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Bidirectional integrative regulation of Cav1.2 calcium channel by microRNA miR-103: role in pain.

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(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 14 September 2010

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

As you can see, while the referees find the topic interesting they also raise major concerns with the analysis that I am afraid preclude publication here at this stage. The referees find that many of the conclusions are at present not sufficiently supported by the data presented and that much further data and extension of the work would be needed to consider publication here. Given that concerns are raised regarding many of the key findings, I am afraid that I cannot offer to commit to a revised version and I therefore see no other choice, but to reject the manuscript at this stage. However, given the potential interest in the findings reported I am not excluding to consider a new submission on this topic should you be able to extend the analysis along the lines indicated by the referees and to provide stronger data in support of the conclusions. For resubmissions we consider the novelty of data at the time of resubmission and may, if needed, bring in new referee(s)

I am sorry that I cannot be more positive at this stage, but I hope that you find the referees comments helpful.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

In the present manuscript, Favereaux et al. explore the role of microRNA miR-103 in pain. The authors show that miR-103 knockdown results in hypersensitivity to pain, and furthermore identify three subunits of the L-type Ca channel Cav1.2 as putative direct targets of miR-103. Describing a function for a specific microRNA in the regulation of neuropathic chronic pain is novel and important, and this study represents a good starting point. However, the presented dataset is too preliminary to provide conclusive evidence for the physiological significance of the described microRNA-based regulation.

Specific concerns:

1. The luciferase assays (Fig. 1c) to demonstrate a direct functional interaction between miR-103 and Cav1.2 subunits are far from convincing. To demonstrate a direct interaction, the authors should mutate the identified seed regions in the 3'UTR of the targets and assess the effects of miR-103 overexpression. In addition, knockdown of miR-103 should be performed to show an effect of endogenous miR-103, again in the context of the wt and mutant reporters. Luciferase RNA levels should be determined to test whether the observed regulation occurs at the level of translation or mRNA stability. Finally, performing these experiments in neurons would substantiate these findings.

2. Immunocytochemistry is used to show regulation of the endogenous Cav1.2 protein by miR-103. This time, miR-103 loss-of-function was performed, but the appropriate control (e.g. scrambled LNA) is missing. To obtain a more quantitative view of the effect on protein expression, the authors should attempt to perform Western blotting of Cav1.2 upon miRNA overexpression/inhibition.

3. In all functional experiments (Fig. 4, 5), no controls are presented to demonstrate the specificity of the microRNA inhibitors used.

4. The data intended to show dendritic localization of miR-103 (Fig. 6) is not conclusive. To suggest a "local control of Cav1.2 synthesis that could underlie changes in its subcellular distribution in pain conditions" based on this data is clearly an overstatement and should be omitted (p. 6). Similarly, stating that localization near the nucleus indicates "miRNAs that were being processed and exported toward the cytoplasm" without any further mechanistic data is not appropriate.

5. The finding that induction of pain decreases endogenous miR-103 levels in vivo (Fig. 7) is very interesting and could potentially provide a mechanism whereby pain stimuli can overcome the inhibitory effect of miR-103. More biochemical experiments are needed to substantiate this point.

Referee #2 (Remarks to the Author):

Using bioinformatics approaches Favereaux and coworkers find that the 3`UTRs of the calcium channel subunits CaV1.2 a1C, beta1, and alpha2delta-1 contain a putative interaction site for a single microRNA (miR-103), and that coexpression of miR-103 with a reporter gene fused to each of the 3´UTRs in COS cells specifically inhibit translation. Then they use immunocytochemical analysis and fluorescent calcium recording to analyze whether overexpression and knockdown of miR-103 alters CaV1.2 expression and calcium signals in cultured spinal cord neurons. Finally they show by intrathecal injection of miR-103KD and miR-103 that expression of the three CaV subunits and pain hypersensitivity inversely correlates with miR-103 expression.

Together with the authors previous findings showing the important role of CaV1.2 regulation in neuropathic pain, the current findings suggest a possible regulatory mechanism. This is an important finding with high potential relevance for clinical application. Unfortunately the impact of the study is severely hampered by poor quality and/or documentation of the immunocytochemical experiments (Fig. 2, 3, and 6). Moreover, some of the claims in the article are not sufficiently supported by experimental data.

Quality of data:

In Figure 2A it is not clear how the labeling intensity was analyzed. Moreover, the low n numbers suggest that only a single experiment has been performed and thus the validity of the statistical significance is questionable. The number of individual cells measured does not represent true n numbers. Also, the quality of the example images is not convincing.

Fig. 3 The used analysis of the cytoplasm/membrane ratio is inadequate to conclude that CaV1.2 membrane targeting was not affected. First of all the presence of large amounts of CaV immunofluorescence in the cytoplasm is unexpected for the native channels and thus worrying. The channels should primarily be present in the membrane. How was the specificity of the antibodies tested in the cell system used? Secondly, the authors do not present any controls demonstrating that the assay indeed detects changes in membrane targeting. Knockdown of the beta subunit should give this result and thus would make a good control experiment.

Fig. 6A-D "The signal was weaker in the dorsal horn ipsilateral to the nerve injury..." The difference shown in the example image (presumable the best the authors obtained) is not convincing. How often has this observation been made? Can this be quantified?

Fig. 6E,F Without controls and quantification the presence of some particles scattered all over the thinsection (nucleus, cytoplasm, plasmamembrane, dendrite) is not conclusive and any conclusions about processing of miRNA in the cytoplasm or local (presumably dendritic) control of CaV1.2-LTC are totally overstated.

Interpretation:

In the abstract the authors claim that "miR-103 simultaneously regulates the expression of the three subunits forming CaV1.2-LTC in a novel integrative regulation"; and again in the results, "These results confirm that the integrative regulation of CaV1.2-LTC by miR-103 is bidirectional...". What they actually show is that miR-103 is capable of regulating three LTC subunits in a reporter gene assay. In neurons they only examined and at best "confirmed" the regulation of a single subunit (CaV1.2 a1C). Thus, the conclusions above are not supported by the experimental evidence.

How specific is the miR103 action on the three calcium channel subunits tested? With the exception of CaV1.3 in the reporter gene assay this question has not been addressed. Without such information the conclusion that the intrathecal injection of miR103 exerts its effects by downregulation of CaV1.2 calcium channels cannot be reached. A broader bioinformatic screen and qRT-PCR analysis (at least including all known CaV subunit genes) and possibly some experiment addressing the specificity of the intrathecal application (e.g. loss of effects in knockout mice) would be necessary to demonstrate specificity and a direct causal relationship of miR-103 induced downregulation of CaV1.2/b1/a2d1 subunits and increased pain relieve.

Minor:

p.3, line 8 "Neuronal CaV1.2-LTC comprises three subunits: CaV1.2, a2d1, b1..." this is not necessarily the case. Depending on the expression pattern in a given neuron CaV1.2 can combine with any auxiliary subunit.

p.4, last line "...due to ion influx through several types of voltage-dependent calcium channels..." This needs to be examined experimentally by measuring the calcium signals while blocking currents with cadmium/lanthanum. Without such an experiment this is only an assumption.

Fig. 1A No mention in legend and/or text that beta1 has two conserved miR103 binding sites

Fig. 2 Results/legend, state the type of neuron used

Fig. 4 and corresponding text in results:
Equating up- or downregulation of CaV1.2 with an increase or decrease of neuronal excitability is problematic as excitability has not been tested directly (e.g. by current clamp recordings). CaV1.2 activation can also be downstream of excitability.

Fig. 5 The caption states that "MiR-103 overexpression ... induces hypersensitivity to pain". However the experiment shows that knockdown reduces the pain threshold. Also provide information on the time course of the experiment.

Referee #3 (Remarks to the Author):

The authors provide potentially interesting findings on the regulation of the calcium channel Cav1.2 by the microRNA miR-103. They show that miR-103 suppresses expression of three subunits of Cav1.2 in vitro, and they provide evidence that miR-103 expression is decreased in the spinal dorsal horn after peripheral nerve injury. Strikingly, intrathecal administration of miR-103 reversed mechanical hypersensitivity induced by nerve injury. However, the authors need to determine whether miR-103 administration affects responses to mechanical, thermal and chemical stimulation in non-nerve injured animals. Also, does miR-103 administration affect cold or heat hypersensitivity after nerve injury, or is its effect restricted to mechanical hypersensitivity?

Resubmission 21 April 2011

Responses to reviewers

Referee #1 (Remarks to the Author):

In the present manuscript, Favereaux et al. explore the role of microRNA miR-103 in pain. The authors show that miR-103 knockdown results in hypersensitivity to pain, and furthermore identify three subunits of the L-type Ca channel Cav1.2 as putative direct targets of miR-103. Describing a function for a specific microRNA in the regulation of neuropathic chronic pain is novel and important, and this study represents a good starting point. However, the presented dataset is too preliminary to provide conclusive evidence for the physiological significance of the described microRNA-based regulation.

Specific concerns:

1. The luciferase assays (Fig. 1c) to demonstrate a direct functional interaction between miR-103 and Cav1.2 subunits are far from convincing. To demonstrate a direct interaction, the authors should mutate the identified seed regions in the 3'UTR of the targets and assess the effects of miR-103 overexpression.

This is done now. We did 3'UTR seed region deletion in the 3 luciferase reporters and assessed the effects of miR-103 overexpression. As expected, it results in a complete abolition of miR103 regulation, demonstrating a direct interaction between miR-103 and Cav1.2 subunits (figure 1C). These results are described page 4 lines 13-16.

In addition, knockdown of miR-103 should be performed to show an effect of endogenous miR-103, again in the context of the wt and mutant reporters.

We knocked-down endogenous miR-103 using miR-103 inhibitors and we observed a moderate but significant upregulation of luciferase, indicating that endogenous miR-103 has an effect and that regulation of Cav1.2-LTC reporter by miR-103 is bidirectional (figure 1C). These results are described page 4 lines 16-20.

Luciferase RNA levels should be determined to test whether the observed regulation occurs at the level of translation or mRNA stability.

We designed primers to PCR amplify luciferase mRNA and quantified by qRT-PCR the effect of miR-103 on Cav1.2-LTC luciferase reporter mRNAs. Our experiments clearly indicate that miR-103 induces target mRNA decay for the 3 Cav1.2-LTC reporters (figure 1E and page 4 lines 22-25). Moreover, we confirmed these *in vitro* results *in vivo* with native miR-103 targets; namely, Cav1.2-LTC subunits' mRNAs (figure 7A).

Finally, performing these experiments in neurons would substantiate these findings.

We agree with the reviewer and we tried to performed luciferase experiments in spinal cord neurons. Unfortunately neurons (especially primary culture from spinal cord) are well known to be hard to transfect. Actually we confirmed that transfection efficiency was too low to produce reliable data. We determine that transfection efficiency is around 1:5000. This technical problem is not uncommon when performing biochemistry on neuronal cultures. As an alternative, we used immunocytochemistry techniques to demonstrate that miR-103 is an endogenous regulator of Cav1.2-LTC in neurons (figure 3).

2. Immunocytochemistry is used to show regulation of the endogenous Cav1.2 protein by miR-103. This time, miR-103 loss-of-function was performed, but the appropriate control (e.g. scrambled LNA) is missing.

As requested by reviewer, we performed scrambled LNA control experiments. Results are presented in figure 2B, 2D and 2F and commented page 5 lines 6-7. Briefly, scrambled LNA had no effect on Cav1.2-LTC expression.

To obtain a more quantitative view of the effect on protein expression, the authors should attempt to perform Western blotting of Cav1.2 upon miRNA overexpression/inhibition.

We have tried to perform these experiments but again due to the very low transfection efficiency of primary spinal cord neuron cultures we, as others, were unable to produce consistent biochemistry data. However, immunocytochemistry experiments enable us to quantify the amount of Cav1.2-LTC channels that are really present at the membrane and therefore the “active” part of the translated Cav1.2-LTC. The western-blot analysis would have told us the level of translation of every subunit but not the amount of functional Cav1.2-LTC at the neuronal membrane.

3. In all functional experiments (Fig. 4, 5), no controls are presented to demonstrate the specificity of the microRNA inhibitors used.

We extended our calcium imaging experiments with scrambled LNA to confirm the specificity of the microRNA inhibitors used. Results are presented in figure 4B and commented page 6 lines 3-4. Briefly, scrambled LNA had no effect on calcium transients. The reviewer’s comment made us realize that our previous legend of the experiment depicted in figure 5 was not clear. In fact we did two groups of animals, one injected with a miR-103 inhibitor and one with a scrambled LNA as inhibitor control. The previous legend “mismatch inhibitor” is now changed to “scrambled miRNA inhibitor”.

4. The data intended to show dendritic localization of miR-103 (Fig. 6) is not conclusive. To suggest a "local control of Cav1.2 synthesis that could underlie changes in its subcellular distribution in pain conditions" based on this data is clearly an overstatement and should be omitted (p. 6). Similarly, stating that localization near the nucleus indicates "miRNAs that were being processed and exported toward the cytoplasm" without any further mechanistic data is not appropriate.

We agree that our conclusions about electron microscopy experiments can be considered as overstatements, therefore we replaced them by this comment: “the dendritic localization of miR-103 and its possible role in local translation remain to be studied.” If needed, we can provide alternative micrographs.

5. The finding that induction of pain decreases endogenous miR-103 levels in vivo (Fig. 7) is very interesting and could potentially provide a mechanism whereby pain stimuli can overcome the inhibitory effect of miR-103. More biochemical experiments are needed to substantiate this point.

We agree that endogenous miR-103 expression may play an important role in pain induction and therefore we used state-of-art method to analyze miR-103 levels: we did qRT-PCR analysis of the mature miR-103, not primary- nor pre-miRNA (figure 7). In addition, we mimiced the onset of pain by applying exogenous miR-103 in naive rats (figure 5). The ultimate demonstration of the key role of miR-103 would be a loss of effect in miR-103 KO mice. Unfortunately, this miR-103 KO mouse is not yet available; therefore we have started to produce it but obviously, we will not have a stable

mouse line to perform experiments before two years. According to the potential impact on pain therapeutic and the finding of a novel integrative silencing of multiple target by single miRNA, we think that our work might be published shortly.

Referee #2 (Remarks to the Author):

Using bioinformatics approaches Favereaux and coworkers find that the 3`UTRs of the calcium channel subunits CaV1.2 a1C, beta1, and alpha2delta-1 contain a putative interaction site for a single microRNA (miR-103), and that coexpression of miR-103 with a reporter gene fused to each of the 3'UTRs in COS cells specifically inhibit translation. Then they use immunocytochemical analysis and fluorescent calcium recording to analyze whether overexpression and knockdown of miR-103 alters CaV1.2 expression and calcium signals in cultured spinal cord neurons. Finally they show by intrathecal injection of miR-103KD and miR-103 that expression of the three CaV subunits and pain hypersensitivity inversely correlates with miR-103 expression.

Together with the authors previous findings showing the important role of CaV1.2 regulation in neuropathic pain, the current findings suggest a possible regulatory mechanism. This is an important finding with high potential relevance for clinical application. Unfortunately the impact of the study is severely hampered by poor quality and/or documentation of the immunocytochemical experiments (Fig. 2, 3, and 6). Moreover, some of the claims in the article are not sufficiently supported by experimental data.

Quality of data:
In Figure 2A it is not clear how the labeling intensity was analyzed. Moreover, the low n numbers suggest that only a single experiment has been performed and thus the validity of the statistical significance is questionable. The number of individual cells measured does not represent true n numbers. Also, the quality of the example images is not convincing.
Fig. 3 The used analysis of the cytoplasm/membrane ratio is inadequate to conclude that CaV1.2 membrane targeting was not affected. First of all the presence of large amounts of CaV immunofluorescence in the cytoplasm is unexpected for the native channels and thus worrying. The channels should primarily be present in the membrane. How was the specificity of the antibodies tested in the cell system used? Secondly, the authors do not present any controls demonstrating that the assay indeed detects changes in membrane targeting. Knockdown of the beta subunit should give this result and thus would make a good control experiment.

We performed a completely new set of immuno-labeling experiments and we hope that the quality of images is now more convincing. In particular, for Cav1.2 immunofluorescence we now provide new confocal images confirming that the native channel is primarily present at the membrane (figure 2A). More details about how labeling intensity was analyzed are provided in materials and methods page 13 lines 13-16. About the n numbers, we now provide in the figure legend (page 19 lines 8-9) the number of individual experiments (4) and the number of quantified neurons (ranging from 11 to 19). About the trafficking assay, we now provide as supplemental data a positive control demonstrating that our assay can actually detect changes in membrane targeting. Briefly, GABAB1 and GABAB2 are two subunits of the heterodimeric GABAB Receptor which is trafficked to the membrane only when the two subunits are associated. When GABAB1 subunit is expressed alone its localization is mainly cytoplasmic, while co-expression of GABAB1 and GABAB2 induced membrane expression of GABAB Receptor. This is shown and quantified in supplementary figure 4.

Fig. 6A-D "The signal was weaker in the dorsal horn ipsilateral to the nerve injury..." The difference shown in the example image (presumable the best the authors obtained) is not convincing. How often has this observation been made? Can this be quantified?

We performed a new set of 5 hybridization experiments and quantified the difference in miR-103 labeling intensity between ipsi and contralateral sides. As shown in figure 4A,B and described in results section (page 6 line 26 – page 7 line 1) miR-103 is less expressed ipsilateral to the lesion. The example image has been changed and its quality enhanced.

Fig. 6E,F Without controls and quantification the presence of some particles scattered all over the thinsection (nucleus, cytoplasm, plasmamembrane, dendrite) is not conclusive and any conclusions

about processing of miRNA in the cytoplasm or local (presumably dendritic) control of CaV1.2-LTC are totally overstated.

We agree that our conclusions about electron microscopy experiments can be considered as overstatements, therefore we replaced them by this comment: “the dendritic localization of miR-103 and its possible role in local translation remain to be studied.” If needed, we can provide alternative micrographs.

Interpretation:

In the abstract the authors claim that "miR-103 simultaneously regulates the expression of the three subunits forming CaV1.2-LTC in a novel integrative regulation"; and again in the results, "These results confirm that the integrative regulation of CaV1.2-LTC by miR-103 is bidirectional...". What they actually show is that miR-103 is capable of regulating three LTC subunits in a reporter gene assay. In neurons they only examined and at best "confirmed" the regulation of a single subunit (CaV1.2 a1C). Thus, the conclusions above are not supported by the experimental evidence.

To confirm that miR-103 is capable of regulating the three Cav1.2-LTC subunits we now extended our immuno-labeling experiments to the a2d1 and b1 subunits. We show that miR-103 knockdown or over-expression induces respectively an up-regulation or a down-regulation of all Cav1.2-LTC subunit (Figure 2C, D, E, F and results section page 5 lines 1-10). Moreover, in the *in vivo* experiment we show by qRT-PCR that exogenous miR-103 applications induce a down-regulation of all Cav1.2-LTC subunit mRNAs (figure 7A). Therefore, we now demonstrate that experimentally modulating miR-103 in neuron cultures impacts all Cav1.2-LTC subunit expression.

How specific is the miR103 action on the three calcium channel subunits tested? With the exception of CaV1.3 in the reporter gene assay this question has not been addressed. Without such information the conclusion that the intrathecal injection of miR103 exerts its effects by downregulation of CaV1.2 calcium channels cannot be reached. A broader bioinformatic screen and qRT-PCR analysis (at least including all known CaV subunit genes) and possibly some experiment addressing the specificity of the intrathecal application (e.g. loss of effects in knockout mice) would be necessary to demonstrate specificity and a direct causal relationship of miR-103 induced downregulation of CaV1.2/b1/a2d1 subunits and increased pain relieve.

We agree that miR-103 action specificity is an important question. Therefore, in this new version of the manuscript we performed a broader bioinformatics analysis to identify miRNA target sites in all voltage gated calcium channels. As shown in supplementary table 2, except Cav1.2-LTC, no other voltage gated calcium channel is targeted by miR-103. In addition, by immunocytochemistry we demonstrated that miR-103 over-expression or knockdown has no effect on the expression of two other calcium channels (Cav2.2 and Cav3.2, supplementary figure 3).

Minor:

p.3, line 8 "Neuronal CaV1.2-LTC comprises three subunits: CaV1.2, a2d1, b1..." this is not necessarily the case. Depending on the expression pattern in a given neuron CaV1.2 can combine with any auxiliary subunit.
We agree that the neuronal Cav1.2-LTC is not necessary composed of a2d1 and b1. Nevertheless, combinatorial subunit associations are still poorly understood and the major form of neuronal Ca1.2-LTC comprises a2d1, the ubiquitous axdy subunit that is essential for the therapeutic effect of anti-allodynic drugs (pregabalin and gabapentin). The main neuronal Cav1.2-LTC also comprises the neuron-specific b1 subunit.

p.4, last line "...due to ion influx through several types of voltage-dependent calcium channels..." This needs to be examined experimentally by measuring the calcium signals while blocking currents with cadmium/lanthanum. Without such an experiment this is only an assumption.
We have modified the text to “…due in part to ion influx through several types of voltage-dependent calcium channels including Cav1.2-LTC” and in addition, using siRNA against Cav1.2 we demonstrate that the decrease in calcium transients was equivalent to that induced by miR-103.

Fig. 1A No mention in legend and/or text that beta1 has two conserved miR103 binding sites.

We have modified both text (page 3 lines 19-20) and legend (page 18 line 15) to mention that beta 1 has two conserved miR103 binding sites.

Fig. 2 Results/legend, state the type of neuron used.
We now state in both text (page 4 line 29) and legend (page 19 line 1) that we used primary spinal neuron cultures.

Fig. 4 and corresponding text in results:
Equating up- or downregulation of CaV1.2 with an increase or decrease of neuronal excitability is problematic as excitability has not been tested directly (e.g. by current clamp recordings). CaV1.2 activation can also be downstream of excitability.

In previous papers, we already addressed this question, thus we added these references to the new version of the manuscript: “Expression of Cav1.2-LTC is determinant for the dorsal horn neuron’s firing properties (Fossat et al, 2010; Morisset & Nagy, 1999). Hence, our results strongly suggest that Cav1.2-LTC regulation by miR-103 controls neuron excitability *in vitro*.”

Fig. 5 The caption states that "MiR-103 overexpression ... induces hypersensitivity to pain". However the experiment shows that knockdown reduces the pain threshold. Also provide information on the time course of the experiment.
We apologize for the typing error in Figure 5, the caption has been corrected to “MiR-103 knockdown in naïve animals induces hypersensitivity to pain.” and a time course for the experiment is now provided.

Referee #3 (Remarks to the Author):

The authors provide potentially interesting findings on the regulation of the calcium channel Cav1.2 by the microRNA miR-103. They show that miR-103 suppresses expression of three subunits of Cav1.2 in vitro, and they provide evidence that miR-103 expression is decreased in the spinal dorsal horn after peripheral nerve injury. Strikingly, intrathecal administration of miR-103 reversed mechanical hypersensitivity induced by nerve injury. However, the authors need to determine whether miR-103 administration affects responses to mechanical, thermal and chemical stimulation in non-nerve injured animals. Also, does miR-103 administration affect cold or heat hypersensitivity after nerve injury, or is its effect restricted to mechanical hypersensitivity?

This neuropathic model was well described in the literature and is known to exhibit cold hypersensitivity. Therefore, we now evaluated cold allodynia in neuropathic animals injected with either miR-103 or mutated miR-103. As a result, miR-103 administration partially abolished cold hypersensitivity (Figure 7 and results section page 7 lines 21-27).

2nd Editorial Decision 16 June 2011

Thank you for submitting your manuscript to the EMBO Journal. This is an invited resubmission of MS 75578 that was rejected post review last year. I asked the original three referees to review the manuscript and both referees #1 and 3 were available to do so. I have now received their comments back and they are listed below. As you can see, both referees appreciate the introduced changes and support publication here. They raise a number of relative minor concerns that should not involve too much additional work to resolve. I would therefore like to invite you to submit a suitably revised manuscript that addresses the last issues.

Once we receive the revised version, we will proceed with the acceptance of the study for publication here.

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://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to seeing the final version.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

the authors have sufficiently addressed most of my concerns. However, a few points still have to be clarified before the paper can be published:

1. In Figure 1C, the amount of seed-mut-miR-103 used has to be stated. For the seed-mut-3'UTR, the basal (w/o microRNA) level is missing. How much of miR-103 was used in this case? The scrambled LNA control is still missing.

2. In Figure 1E, is the seed-mut-miR-103 used as control?

3. In the representative images of Fig.2, transfected neurons should be shown alongside non-transfected neurons to be able to judge the differences in signal intensities between the conditions (as in Fig. 3).

4. In Fig. 2B, D and F, should scrambled miRNA actually read scrambled LNA?

Referee #3 (Remarks to the Author):

The authors have included additional experiments that have sufficiently addressed my original concerns.

I would suggest that they consider two points

1) Fig. 6A shows a sizable increase in miR103 in the ventral horn on the ipsilateral side. The authors should indicate whether this is a consistent finding, and if so this should be quantified, reported and discussed.

2) The writing in the Discussion is loose in a number of places. For example, in the third line the authors write "are few demonstrations of miRNA involvement in central nervous system disorders". But a quick search through pubmed shows there are hundreds papers on this topic - 'miRNA Alzheimer' as a search phrase gives 44 on its own. Therefore the authors need to state more clearly what they mean.

Another example, the authors state "miR-103 has an important impact on neuron excitability". Given that they have not tested 'excitability' but only have done some measurements of intracellular calcium concentration, they need to be clearer here as well.

1st Editorial Decision 04 July 2011

Responses to reviewers

Referee #1 (Remarks to the Author):

The authors have sufficiently addressed most of my concerns. However, a few points still have to be clarified before the paper can be published:

1. In Figure 1C, the amount of seed-mut-miR-103 used has to be stated. For the seed-mut-3'UTR, the basal (w/o microRNA) level is missing. How much of miR-103 was used in this case? The scrambled LNA control is still missing.
We agree with the reviewer that some information is missing for luciferase assay (Figure 1C).The amount of seed-mut-miR-103 used was 750ng, it is now stated in the figure. For the seed-mut-3'UTR, we now present the basal level without miR-103 (labeled: "seed-mut-3'UTR basal"). In the very same experiment, miRNA over-expression was performed with 750ng of miR-103, it is now stated in legend (labeled: "seed-mut-3'UTR-miR-103 – 750ng"). Scrambled LNA control, actually named "scrambled miRNA inhibitor", is now presented as control condition of the miR-103 knockdown experiment.

2. In Figure 1E, is the seed-mut-miR-103 used as control?
Yes, it is. To quantify mRNA decay induced by miR-103 we used seed-mut-miR-103 as control. It is now stated in Figure 1E and in the corresponding text in the result section (page 4 lines 23-24).

3. In the representative images of Fig.2, transfected neurons should be shown alongside non-transfected neurons to be able to judge the differences in signal intensities between the conditions (as in Fig. 3).
This is done now; we added representative images of non-transfected neurons alongside to miR-103 over-expressing neurons to enable readers to compare signal intensities between the conditions (Figure 2).

4. In Fig. 2B, D and F, should scrambled miRNA actually read scrambled LNA?
"Scrambled miRNA" should read "scrambled miRNA inhibitor" (as used in Figures 4B and 5) which is actually a LNA molecule with a scrambled sequence. To be consistent with Figure 4B and 5, we changed "scrambled miRNA" into "scrambled miRNA inhibitor".

Referee #3 (Remarks to the Author):

The authors have included additional experiments that have sufficiently addressed my original concerns.

I would suggest that they consider two points

1) Fig. 6A shows a sizable increase in miR103 in the ventral horn on the ipsilateral side. The authors should indicate whether this is a consistent finding, and if so this should be quantified, reported and discussed.
We agree with the reviewer that the Figure 6A shows a sizeable increase of miR-103 in the ventral horn of the ipsilateral side. However, this finding is not consistent in all the pictures used for the analysis and sometimes an increase was seen on the contralateral side. Therefore, we systematically quantified the miR-103 labeling in both the dorsal and the ventral horn. It appeared that there is no statistical difference in miR-103 expression between ipsilateral and contralateral sides of the ventral horn (paired t-test, p=0.156, n=5).

2) The writing in the Discussion is loose in a number of places. For example, in the third line the authors write "are few demonstrations of miRNA involvement in central nervous system disorders". But a quick search through pubmed shows there are hundreds papers on this topic - 'miRNA Alzheimer' as a search phrase gives 44 on its own. Therefore the authors need to state more clearly what they mean.
We agree with the reviewer that this part of the discussion needed rewriting; in addition, more references are now provided. Page 8 lines 4-10: "The implication of miRNAs in pathological mechanisms is increasingly documented and so are the demonstrations of miRNA involvement in central nervous system disorders. Recently, studies have suggested that deregulated miRNAs expression could contribute to Alzheimer's Disease (for review see (Satoh, 2010)) or Parkinson's Disease (Kim et al, 2007; Wang et al, 2008). The expression of miRNAs is modulated in the context of pain (Aldrich et al, 2009; Bai et al, 2007; Kusuda et al, 2011; Poh et al, 2011; von Schack et al, 2011) but so far the mechanisms involved and the physiological impact remained scarcely explored."

Another example, the authors state "miR-103 has an important impact on neuron excitability". Given that they have not tested 'excitability' but only have done some measurements of intracellular calcium concentration, they need to be clearer here as well.
We agree with the reviewer that our calcium imaging experiments did not directly assess the excitability of spinal neurons. Actually, we showed that regulating Cav1.2 through miR-103 had an important impact on calcium influx through voltage-gated calcium channels, which we showed previously to be an important factor for regulating neuronal excitability. So, we reformulated the sentence on page 8, line 15-18: “Our calcium imaging experiments indicated that regulating Cav1.2 through miR-103 has an important impact on calcium influx through voltage-gated calcium channels, suggesting a significant role in control of membrane potential and firing patterns of spinal neurons (Derjean et al., 2003). This control may constitute a dynamic way of modulating neuronal excitability without altering transcriptional level.”

