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Dear Dr Johnson,

My name is ████████████ and I am a co-author of the publication entitled "HDAC4: a key factor underlying brain developmental alterations in CDKL5 disorder" published by Trazzi et al. in Hum Mol Gen in July 2016 (doi: https://doi.org/10.1093/hmg/ddw231). I write to you in order to request a retraction of this publication, what I motivate with my severe doubts concerning part of the results presented there.

I have been working as a postdoctoral researcher in the Department of Biomedical and Neuromotor Sciences in the laboratory of Prof. Elisabetta Ciani (corresponding author of the mentioned publication) since October 2015. One of my main responsibilities in the laboratory is to plan, conduct and analyze behavioural experiments on mice as I am specialized in rodent behaviour and statistical analysis of biomedical data. In the past few weeks several doubts were raised concerning the quality of the research carried out in the Laboratory of Prof. Ciani within the last two years. Among many other doubts, severe questions arose concerning the mentioned article. They are presented in details below.

As one of the co-authors I am concerned that some of the behavioural results presented in the mentioned article may be based on unconfirmed hypotheses and on data that were not correctly acquired and/ or analysed and/ or presented. As in the laboratory raw data is not freely available to all the lab members (even for the co-authors of the manuscript), it was impossible for me to verify the doubts prior to the publication of the article. Already after publication I was denied to be shown raw data and statistical analyses performed for the experiments presented in the paper in Figure 10 (some of these experiments had been carried out before I joined the laboratory). Moreover, most of the electronic files from behavioural experiments carried out in the past are not properly described and archived. E.g. most of the raw files do not contain animal IDs and only the persons who physically run the experiments have access to information enabling identification of particular experiments and animals. The same concerns apply to statistical analysis, as only the person who performed it has access to the raw files. For these

reasons it was often practically impossible for me to track back specific studies or to verify performed data analyses.

Finally, and this doubt regards <u>all the experiments</u>, not only behavioural ones, it is uncertain and doubtful whether statistical analyses were performed correctly i.e. that the data was checked for meeting the assumptions of the parametrical tests used (e.g., normal distribution, equality of variances). This doubt was raised several times on other occasions by me as well as by the reviewer in a revision of the previous version of the manuscript that, before being published in Hum Mol Gen, had been rejected from publication in another journal.

I hope that my request for the retraction of the article will be considered favourably. I reckon that if, after analysis of such a small portion of raw data, I was able to identify a significant number of severe discrepancies and data flaws, the rest of published results should not be relied upon. Together with a general distrust in a correct use of the statistical methods, it is possible that other findings and/ or conclusions are not trustworthy either. In the reminder of this document I present all the collected proofs of data flaws. I would like to kindly ask you to inform me about the procedure of article retraction from the Hum Mol Gen Journal and about your next steps.

Yours sincerely

Doubts identified during analysis of the behavioural data published by Trazzi et al. in Hum Mol Gen in July 2016

Y maze (Fig. 10 A-B):

Presented Y maze data do not seem to match the raw data in several aspects.

- LMK 235 treated -/Y and +/Y animals were tested in September and November 2015 in separate experiments and pooled for final analysis. In the Materials and Methods section it is stated that there were 10 LMK 235 treated -/Y (12 in raw data) and 8 LMK 235 treated +/Y (8 in raw data). It is not explained why and which animals were removed and/or added to the experiment.

- Control experiment including untreated -/Y and untreated +/Y was run independently from the treated groups (what was not mentioned in Materials and Methods). I could not identify the control animals among experiments run on the age-matched mice that in the past (between February and December 2015). Group means values presented in the results section do not fit to any raw data set.

Morris water maze (Fig. 10 C-D):

Presented water maze data do not seem to match the raw data in several aspects.

- Animal numbers stated in the paper (LMK 235 treated +/Y n = 6 and LMK 235 treated -/Y n = 6) do not correspond to animal numbers used in the study (raw data indicate that there were five LMK 235 treated +/Y mice and five LMK 235 treated -/Y mice).

- The final version on the graph published in Hum Mol Gen (Fig. 10C) is neither consistent with the final draft version sent to me by Prof. Ciani the 17th of May 2016 nor with the graphs provided to Prof. Ciani by Prof. ▓▓▓▓▓▓▓▓ (co-author of the mentioned publication) or me (both me and ▓▓▓▓▓▓▓ independently analyzed the raw data from the experiment). Prof. ▓▓▓▓▓ in a conversation with me the 9th of November 2016 confirmed that the published graph does not correspond to what he had sent to Prof. Ciani and other collaborators the 13th of July 2015. This may suggest that the graph could had been changed without a consent of all the co-authors of the paper.

Below I illustrate discrepancy between the two mentioned graphs.

Morris water maze results (learning curve) sent by Prof. Ciani to the co-authors the 5th of May 2016 and re-sent by her to me later on several other occasions while working on the manuscript:
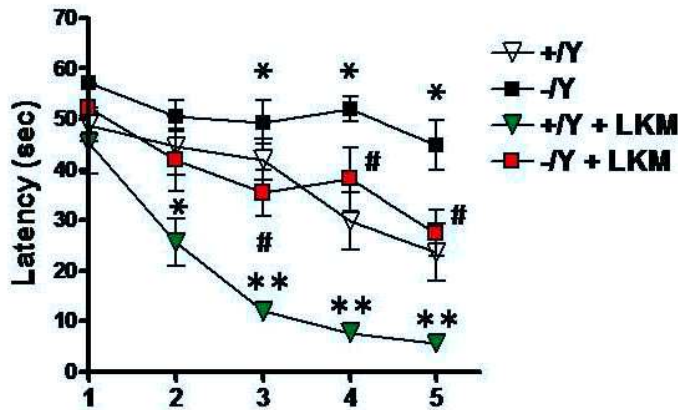
Graph published in the Hum Mol Gen:
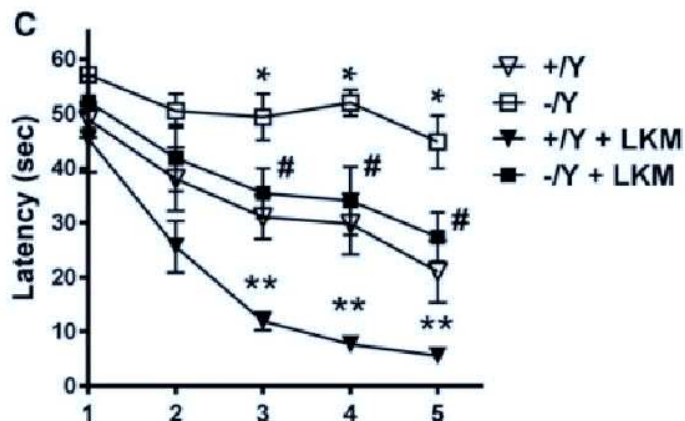
It should be mentioned here that before the publication of the article in Hum Mol Gen, a version of it was rejected from other journal. In that version the first graph shown above (with coloured data points) was included with a following description: "In the MWM test, while Cdkl5 +/Y mice learned to find the platform by the 2nd day, no significant learning was detected in untreated Cdkl5 -/Y mice until the 5th day, clearly indicating a learning deficit in Cdkl5 KO mice". The reviewer pointed out (revision from the 5th of June 2016): "*Also they [authors] state that 'Cdkl5 +/Y learned to find the platform by the 2nd day. However, in the plot of the latency [on] the second day they barely changed their execution, and in fact it is not until the fourth day that their latency becomes clearly reduced. Instead the slope of the learning curve is a bit steeper in the knockout mice. This means that in fact the learning differences between genotypes appear at day four*". In the article published in the Hum Mol Gen the description stayed the same, however the graph was modified so the +/Y curve and the description now correspond. To my best knowledge no more experiments were performed in order to obtain new data points that could lead to such a change of this curve.

- LMK 235 treated -/Y and LMK 235 treated +/Y animals were examined in May 2015 while control experiments including untreated -/Y and untreated +/Y were run independently (what was not mentioned in Materials and Methods). I could not identify these control experiments among any of the experiments run on age matching untreated animals between December 2013 and September 2014 as groups' mean values presented in the result section do not fit to any raw data set.

- Exactly the same controls (untreated -/Y and +/Y animals) were used in the already published paper "Inhibition of GSK3β rescues hippocampal development and learning in a mouse model of CDKL5 disorder" (Fuchs et al. 2015, doi: http://dx.doi.org/10.1016/j.nbd.2015.06.018, Fig. 9A). Curves for untreated -/Y and untreated +/Y overlap between the mentioned publication and the final draft version of the Hum Mol Gen paper sent to the co-authors by Prof. Ciani (first graph above, with coloured data points), but not with the graph eventually published in Hum Mol Gen where +/Y curve seems to be changed for days 3, 4 and 5.

- As confirmed to me by Prof. Rimondini during the conversation the 9th of November 2016, for the probe test (Fig. 10D), the graph represents the "latency to enter the target quadrant", not the "latency to enter the former platform zone" as incorrectly stated in Materials and Methods and figure caption. This error is crucial for understanding the result of this experiment, as presented "latency to enter the target quadrant" is a very uncommon and disputable indicator of memory retention in the water maze, much less common (if actually used at all) than indicators such as percentage of time spent in the former platform quadrant, latency to enter the former platform zone, number of crossings through the former platform zone or mean proximity to the former platform zone (please refer to a comprehensive article "What is the most sensitive measure of water maze probe test performance?" from Paul Frankland's laboratory, doi: http://dx.doi.org/10.3389/neuro.07.004.2009). Analysis of the available data on LMK 235 treated +/Y and LMK 235 treated -/Y done by me revealed that for the probe test: percentage of time spent in the former platform quadrant was 42.9 ± 4.6 for treated +/Y and 28.2 ± 5.7 for treated -/Y (chance level); Latency [in seconds] to enter the former platform zone was 11.2 ± 3.7 and 33.4 ± 10.2, respectively; Number of crossings through the former platform zone was 5.2 ± 0.6 and 1.6 ± 0.9, respectively. These results raise a significant doubt about the efficiency of the LMK 235 treatment on -/Y mice in terms of restoration of memory as measured in the Morris water maze test.

  Below the animals' trajectory during the probe test is presented for LMK 235 treated +/Y and LMK 235 treated -/Y mice, respectively (each plot represents performance of one animal on the course of 60 seconds of the probe trial in the Morris water maze). In each figure the plot in the bottom right corner represents the overlapping tracks obtained for all +/Y mice or all -/Y mice, respectively. The plots were generated by the Ethovision software version 3.0 from the raw files identified as the ones recorded for that experiment. The pictures clearly show memory retention in LMK 235 treated +/Y and no memory retention for LMK 235 treated -/Y mice. Additionally, as no visual (cued) probe was performed (or at least it was not reported in the article and no appropriate file exists in the experiment directory on the computer used for data acquisition), it is not possible to explain the source of low motility also observed in -/Y mice. All these doubts were not mentioned or discussed in the article.

+/Y + LMK 235 (N=5)
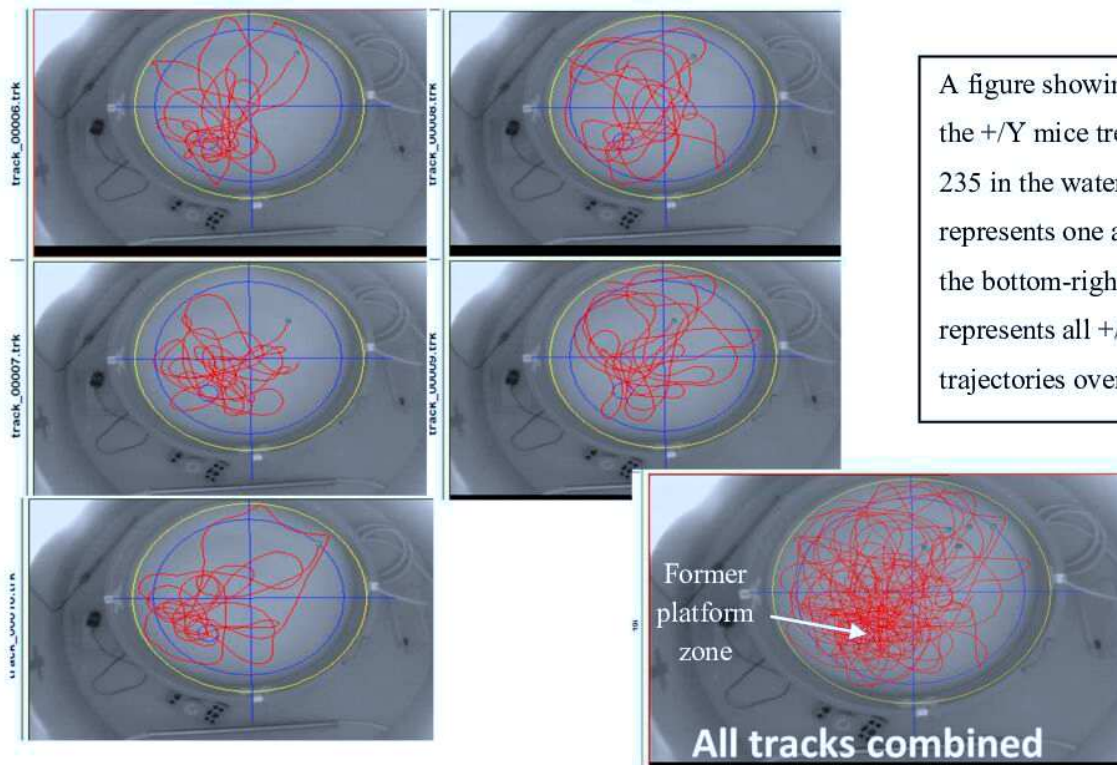
A figure showing trajectory of the +/Y mice treated with LMK 235 in the water maze (each plot represents one animal, the plot in the bottom-right corner represents all +/Y mice's trajectories overlapping).

Former platform zone

All tracks combined

-/Y + LMK 235 (N=5)

A figure showing trajectory of the -/Y mice treated with LMK 235 in the water maze (each plot represents one animal, the plot in the bottom-right corner represents all -/Y mice's trajectories overlapping).

Former platform zone

All tracks combined
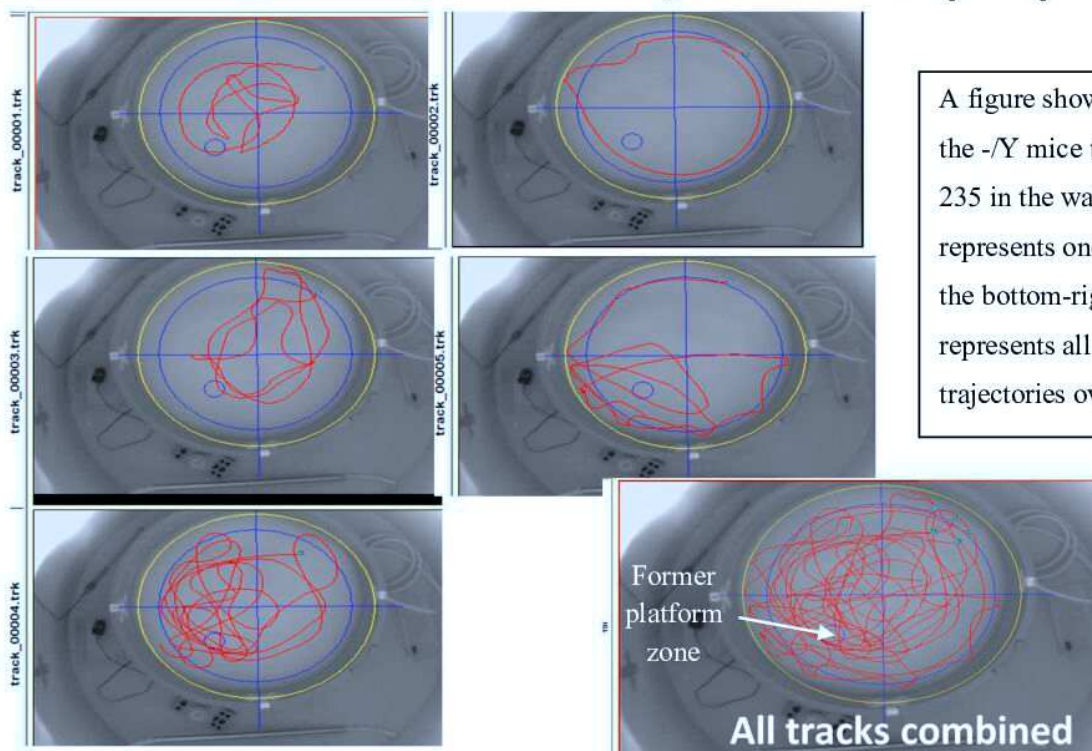
- Lastly, a new experiment designed to prove the effect of LMK 235 compound on restoration of the hippocampus-dependent behaviour, including spatial memory, in -/Y mice and performed in November 2016, failed to prove it. In other words, the positive behavioural effect of the LMK 235 treatment on -/Y mice published in Hum Mol Gen <u>could not be replicated</u>. In the mentioned experiment, again only treated animals were tested, no vehicle or untreated control animals were used (these controls were about to be run separately, at the beginning of the year 2017). It must be mentioned here though, that the experimental design was slightly different: animals were injected with LMK 235 starting from P5 with 14 doses of the drug (every second day). Behavioural experiments were performed around P45. This change might have influenced the overall effect of the drug (the hypothesis was that it will work even better), but in the light of presented doubts it may also prove the lack of effect of LMK 235 treatment on the behaviour of -/Y mice.

CONCLUSIONS

The doubts raised above may suggest that at least part of the data published in the mentioned article are not presented in a correct way, either as an effect of an error or a conscious action. The flaws pointed out above undermine one of the main conclusions of the paper stating that: "HDAC4 inhibition restores neuronal precursor survival, dendritic maturation and hippocampus-dependent learning and memory in the Cdkl5 -/Y mouse", as the raw data fail to prove the restoration of hippocampus-dependent learning and memory in -/Y mice treated with LMK 235.Therefore, I request retraction of the manuscript as the beneficial effect of LMK 235 treatment on learning and memory in -/Y mice, in my opinion, was not proved.

-- Data used to prepare the following report as well as detailed explanation of the process that led to the stated conclusions can be presented by the author if necessary. --