

Dynorphin-Based “Release on Demand” Gene Therapy for Drug-Resistant Temporal Lobe Epilepsy

Alexandra S. Agostinho, Mario Mietzsch, Luca Zangrandi, Iwona Kmiec, Anna Mutti, Larissa Kraus, Pawel Fidzinski, Ulf C. Schneider, Martin Holtkamp, Regine Heilbronn, Christoph Schwarzer.

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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

17th December 2018

Thank you for the submission of your manuscript to EMBO Molecular Medicine and please accept our apologies for the delay in getting back to you. We have now heard back from the three referees whom we asked to evaluate your manuscript.

You will see from the comments pasted below that all referees find the data interesting and promising, however several items need to be critically addressed before publication. Of particular importance we would insist on explaining the discrepancies noted by referee 3 in the experimental procedure. Referee 1 is mainly concerned about long-term effects and would like to see what's happening 3-4 months down the line (not 6 months); safety of the approach should also be better documented (the mechanism of action in the epilepsy context and the lack of toxic effect related to the pathway involved in epilepsy and eventually others, should be studied). Finally some rewriting and provision of controls and better figures are needed.

We would welcome the submission of a revised version within 3 to 4 months for further consideration and would like to encourage you to address all the criticisms raised as suggested to improve conclusiveness and clarity.

REFeree REPORTS

Referee #1 (Comments on Novelty/Model System for Author):

strong unmet medical need.
well characterized model used
large panel of techniques

Referee #1 (Remarks for Author):

The paper by Alexandra S. Agostinho et al, Dynorphin-Based "Drug on Demand" Gene Therapy for Drug-Resistant Temporal Lobe Epilepsy describes a gene therapy approach for a severe form of local epilepsy, called mesial temporal lobe epilepsy (mTLE). This mTLE with hippocampal sclerosis is the hardest to treat and is associated with cognitive deficits.

The dynorphins are a family of endogenous opioids, modulators of neuronal excitability. They are implicated in addiction. They are perceived as natural anticonvulsants.

During burst stimulation, typical for the onset of seizures, dynorphins are released from neurons and bind to kappa opioid receptors (KOR), preventing seizure development.

Preprodynorphin (pDyn) knockout mice show increased susceptibility for the development of epilepsy. Dynorphin levels in humans correlate with increased vulnerability for the disease. In mouse models of mTLE as well as in affected patients, dynorphin levels are reduced in the epileptogenic focus, while KOR are mostly maintained. Altogether, these results suggest that delivering Dyn in affected regions could be of therapeutic value.

The aim of the present study was to replenish the exhausted reservoirs of dynorphins in neurons of the epileptogenic focus. Dynorphin, stored in vesicles can be released and activate KOR to suppress seizures.

The paper is well written but more explanations could be directly put in the figures to help reading;

Authors show nice and documented results in terms of prevention of seizures evidently demonstrated by this gene delivery strategy. The use of two different well-recognized animal models in mouse and rat is convincing in the short term. However there are several important concerns that should be addressed to consider the paper for publication.

Major comments

1) long-term results

Results on spatial learning and memory are not convincing in the long-term (5,5 months) (Figure 2). This is puzzling. This result and the decreased long term production shown in Figure 3G argue for a decreased expression / efficacy in the long-term. This should be more clearly analyzed and discussed. Escape in the long-term? Long term production of pDyn should be evaluated. This is also contrasting with CSF measures (see my comment below discussing results presented page 9).

2) Safety of the approach in naive mice must be addressed more precisely (particularly histology) and in the long-term.

Overexpressing Dyn, even in a restricted portion of the brain could have consequences. Evaluation was made up to 10 weeks after vector administration. This is not sufficient. Long term study is needed.

Cognitive consequences should be evaluated in bilaterally injected animals to increase the sensitivity of the test.

Histology should be much more detailed with long-term (at least 6 months) time points and detailed analysis: neuronal aspect (picnosis, neuronal markers), neuronal count, inflammation (microglia, astrocytes) with high magnification images. Comparison to non-injected animals or contralateral hemisphere should be made. Readouts of Dyn action should be analysed: KOR signaling in particular.

3) This release of the dynorphin is called by authors "on demand". This term leads to misinterpretation and should be changed.

Transgenic Dyn is properly matured and behaves like endogenous counterpart and delivered following stimulation. However the term on demand is confusing and not appropriate. The production of the transgenic dyn is not controlled. Overexpression of the pDyn is permanent. Only the release of mature Dyn vesicles is upon stimulation. This notion should be more clearly explained and it is the opinion of the reviewer that the term « on-demand » is confusing, could be misinterpreted, and should not be used particularly in the title. Since one issue with gene therapy is that gene expression is not regulated and transduced gene is permanently expressed, this is of importance.

Even the release of the mature form stored in vesicles seems to rely on stimulation, transgenic

Dynorphin is produced constitutively under the CAG promoter which raises questions :
 What is the level of production of transgenic Dyn compared to endogenous protein. Total Dyn protein should be quantified.
 Does it accumulate in cells ? in what compartment ? (high quality histology should be performed to document that) How is it eliminated ?
 Authors should investigate the mechanism of action, signal that target correct maturation of pDyn and down signalling mechanism on KOR : show readout of KOR signaling

Since Dynorphins have important functions in the brain, particularly involving reward processing, mood control and the development of addiction, these issues are of major importance in the perspective of a potential therapeutic use.

Specific comments

Injection protocol:

It would help adding a scheme showing the techniques and implanted material. This would help understanding the following Methods: For experiments with subsequent AAV administration, a guide cannula was implanted, which was attached to the hippocampal depth electrode and targeting the hilus of the dentate gyrus.

At the time of viral vector injection, animals were implanted with a guide cannula targeting the hilus of the injected hippocampus and a stimulation electrode in the entorhinal cortex (RC -4.20 mm; ML +3.20 mm; DV -4.90 mm). For

Figure 1:

The cassette presented in a single strand cassette and not a self complementary cassette. This should be modified. The serotype 2 UTR are used and the capsid is 1 . This should be added.

Legend of Figure 1:

It is mentioned that control vectors carry either a truncated, nonfunctional version of the enhanced GFP gene (AAV-ΔGFP), or its functional counterpart (AAV-eGFP; not displayed). Why is eGFP mentioned here ? Where is it used ?

Figure 3: How is measured DynB content in G

page 9:

It is stated that « Dyn expression decreases 6 months after application (Fig 3G) and that the early increase of neuronal Dyn did not lead to increased Dyn levels in the cerebrospinal fluid (CSF) at 1.5 months after AAV-pDyn transduction (Fig. 3H). Interestingly a 2-fold increase of Dyn was observed in the CSF at 6 months after AAV pDyn transduction »

This seems in contradiction With 3G results. Moreover this raises the issue of potentially elevated Dyn level in the CSF. In the context of a supposed limited expression in a restricted region of the brain, how this can be interpreted?

Page 11:

To investigate whether AAV-pDyn derived peptides Dyn peptides suppress seizures-like activity in human hippocampus . How is this experimental design and the amount of Dyn delivered are relevant to the AAV-pDyn delivery? (in the micodyalisis exp the levels are 200 pm).

Graphic summary: should integrate overexpressing Dyn condition in healthy cells

Sup figure 2: images and histological analysis should be strongly improved.

Discussion

It is said in the discussion that « A single dose of 108 AAV vector genomes (gp) per animal was sufficient for long-term suppression of hippocampal HDPs and of generalized seizures. The safety profile of AAV-pDyn is excellent: no signs of toxicity, inflammation or immune reactions were recorded. No adverse effects were observed in healthy mice treated with AAVpDyn, suggesting that unilateral hippocampal overexpression of pDyn does not interfere with emotional control or stress coping abilities, effects attributed to KOR activation in distinct brain regions » This must be moderated with regards to my comments both on the limited tox informations (in particular

histology) and on the short term study only performed.

A comment should be made on other gene therapy approaches for this disease and how AAV-Dyn competes with that.

The conclusion stating that « considering the high medical need for a definite and long-term solution the "drug on demand" concept has enormous advantages over permanent and systemic drug treatment. » is misleading. The « drug » is the AAV-pDYN that could be potentially injected to the patient. The product would be indeed permanently delivered and produced.

This conclusion and the sentence below should be strongly moderated, not to oversell the work. « Timely and spatially restricted peptide release limits the development of tolerance and reduces off-target adverse effects, by taking the brain "offtreatment" in inter-ictal phases. Last but not least, it will be fascinating to see the "peptide on demand" concept being transferred to other targets and disease states, and its realization by focused AAV delivery. »

Again the interesting result of this work is that not only pDyn is produced locally but it is correctly matured and delivered upon epileptic stimulation leading to beneficial effect.

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

The authors employed appropriate model systems on all levels.

Referee #2 (Remarks for Author):

The authors present a range of studies that establish the anti-seizure efficacy and safety of viral vector based preprodynorphin transduction and expression. The studies were well designed in order to address many of the issues inherent to anti-seizure gene therapy. Essentially the diverse findings provided a convincing "proof of principle".

The one minor comment regards the quality of panels A thru C in figure 3. The NeuN immunofluorescence quality is quite low and there are clearly pDYN positive cells that do not co-localize with NeuN. Do the authors have a better example?

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

This is an interesting paper supporting the idea that viral vector delivery of inhibitory neuropeptides to an epileptic focus could be an effective gene therapy approach for drug-resistant focal epilepsy. They use appropriate rodent models, however a weakness of this paper in my opinion is that their reduction in seizures and epileptiform activity is reported for a relatively small number of animals and during short recording sessions.

Referee #3 (Remarks for Author):

Agostinho and colleagues present an interesting paper expanding preclinical data studies that indicate viral vector driven over expression of inhibitory neuropeptides can have therapeutic efficiency in rodent models of temporal lobe epilepsy.

'On demand' release of an anti-seizure peptide has several advantages, namely that it is released only during high frequency neuronal firing. This therefore reduces potential concerns of homeostatic compensation in response to permanently expressed and functional proteins as used in some other gene therapy approaches.

The authors demonstrate that this treatment can also improve memory function in previously epileptic rodents.

Below are my comments on the manuscript presented.

1. Although Fig 5a shows that there is a good spread of transduced cells in the dorsal hippocampus

can the author's state what the viral transduction volume is? Is expression restricted just to the dorsal hippocampus or does it also transduce the ventral hippocampus. 10⁸ viral particles seems quite low, many studies report much higher titre 10¹²/10¹³.

2. How long does it take for virally transduced cells to make the protein of interest? Many groups wait 1-3 weeks after AAV transduction to detect sufficient expression of the transgene protein. However in Fig 1B there appears a significant reduction in epileptiform activity and seizures within 1-2 days.

3. The recording period of EEG is very short. 2hrs for HPD analysis and 48hrs for detecting seizures. Seizure frequency in particular tends to be non-normally distributed. Seizures cluster therefore in a 48hr window seizure frequency could be skewed. A longer baseline record period (7-14 days) and longer post treatment recording sessions (preferably continuous) would be more convincing.

4. Fig 1C shows a blow up of an HPD, can they also provide a blow up of a seizure.

5. Fig 1D - why are the n numbers changing during the course of the experiment - (n=3-7).

6. Fig 1F The colour of the preAAV in AAVDyn rats (blue) is a little confusing as the colour blue is used in other panels to denote AAV-GFP.

7. What is the half-life of norBNI? How long would you expect it to have an effect?

8. If there is a release in suppression of HPDs within 0-2hrs post-injection of norBNI does this indicate that dynorphin is constitutively being released?

9. Fig 1G - Control AAV-GFP data is missing from this figure.

10. Is the dose of kainic acid correct? Pg 18 line 24, '50nl of 1nM KA', most people use 50-100nl of 20mM KA. Also there is a formatting error here, reference 16.

11. 4 electrodes were implanted but analysis only performed on the electrode in the ipsilateral hippocampus? With time there is a development of a more dispersed epileptic network, does dynorphin injection prevent the development of abnormal epileptiform discharges in other brain areas?

12. Does dynorphin overexpression reduce neuropathology? Is there a reduction in sclerosis, granule layer dispersion relative to animals injected with AAV-GFP?

13. Is the conclusion that the recovery in memory impairment is due to reduced seizure and HPDs alone or also due to improvement or prevention of worsening in neuropathology?

Point by point responses to the reviewers' comments:

Reviewer 1:

Referee #1 (Remarks for Author):

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The aim of the present study was to replenish the exhausted reservoirs of dynorphins in neurons of the epileptogenic focus. Dynorphin, stored in vesicles can be released and activate KOR to suppress seizures. The paper is well written but more explanations could be directly put in the figures to help reading;

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This is also contrasting with CSF measures (see my comment below discussing results presented page 9)

ad 1)

The overall performance of mice in the Barnes maze is generally decreasing with age. Therefore, both groups, age-matched healthy controls as well as AAV-pDyn treated epileptic animals were analyzed in parallel. The data show comparable performance of age matched naive and pDyn treated mice. The poor performance of aged mice is most probably due to impairments in vision developing over time in C57BL/6N mice. The time-interval chosen is the latest where C57BL/6N mice are able to learn the task, and AAV-pDyn treated epileptic animals keep up with naive mice. This is now also discussed in the MS (see page 6 line 30 onward). Three months after treatment the data are very similar to the ones observed at the 1 month time-interval.

Dyn levels were measured at 1.5 and 6 months after treatment with AAV-pDyn. These data are depicted in Fig 3 G and H. In tissue samples of older mice we see an overall reduction of dynorphin in the hippocampal tissue as compared to the younger group (Fig. 3 G). However, dynorphin is still over-expressed in the ipsilateral hippocampus of AAV-pDyn treated mice. This is also reflected by a slightly increased level of dynorphin in the CSF of AAV-pDyn treated mice at the 6 months after treatment interval (Fig. 3 H). In fact AAV-pDyn treated

animals show a higher level of Dyn in the CSF at 6 months as compared to younger animals. Therefore, we think that the lastingness of the overexpression is well documented. As stated in the manuscript (page 9, line 22), a transient overexpression which then reduces to a lasting steady level is absolutely normal for AAV-vectors.

2) Safety of the approach in naive mice must be addressed more precisely (particularly histology) and in the long-term.

Overexpressing Dyn, even in a restricted portion of the brain could have consequences. Evaluation was made up to 10 weeks after vector administration. This is not sufficient. Long term study is needed.

Cognitive consequences should be evaluated in bilaterally injected animals to increase the sensitivity of the test. Histology should be much more detailed with long-term (at least 6 months) time points and detailed analysis: neuronal aspect (pyknosis, neuronal markers), neuronal count, inflammation (microglia, astrocytes) with high magnification images. Comparison to non-injected animals or contralateral hemisphere should be made. Readouts of Dyn action should be analysed: KOR signaling in particular

ad 2)

We agree that additional safety tests are needed before AAV-pDyn can go into clinical trials. However, such extensive safety testing will be done on the vector produced under GMP conditions and in a GLP-certified laboratory according to the requirements of the regulatory authorities. In the present manuscript, the first “proof of concept” presentation of the feasibility of an AAV-based gene therapy using dynorphin we address a number of relevant safety issues to exclude overt toxicity, inflammation, impairments of behavioral performance, etc., comparing the injected with the contralateral side (as asked for). The extensive analysis the reviewer asks for is absolutely valid before moving the vector into the clinical study and will be designed in collaboration with regulatory boards, but far beyond the scope of our present study. We now indicate the preliminary character of the safety study. Moreover, we deleted the term long-term from the safety study (page..., line...)

3) This release of the dynorphin is called by authors “on demand”. This term leads to misinterpretation and should be changed.

Transgenic Dyn is properly matured and behaves like endogenous counterpart and is released following stimulation. However the term on demand is confusing and not appropriate. The production of the transgenic dyn is not controlled. Overexpression of the pDyn is permanent. Only the release of mature Dyn vesicles is upon stimulation. This notion should be more clearly explained and it is the opinion of the reviewer that the term “on-demand” is confusing, could be misinterpreted, and should not be used particularly in the title. Since one issue with gene therapy is that gene expression is not regulated and transduced gene is permanently expressed, this is of importance.

ad 3)

The reviewer correctly notes that the AAV vector induces permanent expression of pDyn, which is processed to mature, active dynorphins. By contrast, Dyn peptides are only released upon high-frequency stimulation (like at seizure onset), but not during low frequency signaling. And actually, the seizure onset is the situation of demand. Therefore, we consider “release on demand” to correctly capture this process. However, to avoid misinterpretation, we removed the term from the title and introduce it with a clear definition (page 3 line 18)

Even the release of the mature form stored in vesicles seems to rely on stimulation, transgenic Dynorphin is produced constitutively under the CAG promoter which raises questions:

What is the level of production of transgenic Dyn compared to endogenous protein. Total Dyn protein should be quantified.

The amount of transgenic dynorphin in relation to endogenous can be judged from Fig. 3G. In the dorsal hippocampus, the mean protein level of dynorphin is about twice the content of the contralateral side or of either side in GFP-treated animals.

Does it accumulate in cells ? in what compartment ? (high quality histology should be performed to document that) How is it eliminated ?

Authors should investigate the mechanism of action, signal that target correct maturation of pDyn and down signalling mechanism on KOR : show readout of KOR signaling

It is generally accepted that neuropeptides are stored in large dense core vesicles, which are transported into the axon. This is also what we observed by immunohistochemistry for the vector-derived pDyn. We find accumulation of the peptide in axonal, but not in dendritic compartments. This is shown in supplemental figure 3. For this experiment an antibody also detecting precursor peptides was used. Therefore, we can conclude that there is no accumulation or deposits in somata. Elimination of released neuropeptides occurs through degradation by peptidases. As the peptide produced from the vector is identical to the natural one, it is unlikely, that another process would be involved.

Regarding the mechanism of action: In Fig. 1 F we show the effect of a selective kappa opioid receptor antagonist on the seizure suppression by AAV-pDyn. Blocking kappa opioid receptors leads to a re-occurrence of HPDs and seizures to a level comparable to pre-AAV treatment. This provides solid evidence, that the effect is mediated by activation of kappa opioid receptors. KOR activation leads to activation of inhibitory G-proteins. However, inhibition of cyclase or recruitment of beta-arrestin (which also occurs upon binding of Dyn) is not considered as important as the direct interaction of the beta-gamma subunit with voltage-gated calcium channels (presynaptically) or potassium channels (postsynaptically) this was discussed (page 18, line 6 onwards) and is also indicated in Fig. 6 for illustration. Regarding the correct maturation of pDyn: As stated in the manuscript, the antibody used for the ELISA (data shown in Fig. 3) is highly specific for fully matured dynorphin B. It detects Leumorphin, a larger, yet already active form of Dyn B, but only to 7 % and larger fragments not at all. As we detected DynB release in prodynorphin knockout animals after injection of AAV-pDyn with this antibody, it can be assumed that AAV vector-derived pDyn is indeed matured to functionally active dynorphins. Which proportion of pDyn is processed cannot be concluded. This is now stated in the manuscript (page 11, line 24).

Since Dynorphins have important functions in the brain, particularly involving reward processing, mood control and the development of addiction, these issues are of major importance in the perspective of a potential therapeutic use.

We fully agree that dynorphins play an important role in a number of processes. Exactly this reasoning led us to choose an AAV capsids (serotype 1) that barely spreads beyond the site of injection (see Fig. 4A). In addition, our data show that CSF levels of dynorphins stay very low, also several months after treatment (Fig. 3H), suggesting little to no influence on other mechanisms. This is further supported by the lack of alterations in behavior observed in naive mice treated with AAV-pDyn.

Specific comments

Injection protocole :

It would help adding a scheme showing the technics and implanted material. This would help understanding the following Methods : For experiments with subsequent AAV administration, a guide cannula was implanted, which was attached to the hippocampal depth electrode and targeting the hilus of the dentate gyrus. At the time of viral vector injection, animals were implanted with a guide cannula targeting the hilus of the injected hippocampus and a stimulation electrode in the entorhinal cortex (RC -4.20 mm; ML +3.20 mm; DV -4.90 mm). For

We introduced supplemental figure 5 giving a scheme and showing the material used. The placement of electrodes and microdialysis probes for the release experiments is depicted in Fig. 3.

Figure 1 :

The cassette presented in a single strand cassette and not a self complementary cassette. This should be modified. The serotype 2 UTR are used and the capsid is 1 . This should be added.

The figure and legend was changed according to the reviewers suggestions (Fig. 1A)

Legend of Figure 1 :

It is mentioned that control vectors carry either a truncated, nonfunctional version of the enhanced GFP gene (AAV-ΔGFP), or its functional counterpart (AAV-eGFP; not displayed). Why is eGFP mentioned here ? Where is it used ?

AAV-eGFP was used in the behavioral experiments done on naive animals. All other experiments were conducted using with ΔGFP. The respective figure legends states which control vector eGFP or ΔGFP was used.

Figure 3 : How is measured DynB content in G

Figure 3G: Dyn B is measured by EIA in G, H and K. This is now clearly stated in the figure legend (page 10, line 6)

page 9 :

It is stated that « Dyn expression decreases 6 months after application (Fig 3G) and that the early increase of neuronal Dyn did not lead to increased Dyn levels in the cerebrospinal fluid (CSF) at 1.5 months after AAV-pDyn transduction (Fig. 3H). Interestingly a 2-fold increase of Dyn was observed in the CSF at 6 months after AAV pDyn transduction »

This seems in contradiction With 3G results. Moreover this raises the issue of potentially elevated Dyn level in the CSF. In the context of a supposed limited expression in a restricted region of the brain, how this can be interpreted ?

There is an overall reduction of dynorphin levels in all animals at older age. Still, dynorphin expression in the AAV-pDyn injected hippocampus is increased about 2 fold. As the antibody used in the EIA only detects mature dynorphin B, but not its larger precursors, a reduction of neuronal dynorphin paralleled by increased CSF levels can be explained by increased release, but does not allow conclusions on the intracellular expression level.

Regarding the increased CSF levels: Basal dyn levels in CSF are very low and their 2fold increase still represents non-efficacious concentrations in the pM range (about factor 100 below EC50 in the established GTPγS assay). Thus, already a slight increase in release from a small area may lead to this increase. Considering a total CSF volume of about 30 μl in mice, a femtomole of dynorphin yields a 30 pM concentration.

Page 11 :

To investigate whether AAV-pDyn derived peptides Dyn peptides suppress seizures-like activity in human

hippocampus . How is this experimental design and the amount of Dyn delivered are relevant to the AAV-pDyn delivery? (in the micodyalisis exp the levels are 200 pm).

The CSF levels measured are 200 pM, however this does not reflect the concentration of dynorphins in the synaptic cleft, but represents only a small, highly diluted portion which escapes direct degradation. We applied 600 nM concentrations of Dyn A and B into the medium, and the peptides have to penetrate the slice section and reach the receptors. Therefore, the concentration on the receptor is probably around EC50 up to a factor of 10-20 above. Such concentration can be reached in close vicinity to the release site also by the endogenous and/or vector derived dynorphins.

Graphic summary : should integrate overexpressing Dyn condition in healthy cells

Epilepsy is considered a disturbance of the network, with distinct groups of cells being more or less vulnerable to damage. Neurochemical changes like the reduction of pDyn expression are not necessarily a sign of “unhealthiness” or damage of neurons, but are induced by increased Calcium influx. Therefore, this may well reflect adaptations to altered network activity. In naive animals, endogenous dynorphins are sufficient to yield the full effect on seizure threshold. This is indicated by the fact, that treatment with KOR agonists does not further increase seizure threshold.

The treatment aims at pDyn overexpression in all living neurons, irrespective of the their health state to compensate for reduced pDyn production. The idea of the graphic summary is to illustrate the treatment concept. The bottom panel represents the situation after dynorphin-based gene therapy in epileptic tissue. Therefore, the bottom panel represents healthy cells adapted to a malfunctioning network.

Sup figure 2: images and histological analysis should be strongly improved.

The purpose of this figure is to illustrate the distribution of vector-derived dynorphin in hippocampal neurons. Labeling in the terminal field of mossy fibres is indicative of expression in granule cells, labeling of ipsi- and contralateral projections to the inner molecular layer suggest transduction of mossy cells. In addition, scattered neurons in the polymorph cell layer are labeled. Moreover, the higher magnification images visualize the varicose structure of labeled filaments, characterizing them as axons. As vector-derived and endogenous dynorphin are identical, this experiment was done in pDyn knockout mice. There is no intention to display any potential morphological or histological alterations due to AAV-pDyn injection. This might be part of an extensive safety study, but beyond the aim of a proof of principle study.

Discussion

It is said in the discussion that « A single dose of 108 AAV vector genomes (gp) per animal was sufficient for long-term suppression of hippocampal HDPs and of generalized seizures. The safety profile of AAV-pDyn is excellent: no signs of toxicity, inflammation or immune reactions were recorded. No adverse effects were observed in healthy mice treated with AAVpDyn, suggesting that unilateral hippocampal overexpression of pDyn does not interfere with emotional control or stress coping abilities, effects attributed to KOR activation in distinct brain regions » This must be moderated with regards to my comments both on the limited tox informations (in particular histology) and on the short term study only performed.

We now moderated the text to state that only preliminary tox information is available so far (page 18, lines 7 onwards).

A comment should be made on other gene therapy approaches for this disease and how AAV-Dyn competes with that.

We included a statement on other gene therapy approaches (page 18, line 14 onward).

The conclusion stating that « considering the high medical need for a definite and long-term solution the "drug on demand" concept has enormous advantages over permanent and systemic drug treatment. » is misleading. The « drug » is the AAV-pDYN that could be potentially injected to the patient. The product would be indeed permanently delivered and produced.

The produced product (pDyn) will only be released to the synaptic cleft to reach its site of action (Kappa receptor) upon high frequency stimulation before the onset of seizures, which is "on demand". We replace "drug" by "release".

This conclusion and the sentence below should be strongly moderated, not to oversell the work. « Timely and spatially restricted peptide release limits the development of tolerance and reduces off-target adverse effects, by taking the brain "offtreatment" in inter-ictal phases. Last but not least, it will be fascinating to see the "peptide on demand" concept being transferred to other targets and disease states, and its realization by focused AAV delivery. »

We moderated the statement, which now reads: "Considering the high medical need for a definite and long-term solution the "release on demand" concept has enormous advantages over permanent and systemic drug treatment. Timely and spatially restricted peptide release from a permanently refilled pool limits the development of tolerance and reduces off-target adverse effects, by taking the brain "off-treatment" in inter-ictal phases whilst guaranteeing availability at the onset of seizures."

Again the interesting result of this work is that not only pDyn is produced locally but it is correctly matured and delivered upon epileptic stimulation leading to beneficial effect.

Reviewer 2

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

The authors employed appropriate model systems on all levels.

Referee #2 (Remarks for Author):

The authors present a range of studies that establish the anti-seizure efficacy and safety of viral vector based preprodynorphin transduction and expression. The studies were well designed in order to address many of the issues inherent to anti-seizure gene therapy. Essentially the diverse findings provided a convincing "proof of principle".

The one minor comment regards the quality of panels A thru C in figure 3. The NeuN immunofluorescence quality is quite low and there are clearly pDYN positive cells that do not co-localize with NeuN. Do the authors have a better example?

The distribution of NeuN in the nucleus is not uniform. This produces a grainy impression of the labeling. Moreover, the images are taken from the granule cell layer of 40 μ m sections. In this densely packed layer, cells in different focal planes are labeled. Considering that pDyn is distributed in a different cell compartment than NeuN, it is normal, that mismatches occur, i.e. if a cell on the surface of the section is cut in a way that the nucleus is not present. Therefore, as additional control, we performed co-labeling for pDyn with GFAP, a marker for astrocytes, in parallel. As can be seen from Fig 4 F, the majority of labelled cells sits within the granule cell layer, but does not colocalize with GFAP. Taking both labelings together, we

conclude that a vast majority of transduced cells are actually neurons. This is further supported by the labeling shown in suppl. Fig. 3, displaying dynorphin in the mossy fibres and projections of the mossy cells. Individual axons are labeled in the molecular layer of the dentate gyrus.

Reviewer 3:

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This is an interesting paper supporting the idea that viral vector delivery of inhibitory neuropeptides to an epileptic focus could be an effective gene therapy approach for drug-resistant focal epilepsy. They use appropriate rodent models, however a weakness of this paper in my opinion is that their reduction in seizures and epileptiform activity is reported for a relatively small number of animals and during short recording sessions.

We agree that the number of animals in the EEG recordings is not very high. However, effectiveness of the treatment is also shown in tests of cognitive functions of epileptic animals. This increases the total number of animals per treatment group to over 20.

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Agostinho and colleagues present an interesting paper expanding preclinical data studies that indicate viral vector driven over expression of inhibitory neuropeptides can have therapeutic efficiency in rodent models of temporal lobe epilepsy.

'On demand' release of an anti-seizure peptide has several advantages, namely that it is released only during high frequency neuronal firing. This therefore reduces potential concerns of homeostatic compensation in response to permanently expressed and functional proteins as used in some other gene therapy approaches.

The authors demonstrate that this treatment can also improve memory function in previously epileptic rodents.

Below are my comments on the manuscript presented.

1. Although Fig 5a shows that there is a good spread of transduced cells in the dorsal hippocampus can the author's state what the viral transduction volume is? Is expression restricted just to the dorsal hippocampus or does it also transduce the ventral hippocampus. 10⁸ viral particles seems quite low, many studies report much higher titre 10¹²/10¹³.

Thank you for the comment, indeed 2x10⁹ genomic particles were injected in a total volume of 2 μ L. We apologize for the error. The dose has to be seen in relation to the volume of the relatively small volume of the dorsal hippocampus. The ventral hippocampus was mainly untransduced.

2. How long does it take for virally transduced cells to make the protein of interest? Many groups wait 1-3 weeks after AAV transduction to detect sufficient expression of the transgene protein. However in Fig 1B there appears a significant reduction in epileptiform activity and seizures within 1-2 days.

We used self-complementary (sc)AAV vectors, which express markedly faster than single-stranded AAVs. scAAVs express the protein of interest within 1-2 days after injection. We see the full effect after about a week, which fits to the time-course expected for scAAV.

3. The recording period of EEG is very short. 2hrs for HPD analysis and 48hrs for detecting seizures. Seizure frequency in particular tends to be non-normally distributed. Seizures cluster therefore in a 48hr window seizure frequency could be skewed. A longer baseline record period (7-14 days) and longer post treatment recording sessions (preferably continuous) would be more convincing.

We agree that longer periods of recording would help to reduce the influence of non-normally distributed seizures. However, we had to compromise on this, because we need multiple channel recording to identify HPD (unilateral events) and generalized (events also seen over the motor cortices) seizures. The Neurologger provides 4 channels in a wireless system, but recording time is restricted by battery life and data storage capacity. Multi-channel telemetric systems are too large and heavy for the use in mice. And wired systems providing 24/7 measurement frequently are not stable enough on the thin bone of the mouse skull. To reduce variability, we recorded the 2 hours for HPD analysis always between 2 and 4 pm, when seizures are most frequent. Overall, we see high variability in the GFP treated animals, yet statistical analysis reveals a significant improvement in pDyn treated mice due to the strong reduction of seizures and HPDs.

4. Fig 1C shows a blow up of an HPD, can they also provide a blow up of a seizure.

The requested blow up of a seizure was included as supplemental figure 1.

5. Fig 1D - why are the n numbers changing during the course of the experiment - (n=3-7).

The number of animals is decreasing due to the fact, that some animals lost the implant after some time which precluded further recordings. This is now clearly stated in the text. (page 4 line 14)

6. Fig 1F The colour of the preAAV in AAVDyn rats (blue) is a little confusing as the colour blue is used in other panels to denote AAV-GFP.

We changed the color of pre-AAV the columns in panel F and G to black for increased clarity.

7. What is the half-life of norBNI? How long would you expect it to have an effect?

norBNI is detectable in the CNS of rodents for several days, and fully cleared after one week. Therefore, we allowed one week for washout. 24 hours after application is the commonly used time-interval in studies applying norBNI and generally accepted as displaying full effect, while a delayed onset is frequently reported.

8. If there is a release in suppression of HPDs within 0-2hrs post-injection of norBNI does this indicate that dynorphin is constitutively being released?

The reappearance of HPDs upon blockade of the kappa opioid receptor indicates that HPDs are permanently suppressed by dynorphin. However, this is not depending on a constitutive release of dynorphin, but is most likely achieved by short periods of release at each onset of seizures. The stimulation dependence of the release and the absence of constitutive release was shown in the microdialysis experiments (Fig 3).

9. Fig 1G - Control AAV-GFP data is missing from this figure.

Indeed, a GFP group is missing in these experiments. Unfortunately, the drop-out rate due to death during initial seizures or loss of implant (rats rip of the implant, partially together with parts of the skull) is very high and the first group of 16 rats yielded only the 4 rats, which were treated with AAV-pDyn after stable expression of epileptiform activity. We are aware that this shortcoming. Due to ongoing limitation in our animal house, we could only run a

second round of 10 animals, which resulted in a 100 % loss of animals. However, the development and stability of seizures (and the high drop-out) in this model is well described and we added additional pre-recording data before AAV-pDyn application to compensate for this shortcoming. Irrespective of the lack of GFP animals, we consider these data informative as they, together with the data from human tissue, support the findings in our main model.

10. Is the dose of kainic acid correct? Pg 18 line 24, '50nl of 1nM KA', most people use 50-100nl of 20mM KA. Also there is a formatting error here, reference 16.

We apologize for the mistake, indeed it is 1nmol of KA in a volume of 50 nL. We now corrected this in the text (page 19, line 30).

11. 4 electrodes were implanted but analysis only performed on the electrode in the ipsilateral hippocampus? With time there is a development of a more dispersed epileptic network, does dynorphin injection prevent the development of abnormal epileptiform discharges in other brain areas?

In fact we used all four electrodes for analysis to distinguish between focal seizures (HPDs, occur only ipsilaterally) and secondary generalizing seizures, which can be detected from all channels. Contralateral depth and cortical surface electrodes were used to analyse generalization of seizures. We did not observe the development of a secondary focus in the mouse model. By contrast, rats displayed seizure onset in distinct brain regions.

12. Does dynorphin overexpression reduce neuropathology? Is there a reduction in sclerosis, granule layer dispersion relative to animals injected with AAV-GFP?

We started treatment after the onset of self-sustained seizures. In the model used, the neuronal damage and granule cell dispersion in the ipsilateral hippocampus is very broad, already before the treatment with AAV-pDyn. Contralaterally, we observed very little neuronal damage in both groups. Probably due to the relatively low number of generalized seizures, the progression of neuronal damage in the contralateral hippocampus is very slow. Therefore, analysis of neuronal numbers did not reveal significant differences.

13. Is the conclusion that the recovery in memory impairment is due to reduced seizure and HPDs alone or also due to improvement or prevention of worsening in neuropathology?

We assume that the main effect is due to the reduction in seizures, especially because we see an actual recovery of lost learning abilities and not only a conservation. In mice the interconnection between the hemispheres is strong, making an influence of the ipsilateral hippocampus on the contralateral highly likely. Moreover, neuronal damage in the contralateral hippocampus is mild to almost absent, rendering neuropathological alterations rather unlikely.

2nd Editorial Decision

29th July 2019

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine and for infinite patience during the review process. As a matter of fact, we are indeed still missing referee #1 report but we agree with you at this point, we really cannot justify delaying the manuscript processing any longer.

We have received the enclosed report from referee 3. Along with referee 2 who was already supportive of publication and after extensively discussing this case with my colleagues, we are pleased to inform you that we will be able to accept your manuscript pending the following final editorial amendments.

REFEREE REPORTS

Referee #3 (Remarks for Author):

The authors have addressed my previous review questions. I'm surprised that the dose of KA injected results in SE. However the paper will be of considerable interest to the field.

2nd Revision - authors' response

1st August 2019

[The Authors have satisfied all of the referee reports].

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ▼

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Regine Heilbronn and Christoph Schwarzer

Journal Submitted to: EMBO Molecular Medicine

Manuscript Number: EMM-2018-09963

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if $n < 5$, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

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

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself.

Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ▼ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	power analysis
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	power analysis
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Animals were excluded from analysis when duration of hippocampal paroxysmal discharges within 2 hours recordings was below 100 seconds before viral vector treatment.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	For EEG studies, animals were allocated to viral vector treatment in a way to ensure equal distribution of animals with high and low numbers of seizures in both groups. For behavioural testing animals were distributed in a pseudo-randomized way, splitting litters into different groups. In experiments where vectors were given after initial testing, good and bad learners were equally distributed between groups.
For animal studies, include a statement about randomization even if no randomization was used.	For behavioral experiments animals were distributed in a pseudo-randomized way, splitting litters into different groups.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	The experimenter was blinded to the treatment of animals.
4.b. For animal studies, include a statement about blinding even if no blinding was done	The experimenter was unaware of the treatment of the animals.
5. For every figure, are statistical tests justified as appropriate?	Statistical tests applied were performed according to the advice of a statistician
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	The n is too low to assume normal distribution, therefore this was not assessed
Is there an estimate of variation within each group of data?	We show SD for all data except human slice electrophysiology, in vivo EEG and EIA analysis, where SEM are given.
Is the variance similar between the groups that are being statistically compared?	In the AAV-pDyn treated animals seizure were mostly suppressed, therefore the variability in this group was markedly lower than in control groups.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

<http://www.antibodypedia.com>
<http://1degreebio.org>
<http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor>
<http://grants.nih.gov/grants/olaw/olaw.htm>
<http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm>
<http://ClinicalTrials.gov>
<http://www.consort-statement.org>
<http://www.consort-statement.org/checklists/view/32-consort/66-title>
<http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tum>
<http://datadryad.org>
<http://figshare.com>
<http://www.ncbi.nlm.nih.gov/gap>
<http://www.ebi.ac.uk/ega>
<http://biomodels.net/>
<http://biomodels.net/miriam/>
<http://jij.biochem.sun.ac.za>
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
<http://www.selectagents.gov/>

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	The pDyn antibody used was Serotec AHP377. The antibody specificity can be judged from comparison of the AAV-pDyn injected side to the contralateral side, as the experiment was done in pDyn knockout mice.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	no cell lines used

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Young adult (12-15 weeks) male C57BL6/N mice and pDyn KO mice on the same background (10 generations backcrossing) were used. All animals were either purchased from Charles River or bred in house. Animals were kept in groups of max. 5 mice in type II L Individually ventilated cages with free access to standard rodent chow and water. Light cycle was fixed, lights on from 7 am to 7 pm. Young adult Sprague-Dawley rats (10-12 weeks) were purchased from Charles River and housed in groups of two in type III cages.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All experiments were permitted by the Austrian Animal Experimentation Ethics Board
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLOS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	ARRIVE guidelines were followed

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Experiments were approved by the Ethics Committee of the Charité - Universitätsmedizin Berlin
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	All patients gave written consent prior to surgery.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	no photos
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	human specimens were used for electrophysiology, samples are only viable for a few hours.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	no clinical trial
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	not applicable
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	not applicable

F- Data Accessibility

18. Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	all data are in the manuscript
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	all data are available in the manuscript
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	not applicable
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biocompare (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	not applicable

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	no
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