EMBO Molecular Medicine

Spironolactone is an Antagonist of NRG1-ERBB4 Signaling and Schizophrenia-Relevant Endophenotypes in Mice

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Céline Carret

1st Editorial Decision 29 March 2017

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from the two referees whom we asked to evaluate your manuscript.

You will see from the comments pasted below, that while referee 2 is more succinct than referee 1, they both agree that the study is interesting and should be published. Referee 1 suggests additional experiments to increase the conclusiveness, and both referees would like to see more details and explanations. Please note that EMBO Molecular Medicine encourages a single round of revision and that, as acceptance or rejection of the manuscript will depend on another round of review, your responses should be as complete as possible.

EMBO Molecular Medicine has a "scooping protection" policy, whereby similar findings that are published by others during review or revision are not a criterion for rejection. Should you decide to submit a revised version, I do ask that you get in touch after three months if you have not completed it, to update us on the status.

Please also contact us as soon as possible if similar work is published elsewhere. If other work is published we may not be able to extend the revision period beyond three months.

Please read below for important editorial formatting and consult our author's guidelines for proper formatting of your revised article for EMBO Molecular Medicine.

I look forward to receiving your revised manuscript.

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

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

The approaches are adequate and range from the use of cell culture bases HTS screening to in vivo approaches using models to test schizophrenia-like behavior

Referee #1 (Remarks):

In this study Wehr et al develop a cell based HTS-assay to screen for modulators of NRG1-ERBB4 signaling. This is based on the current view that NRG1-ERBB4 hyperactivity contributes to schizophrenia. The authors specifically test approved drugs that offer the chance for drug repurposing. By this approach they identify spironolactone and provide evidence that it acts as a pan-ERBB antagonist that prevents dimer formation. Most importantly, treating NRG-1 TG mice daily with spironolactone ameliorated schizophrenia-like phenotypes.
In sum, this is a very interesting study that summarizes a lot of work ranging from drug discovery to eventually testing the identified drug in a relevant animal model. Taken into account that spironolactone is an approved drug that has however not been considered in the context of schizophrenia, the presented findings are novel and could have immediate impact in the clinic.
I believe this study will be very interesting to the readers of EMBO Mol. Med.

I only have a few questions remaining:

Major issues:

1. Fig 4. It would be helpful to provide quantification for all investigated proteins in addition to Erbb4 and LimK. Also, please provide the number of animals used for the molecular analysis. This is missing for all immunoblot data and would be important since images are not always high quality.

2. Although the authors convincingly show that spironolactone affects ERBB function in cell culture, many different modes of actions have been assigned to it. Foremost spironolactone is known as MR antagonist and at present its questionable if the therapeutic effect in the NRG-1 TG mice is indeed mediated via the NRG1-ERBB pathway (especially since glucocorticoid signaling has been linked to NRG-1 function) . I suggest two approaches to address this issue.

One possibility would be to measure p-Erbb4 as well as p-LimK etc. levels in animals that were used in the experiments described in Fig5 and EV5. Maybe this is what the authors did but at present it is unclear what treatment paradigm was used to generate the data shown in Fig 4C. Moreover, was really the entire mouse brain used for analysis? The description is misleading and sometimes refers to the prefrontal cortex which would of curse make more sense.

In addition it would be nice to learn more - maybe in the discussion - about the downstream mechanisms of elevated NRG1-ERBB4 signaling in the context of schizophrenia-like phenotypes. In fact considering that NRG1 is overexpressed from early developmental stages and there is evidence that altered NRG-1 function affect brain development and thereby contributes to the pathogenesis of schizophrenia, it is quite remarkable that even 3 weeks of spironolactone treatment in juvenile/adult mice reverse the disease phenotypes. Do the authors think that this correlates with structural changes?

Along this line it is said that age-matched mice were used for behavioral testing (nothing is said about the mice used for immunoblot analysis). However the age appears to vary from 8-16 weeks. The authors should exclude the possibility that the observed effects are linked to the different ages. Can we exclude the possibility that the effect of spironolactone is mainly linked to the treatment of 8 weeks old mice in which post-natal brain development may not have been completed yet? Is it less efficient in 16 month old mice?

Minor issues:

1. page 3: "...principally offers a fast track to the clinic and has been demanded for SZ (Insel, 2012; Lencz & Malhotra, 2015), and also because many pharma companies withdrew from research..."
the "and" appears misplaced.

2. Page 3: "Instead of changes in protein-coding regions..." should be removed.

3. Fig. 1A: Please indicate the type of cells that were used.

4. At what time of the day was spironolactone injected?

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

Extremely well executed project

Referee #2 (Remarks):

This is a very interesting study highly deserving of being published especially after minor revision. My only comments re: 1-Authors should explain the differences in concentrations of spironolactone (10 micromolar in electrophysiology vs.1 in other experiments), 2-explain any sex differences in response to spironolactone

1st Revision - authors' response 29 May 2017

We thank the referees for their constructive comments that greatly improved the quality of the manuscript. All the issues raised by the referees have been addressed (see the point-by-point response below), which prompted us to further analyse existing data that are now included in the updated version of the paper. These data are now shown in Fig. 4C.

In addition, we have expanded our discussion and addressed potential downstream mechanisms of elevated NRG1-ERBB4 signaling in the context of schizophrenia-like phenotypes.

Referee #1

Major issues:
1. Fig 4. It would be helpful to provide quantification for all investigated proteins in addition to Erbb4 and LimK. Also, please provide the number of animals used for the molecular analysis. This is missing for all immunoblot data and would be important since images are not always high quality.

As requested for Fig. 4D, we have now quantified all phospho-protein levels vs. total protein levels of the blots shown in Fig. 4D (was Fig. 4C) using densitometric analysis in ImageJ. As evident from the quantification, phospho-Erk1/2 and phospho-Akt levels were neither changed in Nrg1-tg mice, nor in spironolactone-treated animals (for both controls and Nrg1-tg mice). In addition, we have added information on the number of independent cellular lysates (n=4, Fig. 4A, B) and lysates from animals (n=2, Fig. 4D, E) used for quantification.

Amendments in the figure legend for Fig. 4:
(**B**) Quantification of band intensities for phospho-ERBB4 levels (n=4 per condition) shown in (**A**) using ImageJ. Phosphorylation levels are normalized to protein levels of ERBB4. Data shown as mean, error bars represent SD; t-test, with *, $p \leq 0.05$, and **, $p \leq 0.01$.
(**C**) Experimental design for western blot analysis shown in (**D**) and (**E**). *Nrg1*-tg and wt animals were treated daily with spironolactone (50 mg/kg, s.c.) or vehicle (n=2 per genotype and per treatment) for 21 days.

(**D**) Spironolactone reduces phospho-Erbb4 levels in *Nrg1*-tg mice. Mice were treated with spironolactone for 21 days and sacrificed for western blot analysis. Lysates were probed with indicated antibodies.
(**E**) Quantification of band intensities for phospho-Erbb4 and phospho-Limk1 levels shown in (**D**) using ImageJ. Phosphorylation levels are normalized to protein levels of Erbb4 and Limk1. Data shown as mean, error bars represent SD; t-test, with *, $p \leq 0.05$.

2. Although the authors convincingly show that spironolactone affects ERBB function in cell culture, many different modes of actions have been assigned to it. Foremost spironolactone is known as MR antagonist and at present its questionable if the therapeutic effect in the NRG-1 TG mice is indeed mediated via the NRG1-ERBB pathway (especially since glucocorticoid signaling has been linked to NRG-1 function). I suggest two approaches to address this issue.

One possibility would be to measure p-Erbb4 as well as p-LimK etc. levels in animals that were used in the experiments described in Fig5 and EV5. Maybe this is what the authors did but at present it is unclear what treatment paradigm was used to generate the data shown in Fig 4C.

We thank the referee for this insightful comment and her/his suggestions to clearly present spironolactone-mediated changes of cellular signaling observed in vivo.

Indeed, as the referee already assumed, we have addressed p-Erbb4, p-Erk1/2, p-Akt, and p-Limk levels in spironolactone-treated animals and controls (Fig. 4D, was Fig. 4C). As described in the manuscript, major ERBB4 downstream signaling activities that include MAPK signaling (monitored by p-Erk1/2) and PI3K/Akt signaling (monitored by p-Akt) did not show differences in Nrg1-tg mice as well as spironolactone-treated mice. However, we have, as stated, noticed differences in p-Limk levels in spironolactone-treated mice, a finding that may indeed be linked to cytoskeleton dynamics and the regulation of synaptic spine morphology and function (Meng et al, 2002, 2004) (see below).

The treatment paradigm for the western blots shown in Fig. 4D was chosen alike the paradigm for the behavioral experiments shown in Fig.5 and EV5. To clarify the experimental situation, we added Fig. 4C as experimental outline for Fig. 4D.

Animals for the behavioral and biochemical analysis were, however, cohorted separately. For the biochemical analysis, animals were sacrificed after 21 days of daily spironolactone treatment, which coincided with the start of the behavioral tests. Mice used for biochemistry were also age-matched (11-13 weeks, with males used only), and the prefrontal cortex region was isolated for sample preparation as stated now in the manuscript.

To clarify the description, we have added a paragraph to the section “Supplementary Material and Methods”, subheading “Protein lysates and western blotting” in the Appendix and the main text.
Amendments to the Appendix, section Supplementary Material and Methods, subheading “Protein lysates and western blotting”:

For the biochemical analysis of spironolactone-treated mice (for a precise description of the injection paradigm, see Materials and Methods section, subheading “Mouse behavior analysis”, “Spironolactone treatment”), vehicle control or spironolactone were subcutaneously injected daily for 21 days into age-matched (11-13 weeks) male mice prior to preparation of the mouse prefrontal cortex (n=2 per genotype and treatment). For the generation of lysates, the isolated tissue was immediately placed into cooled (4°C) sucrose buffer (320mM sucrose, 10 mM Tris-HCl, 1 mM NaHCO3, 1 mM MgCl2, supplemented with 10 mM NaF, 1 mM Na2VO4, 1 mM ZnCl2, 4.5 mM Na4P207 as phosphatase inhibitors, and the complete protease inhibitor cocktail (Roche)), homogenized using an ultra-turrax (IKA GmbH, Staufen, Germany), sonicated (3 pulses for 10 sec) and denatured for 10 min at 70°C in LDS sample buffer.
Amendments to the main text:
We tested whether phospho-ERBB4 levels were also regulated by chronic spironolactone treatment in Nrg1-tg mice and injected the drug for 21 consecutive days before sacrificing the mice for biochemical analysis (Fig 4C).
In lysates from mouse prefrontal cortex, phospho-Erbb4 levels were efficiently visualized using the p-ERBB4-Y1284 antibody (Figs 4D and E).

Moreover, was really the entire mouse brain used for analysis? The description is misleading and sometimes refers to the prefrontal cortex which would of course make more sense.

We have corrected the description of the tissue sampling, which indeed was always from frontal regions (see paragraph above)

In addition it would be nice to learn more - maybe in the discussion - about the downstream mechanisms of elevated NRG1-ERBB4 signaling in the context of schizophrenia-like phenotypes. In fact considering that NRG1 is overexpressed from early developmental stages and there is evidence that altered NRG-1 function affect brain development and thereby contributes to the pathogenesis of schizophrenia, it is quite remarkable that even 3 weeks of spironolactone treatment in juvenile/adult mice reverse the disease phenotypes. Do the authors think that this correlates with structural changes?

We like to refer the reviewers comment also to the following paragraph in the Discussion which describes the potential link between Nrg1 function, and a potential impact of spironolactone treatment on the structural changes:
'Our biochemical analysis implicates LIMK1 signaling, but not ERK1/2 nor AKT1 as potential downstream effectors of spironolactone treatment in *Nrg1*-tg mice. As a non-receptor protein serine/threonine kinase, LIMK regulates synaptic spine morphology and function by modulating cytoskeleton dynamics (Meng *et al*, 2002, 2004; Bennett, 2011). Further, LIMK1 has been linked to NRG1 signaling and SZ-relevant endophenotypes in a *Nrg1*-tg mouse model (Yin *et al*, 2013). We show that phospho-LIMK1 levels were upregulated in *Nrg1*-tg mice treated with spironolactone suggesting that LIMK1 activity may possibly integrate spironolactone's inhibitory effect by promoting spine enlargement, and thus synapse formation, through controlling actin cytoskeleton dynamics.'

According to the referee's suggestion, we have substantiated the discussion on structural changes by adding the following sentences to the corresponding paragraph:
Nrg1-tg animals display subtle structural changes related to spine morphology, i.e. the number of bifurcated spines is increased (Agarwal et al, 2014). Therefore, it might be possible that spironolactone treatment reverts this structural endophenotype. Nonetheless, the increased levels of p-LIMK1 rather favors a mechanism compensating for the structural changes in *Nrg1*-tg mice, which may underlie network disturbances in these animals, by stimulating structural plasticity via increased LIMK1 activity. To further explore the mode-of-action of spironolactone in the future, it's impact on structural plasticity should be addressed in additional studies.

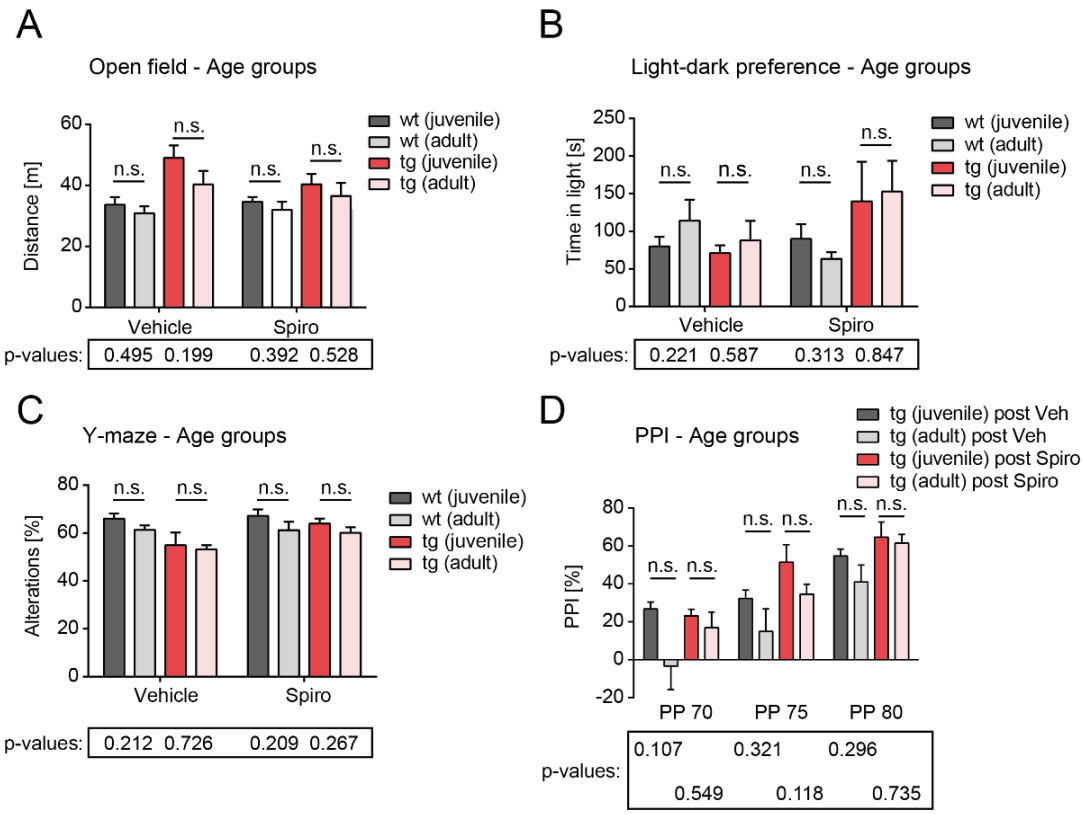
We like to state that these highly interesting follow-up experiments would best be performed with high-resolution imaging of living mice using e.g. a STED setup and is beyond the scope of this manuscript.

Along this line it is said that age-matched mice were used for behavioral testing (nothing is said about the mice used for immunoblot analysis). However the age appears to vary from 8-16 weeks. The authors should exclude the possibility that the observed effects are linked to the different ages. Can we exclude the possibility that the effect of spironolactone is mainly linked to the treatment of 8 weeks old mice in which post-natal brain development may not have been completed yet? Is it less efficient in 16 month old mice?

The referee points out that Nrg1 is a critical regulator of brain development and that Nrg1 overexpression may have different effects on behavior, e.g. in juvenile vs. adult mice. We would like to notice that we consider mice between 8 and 16 weeks as adult mice. Nonetheless, to address any age-related effects on the behavioral effects observed, both for spironolactone-treated and control-treated animals, we grouped mice into juvenile (8-11 weeks) and adult (12-16 weeks) categories. A covariate analysis using ANCOVA was performed for the open field test, Y-maze test and light-dark preference test, which indicated that age had no significant effect on the genotype-dependent treatment response (new Fig S3 in the Appendix). We also stated this in the main manuscript, by adding the following sentence to the results section describing the behavioral results:
As Nrg1 is a critical regulator for brain development, we aimed to exclude age-related consequences on the behavioral effects observed using a covariate analysis, which links age with test performance. To do this, we grouped mice into juvenile (8-11 weeks) and adult (12-16 weeks) categories, and

ANCOVA with the covariate age did not reveal an effect on the genotype-dependent treatment response (Appendix, Fig S3).

In addition, we include below a figure displaying all pairwise t-test p-values between juvenile and adult *Nrg1*-tg mice, which are not significant within each experimental condition for comparisons (as stated in the Appendix Fig S3 descriptions), which further supports the absence of an age-dependent effect. The layout of this figure R1 is corresponding to Fig S3 in the Appendix, with all T-test values depicted.



Minor issues:
1. page 3: "...principally offers a fast track to the clinic and has been demanded for SZ (Insel, 2012; Lencz & Malhotra, 2015), and also because many pharma companies withdrew from research..." the "and" appears misplaced.

Corrected.

2. Page 3: "Instead of changes in protein-coding regions..." should be removed.

As suggested, the part is removed.

3. Fig. 1A: Please indicate the type of cells that were used.

This illustration relates to the repurpose screening, for which PC12 cells were used in a co-culture setting. We have amended the figure legend accordingly. The first sentence now reads:
(**A**) The ERBB4-PIK3R1 split TEV assay monitors NRG1-ERBB4 signaling in PC12 cells.
At what time of the day was spironolactone injected?

As described in the Materials and Methods section, subheading "Spironolactone treatment", mice were injected daily in the afternoon. The description was already described before and reads:
'To avoid injection-induced stress prior to behavioral testing, mice were injected in the afternoon, after the entire cohort has completed the behavioral paradigm.'

Referee #2 (Remarks):
1-Authors should explain the differences in concentrations of spironolactone (10 micromolar in electrophysiology vs.1 in other experiments)

For both electrophysiology and biochemistry experiments, we applied spironolactone at a concentration of 10 µM. To address the effect caused by spironolactone in the presence of canrenone, we have lowered the concentration of spironolactone to 5µM (Fig. 4H). The higher concentrations of spironolactone (5 and 10 µM) in the electrophysiological experiments were chosen to be above the IC50 of about 1 µM as determined by the reporter gene assays to avoid reduced efficacy by higher absorbance and less penetrance of slices. Moreover, the application in the electrophysiological experiments was acute (in the min range) which, together with the absence of toxic effects in the reporter assays (up to 24h incubation), argues against any side effects of the chosen higher concentrations, e.g. by an impact on viability.

2-explain any sex differences in response to spironolactone

In our analyses, both for behavior and biochemistry, we have only used male mice and thus cannot comment on potential sex effects. We kindly ask the referee to refer to the section "Materials and Methods", subheading "Mouse behavior analysis". The description was already described before and reads:

'For behavioral testing, age-matched male mice (8-16 weeks) on C57Bl/6 background that constitutively overexpress the 2×HA-tagged Nrg1 type III b1a isoform (Nrg1-tg) under the control of the mouse Thy1.2 promoter (Velanac et al, 2012) and their wild type (wt) littermates as controls were used.'

2nd Editorial Decision 20 June 2017

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed reports from the referee who was asked to re-assess it. As you will see the reviewer is now globally supportive and I am pleased to inform you that we will be able to accept your manuscript pending following final editorial amendments.

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

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

As mentioned in the original review the manuscript present a very interesting and innovative approach to screen for drugs that might helpt to treat schizophrenia. Via the identification of spironolactone the authors show prove of concept. Using mice for this type of research is adequate.

Referee #1 (Remarks):

I have not further remarks. The authors have adressed all of my previous concerns sufficiently.

2nd Revision - authors' response 26 June 2017

Authors made the requested editorial changes.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Michael Wehr, Moritz Rossner
Journal Submitted to: EMBO Molecular Medicine
Manuscript Number: EMM-2017-07691

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

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

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

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	We did not use power calculations in this study. Sample sizes were defined according to comparable studies from the literature. For the behavioral studies, we have included 12 animals per genotype and treatment. (n=12 per genotype and treatment. (Figure Legend Fig 5, page 34))
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	See 1.a.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Mice were excluded from a data set if mice did not meet baseline test criteria. light dark preference test: Mice that did not enter the dark compartment within 10 min were excluded from the experiment (Appendix, page 12, section Supplementary Materials and Methods, subheading Behavioral tests applied for mouse behavior analysis, light dark preference test)
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Randomization/animal encoding was not done by the experimentor as well as decoding. Animals of differnt groups were randomly mixed with respect to litters and cages.
For animal studies, include a statement about randomization even if no randomization was used.	see 3.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	The investigators for behavioral tests were blind to genotypes and/or spironolactone administration. (page 20)
4.b. For animal studies, include a statement about blinding even if no blinding was done	see 4.
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Assumption of the tests were determined using GraphPad Prism 5.0 and IBM SPSS Statistics v22.
Is there an estimate of variation within each group of data?	Yes
Is the variance similar between the groups that are being statistically compared?	Yes. In those cases where variance was not similar (e.g. significant Levene's test), t-tests with equal variances not assumed were implemented.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

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http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Antibody information can be found in the Appendix, Table S3.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	PC12 tet-OFF cells were obtained from Clontech, 631134. T-47D cells were obtained from ATCC .(ATCC:HTB-133). Cell lines were negative for mycoplasma contamination.

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	We have used age-matched male mice (8-16 weeks) on C57Bl/6 background that constitutively overexpress the 2×HA-tagged Nrg1 type III beta-1a isoform (Nrg1-tg) under the control of the mouse Thy1.2 promoter (Velanac et al, 2012) and their wild type (wt) littermates as controls.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal experiments were conducted in accordance with NIH principles of laboratory animal care and were approved by the Government of Lower Saxony, Germany, in accordance with the German Animal Protection Law.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We confirm compliance.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Screening data is provided as Dataset S1 in Excel.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA