecular organization, and actin cytoskeleton at hippocampal CA3 synapses. J Neurosci, 29, 10869-10882.
Albensi, B.C., Oliver, D.R., Toupin, J. and Odero, G. (2007) Electrical stimulation protocols for hippocampal synaptic plasticity and neuronal hyper-excitability: are they effective or relevant? Exp Neurol, 204, 1-13.
Desai, N.S., Rutherford, L.C. and Turrigiano, G.G. (1999) BDNF regulates the intrinsic excitability of cortical neurons. Learn Mem, 6, 284-291.
Fifkova, E. and Van Harreveld, A. (1977) Long-lasting morphological changes in dendritic spines of dentate granular cells following stimulation of the entorhinal area. J Neurocytol, 6, 211-230.
Frank, C.A., Kennedy, M.J., Goold, C.P., Marek, K.W. and Davis, G.W. (2006) Mechanisms underlying the rapid induction and sustained expression of synaptic homeostasis. Neuron, 52, 663-677.
Minichiello, L., Korte, M., Wolfer, D., Kuhn, R., Unsicker, K., Cestari, V., Rossi-Arnaud, C., Lipp, H.P., Bonhoeffer, T. and Klein, R. (1999) Essential role for TrkB receptors in hippocampus-mediated learning. Neuron, 24, 401-414.
Moser, E.I., Krobert, K.A., Moser, M.B. and Morris, R.G. (1998) Impaired spatial learning after saturation of long-term potentiation. Science, 281, 2038-2042.
Oertner, T.G. and Matus, A. (2005) Calcium regulation of actin dynamics in dendritic spines. Cell Calcium, 37, 477-482.
Paradis, S., Sweeney, S.T. and Davis, G.W. (2001) Homeostatic control of presynaptic release is triggered by postsynaptic membrane depolarization. Neuron, 30, 737-749.
Rial Verde, E.M., Lee-Osbourne, J., Worley, P.F., Malinow, R. and Cline, H.T. (2006) Increased expression of the immediate-early gene arc/arg3.1 reduces AMPA receptor-mediated synaptic transmission. Neuron, 52, 461-474.
Roth-Alpermann, C., Morris, R.G., Korte, M. and Bonhoeffer, T. (2006) Homeostatic shutdown of long-term potentiation in the adult hippocampus. Proc Natl Acad Sci U S A, 103, 11039-11044.
Rutherford, L.C., DeWan, A., Lauer, H.M. and Turrigiano, G.G. (1997) Brain-derived neurotrophic factor mediates the activity-dependent regulation of inhibition in neocortical cultures. J Neurosci, 17, 4527-4535.
Shepherd, J.D., Rumbaugh, G., Wu, J., Chowdhury, S., Plath, N., Kuhl, D., Huganir, R.L. and Worley, P.F. (2006) Arc/Arg3.1 mediates homeostatic synaptic scaling of AMPA receptors. Neuron, 52, 475-484.
Star, E.N., Kwiatkowski, D.J. and Murthy, V.N. (2002) Rapid turnover of actin in dendritic spines and its regulation by activity. Nat Neurosci, 5, 239-246.
Turrigiano, G. (2007) Homeostatic signaling: the positive side of negative feedback. Curr Opin Neurobiol, 17, 318-324.

2nd Editorial Decision 18 March 2010

Thank you for submitting your revised manuscript, and sorry for the slight delay in its re-evaluation. The initially most critical referees 1 and 2 have taken another look at it, and while referee 1 now only wants a few more minor changes before acceptance, referee 2 however remains some major reservations, related to issues that had already been raised during the first round of review. In order to come to a fair and informed decision, I thus solicited extra input from referee 1 on those points. To summarize the outcome of these consultations, s/he agreed that some of the criticisms of referee 2 (esp. regarding LTP/LTD measurements and AMPA/NMDA receptor issues) were well taken, but at the same time not key for supporting the current main conclusions and to some extent also beyond the scope of an already quite comprehensive study. I have therefore come to the conclusion that we should be able to publish your study after a last round of minor modification, and am thus returning the study to you once more with the kind invitation to affect the requested modifications in the text, and to briefly respond to the remaining major points in a point-by-point rebuttal (and, where

appropriate, in the text). Once we will have received this final version, I hope we should then be able to proceed with the acceptance of your paper.

I am looking forward to receiving your final version.

Yours sincerely,

Editor
The EMBO Journal

_____

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

I would like to thank the authors for their extensive revision. In the revised manuscript the authors addressed most of my points and included new experimental data to strengthen their findings. The physiological and behavioural description of the forebrain specific n-cofilin knockout mice will have a strong impact on the models of memory formation and their underlying molecular mechanism. This mouse model has a impressive learning impairment and I hope it will be used in many further studies to dissect different forms of learning.

For the final manuscript I would suggest the following minor changes:

1) Introduction (last sentence): Please specify the receptors as 'AMPA' receptors.
2) Results page 7 line 10: Please add as in hippocampal lysates at 'P50'.
3) Discussion page 18 line 7: Please specify receptor mobility as AMPA receptor mobility.
4) Discussion page 18 line 13: Please add a reference for 'can contribute to mental disorders '(ref)'.
5) It remains difficult to identify the age of the mice in individual experiments. I strongly recommend to mention the age of the animals in each figure legend (or better in the figure directly after the genotype.
6) On page 13, the authors refer to ADF as 'ISOFORM' of n-cofilin, which is not correct since they are encoded by two different genes. This has to be corrected before publication.

Referee #2 (Remarks to the Author):

My major concerns are still on the expression and activity of cofilin in these knockout mice. As shown in Figure 1C, D, the expression of ADF is drastically increased in both cortex and the hippocampus. In addition, phospho-cofilin (Figure 1D) is clearly decreased in the cortex although whether it is also altered in the hippocampus is unknown (Figure 1C). What can one then conclude from these results regarding the level of cofilin and its activity? My guess would be impossible to conclude anything at this stage and if so, it would hard for one to interpret the data obtained from these mice.

I share the concern with the reviewer 1 (#5), which was similar to my comment #9, that the properties of NMDA and AMPA receptors should be investigated in more details. The presented data on AMPA/NMDA ratios only addressed the relative amplitudes and does not tell you anything about the channel properties of these receptors. I also agree that the LTP data should be strengthened, particularly the one induced by 4x100Hz (Figure 4F). Given that it is such a long-term recording, a stable basal line is exceptionally important and this was clearly not achieved in the experiment presented (Figure 4F, please note the increasing basal line recordings). And that is why a control pathway should be included here.

Finally, in my view, the LTD data can be strengthened. Because PPL-LTD can be very complex, to know which form of LTD is affected is important.

2nd Revision - authors' response 25 March 2010

Reply to comments by Referee #1:

1) Introduction (last sentence): Please specify the receptors as 'AMPA' receptors.
We revised this part of the manuscript as proposed by the referee. It now says ëAMPA receptorsí.

2) Results page 7 line 10: Please add as in hippocampal lysates at 'P50'.
We revised this part of the manuscript as proposed by the referee.

3) Discussion page 18 line 7: Please specify receptor mobility as AMPA receptor mobility.
We revised this part of the manuscript as proposed by the referee.

4) Discussion page 18 line 13: Please add a reference for 'can contribute to mental disorders '(ref)'.
We slightly changed the wording (see below) to be more precise and we added another reference. The sentence was changed as follows:
In humans for example, changes in n-cofilin activity can are thought to contribute to mental disorders (Bamburg and Wiggan, 2002), and deletion of the cofilin regulator LIMK-1 has been linked to Williams syndrome (Frangiskakis et al., 1996).

5) It remains difficult to identify the age of the mice in individual experiments. I strongly recommend to mention the age of the animals in each figure legend (or better in the figure directly after the genotype.
As proposed by the referee we now specify the ages of all mice used in the experiments in each figure legend.

6) On page 13, the authors refer to ADF as 'ISOFORM' of n-cofilin, which is not correct since they are encoded by two different genes. This has to be corrected before publication.
The referee is very right with this point. We revised the term ‘isoform’ throughout the manuscript.

Reply to comments by Referee #2:

We very much appreciate the consultation of referee #1 by the editor concerning the criticism by referee #2. The current manuscript comprises an enormous body of data and very interesting conclusions on mechanisms of learning, and we believe that the requests by referee #2 are beyond the scope of the presented work and not essential for the conclusions drawn here.
While we understand the general interest of referee # 2 in control of cofilin/ADF activity, we cannot follow the rational of the comments in the context of the manuscript. Referee #2 is concerned about the levels and activity of cofilin in brain. The phorphorylation phenomena we report here (for the first time) is of general interest in the cofilin/ADF field, but has no implication for the interpretation of the data in our manuscript. Frankly, the relevance of cofilin/ADF phosphorylation in the brain is a completely open question ñ while phosphorylation in cultured cells was shown to be an important mechanism of control, this has to await genetic experiments in the mouse, which are under way in our laboratory, but not relevant for the conclusions made in the manuscript. In the n-cofilin mutant used here there is no expression of n-cofilin and ADF does not compensate. Why referee #2 continues to argue about n-cofilin expression and phosphorylation of a molecule, which in fact is deleted in our mouse model is beyond our comprehension.
Second, referee #2 now moves LTP to the center of attention. We would like to point out that based on the request by referee #2 we have added a completely new set of data in the revised version by including a novel LTP paradigm. Two different paradigms of LTP are now presented, compared and left to the reader for discussion. While electrophysiology is an important aspect of our manuscript,

we do not intend to downplay the other interesting aspects of Cofilin and actin in learning and memory on the expenses of an 'electrophysiology manuscript', that would then be more tailored for a strictly neuronal journal. The extended electrophysiology will certainly be part of a future manuscript for a more specialized journal.

Bamburg, J.R. and Wiggan, O.P. (2002) ADF/cofilin and actin dynamics in disease. Trends Cell Biol, 12, 598-605.
Frangiskakis, J.M., Ewart, A.K., Morris, C.A., Mervis, C.B., Bertrand, J., Robinson, B.F., Klein, B.P., Ensing, G.J., Everett, L.A., Green, E.D., Proschel, C., Gutowski, N.J., Noble, M., Atkinson, D.L., Odelberg, S.J. and Keating, M.T. (1996) LIM-kinase1 hemizygosity implicated in impaired visuospatial constructive cognition. Cell, 86, 59-69.

