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REPORT ON INCONSISTENCIES AND DISAGREEMENT IN EXPERIMENTAL RESULTS AND DATA ANALYSIS IN THE DEPARTMENT OF BIOMEDICAL AND NEUROMOTOR SCIENCES, UNIVERSITY OF BOLOGNA

[redacted]

The following report was created in order to express the doubts concerning the to-be-published paper by Dr Claudia Fuchs et al. as well as other concerns regarding the results obtained in the laboratory in the last 2 years. The authors of the report, as the co-authors of the manuscripts mentioned below, are concerned that some results presented there may be based on unconfirmed hypotheses and on data which were not correctly acquired and/ or analysed and/ or presented.

It should be underlined that in the past [redacted]i and [redacted] communicated orally their doubts to Prof. Ciani, however no action was taken so far.

Since the research of the lab is funded and enabled by the CDKL5 children parents' associations, Telethon foundation, external grants, and the University of Bologna, the authors are convinced that it should be as much transparent as possible, leaving no space for unconfirmed results. Since the lab members have no free access to the experimental data from other researchers of the group, it creates space for potential undetected errors, data manipulation etc. That is why the authors strongly believe that the highlighted doubts should not be left undisputed as well as all the raw data from mentioned and future experiments should be presented to other lab members, without any restrictions.

Additionally, because of a very specific profile of patients the lab's research is targeting, all the group members and donors must be absolutely sure that nothing is going to be published that potentially could lead to an ineffective or even harmful therapy. As there are several concerns regarding datasets presented in the to-be-published paper of Fuchs et al., the authors of the report postulate to repeat all the presented experiments prior to the publication.

Below there is a list of doubts and inconsistencies regarding the research work of the lab. As it also covers results that were already published, the authors insist that all these discrepancies are thoroughly discussed in order to detect potential errors and, if it is a case, to correct them as soon as possible.

1. General doubts about the main hypothesis studied in the laboratory:

There is not enough proof that P-GSK3B protein levels in the P45 Cdkl5 KO mice is lower than for P45 WT mice. On the contrary, there are data suggesting that it is equal or even increased in Cdkl5 KO mice compared to WTs (in contrast to what was published so far by the laboratory). The list of experiments supporting this statement is as follows:

- Phosphoarray on GSK3B (2 independent assays).
- Western blots on GSK3B protein.
- Preliminary results from other research group which show toxicity of the GSK3B inhibitor in the same strain of WTs.
- Corn oil (vehicle) itself proved to have a small positive effect itself (10% on dendrites) however necessary vehicle controls were omitted in the presented study.
- Moreover, it is biologically unlikely that reported phosphorylation levels of GSK3B change drastically as shown, between age of P75 and P90 in the Cdkl5 KO mice.

Presented doubts refer to paragraphs 2., 3., and 4. mentioned below.

2. Doubts about the paper: Inhibiton of GSK3β with Tideglusib rescues hippocampal defects in juvenile, but not in adult Cdkl5 mutant mice (Fuchs et al. to be published)

- Presented water maze data do not match the collected data (Fig. 2). Treated animals were examined in May 2016, control experiment including vehicle treated KO and vehicle treated WT mice was run independently (what was not mentioned in materials and methods and is against good laboratory practice [GLP]). Moreover, although control animals of matching age were tested in similar conditions in December 2013, February 2014 and September 2014, none of those are presented on the graph in the manuscript, indicating that the presented control groups were taken from yet different experiment that could not be identified. Finally, although another experiment run on Tideglusib treated animals (in October 2015) shows the opposite effect of Tideglusib to the one presented in the paper, it is not mentioned in the discussion.
- Passive avoidance data do not match the data collected by N[redacted](Fig. 2).
- Length of dendrites (Fig. 3) - there are 2 different versions of data points for WT group, they are in the same file sent by Dr Claudia Fuchs to [redacted], without explanation why the numbers were changed.
- In the manuscript it is never stated how many animals were used for particular procedures what makes it impossible to verify the composition of the experimental groups.

- Improper data acquisition and archivization was detected:
    - Confocal images: samples acquired with z-stack were compared to samples without z-stack (samples from treated animals).
    - CDs with confocal images, at least part of them, are kept at home, not in the lab.
- In some cases appropriate paired statistical tests should be applied. In the materials and methods section no such tests are mentioned.

3. Doubts about the paper: HDAC4: a key factor underlying brain developmental alterations in CDKL5 disorder (Trazzi et al. 2016)

- Presented water maze data do not match the collected data (Fig. 10). Treated animals were examined in May 2015, control experiment including untreated KO and untreated WT was run independently (what was not mentioned in materials and methods and is against GLP). These controls could not be identified among any of the experiments run on age matching untreated animals either from December 2013, February 2014 or September 2014. Moreover, exactly the same controls were used as in already published paper Inhibition of GSK3β rescues....
- Y maze data do not match the collected data (Fig. 10). Treated animals were tested in September and November 2015. In the materials and methods section it states that there were 10 treated KO (12 in raw data) and 12 treated WT (8 in raw data). It is not explained why and which animals were removed and/or added to the experiment. Control experiment including untreated KO and untreated WT was run independently from the treated groups (what was not mentioned in materials and methods and is against GLP). These controls could not be identified among any experiments that were run in the past.
- In some cases appropriate repeated measures statistical tests should be applied. In the materials and methods section no paired tests are mentioned.

4. Doubts about the paper: Inhibition of GSK3β rescues hippocampal development and learning in a mouse model of CDKL5 disorder (Fuchs et al. 2015)

Presented water maze data do not match the collected data (Fig. 9). Treated and control animals were examined in September 2014 (when no effect of treatment was shown), however these are not the data shown in the paper. Data shown in the paper could not be identified.

5. Doubts about the paper: Characterization Loss of CDKL5 impairs survival and dendritic growth of newborn neurons by altering AKT/GSK-3β signaling (Fuchs et al. 2014)

- Other groups (Prof. M. Giustetto) were not able to repeat the results / had different results concerning p-AKT, VGLUT1, PSD95.
- No one else was able to obtain comparable data involving Caspase-3: **10-fold** difference in the counting, also pointed out by another researcher in the 2014 Telethon convention who asked Dr [redacted] what antibody was used to visualize so many cells.

6. Doubts about the paper: Loss of cdkl5 in forebrain excitatory neurons impairs hippocampal function ([redacted] et al. to be published)

The final version of the paper (approved by the external collaborators and ready to be sent to the Neurobiology of Disease Journal) contained 3 graphs where the non significant differences were marked as significant (Fig. 4D, 5I, 5K). In one case (Fig. 5K) an analysis of the raw data revealed that the graph represents values as if 2 animals out of 6 were removed (1 KO and 1 WT) in order to obtain significant effect. As these facts were reported to the PI by the co-authors prior to sending for publication, more experiments are about to be performed to confirm the results.

7. Doubts concerning other projects from the lab:

- GENE THERAPY

The particle produced by Dr Fuchs during the internship in the USA (2015) did not prove to work. After several months and many additional experiments performed in the laboratory in Bologna by Dr [redacted], it occurred that the particle that Dr Fuchs provided was never shown to be functional. The whole experimental work had to be repeated by [redacted]. Dr Fuchs was not able to provide explanations for the exact experimental protocols and steps she has undertaken, but claimed that she run all the necessary controls to prove that the particle was functional. Moreover, Dr Fuchs was not able to explain the cloning strategy she had been supposed to apply during her internship. Dr Fuchs also claimed that her constructs were sequenced during her internship, however one year later she declined it. Verification of her experiments was additionally difficult as Dr Fuchs claimed that **the original data from experiments were left in the lab in the USA**. Since one of the projects of the lab was based on the mentioned particle, it has been delayed for one year already according to the previous research plan.

- PROTEIN THERAPY

- Spines counting performed in a blinded manner did not prove the results obtained when the genotype and treatments were known to the researcher.
- P-AKT optical density in brain slices (images shown to *Kininska* or *Amicus* experts and part of the Patent data) was not confirmed in various, independent, western blot analysis: neither the putative positive effect of the treatment, nor the difference between untreated KO and WT mice at that age (6 months).
- TAT-CDKL5 diffusion in the brain after ICV injection: images were presented with concentrical diffusion from the ventricle to the other brain areas, which is biologically doubtful considering the very low levels of protein and that the optical signal was amplified.

CONCLUSIONS

Taking into account the number and severity of doubts listed above as well as having in mind the best interest of the CDKL5 patients, the authors of this report claim that immediate actions are taken in order to verify the results and correct potential errors.

First of all we insist on MAKING AVAILABLE ALL THE RAW DATA CONCERNING THE EXPERIMENTS DISCUSSED IN THIS REPORT, with all necessary explanations about the changes in the data, removal/ addition of data points, statistical tests used etc. It should be presented in a form that can be understood without further explanations from the side of the person who makes the data available.

Secondly, we insist on REPEATING THE ENTIRE EXPERIMENT that led to the conclusions stated in the to-be-published manuscript by Fuchs et al. sent to the lab members in July 2016.

* *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 authors when necessary* *