

# Gene therapy for epilepsy targeting Neuropeptide Y and its Y2 receptor to dentate gyrus granule cells

Stefano Cattaneo, Barbara Bettegazzi, Lucia Crippa, Laila Asth, Maria Regoni, Marie Soukupova, Silvia Zucchini, Alessio Cantore, Franca Codazzi, Flavia Valtorta, and Michele Simonato

Corresponding author(s): Michele Simonato ([simonato.michele@hsr.it](mailto:simonato.michele@hsr.it))

---

## Review Timeline:

Submission Date:	20th Dec 23
Editorial Decision:	26th Jan 24
Revision Received:	12th Jul 24
Editorial Decision:	2nd Aug 24
Revision Received:	15th Aug 24
Accepted:	22nd Aug 24

---

Editor: Esther Schnapp

**Transaction Report: This manuscript was transferred to EMBO reports following peer review at EMBO Molecular Medicine.**

(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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

### Referee #1

There are several papers showing an anticonvulsive effect of combined injections of NPY and Y2 vectors in other animal models. This paper, however uses CamKinase IIa promoters to allow expression only on glutamatergic neurons.

The authors use intrahippocampal injections of NPY and Y2 receptor vectors on CamKinase IIa promoter and test this in on epileptic activity in synapsin triple KO mice.

This approach has been well established (several times) for NPY vectors and NPY + Y2 vectors in other animal models and using other promoters. The use of CamKinase II promoters used here, however, has the purpose to restrict expression to glutamatergic neurons.

Although the findings are not entirely novel, the paper shows a very nice analysis of the sites of vector expression and documents the anti-seizure effect of a combination of Y2 and NPY expressing vectors.

The experiments document expression of the vectors mainly (or exclusively) in mossy fibers. Surprisingly, they are obviously not expressed in mossy cells of the dentate gyrus. Why? Otherwise one would find NPY and Y2 in the inner molecular layer.

Figure 5. What does the difference in NPY expression between B and D mean? At p100 there was a high expression of NPY in mossy fibers (B), not at p120? How does NPY expression in mossy fibers relate to recent seizure activity? Is this expression typical for all animals?

Figure 6. To which time interval does "number of seizures" refer to? Video recording started two weeks after vector injection. How long did the recording last?

6E. Would in the Y2-NPY group the number of seizure-free mice go further down after 30 days? I think one should investigate this.

### Referee #2

The choice of Synapsin TKO as model raises questions. It is not a recognized model of drug-resistant epilepsy, and its translational potential is unclear. The authors should have used other models where AAV-NPY-Y2 has already been tested

Cattaneo and colleagues devised a combinatorial approach involving lentivirus delivery of NPY and its receptor Y2, driven by a CamkII promoter. They demonstrated NPY and Y2 expression both in vitro and in vivo. Additionally, they observed no expression in astrocytes and inhibitory neurons in vivo. Subsequently, they administered this lentiviral tool to Synapsin TKO mice

before the onset of seizures, revealing a reduction in seizures and an increase in seizure-free animals.

To the best of my knowledge, this marks the first gene therapy approach for Synapsin TKO mice. Nevertheless, caution is necessary before characterizing it as a "promising treatment option for drug-resistant epilepsy." Several major concerns arise:

1. The statement, "However, none so far included a built-in auto-regulatory system, and each, therefore, may lead to excessive inhibition," is inaccurate. Previous auto-regulatory gene therapies for epilepsy, such as those conducted by Kullmann's lab at UCL, and those including work on NPY and Y2 co-expression using a single AAV (e.g., Melin et al., 2019, 2023, and Szczygie et al., 2020).
2. The choice of Synapsin TKO as model raises questions. It is not a recognized model of drug-resistant epilepsy, and its translational potential is unclear. Why did the authors not opt for other models where AAV-NPY-Y2 has already been tested?
3. In terms of translation, lentiviral delivery in the human hippocampus may not be a realistic therapy due to the limited virus spread. Why was AAV not used, especially considering its previous use with NPY-Y2? The novelty here seems to lie in the specific excitatory promoter.
4. The specificity of CamKII to excitatory neurons, particularly the lack of proper quantification, including inhibitory neurons and the percentage of transduced cells, needs clarification. Literature reports mixed results.
5. Could the observed effect be a mere delay in epileptogenesis? A longitudinal study is crucial to explore this possibility, as the two-week recording period appears short given the dynamics shown in Figure 6E.
6. The use of T2A for NPY-Y2, as previously done by Melin et al., renders the discussion on this matter redundant. It would be more beneficial to focus on how this therapy fits into the context of other developed gene therapies.
7. Although the authors discussed the self-regulated approach, direct evidence supporting this claim is lacking in the present work.
8. If the authors aim to establish the superiority of this therapy over NPY alone, the addition of another group to the seizure analysis for a direct comparison is essential

### **Referee #3**

The manuscript describes the result of a newly developed lentiviral vector which enables co-expression of anti-convulsant neuropeptide NPY and its autoreceptor Y2 genes in the epileptic hippocampus, and evaluates its ability to suppress the seizures *in vivo*. The critical feature of this viral vector is its use of CamKIIa promoter driving both transgenes in the same excitatory neurons, which allows autoregulation of neural excitability through Y2 receptor-mediated inhibition. Telemetry video-EEG monitoring was conducted to detect seizures in the TKO mice expressing NPY/Y2 transgenes in dentate gyrus granule cells, and a clear reduction of the seizure occurrence in these mice was demonstrated.

This is a well thought out and seems to be a well carried out study. The idea of "autoregulation" of the epileptic brain activity is novel and very interesting. The choice of the model organism

(i.e., mouse) is adequate, the statistical analysis methods used are adequate, and their usage of lentiviral vector for gene therapy seems to be reasonable and less hazardous compared to other viral vectors.

The main concern I have is that a few important control experiments are missing in this study. It weakens the overall impact of the core message "autoregulation of the epileptic activity". I would like the authors discuss more thoroughly the mechanism how the autoregulation is realized in their model system to strengthen the core message.

Major points;

1) "Necessity of combinatorial gene expression"

Authors demonstrated that expressing NPY and Y2R transgenes suppressed the seizures very well (Fig.6) and argue that the combinatorial expression is important. Unfortunately, however, the authors didn't show that expressing either of the two genes alone is NOT sufficient for suppressing seizures. Induction of the Y2R gene alone in the excitatory neurons may be sufficient for suppressing seizures, considering the low but constitutive expression of the endogenous NPY peptides in the hippocampus, and the increase in its expression level at the period of seizure onset (Fig. 5A). If that is the case, expressing a single gene, for example, a constitutively active form of the Y2R gene alone in place of the wild-type counterpart, seems to be a simpler and more secured way to suppress the seizures.

Forced expression of NPY gene in the excitatory neurons may also be sufficient. At least, authors could have examined the effect from NPY alone (LV\_NPY) in vivo and evaluated its efficacy of suppression. I would like the authors to add a few control experiments to strengthen the argument that the combinatorial gene expression is critical.

2) "Autoregulation on the seizure activity"

Authors argue that combinatorial expression of the NPY and Y2R genes in Hippocampal excitatory neurons exerts an "auto-feedback" regulation on the epileptic activity. When it comes to autoregulation, one would imagine a series of consecutive events in the hippocampus; 1) NPY release triggered by a spontaneous epileptic activity in the excitatory neurons, 2) Y2R activation by the induced NPY, 3) hyperpolarization of the membrane potential due to the K-channel opening after Y2R activation or whatever, and 4) premature shut-down of the epileptic activity. If the scenario is right, frequency of the seizure events in TKO mice would not be influenced much by expressing the NPY-Y2R transgenes, but the seizure length should be shortened drastically. The results shown in figure 6 C.D, however, are not consistent with the scenario. Detectable seizure was nearly absent in TKO/LV\_NPY\_Y2R mice. I wonder how and when the NPY-Y2R system was activated without triggered by seizure activity in these seizure-free animals. I'd like the authors discuss more about the mechanism how the NPY-Y2R system autoregulates the spontaneous seizures in TKO mice.

Another scenario is that TKO/LV\_NPY\_Y2R mice still had sub-threshold (undetectable) seizure activities frequently, which continuously activated the NPY/Y2R system in these mice. If that is the case, expressing a constitutively active form of the Y2R gene alone would be sufficient for suppressing the seizures.

3) "interference on the normal hippocampal function"

I agree with the authors that forced expression of the NPY-Y2R transgenes in the hippocampal

excitatory neurons suppress the seizures quite well (Fig. 6). Now, I would like the authors to discuss the safety of the LV\_NPY\_Y2R.

It would be very nice if the authors can show that forced expression of the NPY-Y2R genes does not perturb the normal activity of the healthy excitatory neurons. Did authors observe any sign of behavioral abnormality in LV\_NPY\_Y2R mice? Authors should report detailed non-ictal behaviors/observations of the TKO/LV\_NPY\_Y2R mice. Autoregulating the epileptic activity as necessary seems to be definitely a better strategy than continuously lowering the neural activity using a constitutively active form of Y2R gene, which presumably has deleterious effects on the normal function of the hippocampus. Authors should discuss the merits and demerits of combinatorial gene expression strategy in comparison with single gene strategy, such as a constitutively active form of Y2R.

Minor points;

Fig.2 D. The bands for pre-processed NPY and mature NPY should be annotated and indicated by arrows. The unit (kDa ?) should also be indicated. The two lanes for LV\_Y2-NPY are redundant.

Fig6 E,F. Did the authors also check whether the NPY/Y2R transgenes were successfully expressed or not in the TKO/LV\_NPY\_Y2R mice with seizures (n = 4)? Would the failure of suppressing the seizure in these mice be accounted for simply by a failure of NPY/Y2R transgene expression in the hippocampus?

The success of the combinatorial gene therapy using NPY-Y2R system is clear. The manuscript as in the current form is worth while publishing at least as a short report. For a full report, authors may want to consider the concerns above, and revise the manuscript.

Cattaneo et al.

“Combinatorial gene therapy for epilepsy based on Neuropeptide Y and its Y2 receptor”  
Ms number **EMBOR-2023-58691-T** (transfer from EMBO Mol Med EMM-2023-18970)

### Point-by-point response to Reviewers

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

There are several papers showing an anticonvulsive effect of combined injections of NPY and Y2 vectors in other animal models. This paper, however uses CamKinase IIa promoters to allow expression only on glutamatergic neurons.

#### Referee #1 (Remarks for Author):

The authors use intrahippocampal injections of NPY and Y2 receptor vectors on CamKinase IIa promoter and test this in on epileptic activity in synapsin triple KO mice.

This approach has been well established (several times) for NPY vectors and NPY + Y2 vectors in other animal models and using other promoters. The use of CamKinaseII promoters used here, however, has the purpose to restrict expression to glutamatergic neurons.

Although the findings are not entirely novel, the paper shows a very nice analysis of the sites of vector expression and documents the anti-seizure effect of a combination of Y2 and NPY expressing vectors.

The experiments document expression of the vectors mainly (or exclusively) in mossy fibers. Surprisingly, they are obviously not expressed in mossy cells of the dentate gyrus. Why? Otherwise one would find NPY and Y2 in the inner molecular layer.

RE: Indeed, some degree of expression in mossy cells was detected, with labeling of the inner molecular layer, which was more obviously detectable contralaterally. This information was reported and shown in the original manuscript (page 6, first 2 lines, and Fig. 3D).

ACTION: To refine this observation, we may perform immunofluorescence labeling of mossy cells in LV\_EGFP animals using an antibody against mGluR2/3 (Bui et al., Science 2018).

Figure 5. What does the difference in NPY expression between B and D mean?

RE: Panel B represents the quantitative measurements of NPY hippocampal levels that were performed in 5 animals per group per time point at four different timepoints (p30/60/90/120). Panel D is a representative image that qualitatively indicates the localization of NPY upregulation, that is, mainly in the mossy fiber terminals of the TKO mice.

At p100 there was a high expression of NPY in mossy fibers (B), not at p120?

RE: Indeed, we detected increased NPY expression at p90, when most of the animals were experiencing spontaneous seizures, while NPY levels returned to baseline by p120. In other words, increased NPY expression follows the onset of spontaneous seizures and precedes their attenuation. This suggests that NPY overexpression may represent an adaptive mechanism to seizure activity, that is set in place to counteract seizures themselves (which in fact decrease after p90 in TKO mice).

How does NPY expression in mossy fibers relate to recent seizure activity?

RE: In keeping with findings in other models (Vezzani et al., Neuropeptides 2004; Cattaneo et al., Front Mol Neurosci 2021), we hypothesize that reactive NPY overexpression reflects

intensity of seizure activity also in the TKO model. However, we did not measure the correlation between seizure activity and NPY expression, as this was beyond the goals of the present study.

**ACTION:** This point may be briefly discussed in the revised manuscript.

Is this expression typical for all animals?

**RE:** Yes, this pattern is observed in all animals that were analyzed.

Figure 6. To which time interval does "number of seizures" refer to? Video recording started two weeks after vector injection. How long did the recording last?

**RE:** The time interval of "number of seizures" refers to the three weeks of recording.

**ACTION:** This may be better specified in the figure legend.

6E. Would in the Y2-NPY group the number of seizure-free mice go further down after 30 days? I think one should investigate this.

**RE:** We agree that it would be interesting to know whether seizure freedom is maintained for a longer time. Unfortunately, we could not prolong the recording period over 3 weeks because of limited duration of the telemetry battery. Please remember that the cost of recording animals in the telemetry system is very high. We are aware of the need of refining our observations in the future, in longer time periods and in other epilepsy models.

**ACTION:** We will explicitly state this need in the revised manuscript.

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

The choice of Synapsin TKO as model raises questions. It is not a recognized model of drug-resistant epilepsy, and its translational potential is unclear. The authors should have used other models where AAV-NPY-Y2 has already been tested

**RE:** The multiple reasons why we selected the TKO model for this study are described in the original manuscript (page 6, last 3 lines to page 7, end of first paragraph). As stated above, while we are aware of the need of extending our observations to other models, we thought that this was a good model to start testing our new vector. Incidentally, please note that this point was raised only by Referee #2, whereas Referee #1 did not comment on it, and Referee #3 explicitly stated that "The choice of the model organism is adequate".

**Referee #2** (Remarks for Author):

Cattaneo and colleagues devised a combinatorial approach involving lentivirus delivery of NPY and its receptor Y2, driven by a CamkII promoter. They demonstrated NPY and Y2 expression both in vitro and in vivo. Additionally, they observed no expression in astrocytes and inhibitory neurons in vivo.

Subsequently, they administered this lentiviral tool to Synapsin TKO mice before the onset of seizures, revealing a reduction in seizures and an increase in seizure-free animals.

To the best of my knowledge, this marks the first gene therapy approach for Synapsin TKO mice. Nevertheless, caution is necessary before characterizing it as a "promising treatment option for drug-resistant epilepsy."

RE: We agree with the Referee. Our statement in the Abstract was excessively optimistic, while our view is better expressed elsewhere in the manuscript.

ACTION: We will rephrase this sentence appropriately.

Several major concerns arise:

1. The statement, "However, none so far included a built-in auto-regulatory system, and each, therefore, may lead to excessive inhibition," is inaccurate. Previous auto-regulatory gene therapies for epilepsy, such as those conducted by Kullmann's lab at UCL, and those including work on NPY and Y2 co-expression using a single AAV (e.g., Melin et al., 2019, 2023, and Szczygie et al., 2020).

RE: We agree that our statement is inaccurate. Please note, however, that whereas the Kullmann's lab (Qiu et al., Science 2022) proposed a regulated system based on the expression of therapeutic genes under the control of an activity-dependent promoter (that is, a regulation at transcriptional level), our approach entails an autoregulation in which transgenes are continuously expressed, not altering normal neuronal function but activating an auto-regulatory loop in case of hyper-activity. With reference instead to previous studies on NPY/Y2, as recognized by all Referees, the novelty is the expression of transgenes in excitatory neurons by using the CaMKII promoter (by the way, this is a necessary prerequisite for our auto-regulatory strategy). In sum, we believe that it is fair to say (words of Reviewer #3) that the study "can be another excellent work supporting the concept in a complementary fashion".

ACTION: We will revise the manuscript in order to make the novelty clearer, with reference in particular to the studies mentioned by the Referee.

2. The choice of Synapsin TKO as model raises questions. It is not a recognized model of drug-resistant epilepsy, and its translational potential is unclear. Why did the authors not opt for other models where AAV-NPY-Y2 has already been tested?

Please see above, our response to the Comments on Novelty of this Referee.

RE: The multiple reasons why we selected the TKO model for this study are described in the original manuscript (page 6, last 3 lines to page 7, end of first paragraph). As stated above, while we are aware of the need of extending our observations to other models, we thought that this was a good model to start testing our new vector.

Incidentally, please note that this point was raised only by Referee #2, whereas Referee #1 did not comment on it, and Referee #3 explicitly stated that "The choice of the model organism is adequate".

ACTION: We will explicitly state in the revised manuscript that it will be needed in the future to extend our observations to other models.

3. In terms of translation, lentiviral delivery in the human hippocampus may not be a realistic therapy due to the limited virus spread. Why was AAV not used, especially considering its previous use with NPY-Y2? The novelty here seems to lie in the specific excitatory promoter.

RE: The choice of primarily using lenti- rather than AAV vectors was indeed based on the idea to use a specific excitatory promoter (CamKII), which implies a relatively large expression cassette that could hardly fit in AAV. In addition, we aimed at restricting the expression, for



what possible, to granule cells. While AAV vectors certainly ensure a greater spread, one should also take into account that they must be used at high titers, which raise some concerns (e.g., BBB-breakdown and DNA interference - Guo et al., Mol Ther Meth & Clin Devel 2023). A comment of Referee #3 is perfectly in line with these considerations: “usage of lentiviral vector for gene therapy seems to be reasonable and less hazardous compared to other viral vectors”. Note also that a currently approved gene therapy clinical trial for focal epilepsy uses lentivectors (ClinicalTrials.gov ID NCT04601974); thus, it is not demonstrated that the limited spread of the virus cannot ensure a therapeutic effect.

**ACTION:** We may better explain the reasons for choosing a lentivector platform for the present study.

4. The specificity of CamKII to excitatory neurons, particularly the lack of proper quantification, including inhibitory neurons and the percentage of transduced cells, needs clarification. Literature reports mixed results.

**RE:** We have tested the specificity of CamKII in excitatory neurons by verifying lack of expression in either parvalbumin-positive and NPY-positive inhibitory neurons, and in astrocytes (Fig. 3).

**ACTION:** We are willing to replicate and extend these data in a new cohort of animals, by quantifying not only the lack of expression in astrocytes (using GFAP as well as S100b immunofluorescence) and in PV- and NPY-positive interneurons, but also by verifying it in other GABAergic populations (e.g., SOM) and all GABAergic interneurons (using GAD65-67 immunofluorescence). These data will help to shed more light on mixed results obtained so far with the use of this specific promoter.

5. Could the observed effect be a mere delay in epileptogenesis? A longitudinal study is crucial to explore this possibility, as the two-week recording period appears short given the dynamics shown in Figure 6E.

**RE:** This is indeed a possibility, even if our recording period was 3 and not 2 weeks. As described above (our response to Referee #1, point 6A), we did not prolong the recording period over 3 weeks because of limited battery duration and cost of the telemetry probe. Nonetheless, we are aware of the need of refining our observations in the future, in longer time periods and in other epilepsy models.

**ACTION:** We may add a comment in the Discussion stating the need to extend our finding in a longer timeframe of observation.

6. The use of T2A for NPY-Y2, as previously done by Melin et al., renders the discussion on this matter redundant. It would be more beneficial to focus on how this therapy fits into the context of other developed gene therapies.

**RE:** We respectfully disagree with the reviewer's comment. IRES, used by Melin et al. (2023) and T2A elements, used in the present work to simultaneously express two or more genes, are structurally different in terms of translational mechanism. We believe that is indeed relevant to discuss the topic not only with reference to our work, but also for potential future gene therapy products that might need to obtain the simultaneous expression of two genes.

7. Although the authors discussed the self-regulated approach, direct evidence supporting this claim is lacking in the present work.

RE: Since both transgenes are expressed by each single viral particle, they must be expressed together in each transduced cell. We have evidence of localization of both NPY and Y2R in mossy fiber terminals, i.e., of the anatomical substrate of autoregulation. In addition, we believe that the most parsimonious interpretation of the observation that NPY extracellular levels in vitro are increased much less by the vector expressing NPY and Y2R than by the vector expressing NPY alone is the existence of an autofeedback. This having said, we acknowledge that we need further verification that an auto-inhibitory pre-synaptic feedback loop on glutamate release can be established.

ACTION: We plan to perform experiments to show that neurons simultaneously over-expressing NPY and Y2 receptor release a lower amount of glutamate. Using cultured hippocampal neurons, transduced with either EGFP, NPY alone or Y2\_NPY expressing vectors, we will i) directly measure the amount of glutamate released by neurons in which synaptic activity is stimulated (e.g., by high K<sup>+</sup> or by treatment with 4-amino pyridine); ii) measure the vesicular release of glutamate, exploiting the properties of the FM1-43 dye (Amaral et al., Methods Mol Biol 2011), since the loading and loss of the fluorescent dye provide a reliable measurement of synaptic vesicle retrieval and release in cultured neurons (Sambri et al., EBioMedicine 2020).

8. If the authors aim to establish the superiority of this therapy over NPY alone, the addition of another group to the seizure analysis for a direct comparison is essential

RE: We are sorry that we did not make ourselves sufficiently clear on this point; we did not expect our approach to be superior to previous ones in terms of seizure control. Previous studies overexpressed NPY and/or Y2 in a non-cell specific, non-regulated manner, ensuring a constant and widespread increase of this inhibitory signal. We instead aimed at restricting expression to excitatory neurons (and mainly to a population of excitatory neurons, dentate gyrus granule cells), in an auto-regulated manner. Therefore, we wanted to test if this approach was sufficient to produce effects on seizures, not superior to other approaches.

ACTION: The manuscript will be revised to make this point clearer.

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

The manuscript describes the result of a newly developed lentiviral vector which enables co-expression of anti-convulsant neuropeptide NPY and its autoreceptor Y2 genes in the epileptic hippocampus, and evaluates its ability to suppress the seizures in vivo. The critical feature of this viral vector is its use of CamKIIa promotor driving both transgenes in the same excitatory neurons, which allows autoregulation of neural excitability through Y2 receptor-mediated inhibition. Telemetry video-EEG monitoring was conducted to detect seizures in the TKO mice expressing NPY/Y2 transgenes in dentate gyrus granule cells, and a clear reduction of the seizure occurrence in these mice was demonstrated.

This is a well thought out and seems to be a well carried out study. The idea of "autoregulation" of the epileptic brain activity is novel and very interesting. The choice of the model organism (i.e., mouse) is adequate, the statistical analysis methods used are adequate, and their usage of lentiviral vector for gene therapy seems to be reasonable and less hazardous compared to other viral vectors.

The main concern I have is that a few important control experiments are missing in this study. It weakens the overall impact of the core message "autoregulation of the epileptic activity". I

would like the authors discuss more thoroughly the mechanism how the autoregulation is realized in their model system to strengthen the core message.

RE: Since both transgenes are expressed by each single viral particle, they must be expressed together in each transduced cell. We present in vitro and in vivo evidence that this is indeed the case. In addition, we believe that the most parsimonious interpretation of the observation that NPY extracellular levels in vitro are increased much less by the vector expressing NPY and Y2R than by the vector expressing NPY alone is the existence of an autofeedback. This having said, we acknowledge that we need further verification that an auto-inhibitory pre-synaptic feedback loop on glutamate release can be established.

ACTION: Not only we will discuss more thoroughly the mechanism by which the autoregulation is realized, but we will also perform in vitro experiments to verify that neurons simultaneously over-expressing NPY and Y2 receptor release a lower amount of glutamate (see our response to Referee #2, point 7).

Major points;

1) "Necessity of combinatorial gene expression"

Authors demonstrated that expressing NPY and Y2R transgenes suppressed the seizures very well (Fig.6) and argue that the combinatorial expression is important. Unfortunately, however, the authors didn't show that expressing either of the two genes alone is NOT sufficient for suppressing seizures. Induction of the Y2R gene alone in the excitatory neurons may be sufficient for suppressing seizures, considering the low but constitutive expression of the endogenous NPY peptides in the hippocampus, and the increase in its expression level at the period of seizure onset (Fig. 5A). If that is the case, expressing a single gene, for example, a constitutively active form of the Y2R gene alone in place of the wild-type counterpart, seems to be a simpler and more secured way to suppress the seizures.

Forced expression of NPY gene in the excitatory neurons may also be sufficient. At least, authors could have examined the effect from NPY alone (LV\_NPY) in vivo and evaluated its efficacy of suppression. I would like the authors to add a few control experiments to strengthen the argument that the combinatorial gene expression is critical.

RE: We apologize for not making ourselves sufficiently clear on the point that we did not expect our approach to be superior to previous ones in terms of seizure control. Previous studies overexpressed NPY and/or Y2 in a non-cell specific, non-regulated manner, ensuring a constant and widespread increase of this inhibitory signal. We instead aimed at restricting expression to excitatory neurons (and mainly to a population of excitatory neurons, dentate gyrus granule cells), in an auto-regulated manner. Therefore, we wanted to test if this approach was sufficient to produce effects on seizures, not superior to other approaches.

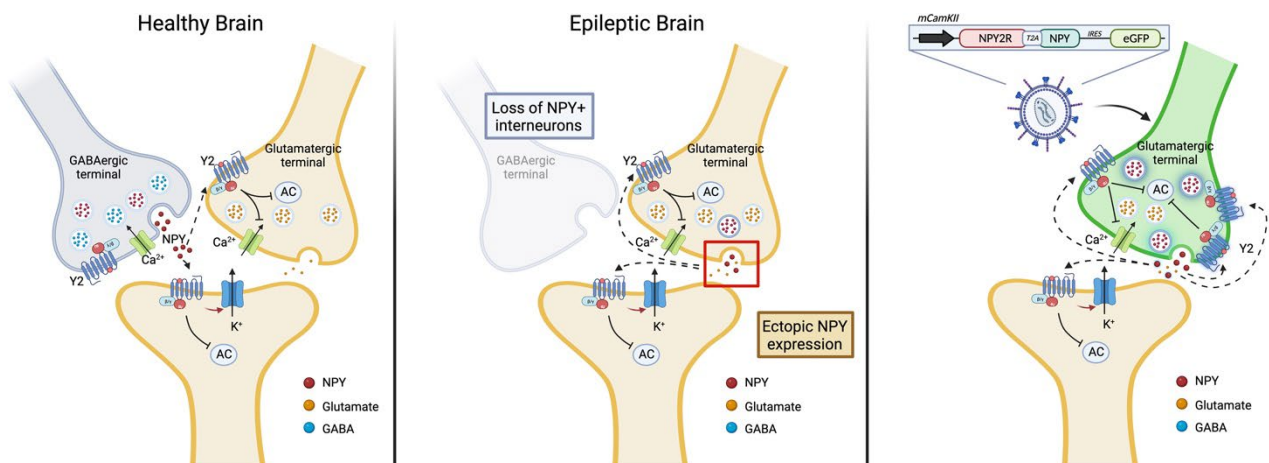
ACTION: We will rephrase key sentences in the manuscript to make our point clearer.

2) "Autoregulation on the seizure activity"

Authors argue that combinatorial expression of the NPY and Y2R genes in Hippocampal excitatory neurons exerts an "auto-feedback" regulation on the epileptic activity. When it comes to autoregulation, one would imagine a series of consecutive events in the hippocampus; 1) NPY release triggered by a spontaneous epileptic activity in the excitatory neurons, 2) Y2R activation by the induced NPY, 3) hyperpolarization of the membrane potential due to the K-channel opening after Y2R activation or whatever, and 4) premature

shut-down of the epileptic activity. If the scenario is right, frequency of the seizure events in TKO mice would not be influenced much by expressing the NPY-Y2R transgenes, but the seizure length should be shortened drastically. The results shown in figure 6 C.D, however, are not consistent with the scenario. Detectable seizure was nearly absent in TKO/LV\_NPY\_Y2R mice. I wonder how and when the NPY-Y2R system was activated without triggered by seizure activity in these seizure-free animals. I'd like the authors discuss more about the mechanism how the NPY-Y2R system autoregulates the spontaneous seizures in TKO mice. Another scenario is that TKO/LV\_NPY\_Y2R mice still had sub-threshold (undetectable) seizure activities frequently, which continuously activated the NPY/Y2R system in these mice. If that is the case, expressing a constitutively active form of the Y2R gene alone would be sufficient for suppressing the seizures.

RE: Existing data (see Krook-Magnuson et al., J Physiol 2015) support the notion (in keeping with the so called dentate 'gate' hypothesis) that preventing granule cell hyperexcitability can prevent occurrence of spontaneous seizures. Thus, our hypothesis is that an NPY-Y2R autoregulatory loop, by avoiding excessive granule cell activity and glutamate release, can prevent seizures. This implies that seizure frequency should be reduced whereas rare, occasional escape to control should evoke nearly "normal" seizures. ACTION: We will discuss more carefully this hypothesis and the hypothesis outlined by the Referee. We may also add a graphical abstract like the one below.



### 3) "interference on the normal hippocampal function"

I agree with the authors that forced expression of the NPY-Y2R transgenes in the hippocampal excitatory neurons suppress the seizures quite well (Fig. 6). Now, I would like the authors to discuss the safety of the LV\_NPY\_Y2R.

It would be very nice if the authors can show that forced expression of the NPY-Y2R genes does not perturb the normal activity of the healthy excitatory neurons. Did authors observe any sign of behavioral abnormality in LV\_NPY\_Y2R mice? Authors should report detailed non-ictal behaviors/observations of the TKO/LV\_NPY\_Y2R mice. Autoregulating the epileptic activity as necessary seems to be definitely a better strategy than continuously lowering the neural activity using a constitutively active form of Y2R gene, which presumably has deleterious effects on the normal function of the hippocampus. Authors should discuss the merits and demerits of combinatorial gene expression strategy in comparison with single gene strategy, such as a constitutively active form of Y2R.

RE: We agree with the Referee that this is an important point.

ACTION: To test the safety of our LV platform, we will perform electrophysiological experiments on transduced primary hippocampal neurons and measure to which extent the overexpression of NPY-Y2, as compared with NPY alone, might influence normal neuronal activity. We will measure the basic electrophysiological properties of neurons and their spontaneous excitatory/inhibitory post-synaptic activity.

Minor points;

Fig.2 D. The bands for pre-processed NPY and mature NPY should be annotated and indicated by arrows. The unit (kDa ?) should also be indicated. The two lanes for LV\_Y2-NPY are redundant.

RE: We thank the Referee for this suggestion.

ACTION: We will modify the image according to his/her suggestion.

Fig6 E,F. Did the authors also check whether the NPY/Y2R transgenes were successfully expressed or not in the TKO/LV\_NPY\_Y2R mice with seizures (n = 4)? Would the failure of suppressing the seizure in these mice be accounted for simply by a failure of NPY/Y2R transgene expression in the hippocampus?

RE: We thank the Referee for this valuable suggestion.

ACTION: We will extend the analysis on the brains of the animals employed in Figure 6 to verify the successful expression of transgenes.

**Referee #3** (Remarks for Author):

The success of the combinatorial gene therapy using NPY-Y2R system is clear. The manuscript as in the current form is worth while publishing at least as a short report. For a full report, authors may want to consider the concerns above, and revise the manuscript.

RE: We thank the Referee for this comment, that we endorse completely. Indeed, we view this manuscript as the conceptual basis for future studies. We feel that the results are worthwhile communicating while embarking in further necessary but complex, long, and costly experiments.

Dear Michele,

Thank you for the transfer of your manuscript with referee reports to EMBO reports, and for your proposed revision plan.

I think your suggestions for how to revise your study are good, and I am happy to invite you to revise it with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of major revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (27th Apr 2024). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

**IMPORTANT NOTE:** we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

- 1) A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.
- 2) Your manuscript contains statistics and error bars based on  $n=2$ . Please use scatter blots in these cases. No statistics should be calculated if  $n=2$ .

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure). See [https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress\\_Figure\\_Guidelines\\_061115-1561436025777.pdf](https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf) for more info on how to prepare your figures.

3) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as "Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called \*Appendix\*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here:

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

4) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

5) a complete author checklist, which you can download from our author guidelines . Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

6) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (. Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines

7) Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database (see <https://www.embopress.org/page/journal/14693178/authorguide#datadeposition>). Please remember to provide a reviewer password if the datasets are not yet public. The accession numbers and database should be listed in a formal "Data Availability" section placed after Materials & Method (see also <https://www.embopress.org/page/journal/14693178/authorguide#datadeposition>). Please note that the Data Availability Section is restricted to new primary data that are part of this study. \* Note - All links should resolve to a page where the data can be accessed. \*

If your study has not produced novel datasets, please mention this fact in the Data Availability Section.

8) At EMBO Press we ask authors to provide source data for the main manuscript figures. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

9) Our journal also encourages inclusion of \*data citations in the reference list\* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at <https://www.embopress.org/page/journal/14693178/authorguide#referencesformat>

10) Regarding data quantification (see Figure Legends:  
<https://www.embopress.org/page/journal/14693178/authorguide#figureformat>)

The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.),
- If the data are obtained from n {less than or equal to} 2, use scatter blots showing the individual data points.

Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

- Please also include scale bars in all microscopy images.

11) The journal requires a statement specifying whether or not authors have competing interests (defined as all potential or actual interests that could be perceived to influence the presentation or interpretation of an article). In case of competing interests, this must be specified in your disclosure statement. Further information: <https://www.embopress.org/competing-interests>

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File (RPF) to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know ([emboreports@embo.org](mailto:emboreports@embo.org)). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

I look forward to seeing a revised form of your manuscript when it is ready.

Best regards,  
Esther

Esther Schnapp, PhD  
Senior Editor  
EMBO reports

Cattaneo et al.

“Combinatorial gene therapy for epilepsy based on Neuropeptide Y and its Y2 receptor”  
Ms number **EMBOR-2023-58691-T** (transfer from EMBO Mol Med EMM-2023-18970)

## Point-by-point response to Reviewers

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

There are several papers showing an anticonvulsive effect of combined injections of NPY and Y2 vectors in other animal models. This paper, however uses CamKinase IIa promoters to allow expression only on glutamatergic neurons.

### Referee #1 (Remarks for Author):

The authors use intrahippocampal injections of NPY and Y2 receptor vectors on CamKinase IIa promoter and test this in on epileptic activity in synapsin triple KO mice. This approach has been well established (several times) for NPY vectors and NPY + Y2 vectors in other animal models and using other promoters. The use of CamKinaseII promoters used here, however, has the purpose to restrict expression to glutamatergic neurons. Although the findings are not entirely novel, the paper shows a very nice analysis of the sites of vector expression and documents the anti-seizure effect of a combination of Y2 and NPY expressing vectors.

The experiments document expression of the vectors mainly (or exclusively) in mossy fibers. Surprisingly, they are obviously not expressed in mossy cells of the dentate gyrus. Why? Otherwise one would find NPY and Y2 in the inner molecular layer.

**PROPOSAL OF RESPONSE TO EMBO REPORT EDITORS** (approved with mail from Esther Schnapp dated January 26, 2024)

*Indeed, some degree of expression in mossy cells was detected, with labeling of the inner molecular layer, which was more obviously detectable contralaterally. This information was reported and shown in the original manuscript (page 6, first 2 lines, and Fig. 3D).*

*ACTION: To refine this observation, we may perform immunofluorescence labeling of mossy cells in LV\_EGFP animals using an antibody against mGluR2/3 (Bui et al., Science 2018).*

### RESPONSE

*We attempted to perform the mGluR2/3 staining using two commercially available antibodies and multiple experimental procedures, but failed to observe a specific signal with immunofluorescence, as per Bui et al. (2018). While we agree that this would have been a useful refinement, we trust that the original observation (**now at page 7, 2<sup>nd</sup> paragraph, lines 6-8, and Fig. 3D**) is sufficient for addressing the Reviewer's comment.*

Figure 5. What does the difference in NPY expression between B and D mean?

### PROPOSAL OF RESPONSE

*Panel B represents the quantitative measurements of NPY hippocampal levels that were performed in 5 animals per group per time point at four different timepoints (p30/60/90/120). Panel D is a representative image that qualitatively indicates the localization of NPY upregulation, that is, mainly in the mossy fiber terminals of the TKO mice.*



## RESPONSE

*As above, in the proposal.*

At p100 there was a high expression of NPY in mossy fibers (B), not at p120?

## PROPOSAL OF RESPONSE

*Indeed, we detected increased NPY expression at p90, when most of the animals were experiencing spontaneous seizures, while NPY levels returned to baseline by p120. In other words, increased NPY expression follows the onset of spontaneous seizures and precedes their attenuation. This suggests that NPY overexpression may represent an adaptive mechanism to seizure activity, that is set in place to counteract seizures themselves (which in fact decrease after p90 in TKO mice).*

## RESPONSE

*As above, in the proposal.*

How does NPY expression in mossy fibers relate to recent seizure activity?

## PROPOSAL OF RESPONSE

*In keeping with findings in other models (Vezzani et al., Neuropeptides 2004; Cattaneo et al., Front Mol Neurosci 2021), we hypothesize that reactive NPY overexpression reflects intensity of seizure activity also in the TKO model. However, we did not measure the correlation between seizure activity and NPY expression, as this was beyond the goals of the present study.*

*ACTION: This point may be briefly discussed in the revised manuscript.*

## RESPONSE

*A sentence has been added as a comment to our finding of increased NPY levels at p90-p100 in the TKO model (page 9, 2<sup>nd</sup> paragraph, lines 2-4).*

Is this expression typical for all animals?

## PROPOSAL OF RESPONSE

*Yes, this pattern is observed in all animals that were analyzed.*

## RESPONSE

*As above, in the proposal.*

Figure 6. To which time interval does "number of seizures" refer to? Video recording started two weeks after vector injection. How long did the recording last?

## PROPOSAL OF RESPONSE

*The time interval of "number of seizures" refers to the three weeks of recording.*

*ACTION: This may be better specified in the figure legend.*

## RESPONSE

*A sentence has been added to specify this important point (page 9, 4<sup>th</sup> paragraph, lines 1-2).*

6E. Would in the Y2-NPY group the number of seizure-free mice go further down after 30 days? I think one should investigate this.

#### PROPOSAL OF RESPONSE

*We agree that it would be interesting to know whether seizure freedom is maintained for a longer time. Unfortunately, we could not prolong the recording period over 3 weeks because of limited duration of the telemetry battery. Please remember that the cost of recording animals in the telemetry system is very high. We are aware of the need of refining our observations in the future, in longer time periods and in other epilepsy models.*

*ACTION: We will explicitly state this need in the revised manuscript.*

#### RESPONSE

*A sentence has been added in the Discussion to mention this limitation of our study (page 14, 3<sup>d</sup> paragraph, lines 3-5).*

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

The choice of Synapsin TKO as model raises questions. It is not a recognized model of drug-resistant epilepsy, and its translational potential is unclear. The authors should have used other models where AAV-NPY-Y2 has already been tested

#### PROPOSAL OF RESPONSE

*The multiple reasons why we selected the TKO model for this study are described in the original manuscript (page 6, last 3 lines to page 7, end of first paragraph). As stated above, while we are aware of the need of extending our observations to other models, we thought that this was a good model to start testing our new vector. Incidentally, please note that this point was raised only by Referee #2, whereas Referee #1 did not comment on it, and Referee #3 explicitly stated that "The choice of the model organism is adequate".*

#### RESPONSE

*As above, in the proposal. The statement on the reasons to select the TKO model can be found in the revised manuscript at (page 8, 2<sup>nd</sup> paragraph, lines 5 to end).*

**Referee #2** (Remarks for Author):

Cattaneo and colleagues devised a combinatorial approach involving lentivirus delivery of NPY and its receptor Y2, driven by a CamkII promoter. They demonstrated NPY and Y2 expression both in vitro and in vivo. Additionally, they observed no expression in astrocytes and inhibitory neurons in vivo. Subsequently, they administered this lentiviral tool to Synapsin TKO mice before the onset of seizures, revealing a reduction in seizures and an increase in seizure-free animals.

To the best of my knowledge, this marks the first gene therapy approach for Synapsin TKO mice. Nevertheless, caution is necessary before characterizing it as a "promising treatment option for drug-resistant epilepsy."

#### PROPOSAL OF RESPONSE

*We agree with the Referee. Our statement in the Abstract was excessively optimistic, while our view is better expressed elsewhere in the manuscript.*

*ACTION: We will rephrase this sentence appropriately.*

#### RESPONSE

*The sentence has been rewritten (page 2, last 3 lines of the Abstract).*

Several major concerns arise:

1. The statement, "However, none so far included a built-in auto-regulatory system, and each, therefore, may lead to excessive inhibition," is inaccurate. Previous auto-regulatory gene therapies for epilepsy, such as those conducted by Kullmann's lab at UCL, and those including work on NPY and Y2 co-expression using a single AAV (e.g., Melin et al., 2019, 2023, and Szczygie et al., 2020).

#### PROPOSAL OF RESPONSE

*We agree that our statement is inaccurate. Please note, however, that whereas the Kullmann's lab (Qiu at al., Science 2022) proposed a regulated system based on the expression of therapeutic genes under the control of an activity-dependent promoter (that is, a regulation at transcriptional level), our approach entails an autoregulation in which transgenes are continuously expressed, not altering normal neuronal function but activating an auto-regulatory loop in case of hyper-activity. With reference instead to previous studies on NPY/Y2, as recognized by all Referees, the novelty is the expression of transgenes in excitatory neurons by using the CaMKII promoter (by the way, this is a necessary prerequisite for our auto-regulatory strategy). In sum, we believe that it is fair to say (words of Reviewer #3) that the study "can be another excellent work supporting the concept in a complementary fashion".*

*ACTION: We will revise the manuscript in order to make the novelty clearer, with reference in particular to the studies mentioned by the Referee.*

#### RESPONSE

*The statement mentioned by the Reviewers has been corrected (page 3, 3<sup>rd</sup> paragraph, lines 4-6). In addition, we rephrased several sentences in the 3<sup>rd</sup> paragraph of page 4 to emphasize the differences between our approach and those used previously.*

2. The choice of Synapsin TKO as model raises questions. It is not a recognized model of drug-resistant epilepsy, and its translational potential is unclear. Why did the authors not opt for other models where AAV-NPY-Y2 has already been tested?

#### PROPOSAL OF RESPONSE

*Please see above, our response to the Comments on Novelty of this Referee.*

*The multiple reasons why we selected the TKO model for this study are described in the original manuscript (page 6, last 3 lines to page 7, end of first paragraph). As stated*

above, while we are aware of the need of extending our observations to other models, we thought that this was a good model to start testing our new vector. Incidentally, please note that this point was raised only by Referee #2, whereas Referee #1 did not comment on it, and Referee #3 explicitly stated that “The choice of the model organism is adequate”.

*ACTION:* We will explicitly state in the revised manuscript that it will be needed in the future to extend our observations to other models.

## RESPONSE

As above, in the proposal. The statement on the reasons to select the TKO model can be found in the revised manuscript at [page 8, 2<sup>n</sup> paragraph, lines 5 to end](#). A statement of the future need to extend the observations to other epilepsy models has also been added in the Discussion ([page 14, 3<sup>rd</sup> paragraph, lines 3-6](#)).

3. In terms of translation, lentiviral delivery in the human hippocampus may not be a realistic therapy due to the limited virus spread. Why was AAV not used, especially considering its previous use with NPY-Y2? The novelty here seems to lie in the specific excitatory promoter.

## PROPOSAL OF RESPONSE

The choice of primarily using lenti- rather than AAV vectors was indeed based on the idea to use a specific excitatory promoter (CamKII), which implies a relatively large expression cassette that could hardly fit in AAV. In addition, we aimed at restricting the expression, for what possible, to granule cells. While AAV vectors certainly ensure a greater spread, one should also take into account that they must be used at high titers, which raise some concerns (e.g., BBB-breakdown and DNA interference - Guo et al., *Mol Ther Meth & Clin Devel* 2023). A comment of Referee #3 is perfectly in line with these considerations: “usage of lentiviral vector for gene therapy seems to be reasonable and less hazardous compared to other viral vectors”. Note also that a currently approved gene therapy clinical trial for focal epilepsy uses lentivectors ([ClinicalTrials.gov ID NCT04601974](#)); thus, it is not demonstrated that the limited spread of the virus cannot ensure a therapeutic effect.

*ACTION:* We may better explain the reasons for choosing a lentivector platform for the present study.

## RESPONSE

We added a few sentences to explain the reasons why, in our experimental settings, a lentivector platform was preferable over AAV ([page 4, 2<sup>nd</sup> paragraph, lines 3 to end](#)).

4. The specificity of CamKII to excitatory neurons, particularly the lack of proper quantification, including inhibitory neurons and the percentage of transduced cells, needs clarification. Literature reports mixed results.

## PROPOSAL OF RESPONSE

We have tested the specificity of CamKII in excitatory neurons by verifying lack of expression in either parvalbumin-positive and NPY-positive inhibitory neurons, and in astrocytes (Fig. 3).

*ACTION:* We are willing to replicate and extend these data in a new cohort of animals, by quantifying not only the lack of expression in astrocytes (using GFAP as well as

*S100b immunofluorescence) and in PV- and NPY-positive interneurons, but also by verifying it in other GABAergic populations (e.g., SOM) and all GABAergic interneurons (using GAD65-67 immunofluorescence). These data will help to shed more light on mixed results obtained so far with the use of this specific promoter.*

#### RESPONSE

*Data have been extended and quantified (Results: page 7, 3<sup>rd</sup> paragraph; Methods: page 22, "Imaging and analysis"). A new figure has been added with a detailed analysis of EGFP expression in interneurons (Figure 4).*

5. Could the observed effect be a mere delay in epileptogenesis? A longitudinal study is crucial to explore this possibility, as the two-week recording period appears short given the dynamics shown in Figure 6E.

#### PROPOSAL OF RESPONSE

*This is indeed a possibility, even if our recording period was 3 and not 2 weeks. As described above (our response to Referee #1, point 6A), we did not prolong the recording period over 3 weeks because of limited battery duration and cost of the telemetry probe. Nonetheless, we are aware of the need of refining our observations in the future, in longer time periods and in other epilepsy models.*

*ACTION: We may add a comment in the Discussion stating the need to extend our finding in a longer timeframe of observation.*

#### RESPONSE

*A sentence has been added in the Discussion to mention this limitation of our study (page 14, 3<sup>rd</sup> paragraph, lines 3-6).*

6. The use of T2A for NPY-Y2, as previously done by Melin et al., renders the discussion on this matter redundant. It would be more beneficial to focus on how this therapy fits into the context of other developed gene therapies.

#### PROPOSAL OF RESPONSE

*We respectfully disagree with the reviewer's comment. IRES, used by Melin et al. (2023) and T2A elements, used in the present work to simultaneously express two or more genes, are structurally different in terms of translational mechanism. We believe that is indeed relevant to discuss the topic not only with reference to our work, but also for potential future gene therapy products that might need to obtain the simultaneous expression of two genes.*

#### RESPONSE

*As above, in the proposal. In addition, a more extensive description of how our gene therapy approach compares with other developed gene therapies has been added in the Introduction (page 4, last paragraph).*

7. Although the authors discussed the self-regulated approach, direct evidence supporting this claim is lacking in the present work.

#### PROPOSAL OF RESPONSE

Since both transgenes are expressed by each single viral particle, they must be expressed together in each transduced cell. We have evidence of localization of both NPY and Y2R in mossy fiber terminals, i.e., of the anatomical substrate of autoregulation. In addition, we believe that the most parsimonious interpretation of the observation that NPY extracellular levels in vitro are increased much less by the vector expressing NPY and Y2R than by the vector expressing NPY alone is the existence of an autofeedback. This having said, we acknowledge that we need further verification that an auto-inhibitory pre-synaptic feedback loop on glutamate release can be established.

*ACTION:* We plan to perform experiments to show that neurons simultaneously over-expressing NPY and Y2 receptor release a lower amount of glutamate. Using cultured hippocampal neurons, transduced with either EGFP, NPY alone or Y2\_NPY expressing vectors, we will i) directly measure the amount of glutamate released by neurons in which synaptic activity is stimulated (e.g., by high K<sup>+</sup> or by treatment with 4-amino pyridine); ii) measure the vesicular release of glutamate, exploiting the properties of the FM1-43 dye (Amaral et al., *Methods Mol Biol* 2011), since the loading and loss of the fluorescent dye provide a reliable measurement of synaptic vesicle retrieval and release in cultured neurons (Sambri et al., *EBioMedicine* 2020).

## RESPONSE

We performed the planned experiments on glutamate release, that are now described and discussed in the revised manuscript (Results: page 6, last paragraph ending at page 7; Methods, page 20; Figure 2G; Discussion: page 11, 1<sup>st</sup> paragraph, lines 9 to end). We also attempted to measure the vesicular release of glutamate using FM1-43. Unfortunately, this technique did not prove sufficiently specific and sensitive in our cultures. The reason for this unexpected outcome is unclear; the technique has been validated in peripheral neurons and embryonic cultures, but never tested in post-natal, central neuronal cultures like ours. While we believe that the level of evidence in favor of autoregulation is reasonable and maybe sufficient, we prudentially avoided to present it as solid and conclusive (Results: page 7, lines 3-4; Discussion: page 11, 1<sup>st</sup> paragraph, lines 9 to end).

8. If the authors aim to establish the superiority of this therapy over NPY alone, the addition of another group to the seizure analysis for a direct comparison is essential

## PROPOSAL OF RESPONSE

We are sorry that we did not make ourselves sufficiently clear on this point; we did not expect our approach to be superior to previous ones in terms of seizure control. Previous studies overexpressed NPY and/or Y2 in a non-cell specific, non-regulated manner, ensuring a constant and widespread increase of this inhibitory signal. We instead aimed at restricting expression to excitatory neurons (and mainly to a population of excitatory neurons, dentate gyrus granule cells), in an auto-regulated manner. Therefore, we wanted to test if this approach was sufficient to produce effects on seizures, not superior to other approaches.

*ACTION:* The manuscript will be revised to make this point clearer.

## RESPONSE

*We revised the manuscript as described in the proposal. In particular, we added a few sentences in the Discussion to clarify this point (page 12, 1<sup>st</sup> paragraph).*

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

The manuscript describes the result of a newly developed lentiviral vector which enables co-expression of anti-convulsant neuropeptide NPY and its autoreceptor Y2 genes in the epileptic hippocampus, and evaluates its ability to suppress the seizures in vivo. The critical feature of this viral vector is its use of CamKIIa promotor driving both transgenes in the same excitatory neurons, which allows autoregulation of neural excitability through Y2 receptor-mediated inhibition. Telemetry video-EEG monitoring was conducted to detect seizures in the TKO mice expressing NPY/Y2 transgenes in dentate gyrus granule cells, and a clear reduction of the seizure occurrence in these mice was demonstrated.

This is a well thought out and seems to be a well carried out study. The idea of "autoregulation" of the epileptic brain activity is novel and very interesting. The choice of the model organism (i.e., mouse) is adequate, the statistical analysis methods used are adequate, and their usage of lentiviral vector for gene therapy seems to be reasonable and less hazardous compared to other viral vectors.

The main concern I have is that a few important control experiments are missing in this study. It weakens the overall impact of the core message "autoregulation of the epileptic activity". I would like the authors discuss more thoroughly the mechanism how the autoregulation is realized in their model system to strengthen the core message.

**PROPOSAL OF RESPONSE**

*Since both transgenes are expressed by each single viral particle, they must be expressed together in each transduced cell. We present in vitro and in vivo evidence that this is indeed the case. In addition, we believe that the most parsimonious interpretation of the observation that NPY extracellular levels in vitro are increased much less by the vector expressing NPY and Y2R than by the vector expressing NPY alone is the existence of an autofeedback. This having said, we acknowledge that we need further verification that an auto-inhibitory pre-synaptic feedback loop on glutamate release can be established.*

*ACTION: Not only we will discuss more thoroughly the mechanism by which the autoregulation is realized, but we will also perform in vitro experiments to verify that neurons simultaneously over-expressing NPY and Y2 receptor release a lower amount of glutamate (see our response to Referee #2, point 7).*

**RESPONSE**

*We performed the planned experiments on glutamate release, that are now described and discussed in the revised manuscript (Results: page 6, last paragraph ending at page 7; Methods, page 20; Figure 2G; Discussion: page 11, 1<sup>st</sup> paragraph, lines 9 to end).*

Major points;

1) "Necessity of combinatorial gene expression"

Authors demonstrated that expressing NPY and Y2R transgenes suppressed the seizures very well (Fig.6) and argue that the combinatorial expression is important. Unfortunately, however, the authors didn't show that expressing either of the two genes alone is NOT sufficient for suppressing seizures. Induction of the Y2R gene alone in the excitatory neurons may be sufficient for suppressing seizures, considering the low but constitutive expression of

the endogenous NPY peptides in the hippocampus, and the increase in its expression level at the period of seizure onset (Fig. 5A). If that is the case, expressing a single gene, for example, a constitutively active form of the Y2R gene alone in place of the wild-type counterpart, seems to be a simpler and more secured way to suppress the seizures. Forced expression of NPY gene in the excitatory neurons may also be sufficient. At least, authors could have examined the effect from NPY alone (LV\_NPY) in vivo and evaluated its efficacy of suppression. I would like the authors to add a few control experiments to strengthen the argument that the combinatorial gene expression is critical.

#### PROPOSAL OF RESPONSE

*We apologize for not making ourselves sufficiently clear on the point that we did not expect our approach to be superior to previous ones in terms of seizure control. Previous studies overexpressed NPY and/or Y2 in a non-cell specific, non-regulated manner, ensuring a constant and widespread increase of this inhibitory signal. We instead aimed at restricting expression to excitatory neurons (and mainly to a population of excitatory neurons, dentate gyrus granule cells), in an auto-regulated manner. Therefore, we wanted to test if this approach was sufficient to produce effects on seizures, not superior to other approaches.*

*ACTION: We will rephrase key sentences in the manuscript to make our point clearer.*

#### RESPONSE

*We revised the manuscript as described in the proposal. In particular, we added a few sentences in the Discussion to clarify this point (page 12, 1<sup>st</sup> paragraph).*

#### 2) "Autoregulation on the seizure activity"

Authors argue that combinatorial expression of the NPY and Y2R genes in Hippocampal excitatory neurons exerts an "auto-feedback" regulation on the epileptic activity. When it comes to autoregulation, one would imagine a series of consecutive events in the hippocampus; 1) NPY release triggered by a spontaneous epileptic activity in the excitatory neurons, 2) Y2R activation by the induced NPY, 3) hyperpolarization of the membrane potential due to the K-channel opening after Y2R activation or whatever, and 4) premature shut-down of the epileptic activity. If the scenario is right, frequency of the seizure events in TKO mice would not be influenced much by expressing the NPY-Y2R transgenes, but the seizure length should be shortened drastically. The results shown in figure 6 C,D, however, are not consistent with the scenario. Detectable seizure was nearly absent in TKO/LV\_NPY\_Y2R mice. I wonder how and when the NPY-Y2R system was activated without triggered by seizure activity in these seizure-free animals. I'd like the authors discuss more about the mechanism how the NPY-Y2R system autoregulates the spontaneous seizures in TKO mice.

Another scenario is that TKO/LV\_NPY\_Y2R mice still had sub-threshold (undetectable) seizure activities frequently, which continuously activated the NPY/Y2R system in these mice. If that is the case, expressing a constitutively active form of the Y2R gene alone would be sufficient for suppressing the seizures.

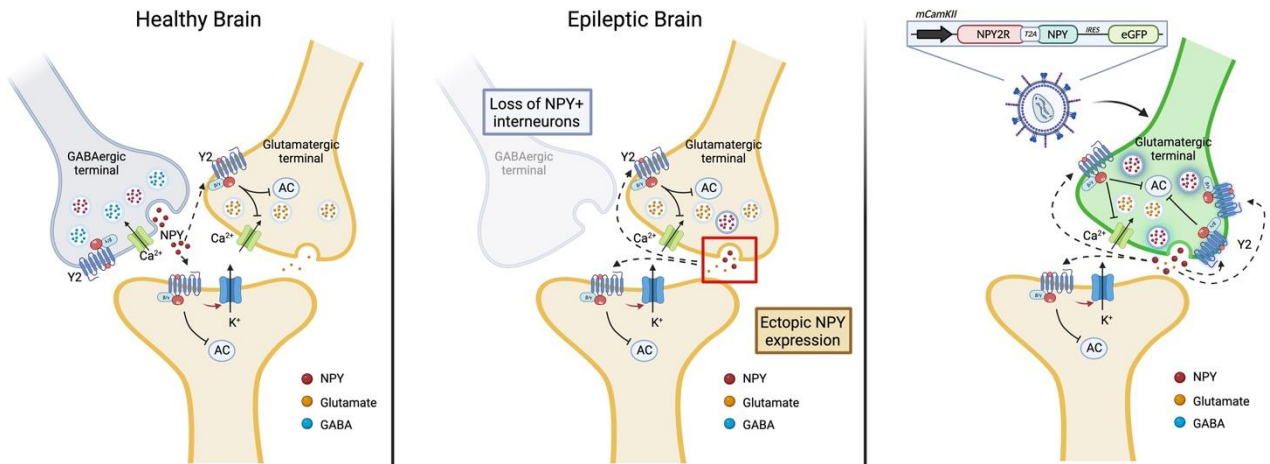
#### PROPOSAL OF RESPONSE

*Existing data (see Krook-Magnuson et al., J Physiol 2015) support the notion (in keeping with the so called dentate 'gate' hypothesis) that preventing granule cell hyperexcitability can prevent occurrence of spontaneous seizures. Thus, our*



*hypothesis is that an NPY-Y2R autoregulatory loop, by avoiding excessive granule cell activity and glutamate release, can prevent seizures. This implies that seizure frequency should be reduced whereas rare, occasional escape to control should evoke nearly "normal" seizures.*

*ACTION: We will discuss more carefully this hypothesis and the hypothesis outlined by the Referee. We may also add a graphical abstract like the one below.*



## RESPONSE

*This hypothesis has been discussed (page 14, 2<sup>nd</sup> paragraph) and the graphical abstract has been added as Figure 8. Needless to say, should the Editor believe that this figure is redundant, we are ready to delete it or to add it to the manuscript as supplementary material.*

### 3) "interference on the normal hippocampal function"

I agree with the authors that forced expression of the NPY-Y2R transgenes in the hippocampal excitatory neurons suppress the seizures quite well (Fig. 6). Now, I would like the authors to discuss the safety of the LV\_NPY\_Y2R.

It would be very nice if the authors can show that forced expression of the NPY-Y2R genes does not perturb the normal activity of the healthy excitatory neurons. Did authors observe any sign of behavioral abnormality in LV\_NPY\_Y2R mice? Authors should report detailed non-ictal behaviors/observations of the TKO/LV\_NPY\_Y2R mice. Autoregulating the epileptic activity as necessary seems to be definitely a better strategy than continuously lowering the neural activity using a constitutively active form of Y2R gene, which presumably has deleterious effects on the normal function of the hippocampus. Authors should discuss the merits and demerits of combinatorial gene expression strategy in comparison with single gene strategy, such as a constitutively active form of Y2R.

## PROPOSAL OF RESPONSE

*We agree with the Referee that this is an important point.*

*ACTION: To test the safety of our LV platform, we will perform electrophysiological experiments on transduced primary hippocampal neurons and measure to which extent the overexpression of NPY-Y2, as compared with NPY alone, might influence normal*

*neuronal activity. We will measure the basic electrophysiological properties of neurons and their spontaneous excitatory/inhibitory post-synaptic activity.*

#### RESPONSE

*We performed the planned experiments, which confirmed the absence of electrophysiological alterations in neurons transduced with LV\_NPY or LV\_Y2-NPY (Results: page 6, 3<sup>rd</sup> paragraph; Figure EV1; Methods: page 20).*

Minor points;

Fig.2 D. The bands for pre-processed NPY and mature NPY should be annotated and indicated by arrows. The unit (kDa ?) should also be indicated. The two lanes for LV\_Y2-NPY are redundant.

#### PROPOSAL OF RESPONSE

*We thank the Referee for this suggestion.*

*ACTION: We will modify the image according to his/her suggestion.*

#### RESPONSE

*Changes in Figure 2D have been made.*

Fig6 E,F. Did the authors also check whether the NPY/Y2R transgenes were successfully expressed or not in the TKO/LV\_NPY\_Y2R mice with seizures (n = 4)? Would the failure of suppressing the seizure in these mice be accounted for simply by a failure of NPY/Y2R transgene expression in the hippocampus?

#### PROPOSAL OF RESPONSE

*We thank the Referee for this valuable suggestion.*

*ACTION: We will extend the analysis on the brains of the animals employed in Figure 6 to verify the successful expression of transgenes.*

#### RESPONSE

*As agreed, we extended analysis to all brains. Indeed, there may be a slightly lower expression of at least Y2R in animals that were having seizures. While this would reinforce the concept of efficacy of the treatment, we believe that the numbers are too low to reach firm conclusions. Therefore, we modified the paragraph only to specify that all brains were analyzed (page 10, 2<sup>nd</sup> paragraph), but would be reluctant to add this other piece of information.*

**Referee #3** (Remarks for Author):

The success of the combinatorial gene therapy using NPY-Y2R system is clear. The manuscript as in the current form is worth while publishing at least as a short report. For a full report, authors may want to consider the concerns above, and revise the manuscript.

#### PROPOSAL OF RESPONSE

*We thank the Referee for this comment, that we endorse completely. Indeed, we view this manuscript as the conceptual basis for future studies. We feel that the results are*

*worthwhile communicating while embarking in further necessary but complex, long, and costly experiments.*

**RESPONSE**

*As above.*

Dear Michele,

Thank you for the submission of your revised manuscript and also for your comments on the referee reports. I paste the reports again below. I understand your arguments and I agree with you that delaying the publication of your work by another 6 months is not a viable solution now. I would therefore like to invite you to address all final referee comments in the ms text and in a final point-by-point response. Please also rewrite the ms title and abstract to clearly point out the main advance your study provides. The description of your findings in the abstract needs to be written in present tense, as per journal policy.

A few editorial requests will also need to be addressed before we can proceed with the official acceptance of your manuscript:

- Please remove the author credits from the ms file. All credits need to be entered in our online ms submission system.
- Please add callouts for the individual panels of Figure 4 and Dataset EV1. These are currently missing.
- Dataset EV1 is a regular table, please re-name it Table 1 or Table EV1 and correct both the file name and the callouts in the ms text.
- The manuscript sections should be in the following order: Title page - Abstract & Keywords - Introduction - Results - Discussion - Methods - Data Availability - Acknowledgments - Disclosure Statement & Competing Interests - References - Figure Legends - (Main Tables with legends) - Expanded View Figure Legends.
- Materials and Methods should be called Methods
- Please note that the legends for figures 4b-g" are not provided in a sequential manner (legend for figures 4c-c", e-e", g-g" are provided before legend of figures 4b, d, f). This needs to be rectified.
- Please note that the exact p values are not provided in the legends of figures 2e-g; 5g; 6b; 7c-d.
- Please note that the error bars are not defined in the legend of figure 5g.

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is exactly 550 pixels wide and 200-600 pixels high (the height is variable). The synopsis image should provide a sketch of the major findings, like a graphical abstract. Please note that text needs to be readable at the final size. Please send us this information along with the final manuscript.

I look forward to seeing a final version of your manuscript as soon as possible.

Best regards,  
Esther

Esther Schnapp, PhD  
Senior Editor  
EMBO reports

Referee #1:

I greatly appreciate the responses to my discussion points, which have significantly strengthened the manuscript. However, the model used, the evidence for autoregulatory mechanisms, and the translatability of the viral vector remain weaknesses. While lentivirus may be effective for small cortical malformations in humans, it is less likely to work in the hippocampus. I have two minor points to add:

1. Autoregulatory mechanisms: The paper I referenced is Lieb et al. (2018) in Nature Medicine, where an autoregulatory gene therapy based on glutamate levels was shown to be effective for focal epilepsy.
2. hCAMK2A specificity: I appreciate the new analysis on promoter specificity, and the results are intriguing. It might be beneficial to include a discussion of studies using AAVs, which have shown different results. This addition would help differentiate between lentivirus and AAV transduction.

Referee #2:

The authors use a mouse model of triple synapsin KO mice showing seizures. They inject lentiviral NPY/Y2 vectors on the CamKinasell promoter (targeting vector expression in principal neurons) for antagonizing seizures. They carefully characterize the targeting of the vectors in control mice (eg. expression on mossy fibers) and characterize the anti-convulsive effect.

Major comments:

A considerable drawback of the model is that the time period when seizures occur is obviously limited. Unfortunately, the authors performed their experiments (vector injections) not at the period of maximal seizures but the time before:

Thus, the maximal increase in endogenous NPY occurs P90 (Fig. 6 B') indicating that this was the time of maximal seizure activity. Telemetric recording (shown in Fig. 7) started on P60 (Fig. 7A) and lasted for 19 days (until P 79; Fig. 7E). The untreated mice had no seizures at the beginning; at the end of this period, practically all of them developed seizures. By this, around 60% of seizures were prevented by the LV-NPY-Y2 (lentivirus-NPY-Y2 receptor) injection (Fig. 7F). The authors performed their experiments when the animals show sub-maximal spontaneous seizures. In my opinion the interesting time period, however, would have been the time just afterwards (P75 to P100) when seizure activity was highest (Fig. 6B'). The authors only had to start their experiment (according to the results shown in Fig. 6B) two weeks later. It is interesting that the spontaneous seizures decline afterwards (P120; Fig 6 B'). Why is this so?

Minor Comments:

1. Fig. 7D: Mean seizure duration: this is somewhat misleading since only mice with seizures are included. This should be mentioned.
2. Fig. 8: The situation in the epileptic brain is not correctly shown. It implies that all NPY neurons degenerate. After status epilepticus somatostatin neurons degenerate in the tip of the hilus. Loss in NPY neurons is rather limited. In contrast, most NPY neurons up-regulate the neuropeptide (and GADs). This has been frequently shown (see also Fig. 6B of this paper). The paper referenced to (Housko et al) refers to a model of traumatic brain injury (TBI) in which 40 % of NPY neurons degenerated. This model is characterized by severe general brain damage (interneurons and principal neurons), although the rats show no epilepsy but only a reduced seizure threshold (presumably due to this general damage, including PV neurons). After electrical stimulation of the amygdala (inducing a status epilepticus) around 35 % of NPY degenerated in the hilus. Thus, it is not correct to refer to "loss of NPY + interneurons". Depending on the model the majority of interneurons survive. GABA-ergic terminal of the "healthy brain": NPY is co-stored with GABA in large dense core vesicles, GABA is contained also (without neuropeptides) in small synaptic vesicles.

I would include some explanations into legend (notably concerning the third neuron below).

2. Mention in the Methods section when you started the recording and how long it lasted
3. Legend to Fig 2: F-F': I would refer to NPY as peptide or neuropeptide not as protein.

In Conclusion:

1. The manuscript is not entirely novel: Combination of NPY and Y2 vectors have been shown before to have anticonvulsive effects (as NPY vectors alone).
2. The animal model may not be optimal. In my opinion it has also not been use optimal.
3. The use of a CamKinasell promoter for these vectors to selectively target principal neurons is novel
4. Characterization of vector targeting and vector expression is perfect
5. Also characterization of the animal model and of seizures is perfect

Referee #3:

Rev #3, Comments on Cattaneo et al., revision

The authors now provide a revision with additional necessary control experiments (Fig. 2G, Fig.EV1). I'm pleased to read the revision. However, I still have questions and comments on the revision. I'd like the authors to consider the comments below and reflect them in the revision.

1) Fig 2G.

The authors removed Mg<sup>2+</sup> from the medium to increase the excitability of the hippocampal primary neuron culture. I don't quite understand the principle of the method. Please provide with relevant references. Independent experimental confirmation of a hyperexcitation by this manipulation may be necessary.

## 2) Fig EV1

The authors demonstrated that electrophysiological properties of isolated neurons transfected with the transgenes seem to be normal. However, consequences of introducing NPY-Y2R transgenes may become apparent only when the transfected neurons are integrated in the hippocampal neural circuits and operating there. Have authors monitored the wild type mouse injected with LV\_NPY\_Y2 vector (Figure 5) for their health and behavior? Any abnormality in, e.g., body weight increase, aggressiveness, feeding behavior, sleep-awake cycle, EEG patterns?

## 3) Practical merit of Lenti-virus vector.

In addition to the minimal spared of the virus vector in the brain tissue, I would also like the authors mention about the superior stability of the Lentivirus vector. These vectors are inserted into the chromosomes, once integrated, work permanently unlike other episomal AAV vectors which would be eventually lost in the cytoplasm. Lenti-virus vectors also have a high affinity to stem cells. Gene therapy with Lenti-vectors, therefore, eliminates the necessity of repeated injections into the body.

## 4) Adequacy of TKO mice as an epilepsy model animal (related to rev#2 comment).

As stated in the manuscript, it has been reported that the GABAergic signaling is impaired in TKO mice, and the failure of inhibition is the primary cause of the epileptic phenotype seen in these mutant mice. In this point, TKO mice is an adequate epilepsy model. However, I'd like to also remind the authors of the fact that synapsin II is expressed in excitatory neurons, therefore, glutamatergic synaptic signaling pathway is also impaired in these mice. In this regard, presentation of the new figure 8 needs a caution. Especially, in the middle panel titled "epileptic brain", the glutamatergic terminal is drawn as if it is operating normally. However, in the TKO mice used in this study, formation of the neurotransmitter vesicle pool at the terminal is presumably impaired. It is even possible that the NPY\_Y2R auto-inhibition works well only in these synapsin II-missing excitatory synapses.

I'd like the authors to mention about this possibility in discussion.

## 5) Fig.2 D

A label for the molecular size unit ("kDa") is still missing (in other similar figures as well). The unit should be spelled out somewhere in the figure or mentioned in the legend.

## 6) Fig.2 G

This new experiment needs a control condition in which the basal glutamate release is measured in the normal medium (Mg2+ present), to demonstrate that the Mg2+ manipulation really induced a Glutamate release.

## Cross-comments from referee 1:

I still believe that the model used (not translatable to human pathology, no patients with TKO for synapses) is a big weakness of this manuscript. And I agree with Ref#2 that also the time of intervention is not relevant to a potential gene therapy. So, I agree that showing an effect at the chronic phase of the pathology can have a more translational impact.

## Cross-comments from referee 2:

The work mainly replicates that of Ledri et al and of others using a different vector and a different animal model. I think per se this can be published. The present work, however, suffers from the lack of demonstration of a long-time effect and from the use of a sub-optimal timing. In Ledri's work the effect on seizure development has been well demonstrated.

I agree with Referee 1. I think it is crucial to investigate the optimal time period (chronic phase). The additional experiment would also extend the whole time frame.

## Cross-comments from referee 3:

It is unfortunate that the authors ended the recording (~P81), before the period of peak seizure activity (~P90). However, the anti-seizure effect is clearly demonstrated (Fig7E), even in the recording period. As the significant difference has been properly demonstrated between the age(day)-matched animals with/without Y2-NPY transgenes, I'm fine without a new experiment to be conducted at the period of maximal seizures.

## Response to Editor and Referees comments

### Editor

Thank you for the submission of your revised manuscript and also for your comments on the referee reports. I paste the reports again below. I understand your arguments and I agree with you that delaying the publication of your work by another 6 months is not a viable solution now. I would therefore like to invite you to address all final referee comments in the ms text and in a final point-by-point response. Please also rewrite the ms title and abstract to clearly point out the main advance your study provides. The description of your findings in the abstract needs to be written in present tense, as per journal policy.

*The abstract has been rewritten as indicated.*

A few editorial requests will also need to be addressed before we can proceed with the official acceptance of your manuscript:

Please remove the author credits from the ms file. All credits need to be entered in our online ms submission system.

*The credits have been removed.*

Please add callouts for the individual panels of Figure 4 and Dataset EV1. These are currently missing.

*The callouts have been added at page 7 and 17*

Dataset EV1 is a regular table, please re-name it Table 1 or Table EV1 and correct both the file name and the callouts in the ms text.

*We re-named and inserted the Table as Table 1 at the bottom of the main manuscript file, corrected the callouts.*

The manuscript sections should be in the following order: Title page - Abstract & Keywords - Introduction - Results - Discussion - Methods - Data Availability - Acknowledgments - Disclosure Statement & Competing Interests - References - Figure Legends - (Main Tables with legends) - Expanded View Figure Legends.

*We reordered the manuscript sections.*

Materials and Methods should be called Methods

*We renamed the section.*

Please note that the legends for figures 4b-g" are not provided in a sequential manner (legend for figures 4c-c", e-e", g-g" are provided before legend of figures 4b, d, f). This needs to be rectified.

*We corrected the legend for figure 4.*

Please note that the exact p values are not provided in the legends of figures 2e-g; 5g; 6b; 7c-d.

*We provided the exact p values where requested.*

Please note that the error bars are not defined in the legend of figure 5g.

*The error bars are now defined in figure 5g*

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is exactly 550 pixels wide and 200-600 pixels high (the height is variable). The synopsis image should provide a sketch of the major findings, like a graphical abstract. Please note that text needs to be readable at the final size. Please send us this information along with the final manuscript.

*Here and attached we provide the short summary, key results and attached the synopsis image.*

### **Summary of the findings and their significance**

We designed a lentiviral vector co-expressing NPY with its inhibitory receptor Y2 in excitatory hippocampal cells, that allows inhibitory autoregulation of glutamate release. We demonstrate that efficient and selective overexpression of both genes in granule cell mossy fiber terminals is sufficient to induce a dramatic reduction in the frequency and duration of seizures in the synapsin triple KO model of epilepsy.

### **Key results**

- Development of a LV vector with minimal CamKinasell promoter driving expression of NPY and its receptor Y2
- Efficient and nearly selective *in vivo* overexpression of NPY and Y2 receptor in granule cell mossy fiber terminals following vector administration in the dentate gyrus
- Reduction in the frequency and duration of seizures in the synapsin triple KO model of epilepsy

### **Referee 1**

I greatly appreciate the responses to my discussion points, which have significantly strengthened the manuscript. However, the model used, the evidence for autoregulatory mechanisms, and the translatability of the viral vector remain weaknesses. While lentivirus may be effective for small cortical malformations in humans, it is less likely to work in the hippocampus. I have two minor points to add: 1. Autoregulatory mechanisms: The paper I referenced is Lieb et al. (2018) in Nature Medicine, where an autoregulatory gene therapy based on glutamate levels was shown to be effective for focal epilepsy.

*We thank the reviewer for this annotation, we added a reference to Lieb et al. in the introduction at page 5.*

2. hCAMK2A specificity: I appreciate the new analysis on promoter specificity, and the results are intriguing. It might be beneficial to include a discussion of studies using AAVs, which have shown different results. This addition would help differentiate between lentivirus and AAV transduction.

*We thank the reviewer for this suggestion, we added some observations about this in the discussion at page 12 and 13.*



## Referee 2

The authors use a mouse model of triple synapsin KO mice showing seizures. They inject lentiviral NPY/Y2 vectors on the CamKinasell promoter (targeting vector expression in principal neurons) for antagonizing seizures. They carefully characterize the targeting of the vectors in control mice (eg. expression on mossy fibers) and characterize the anti-convulsive effect.

Major comments:

A considerable drawback of the model is that the time period when seizures occur is obviously limited. Unfortunately, the authors performed their experiments (vector injections) not at the period of maximal seizures but the time before:

Thus, the maximal increase in endogenous NPY occurs P90 (Fig. 6 B') indicating that this was the time of maximal seizure activity. Telemetric recording (shown in Fig. 7) started on P60 (Fig. 7A) and lasted for 19 days (until P 79; Fig. 7E). The untreated mice had no seizures at the beginning; at the end of this period, practically all of them developed seizures. By this, around 60% of seizures were prevented by the LV-NPY-Y2 (lentivirus-NPY-Y2 receptor) injection (Fig. 7F). The authors performed their experiments when the animals show sub-maximal spontaneous seizures. In my opinion the interesting time period, however, would have been the time just afterwards (P75 to P100) when seizure activity was highest (Fig. 6B'). The authors only had to start their experiment (according to the results shown in Fig. 6B) two weeks later.

It is interesting that the spontaneous seizures decline afterwards (P120; Fig 6 B'). Why is this so?

*We thank the reviewer for this observation. However, we would like to point out that we actually applied our treatment at the time of peak seizure activity. We realize that we may not have made ourselves clear for the rationale of using the time point that we employed in the study, and this may have been misleading to the readers. Based on the literature, the susceptibility to seizures (that is, sensitivity to a seizure-provoking maneuver) in this model reaches a peak between P60 and P80 (Cambiaghi et al., Epil Res 2013 and at page 9). This is exactly the time window that we chose for 24/7 (24 h per day, 7 day a week) video-EEG monitoring. In fact, and in line with these findings, we observed spontaneous seizures in the control group within this time window. Seizures were observed in all the time window and did not display any tendency to increase in frequency, severity or duration in time (see the figure below). In particular, we would like to specify that the concept of "time to first seizure" (Fig. 7E) refers to the first occurrence of a seizure after a treatment, not in life. Regarding the peak of NPY expression (P90) that we report in Fig. 6, as we describe in the original manuscript (page 9, second paragraph), literature data suggest that increased NPY expression occurs as a reaction to seizure activity, indicating that the highest seizure activity in our model occurs before P90. This is the reason why we decided to anticipate its overexpression, ensuring that NPY is overexpressed while animals are in chronic epilepsy period.*

*We rewrote the paragraph at page 9 in a manner that should be clearer to the readers, by correcting a few wrong or misleading sentences.*

*The reason for the bell-shaped behavior in seizure frequency in TKO animals is not*

*clear, however the decline in spontaneous seizure activity in the TKO mice after P120 could be the result of the development of compensatory mechanisms, involving synaptic mechanisms differentially affecting excitatory and inhibitory neurons, during the chronicization of the epileptic phenotype.*

*Figure for referees not shown.*

Minor Comments:

1. Fig. 7D: Mean seizure duration: this is somewhat misleading since only mice with seizures are included. This should be mentioned.

*We thank the reviewer for the observation, we corrected the figure legend at page 28.*

2. Fig. 8: The situation in the epileptic brain is not correctly shown. It implies that all NPY neurons degenerate. After status epilepticus somatostatin neurons degenerate in the tip of the hilus. Loss in NPY neurons is rather limited. In contrast, most NPY neurons up-regulate the neuropeptide (and GADs). This has been frequently shown (see also Fig. 6B of this paper). The paper referenced to (Housko et al) refers to a model of traumatic brain injury (TBI) in which 40 % of NPY neurons degenerated. This model is characterized by severe general brain damage (interneurons and principal neurons), although the rats show no epilepsy but only a reduced seizure threshold (presumably due to this general damage, including PV neurons). After electrical stimulation of the amygdala (inducing a status epilepticus) around 35 % of NPY degenerated in the hilus. Thus, it is not correct to refer to "loss of NPY + interneurons". Depending on the model the majority of interneurons survive. GABA-ergic terminal of the "healthy brain": NPY is co-stored with GABA in large dense core vesicles, GABA is contained also (without neuropeptides) in small synaptic vesicles. I would include some explanations into legend (notably concerning the third neuron below).

*We thank the reviewer for the suggestion, we included a legend for figure 8 taking into account all these observations (page 29).*

2. Mention in the Methods section when you started the recording and how long it lasted

*We modified the methods section, at page 24.*

3. Legend to Fig 2: F-F': I would refer to NPY as peptide or neuropeptide not as protein.

*We modified the legend, at page 26.*

In Conclusion:

1. The manuscript is not entirely novel: Combination of NPY and Y2 vectors have been shown before to have anticonvulsive effects (as NPY vectors alone).
2. The animal model may not be optimal. In my opinion it has also not been use optimal.
3. The use of a CamKinasell promoter for these vectors to selectively target principal neurons is novel
4. Characterization of vector targeting and vector expression is perfect
5. Also characterization of the animal model and of seizures is perfect

### Referee 3

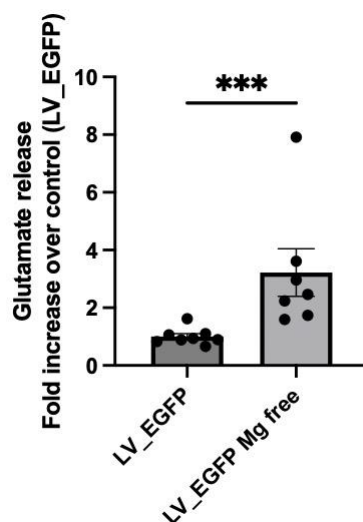
Rev #3, Comments on Cattaneo et al., revision

The authors now provide a revision with additional necessary control experiments (Fig. 2G, Fig.EV1). I'm pleased to read the revision. However, I still have questions and comments on the revision. I'd like the authors to consider the comments below and reflect them in the revision.

1) Fig 2G.

The authors removed Mg<sup>2+</sup> from the medium to increase the excitability of the hippocampal primary neuron culture. I don't quite understand the principle of the method. Please provide with relevant references. Independent experimental confirmation of a hyperexcitation by this manipulation may be necessary.

*The incubation of cultured neurons or tissue slices in a Mg<sup>2+</sup>-free solution, allowing the activation of NMDA receptors due to the removal of the Mg<sup>2+</sup> blockade of the channel, is a very common stimulus used to induce in vitro a SE-like condition (see for example Hogg et al. 2019, Mele et al. 2021, Mangan et al. 2004). The references were added in the results section, page 7. The figure below (now added to the manuscript as Figure EV2) shows that Mg<sup>2+</sup> removal increases glutamate release in neurons transduced with LV\_EGFP.*



## 2) Fig EV1

The authors demonstrated that electrophysiological properties of isolated neurons transfected with the transgenes seem to be normal. However, consequences of introducing NPY-Y2R transgenes may become apparent only when the transfected neurons are integrated in the hippocampal neural circuits and operating there. Have authors monitored the wild type mouse injected with LV\_NPY\_Y2 vector (Figure 5) for their health and behavior? Any abnormality in, e.g., body weight increase, aggressiveness, feeding behavior, sleep-awake cycle, EEG patterns?

*The observation of the reviewer is obviously correct. We did not monitor the animals' health by using specific tests. However, we did not observe any change in their gross behavior (including aggressiveness and feeding behavior), weight curve and EEG patterns (including that during sleep). We added this info in the Results section (page 8).*

## 3) Practical merit of Lenti-virus vector.

In addition to the minimal spared of the virus vector in the brain tissue, I would also like the authors mention about the superior stability of the Lentivirus vector. These vectors are inserted into the chromosomes, once integrated, work permanently unlike other episomal AAV vectors which would be eventually lost in the cytoplasm. Lenti-virus vectors also have a high affinity to stem cells. Gene therapy with Lenti-vectors, therefore, eliminates the necessity of repeated injections into the body.

*We thank the reviewer for the observation, we added a sentence at page 4.*

## 4) Adequacy of TKO mice as an epilepsy model animal (related to rev#2 comment).

As stated in the manuscript, it has been reported that the GABAergic signaling is impaired in TKO mice, and the failure of inhibition is the primary cause of the epileptic phenotype seen in these mutant mice. In this point, TKO mice is an adequate epilepsy model. However, I'd like to also remind the authors of the fact that synapsin II is expressed in excitatory neurons, therefore, glutamatergic synaptic signaling pathway is also impaired in these mice. In this regard, presentation of the new figure 8 needs a caution. Especially, in the middle panel titled "epileptic brain", the glutamatergic terminal is drawn as if it is operating normally. However, in the TKO mice used in this study, formation of the neurotransmitter vesicle pool at the terminal is presumably impaired. It is even possible that the NPY\_Y2R auto-inhibition works well only in these synapsin II-missing excitatory synapses. I'd like the authors to mention about this possibility in discussion.

*We added this information at page 8 of the revised manuscript.*

## 5) Fig.2 D

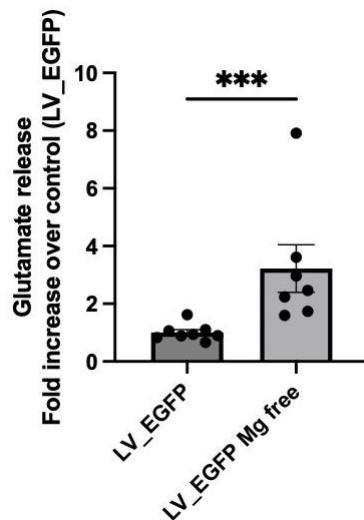
A label for the molecular size unit ("kDa") is still missing (in other similar figures as well). The unit should be spelled out somewhere in the figure or mentioned in the legend.

*We modified the figures according to the reviewer's indication and added the molecular weight labeling.*

## 6) Fig.2 G

This new experiment needs a control condition in which the basal glutamate release is measured in the normal medium (Mg<sup>2+</sup> present), to demonstrate that the Mg<sup>2+</sup> manipulation really induced a Glutamate release.

*As said, the incubation of cultured neurons or tissue slices in a Mg<sup>2+</sup>-free solution, allowing the activation of NMDA receptors due to the removal of the Mg<sup>2+</sup> blockade of the channel, is a very common stimulus used to induce in vitro a SE-like condition (see for example Hogg et al. 2019, Mele et al. 2021, Mangan et al. 2004). The figure below (now added to the manuscript as Figure EV2) shows that Mg<sup>2+</sup> removal increases glutamate release in neurons transduced with LV\_EGFP.*



Cross-comments from referee 1:

I still believe that the model used (not translatable to human pathology, no patients with TKO for synapses) is a big weakness of this manuscript. And I agree with Ref#2 that also the time of intervention is not relevant to a potential gene therapy. So, I agree that showing an effect at the chronic phase of the pathology can have a more translational impact.

Cross-comments from referee 2:

The work mainly replicates that of Ledri et al and of others using a different vector and a different animal model. I think per se this can be published. The present work, however, suffers from the lack of demonstration of a long-time effect and from the use of a sub-optimal timing. In Ledri's work the effect on seizure development has been well demonstrated.

I agree with Referee 1. I think it is crucial to investigate the optimal time period (chronic phase). The additional experiment would also extend the whole time frame.

Cross-comments from referee 3:

It is unfortunate that the authors ended the recording (~P81), before the period of peak seizure activity(~P90). However, the anti-seizure effect is clearly demonstrated (Fig7E), even in the recording period. As the significant difference has been properly

demonstrated between the age(day)-matched animals with/without Y2-NPY transgenes, I'm fine without a new experiment to be conducted at the period of maximal seizures.

#### RESPONSE to the Cross Comments

*We would like to point out that we actually applied our treatment at the time of peak seizure activity. We realize that we may not have made ourselves clear for the rationale of using the time point that we employed in the study, and this may have been misleading to the readers. Thus, the paragraph at page 9 has been extensively rewritten. Based on the literature, the susceptibility to seizures (that is, sensitivity to a seizure-provoking maneuver) in this model reaches a peak between P60 and P80 (Cambiaghi et al., Epil Res 2013 and at page 9). This is exactly the time window that we chose for 24/7 (24 h per day, 7 day a week) video-EEG monitoring. In fact, and in line with these findings, we observed spontaneous seizures in the control group within this time window. Seizures were observed in all the time window and did not display any tendency to increase in frequency, severity or duration in time (see the figure below). In particular, we would like to specify that the concept of "time to first seizure" (Fig. 7E) refers to the first occurrence of a seizure after a treatment, not in life. Regarding the peak of NPY expression (P90) that we report in Fig. 6, as we describe in the manuscript (page 9, second paragraph), literature data suggest that increased NPY expression occurs as a reaction to seizure activity, indicating that the highest seizure activity in our model occurs before P90. This is the reason why we decided to anticipate its overexpression, ensuring that NPY is overexpressed while animals are in chronic epilepsy period.*

*Regarding the translatability issue: based on the above, we believe that our experimental design is correct for testing effects in a chronic phase of epilepsy. Note also that our present work includes one of the longer-lasting video-EEG monitoring among those on NPY-Y2 gene therapy. There is no single animal model that is fully validated for translation in human epilepsy, except for antiseizure effects. While we acknowledge the limitations of the TKO model, we also recognize that it holds many interesting features that led us to choose it as a first testing model. Note that aim of this work was not to provide immediately translatable evidence for a novel approach. Admittedly, we stated something like that in a couple of sentences of the original manuscript, which was correctly noted by the Referees, leading us to a more accurate definition of the aims and a more conservative conclusive statement ("these results suggest that an auto-regulated gene therapy approach based on co-expression of NPY and Y2R in hippocampal GCs is feasible and worth further investigation"). In other words, the aim of our work was to provide initial evidence that the well-known NPY-Y2 approach to the gene therapy of epilepsy may be refined by targeting as specifically as possible a relatively small subset of neurons, instead of the widespread and non-specific approaches used so far.*

Prof. Michele Simonato  
IRCCS Ospedale San Raffaele  
Milan  
Italy

Dear Prof. Simonato,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

Your manuscript will be processed for publication by EMBO Press. It will be copy edited and you will receive page proofs prior to publication. Please note that you will be contacted by Springer Nature Author Services to complete licensing and payment information.

You may qualify for financial assistance for your publication charges - either via a Springer Nature fully open access agreement or an EMBO initiative. Check your eligibility: <https://www.embopress.org/page/journal/14693178/authorguide#chargesguide>

Should you be planning a Press Release on your article, please get in contact with [embo\\_production@springernature.com](mailto:embo_production@springernature.com) as early as possible in order to coordinate publication and release dates.

If you have any questions, please do not hesitate to contact the Editorial Office. Thank you for your contribution to EMBO Reports.

Best regards,  
Esther

Esther Schnapp, PhD  
Senior Editor  
EMBO reports

-----

>>> Please note that it is EMBO Reports policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. If you do NOT want this, you will need to inform the Editorial Office via email immediately. More information is available here: [https://www.embopress.org/transparent-process#Review\\_Process](https://www.embopress.org/transparent-process#Review_Process)

## EMBO Press Author Checklist

Corresponding Author Name: Michele Simonato
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2023-58691-T

### USEFUL LINKS FOR COMPLETING THIS FORM

- [The EMBO Journal - Author Guidelines](#)
- [EMBO Reports - Author Guidelines](#)
- [Molecular Systems Biology - Author Guidelines](#)
- [EMBO Molecular Medicine - Author Guidelines](#)

### Reporting Checklist for Life Science Articles (updated January)

This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: [10.31222/osf.io/9sm4x](https://doi.org/10.31222/osf.io/9sm4x)). Please follow the journal's guidelines in preparing your

**Please note that a copy of this checklist will be published alongside your article.**

### Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if  $n < 5$ , the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

#### 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  $\chi^2$  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.

**Please complete ALL of the questions below.**  
**Select "Not Applicable" only when the requested information is not relevant for your study.**

### Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	
Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For <b>antibodies</b> provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Reagents and Tools Table
DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
<b>Short novel DNA or RNA including primers, probes:</b> provide the sequences.	Yes	Reagents and Tools Table - Table 1
Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
<b>Cell lines:</b> Provide species information, strain. Provide accession number in repository <b>OR</b> supplier name, catalog number, clone number, and/OR RRID.	Yes	Reagents and Tools Table, Materials and Methods
<b>Primary cultures:</b> Provide species, strain, sex of origin, genetic modification status.	Yes	Reagents and Tools Table, Materials and Methods
Report if the cell lines were recently <b>authenticated</b> (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Materials and Methods
Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
<b>Laboratory animals or Model organisms:</b> Provide species, strain, sex, age, genetic modification status. Provide accession number in repository <b>OR</b> supplier name, catalog number, clone number, <b>OR</b> RRID.	Yes	Materials and Methods
<b>Animal observed in or captured from the field:</b> Provide species, sex, and age where possible.	Not Applicable	N/A
Please detail <b>housing and husbandry conditions</b> .	Yes	Materials and Methods
Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
<b>Plants:</b> provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	N/A
<b>Microbes:</b> provide species and strain, unique accession number if available, and source.	Not Applicable	Materials and Methods
Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	N/A
Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Not Applicable	N/A

### Design



<b>Study protocol</b>	<b>Information included in the manuscript?</b>	<b>In which section is the information available?</b> (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been <b>pre-registered</b> , provide DOI in the <b>manuscript</b> . For clinical trials, provide the trial registration number <b>OR</b> cite DOI.	Not Applicable	N/A
Report the <b>clinical trial registration number</b> (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	N/A

<b>Laboratory protocol</b>	<b>Information included in the manuscript?</b>	<b>In which section is the information available?</b> (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if <b>external detailed step-by-step protocols</b> are available.	Not Applicable	N/A

<b>Experimental study design and statistics</b>	<b>Information included in the manuscript?</b>	<b>In which section is the information available?</b> (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about <b>sample size</b> estimate even if no statistical methods were used.	Yes	Materials and Methods
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. <b>randomization procedure</b> )? If yes, have they been described?	Yes	Materials and Methods
Include a statement about <b>blinding</b> even if no blinding was done.	Yes	Materials and Methods
Describe <b>inclusion/exclusion criteria</b> if samples or animals were excluded from the analysis. Were the criteria pre-established?	Yes	Materials and Methods
If sample or data points were omitted from analysis, report if this was due to <b>attrition or intentional exclusion</b> and provide justification.	Yes	Materials and Methods
For every figure, are <b>statistical tests</b> justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materials and Methods

<b>Sample definition and in-laboratory replication</b>	<b>Information included in the manuscript?</b>	<b>In which section is the information available?</b> (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was <b>replicated</b> in laboratory.	Yes	Figures
In the figure legends: define whether data describe <b>technical or biological replicates</b> .	Yes	Figures

## Ethics

<b>Ethics</b>	<b>Information included in the manuscript?</b>	<b>In which section is the information available?</b> (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving <b>human participants</b> : State details of <b>authority granting ethics approval</b> (IRB or equivalent committee(s), provide reference number for approval).	Not Applicable	N/A
Studies involving <b>human participants</b> : Include a statement confirming that <b>informed consent</b> 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.	Not Applicable	N/A
Studies involving <b>human participants</b> : For publication of <b>patient photos</b> , include a statement confirming that consent to publish was obtained.	Not Applicable	N/A
Studies involving experimental <b>animals</b> : State details of <b>authority granting ethics approval</b> (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations).	Yes	Materials and Methods
Studies involving <b>specimen and field samples</b> : State if relevant <b>permits</b> obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	N/A

<b>Dual Use Research of Concern (DURC)</b>	<b>Information included in the manuscript?</b>	<b>In which section is the information available?</b> (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of <b>select agents and toxins</b> (CDC): <a href="https://www.selectagents.gov/sat/list.htm">https://www.selectagents.gov/sat/list.htm</a>	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the <b>authority granting approval and reference number</b> for the regulatory approval provided in the manuscript?	Not Applicable	

## Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

<b>Adherence to community standards</b>	<b>Information included in the manuscript?</b>	<b>In which section is the information available?</b> (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., <b>ICMJE, MIBBI, ARRIVE, PRISMA</b> ) have been followed or provided.	Not Applicable	
For <b>tumor marker prognostic studies</b> , we recommend that you follow the <b>REMARK</b> reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For <b>phase II and III randomized controlled trials</b> , please refer to the <b>CONSORT</b> flow diagram (see link list at top right) and submit the <b>CONSORT</b> 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	

## Data Availability

<b>Data availability</b>	<b>Information included in the manuscript?</b>	<b>In which section is the information available?</b> (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have <b>primary datasets</b> been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Not Applicable	
Were <b>human clinical and genomic datasets</b> deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are <b>computational models</b> that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective <b>data citations in the reference list</b> .	Not Applicable	