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Systemic low molecular weight drug delivery to pre-selected neuronal regions

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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 10 December 2010

Thank you for the submission of your manuscript "Systemic low molecular weight drug delivery to pre-selected neuronal regions" to EMBO Molecular Medicine. We have now finally heard back from the three referees whom we asked to evaluate your manuscript. You will see that they find the topic of your manuscript potentially interesting. However, they also raise significant concerns about the study, which should be addressed in a major revision of the manuscript.

You will see that reviewer #1 is positive about the study, while reviewers #2 and #3 are more reserved. In particular, reviewer #2 feels that it is crucial to much better demonstrate the locally restricted modulation of the BBB and iBRB and makes suggestions how this could be achieved. Importantly, reviewer #3 highlights the issues of vector toxicity and innate immune responses to the vector and we feel that experiments addressing these issues would significantly improve the clinical impact of the findings.

Given the balance of these evaluations, we feel that we can consider a revision of your manuscript if you can convincingly address the issues that have been raised within the space and time constraints outlined below.

Revised manuscripts should be submitted within three months of a request for revision. They will otherwise be treated as new submissions, unless arranged otherwise with the editor.

I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1 (Remarks to the Author):

The concept of this mansuscript is both novel and very interesting. The data presented is really outstanding and a well written manuscript. Overall, very good work with minor revisions noted. The authors suggest a basic science application for the work, but focus on a therapeutic line of investigation and this should be altered in text.

There is no discussion of inducible systems that may actually be used in humans. My understanding is that the tet elements would be too immunogenic for human use.

The authors also discuss AAV stability in neural tissue. The efficacy of their transient disruption depends on aav transduction of the endothelial cells. A discussion on vector persistence in those cells is needed.

Finally, there is no discussion on other methods of bbb disruption. Why is this method better than focused ultra sound? or other methods- just a greater discussion will suffice

Referee #2 (Remarks to the Author):

1. General comment - The overall focus of this study is to describe a strategy to selectively open localized regions of the blood brain barrier (BBB) and inner blood-retinal barrier (IRRB) using an AAV9 serotype capsid expressing an AAV2 expression cassette encoding a deoxycline-inducible promoter driving an shRNA targeting claudin-5, an important component of the BBB and iRRB. By introducing the vector in a selected region of the brain or retina, if the vector is successful in suppressing claudin-5 levels in the microvasculature of these selected regions for the period of time it is expressed, this should allow low molecular weight compounds administered systemically to enter the selected region of the brain, thus proving a means of selectively opening these barriers to systemic low molecular weight therapies. As a model, the investigators then use this approach to treat laser induced choroidal neovascularization. The following may help clarify the study.

2. Results p5, parag 1 "these viruses have recently been shown to transduce endothelial cells of the neuronal microvasculature" should be referenced. These vectors also transduce neurons and likely other cns cell types; does the expression co claudin5 effect the function of these cells?

3. Figure 1a, b - if the vectors have been published, the a and b panels should be put into the supplementary materials. In addition, as it stands, both panels are meaningless because they are labeled with abbreviations for which there is no information in the text nor legends.

4. Figure 1c, the purity of the vector can be stated in the text; the panel should be deleted or put in supplementary materials

5. Figure 1G, H - while the anecdotal pictures of decreased claudin5 expression are convincing, this should be quantified by blinding the observers to a histologic rating scale (eg, 0-4), with appropriate statistics.

6. Figure 2 - the investigators make a major point in their abstract, introduction and discussion of the advantage of their method is to allow localized regions of the BBB and iRRB to be genetically modified and hence allowing localized regions to receive the systemically administered low molecular weight, but it looks from the data that this effects most all of the retina. This may be secondary to the fact that it is a mouse, but it is not convincing to claim regionality in retina with this data.

7. General comment and control - while the aav-deoxycline-claudin5 strategy is elegant; is it necessary - could that same thing be achieved with the classic use of mannitol but on a local level eg, 1 retina or local cns? This is an important control ie, to show that the aav-deoxycline-claudin5 strategy is really better than existing technology.

8. Figure 3b - the data showing localization needs to have other regions of the brain to show it is negative; this is the major focus of the strategy and it needs both positive and negative controls. Also supporting this, the investigators need to show that in different regions of the brain there are (relevant to the site of administration) different levels of claudin5 expression, and that this correlates with leak as they predict.

9. Figure 4 is very nice data showing local (right vs left eye) effects, but couldn't the same be achieved with local mannitol?

10. Figure 5 - same comments as for figure 4; the data is very nice, but is the gene therapy strategy really necessary?

11. Similar to comment 6, the "sell" of this strategy is local, but all the investigators have really shown is very little cns data, and right left eye data. The overall approach is elegant, but the demonstration of its utility is limited. The approach would be much more convincing if regionality could be shown in the cns. This probably would require a larger animal model.

Referee #3 (Novelty/Model system Comments for Author):

The AAV-murine model may not be appropriate for human therapy since humans have been exposed to AAV and therefore may have an immune response to the vector which mice do not.

Referee #3 (Remarks to the Author):

This manuscript describes a novel approach for opening the BBB and iBBB to small molecular weight drugs which normally cannot traverse these barriers. The authors incorporated a shRNA targeting Claudin-5, which is a constituent protein of the BBB vasculature. Down-regulation of this protein via an Doxycycline inducible shRNA resulted in opening the BBB and iBBB to small therapeutic drugs, thereby highlighting the therapeutic potential of this approach for clinical use. Despite the novelty of the approach and the validated permeation of the BBB by claudin-5 knockdown, there are some concerns about the potential use of such a strategy in a clinical setting. These are listed below and need to be addressed.

1. AAV delivered shRNAs have been shown to cause liver toxicity and even mortality in mice (Grimm et al, Nature). The toxicity appears to be associated with competition between the high levels of shRNA and endogenous pre-miRNAs for exportin 5 and possibly for other downstream members of the RNAi pathway. In the present study the levels of shRNA expression are not monitored, and the potential for interfering with the endogenous miRNA pathway is not addressed. There needs to be an assessment of this strategy on miRNA function in the tissues where the AAV delivered shRNA is expressed, especially given the long period of induction.

2. What is the rate of reconstitution of the claudin-5 levels in the vasculature and retinal tissue once the shRNA induction is cessated? A concern here is that the longevity of the shRNA guide strand within RISC may lead to a too prolonged period of inhibition of claudin-5 which could have serious negative consequences in a patient. The rebound of claudin5 needs to be carefully addressed here.

3. AAV can be long lived in the transduced tissue, especially if the tissue is not dividing or dividing slowly. Is this study based upon this fact, that is, will the longevity of AAV with the inducible shRNA be useful for a chronic disease? The authors should comment on this, and also show that readministering of Dox results in a renewed permeation of the BBB or iBBB (this relates to comment 2).

4, Given the false positive results with siRNAs targeting VegF or VegFR for macular degeneration, induction of gamma interferon and IL12, thereby abrograting neo vascularization by a non-siRNA specific mechanism, the authors need to determine whether or not the AAV infection and shRNA induced expression are triggering type 1 or gamma IFN expression.

1st Revision - authors' response 21 December 2010

Referee #1 (Remarks to the Author):

The concept of this mansuscript is both novel and very interesting. The data presented is really outstanding and a well written manuscript. Overall, very good work with minor revisions noted. The authors suggest a basic science application for the work, but focus on a therapeutic line of investigation and this should be altered in text.
There is no discussion of inducible systems that may actually be used in humans. My understanding is that the tet elements would be too immunogenic for human use.

The tet elements have been used previously in a long term safety study in the retinas of macaques and have shown to be an efficient and safe means to regulate gene expression (Steiger K et al., 2006). Repeated induction of gene expression was also observed for 2.5 years after initial injection. We have now incorporates this into the discussion of the manuscript as follows: *"Tet elements have also been used previously in a long term safety study in the retinas of macaques and have shown to be an efficient and safe means to regulate gene expression. Repeated induction of gene expression was also observed for 2.5 years after initial injection".*

The authors also discuss AAV stability in neural tissue. The efficacy of their transient disruption depends on aav transduction of the endothelial cells. A discussion on vector persistence in those cells is needed.

To the best of our knowledge AAV transduction is permanent which in the case of our therapeutic strategy is ideal and in relation to endothelial cells associated with the brain and retina, these cells are non-replicative and will maintain episomal AAV DNA in their nuclei. This is now discussed in the manuscript as follows: *"All evidence to date indicates that AAV infection of neuronal tissues is long lasting or may even be permanent and hence repeated injections of this inducible barrier-modulating agent should not be required (Maguire et al., 2008; Bainbridge et al., 2008; Hauswirth et al., 2008). Indeed, endothelial cells of the iBRB and BBB, while possessing high turnover rates and replication potential do not in fact undergo nuclear division and AAV transduction should persist long term following one injection".*

Finally, there is no discussion on other methods of bbb disruption. Why is this method better than focused ultra sound? or other methods- just a greater discussion will suffice.

This method has distinct advantages over BBB disruption using ultrasound or mannitol in that it is size-selective to low molecular weight molecules. The two methods outlined above are not size-selective and will allow for extravasation of plasma constituents such as albumin or IgG. The presence of albumin can induce astrogliosis in neuronal tissues which in itself can cause further inflammatory responses. Moreover, the use of intra-carotid mannitol which is used clinically to break down the BBB for drug delivery in Glioblastoma treatment can induce severe seizures in patients and is neither size-selective nor short-term, with effects being manifested for days after the carotid infusion. This has now been incorporated into the Discussion.

Referee #2 (Remarks to the Author):

1. General comment - The overall focus of this study is to describe a strategy to selectively open localized regions of the blood brain barrier (BBB) and inner blood-retinal barrier (IRRB) using an AAV9 serotype capsid expressing an AAV2 expression cassette encoding a deoxycline-inducible promoter driving an shRNA targeting claudin-5, an important component of the BBB and iRRB. By

introducing the vector in a selected region of the brain or retina, if the vector is successful in suppressing claudin-5 levels in the microvasculature of these selected regions for the period of time it is expressed, this should allow low molecular weight compounds administered systemically to enter the selected region of the brain, thus proving a means of selectively opening these barriers to systemic low molecular weight therapies. As a model, the investigators then use this approach to treat laser induced choroidal neovascularization. The following may help clarify the study.

2. Results p5, parag 1 "these viruses have recently been shown to transduce endothelial cells of the neuronal microvasculature" should be referenced. These vectors also transduce neurons and likely other cns cell types; does the expression co claudin5 effect the function of these cells?

These papers have now been referenced. We have not observed any adverse effects on neuronal function after AAV transduction as manifested by normal electrophysiological readouts from the retina. Moreover, retinal histology is the same in mice receiving NT AAV and CLDN5 AAV's.

3. Figure 1a, b - if the vectors have been published, the a and b panels should be put into the supplementary materials. In addition, as it stands, both panels are meaningless because they are labeled with abbreviations for which there is no information in the text nor legends.

Panels A and B describe the novel constructs containing claudin-5 shRNA under the control of a doxycyline inducible promoter. This is the first ever report of AAV2/9 expressing shRNA under the control of doxycycline. All abbreviations have now been included in the legend of the figure.

4. Figure 1c, the purity of the vector can be stated in the text; the panel should be deleted or put in supplementary materials

SDS-PAGE analysis of viral purity has now been moved to supplementary data.

5. Figure 1G, H - while the anecdotal pictures of decreased claudin5 expression are convincing, this should be quantified by blinding the observers to a histologic rating scale (eg, 0-4), with appropriate statistics.

Immunohistochemical staining of claudin-5 expression in retinal flatmounts is essentially qualitative in nature and we believe that the quantitative aspect of claudin-5 expression is profound in the qPCR and Western data. Moreover, the histological analysis of claudin-5 in retinal cryosections and flatmounts is performed in blinded conditions whereby the stained slides are assigned to be either NT AAV or CLDN5 AAV injected, i.e., suppression is either observed or it isn't, as there is not a large degree of discrepancy between eyes.

6. Figure 2 - the investigators make a major point in their abstract, introduction and discussion of the advantage of their method is to allow localized regions of the BBB and iRRB to be genetically modified and hence allowing localized regions to receive the systemically administered low molecular weight, but it looks from the data that this effects most all of the retina. This may be secondary to the fact that it is a mouse, but it is not convincing to claim regionality in retina with this data.

The advantage of our technique is that the inner blood-retina barrier can be modulated to the exculsion of the brain, and vice-versa. Modlation of the iBRB results in barrier permeability in those regions of the retina transdcued with viurs. Even if this if not the entire retina, it is more than sufficient to open up the retina and indeed the vitreous, to peripherally administered low molecular weight drugs. However, regional modulation in the brain would be a distinct advantage, and this is demonstrated in the manuscipt by using the hippocampus as a target. We have now included a figure (Supplementary Figure 3) showing a 3-dimensional re-construction of the MRI data post injection of Gd-DTPA which shows a clear example of the "spread" of extravasation of Gd-DTPA post-suppression of claudin-5. The extravasation will extend into the vitreous, but as outlined from the figure it is not "pan-retinal" and is more than likely due to the transduction efficiency of the AAV.

7. General comment and control - while the aav-deoxycline-claudin5 strategy is elegant; is it necessary - could that same thing be achieved with the classic use of mannitol but on a local level eg, 1 retina or local cns? This is an important control ie, to show that the aav-deoxycline-claudin5 strategy is really better than existing technology.

The classic use of mannitol infusion to induce BBB breakage is not size-selective and has been described to have very short lived effects. Indeed as mentioned above, mannitol infusion can induce seizures in patients. To the best of our knowledge mannitol has never been used locally to induce BBB or iBRB opening as it works under the premise that systemic injection of high concentrations of mannitol causes shrinkage of endothelial cells and subsequent "breakage" of the tight junctions. Indeed, the use of mannitol in mice is notoriously difficult and it would not be an appropriate control as there are few reports describing it's potency. Moreover, there is growing anecdotal evidence that intra-carotid infusion of any solution may induce a physiological effect similar to mannitol infusion, most likely due to the increased shear stress on endothelial cells that the infusion will generate. Hypothetically, mannitol infusion should be a great positive control but in reality, it's use is fraught with difficulties, both technically and physiologically. These aspects are now discussed in our revised manuscript.

8. Figure 3b - the data showing localization needs to have other regions of the brain to show it is negative; this is the major focus of the strategy and it needs both positive and negative controls. Also supporting this, the investigators need to show that in different regions of the brain there are (relevant to the site of administration) different levels of claudin5 expression, and that this correlates with leak as they predict.

We have now included in the supplementary data (Supplementary Figure 5) a 3D rendered image of an MRI scan of a mouse receiving claudin-5 AAV in its right hippocampus. The specificity is manifested by distinct contrasting solely at the site of CLDN5 AAV injection, with contrasting (dark) not observed in other regions of the brain.

9. Figure 4 is very nice data showing local (right vs left eye) effects, but couldn't the same be achieved with local mannitol?

This would not be an option. Local mannitol is not a technique that could be used to enhance drug delivery to the retina. Mannitol has previously been reported to be used systemically and even then, its use in mice is severely limited. Even if this were a viable option, the mannitol would have to be introduced repeatedly into the retina by intraocular injection and each manipulation of this sort would carry with it a risk of panophthalmitis, retinal haemhorrage or retinal detachement and could never be used routinely in human subjects.

10. Figure 5 - same comments as for figure 4; the data is very nice, but is the gene therapy strategy really necessary?

The gene therapy strategy would negate the need for regular intraocular injections of medications. Currently these include the use of monoclonal antibodies targeting VEGF in a limited number of patients with exudative forms of age-related macular degeneration, although as previoulsly stated, such injections are invasive and carry with them the risk of ocular damage. Our therapeutic strategy would involve a one off injection of AAV expressing the inducible claudin 5 shRNA followed by a therapeutic regimen involving systemic (oral) administration of an FDA/EMEA approved VEGFR antagonist at doses significantly lower than are currently used clinically for non-ocular or non-neuronal conditions..

11. Similar to comment 6, the "sell" of this strategy is local, but all the investigators have really shown is very little cns data, and right left eye data. The overall approach is elegant, but the demonstration of its utility is limited. The approach would be much more convincing if regionality could be shown in the cns. This probably would require a larger animal model.

The retina has previously been described by physiologist, John Dowling, as *an approachable part of the brain* and an external portion of the CNS and is an ideal model system for neuroscience

research. Indeed, the microvasculature of the iBRB is almost identical to the microvasculature of the BBB and the advantage of using the retina as a model organ is that we can very accurately and non-invasively measure electrophysiological readout during experimental analyses.

Referee #3 (Novelty/Model system Comments for Author):

The AAV-murine model may not be appropriate for human therapy since humans have been exposed to AAV and therefore may have an immune response to the vector which mice do not.

AAV is highly non-immunogenic. It is currently in use in a number of clinical trials for Leber Congenital Amaurosis (LCA) and to date, inflammation of the retina has not been observed in humans. AAV is also in use clinically for the treatment of Parkinson disease, cystic fibrosis and muscular dystrophy to name just a few. This is the most appropriate vector for use clinically as it is non-immunogenic, does not integrate into the host genome and has a proven track record of FDA and EMEA directed saftey/toxicology.

Referee #3 (Remarks to the Author):

This manuscript describes a novel approach for opening the BBB and iBBB to small molecular weight drugs which normally cannot traverse these barriers. The authors incorporated a shRNA targeting Claudin-5, which is a constituent protein of the BBB vasculature. Down-regulation of this protein via an Doxycycline inducible shRNA resulted in opening the BBB and iBBB to small therapeutic drugs, thereby highlighting the therapeutic potential of this approach for clinical use. Despite the novelty of the approach and the validated permeation of the BBB by claudin-5 knockdown, there are some concerns about the potential use of such a strategy in a clinical setting. These are listed below and need to be addressed.
1. AAV delivered shRNAs have been shown to cause liver toxicity and even mortality in mice (Grimm et al, Nature). The toxicity appears to be associated with competition between the high levels of shRNA and endogenous pre-miRNAs for exportin 5 and possibly for other downstream members of the RNAi pathway. In the present study the levels of shRNA expression are not monitored, and the potential for interfering with the endogenous miRNA pathway is not addressed. There needs to be an assessment of this strategy on miRNA function in the tissues where the AAV delivered shRNA is expressed, especially given the long period of induction.

We are fully aware of the concerns associated with persistent and high level expression of shRNA in mammalian tissue and this is the central reason we were stimulated to generate an inducible vector so that we could control shRNA expression. Therapeutically, our shRNA would be expressed for very short periods of time and given the half life of claudin-5 being approximately 30 hours, the barrier will reform once shRNA expression is suppressed by removing doxycycline. Given the lack of impact on retinal electrophysiology and retinal histology, it is highly unlikely that we are expressing shRNA in sufficient quantities to interfere with the endogenous miRNA pathways. Indeed, miRNAs are involved in the control of expression of up to 30 % of genes and from a previous microarray study of globally expressed neuronal transcripts i (Campbell *et al.,* 2009) we have observed minimal disturbance of gene regulation when claudin-5 is suppressed using siRNA. We have now however included in the discussion a paragraph addressing the issue of interfering with the miRNA pathway.

2. What is the rate of reconstitution of the claudin-5 levels in the vasculature and retinal tissue once the shRNA induction is cessated? A concern here is that the longevity of the shRNA guide strand within RISC may lead to a too prolonged period of inhibition of claudin-5 which could have serious negative consequences in a patient. The rebound of claudin5 needs to be carefully addressed here.

Claudin-5 has an approximate half-life of 30 hours and from our previous work involving claudin-5 siRNA, suppression effects are manifested 24 hours after siRNA delivery, with maximum suppression observed at 48 hours. 72 hours post siRNA delivery, levels of claudin-5 are increased again, with normal levels observed 96 hours- 1week post delivery. Given the half-life of doxycycline being 18-22 hours, once dox is not administered, the levels of claudin-5 return to normal 3-5 days post administration (this has now been included in the discussion). We have shown

the inducibility of the vector in supplementary figure 3, where the mice were administered doxycycline for 2 weeks and then returned to normal drinking water. Analysed 1 week later, mice had an intact iBRB, with normal levels of claudin-5.

3. AAV can be long lived in the transduced tissue, especially if the tissue is not dividing or dividing slowly. Is this study based upon this fact, that is, will the longevity of AAV with the inducible shRNA be useful for a chronic disease? The authors should comment on this, and also show that readministering of Dox results in a renewed permeation of the BBB or iBBB (this relates to comment 2).

Persistent AAV transduction is the central reason we chose this vector as it is ideal for chronic situations. Endothelial cells at the iBRB and BBB will have high regenerative capacities, yet they will not have nuclear division so the episomal viral DNA should persist indefinitely in transduced cells. This would negate the need for repeated intra-ocular injection of the AAV and in principle, one injection should allow for inducible modulation of the iBRB in individuals for the remainder of their lives. Indeed in the Briard dogs that were used for the experimental work-up of the vectors used in the 2008/2009 clinical trial for LCA, those animals have been shown to have persistent GFP expression in their eyes 10 years after the initial sub-retinal inoculation of AAV. To the best of our knowledge AAV transduction is permanent which in the case of our therapeutic strategy is ideal.

4, Given the false positive results with siRNAs targeting VegF or VegFR for macular degeneration, induction of gamma interferon and IL12, thereby abrograting neo vascularization by a non-siRNA specific mechanism, the authors need to determine whether or not the AAV infection and shRNA induced expression are triggering type 1 or gamma IFN expression.

The results that referee #3 is referring to relate to a paper in 2008 whereby siRNA's of 21 nucleotides or greater in length were shown to activate TLR3 (Kleinman *et al.,* 2008). Rather than being "false positive" results, the siRNA's directed against VEGF or VEGFR did cause suppression but they also activated TLR-3 as an "off-target" effect, causing IL-12 and IFN-gamma up-regulation which was shown to be a central reason for inhibition of CNV. Our shRNAs generate siRNAs of 19 nucleotides in length and are below the size threshold for TLR3 activation. Moreover, it is clearly stated in the paper cited that the TLR3 activation is specifically cell surface TLR3 and not endosomal TLR3. Following escape from the endosome, AAV particles deliver their ssDNA to the nucleus where it is made into dsDNA and then transcribed to give the hairpin shRNA. This is then exported via Exportin-5 to the cytoplasm where it is cleaved by Dicer to give a 19 base-pair siRNA the guide strand of which is then incorporated into RISC and RNA interference ensues. Thus, siRNA originating from AAV will never encounter cell surface (or even endosomal) TLR3. However, to this end, we have performed qPCR of retinas of eyes transduced with NT AAV or CLDN5 AAV in order to analyse levels of IL12 and the pro-inflammatory cytokine RANTES. In both RNA samples, the transcript levels of these cytokines was at inherently basal levels with very high Ct values indicative of no activation of cytokine secretion. This is likely due to the different cellular localisations of TLR3 and AAV-delivered siRNA and the fact that it is specifically cell surface TLR3 that will induce the anti-neovascularisation effect observed by Kleinman *et al.* 2008.

References

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Stieger K, Le Meur G, Lasne F, Weber M, Deschamps JY, Nivard D, Mendes-Madeira A, Provost N, Martin L, Moullier P, Rolling F. Long-term doxycycline-regulated transgene expression in the retina of nonhuman primates following subretinal injection of recombinant AAV vectors. Mol Ther. 2006 May;13(5):967-75

2nd Editorial Decision 03 February 2011

Thank you for the submission of your revised manuscript "Systemic low molecular weight drug delivery to pre-selected neuronal regions" to EMBO Molecular Medicine. We have now finally received the enclosed reports from the referees whom we asked to re-assess it.

As you will see, Reviewers #1 and #3 acknowledge that the manuscript was significantly improved during revision and indicate that it is suitable for publication. However, Reviewer #2 still raises concerns. His/her major concern is the lack of a direct comparison between the use of mannitol infusion and the gene therapy approach to demonstrate that the new approach is superior to the existing one.

We agree that it would be ideal to perform these experiments, however, we realize that the addition of more in vivo data would be time-consuming. We also understand your arguments why the use of mannitol might not be warranted as a control in this case. Thus, after discussion with the Chief Editor and in view of the positive comments from the other two Reviewers, we feel that, on balance, we can continue to consider your manuscript further. We would however strongly encourage you to include a brief discussion on why the comparison between your gene therapy approach and mannitol infusion was not performed.

A small concern is the font size of labeling in some figures, which will be difficult to read when the figures have been incorporated into the text. Please increase the font size in the labeling of y-axes in all graphs as well as labeling in Fig 1C, Fig 2, Fig 3G (all axes), Fig 5 C and F.

Please also include 'The paper explained' and 'For more information' (please see instructions below).

Revised manuscripts should be submitted within one months of a request for revision; they will otherwise be treated as new submissions, unless arranged otherwise with the editor.

I look forward to reading a new revised version of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1 (Remarks to the Author):

I am fully supportive of this manuscript and the authors have done an outstanding job addressing the minor concerns from my standpoint. AAV vectors have been gaining particular attention clinically, with certain successes even in early trials. The authors have adequately addressed the other reviews in my opinion.

Referee #2 (Novelty/Model system Comments for Author):

See below - Remarks to be sent to the author

Referee #2 (Remarks to the Author):

The following comments are the responses to review #2, point by point with the same numbering used by the investigators

1,2. adequate response

3. this is now clarified

4. adequate

5. The review asked for quantitative, blinded assessment of the flatmounts, but the investigators argue the immunohistochemistry is qualitative and either positive or negative. Their argument is circular re "suppression is either observed or it isn't"; it is unclear why they are unwilling to do the extra work to make it a better study

6. As with #5, the argument is anecdotal rather than quantitative

7. Mannitol is the classic to open the blood brain barrier; it is unclear why the investigators are unwilling to do the experiment

8. While the anecdote is consistent with their hypothesis, anecdotes are not science; it should be repeated with quantification

9. same as 7

10. Again, there is no data in the paper to argue whether gene therapy is really necessary

11. Since the investigators refuse to do additional studies, all we have is an argument as to why they do not want to do additional studies

Referee #3 (Remarks to the Author):

In this revised manuscript the authors have attempted to address the critiques of the previous review and in so doing have included some additional experimental data which strengthens the manuscript. Overall this work is novel in the selective opening of the BBB by the claudin shRNA under inducible control. This is an approach that could be used from chronic illness and drug treatment in the CNS.

2nd Revision - authors' response 04 February 2011

Thank you very much for sending the reviews of our revised manuscript "Systemic low molecular weight drug delivery to pre-selected neuronal regions" and for giving us the opportunity to submit a revised version. As requested, we have now included a brief discussion on why our gene therapy approach to localised and selective barrier modulation was not directly compared to BBB disruption using mannitol (see below). We have also increased the font sizes in the y-axes of all graphs and we have increased the font size in the labelling of all figures as requested. We have also now included a section on "The paper explained" (see below) and we hope that these amendments to the manuscript will suffice. Please let us know if you require any further information.

"A direct comparison of mannitol infusion and AAV-mediated barrier modulation was not performed during the course of this study given the disparity between the two approaches. Mannitol infusion causes global BBB disruption, for short periods of time, and this is neither size-selective, nor is it localized to distinct neuronal regions. Our AAV approach allows for periodic and localized barrier modulation specifically at the site of injection of the virus and has been designed in such a way that barriers only become reversibly permeable to low molecular weight material. While the use of mannitol has been described in relation to attempts to improve access of cytotoxic drugs to the brain for treatment of GBM, its administration must be performed under highly specialized surgical conditions and it cannot be used for chronic conditions such as those described here in view of the fact that repeated administration may produce abnormal neurological and renal side-effects".

