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14. ABSTRACT

Our two laboratories – Klann and Bagni - are committed to understanding the detailed molecular abnormalities associated with developmental disabilities and how these result in synaptic dysfunction and aberrant behavior. Our overall hypothesis is that repetitive and perseverative behaviors exhibited by FXS patients that can be recapitulated in the FXS model mice are caused by affected cortico-striatal synapses. To test this hypothesis, we propose two specific aims: **1) To determine cortico-striatal synaptic composition, function and plasticity in FXS model mice; 2) To determine whether altered cortico-striatal synaptic plasticity and repetitive/perseverative behaviors displayed by FXS model mice are reversed by novel cap-dependent translation inhibitors.** Our specific tasks are centered on a proteomic study of FXS striatal synapses by using a transgenic mouse model that allows capturing "native" synapses. Purified synapse will be analyzed by mass spectrometry and the data will be validated using biochemical and cellular methods. The comparison of the synaptic proteome between the wild type and the FXS mice during development will identify which complexes are affected in FXS and possibly in other synaptopathies. These data will complement the electrophysiological and behavioral studies performed by the coordinator.

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Introduction

This joint project, coordinated by the group of Dr. Eric Klann at the New York University, NY, aims at understanding, at the molecular level, the abnormalities of neurodevelopmental disorders such the Fragile X Syndrome (FXS). We are studying how these abnormalities result in synaptic dysfunction and aberrant behavior. Our overall hypothesis is that repetitive and perseverative behaviors exhibited by FXS patients that can be recapitulated in the FXS model mice are caused by affected cortico–striatal synapses.

To test this hypothesis we propose two specific aims: **1) To determine cortico–striatal synaptic composition, function and plasticity in FXS model mice; 2) To determine whether altered cortico–striatal synaptic plasticity and repetitive/perseverative behaviors displayed by FXS model mice are reversed by novel cap-dependent translation inhibitors.**

The specific contribution of the laboratory of Dr. Bagni is centered on a cutting-edge technique to purify post-synaptic densities, i.e., the crucial receptor field of glutamatergic synapses. Post-synaptic densities will be purified from brain cortices and striata and analyzed by mass spectrometry. These data will be validated using biochemical and cellular methods. The comparison of the synaptic proteome between the wild type and the FXS mice during development will identify which complexes are affected in FXS and possibly in other synaptopathies. These data will complement the electrophysiological and behavioural studies performed by the coordinator.

Key Words

Fragile X syndrome (FXS), synaptic proteome, synaptic structure, postsynaptic density, PSD-95, NMDA receptors, striatum, cortex, hippocampus.

Accomplishments

The accomplishments during year 3 are described, task-by-task, in the next section. A summary is given at the end of the following section.

Major goals of the project (year 3)

Task 1-Subtask 2: *Breed and generate sufficient numbers of Fmr1 knockout/PSD-95TAP mice*

Breeding of the mouse colony will continue up to the end of the project as stated in previous reports. We still need $Fmr1^{-/y}$ x $PSD95^{TAP/TAP}$ mice and the respective controls for the preparation of the PSD samples to be analysed by phosphor-proteomics and validation of the obtained results.

Task 1-Subtask 3: *Measure levels of NMDA receptor subunits and complexes at cortico-striatal synapses in FXS model mice and their wild-type littermates*

This task has been completed (see Figure 1 and manuscript in preparation). We performed protein quantification through WES and Western blotting for the NMDA-R

subunit NR1 in addition to the NR2B that was named in the initial project because the proteomic analysis of the post-synaptic density (task 1-Subtask 4) indicated that the NMDA receptor NR1 is dysregulated. We analysed the levels of these receptors in striatal post-synaptic densities because 1) cortico-striatal projections form glutamatergic synapses in the striatum; 2) the striatum appears the brain region with most dysregulated proteins during development (see Subtask 4 and report year 2). Both NR1 and NR2B are

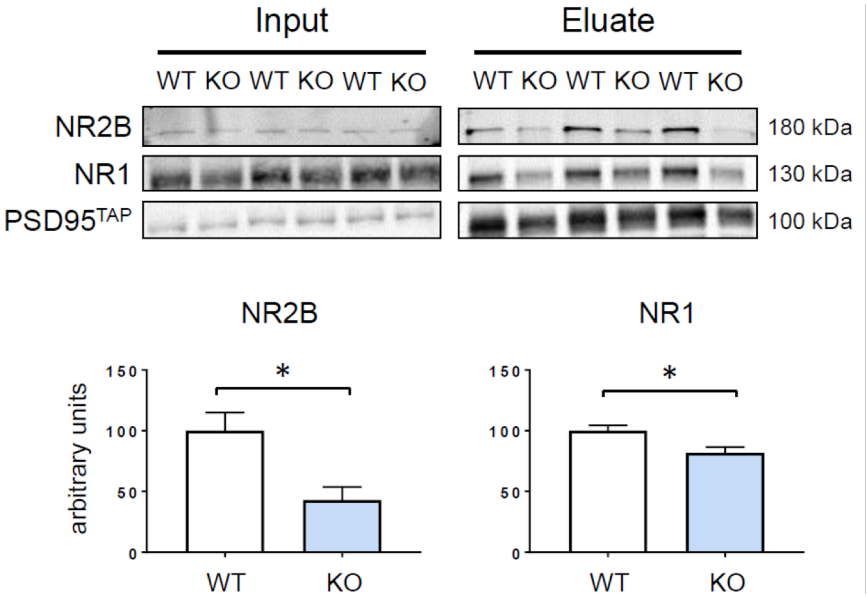

Figure 1. NMDA-Rs are dysregulated in post-synaptic densities purified from the Fmr1$^{-/y}$ striatum. Upper panel: Western blotting analysis of the indicated proteins in total extract (left) and purified post-synaptic densities (right) in the indicated genotypes. Lower panel: the signal in the purified PSDs (panel eluate) was quantified for NR1 and NR2B and normalized for the respective PSD95^{TAP} signal. Data are presented as mean ± SEM (n=3, *, p<0.05)

significantly reduced (Figure 1), suggesting that FXS synapses have an impaired structural and functional plasticity.

Task 1-Subtask 4: *Isolation of the PSD-95 interactome from the cortical, hippocampal, and cortico-striatal regions of the Fmr1 knockout/ PSD-95^{TAP} mice and PSD-95^{TAP} control mice at different developmental stages*

As stated in the previous report, the subtask consisting of tandem-affinity purification and mass spectrometry analysis has been completed. The bioinformatical analysis to evaluate differences between developmental stages and genotypes had been concluded (see manuscript in preparation). Currently we are completing the validation of the data (described under task 1-Subtask 11). Furthermore, in the context of the current collaboration with Dr. Seth Grant University of Edinburgh/UK whose lab generated the PSD-95 TAP mice, we started a bioinformatical analysis to do gene-disease association studies on our dataset.

Furthermore, we have performed the analysis of the phospho-peptides that were detected in the mass spectrometry (described in the previous report). From this dataset, we were able to extract quantitative information about 277 unique phosphorylation sites in 76 proteins. We detected striking differences during development (Figure 2A), and significant differences in developmental regulation between regions (Figure 2B). In contrast, no

statistically significant differences were observed between WT and Fmr$^{-/y}$ PSDs. Note, however, that only the most abundant phosphoproteins and their phosphorylation sites could be detected in this analysis.

To obtain data also on less abundant – *e.g.*, regulatory – proteins, we are now proceeding with a dedicated phospho-proteomic analysis as it was envisioned for the last year of the project. In such an analysis, the tryptic peptides are fractionated by metal-affinity chromatography. The mass spectrometry is then performed on the fraction that is highly enriched in phosphopeptides and therefore yields much more information on these peptides. This part will be performed at the expert Protein Analysis Facility of the University of Lausanne (https://www.unil.ch/paf/en/home.html).

To perform this analysis, we have bred more mice and are now preparing a new set of purified post-synaptic densities. We envision that both purification and analysis will be completed before the end of the project. Validation of any changes that should be identified in the analysis will then be validated in a follow-up period outside of this project.

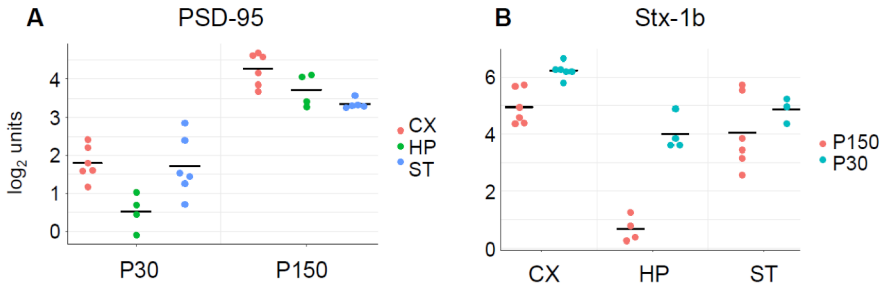

Figure 2. Phosphorylation changes of PSD-associated proteins. Two examples are shown for proteins that are differentially phosphorylated during development (panel A: PSD-95, phosphorylated at position 553) or between regions (panel B: syntaxin 1b at P150, phosphorylated at position 14). PSD-95 phosphorylation is upregulated during development by at least 1.5 $\log_2$ units (~3-fold) in all regions, whereas syntaxin 1b is downregulated by two $\log_2$ units (4-fold) specifically in the hippocampus at P150.

Task 1-Subtask 7: *Protein composition analysis of the PSD-95 interactome from Fmr1 knockout/PSD-95^{TAP} mice and PSD-95^{TAP} control mice at two different developmental stages*

This task has been completed. While there are several proteins that are dysregulated between P30 and P150 (see for example the solute channel Slc25a1, Figure 3A), there is no significant statistical interaction between age and genotype, which would have indicated that FMRP regulates protein expression differently at P30 and P150. The most significant interaction was observed for actinin Actn1 (FDR = 0.08, Fig. 3B), but this result is confounded by the fact that the protein was detected only in cortical samples at P30, and only in striatal samples at P150. Therefore we conclude that FMRP regulation of synaptic proteins strongly depends on the brain region but, somewhat surprisingly, not on the developmental stage.

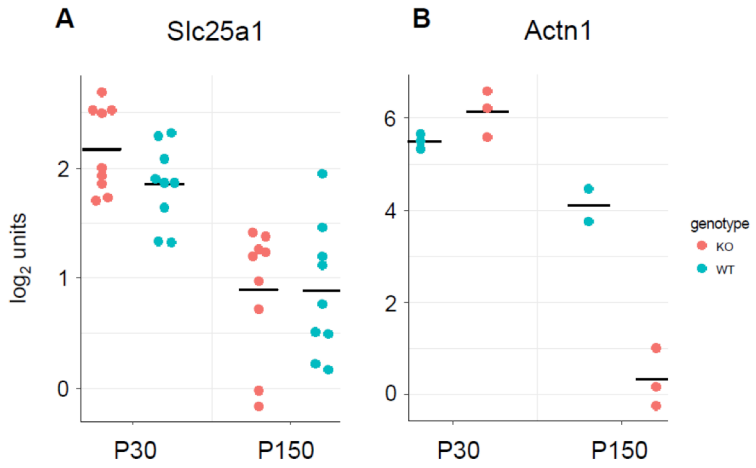

Figure 3. Development-specific expression of PSD-associated proteins. Two examples are shown for proteins that show development-specific expression (Slc25a1, A) and a near-significant interaction between age and genotype (Actn1, B). For Slc25a1, data from the three different regions are reported, while actinin1 was detected only in one region, hence less data points are available.

Task 1-Subtask 11: *Validation (protein levels, subcellular localization in vitro and ex vivo) of selected dysregulated proteins in the Fmr1 knockout mice at two developmental stages*

We have started this task. Importantly, several data points of the mass spectrometry could be validated: NR1 and NR2B (Figure 1) as well as β-actin (Figure 2) differ in their association with striatal post-synaptic densities. We have additional 19 more proteins that are dysregulated. We selected 6 of them for which commercial antibodies were available. However, 5 out the six antibodies fail to detect it in the purified PSDs but readily revealed their targets in total extracts (data not shown). We conclude that these proteins are very low abundant in the pure PSDs and can be detected only by mass spectroscopy, which is more sensitive than Western blotting. We are currently testing if these 5 proteins can be detected and are dysregulated in synaptoneurosomes, as these preparations contain higher concentrations of synaptic proteins compared to the highly purified PSD preparations tested so far.

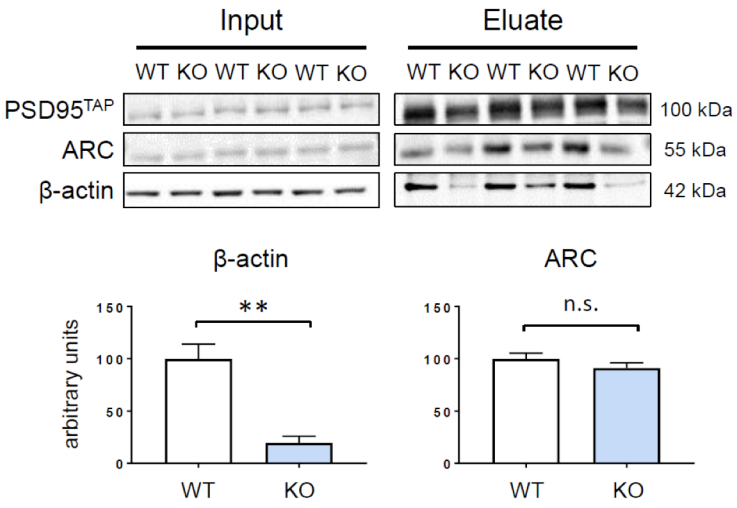

Figure 4. β-actin, but not ARC, differs in association with post-synaptic densities purified from the Fmr1$^{-/y}$ striatum. Upper: Western blot analysis of the indicated proteins in the total extract (left) and in purified post-synaptic densities (right) with the indicated genotypes. Lower: the bands in the purified PSDs were quantified and the actin/ARC was normalised for the respective PSD95^{TAP} signal. Data are presented as mean±SEM (n=3, **, p<0.01, n.s., not significant)

Task 2-Subtask 2: *Determine whether inhibitors of eIF4E and eIF4A have a (positive) effect on the corticostriatal PSD-95 interactome protein composition, modification, and localization.*

This subtask was envisioned to be completed in the last year; work on the task is currently on progress.

Summary of accomplishments

- The NMDA receptors have been shown to be dysregulated in the striatum, where the cortical projections arrive.
- Analysis of the PSD proteome has been completed for three brain regions and at two developmental stages. Strikingly, FMRP-dependent regulation is mostly observed in the striatum. In addition, there is little if any difference in FMRP regulation between the two ages that we analysed.
- The proteomic data has been validated for selected high-abundance proteins- 3 of them so far.
- A preliminary phospho-proteome of the PSD has been analysed. 76 phosphoproteins were reliably detected, some of which change in phosphorylation levels between brain regions. Age has less effect, the *Fmr1* genotype has none. We are currently preparing samples for a dedicated phospho-proteome in order to obtain better data on more phosphoproteins.

Impact

Our laboratories are committed to understanding the detailed molecular abnormalities associated with developmental disabilities and how these result in synaptic dysfunction and aberrant behavior. Thus, military families with members afflicted with these disorders will benefit from these studies.

Our studies provide information on the structural composition of the synapse during development and on the fine regulation (phosphorylation) of the synapses in FXS and non-affected mouse models. We have elucidated which brain region shows a higher change in structural composition of the synapse. The striatum is involved in FXS related behaviors such as repetitive/perseverative behavior. Importantly, such behavior is also observed in individuals with traumatic brain injury.

In the long term, our studies will provide information for the design and use of novel compounds to therapeutically target pathways affected in FXS and other developmental or disabilities due to traumatic events.

Changes/Problems

There are no problems or changes in the last phase of the project.

Products

Publications, 3rd year only:

The following paper is currently in preparation:

Fernandez F*, Gastaldo D*, Mercaldo V, Grant SGN, Achsel T, and **Bagni C**. (2017). "Region-specific composition of post-synaptic densities in mouse brain." *: these authors contributed equally to the work.

The following paper was published under this DoD-funded grant as collaboration between the laboratories of Dr. Klann and Dr. Bagni:

Santini E, Huynh TN, Longo F, Koo SY, Mojica E, D'Andrea L, **Bagni C**, and **Klann E**. (2017). "Reducing eIF4E-eIF4G interactions restores the balance between protein synthesis and actin dynamics in fragile X syndrome model mice." *Science Signalling* 10(504).

The following two reviews/comments were produced in the context of this project, and DoD funding was acknowledged accordingly:

Salinas PC, and **Bagni C**. (2017). "Gender Equality from a European Perspective: Myth and Reality." *Neuron* 96: 721-729.

Borrie SC, Brems H, Legius E, and **Bagni C**. (2017). " Cognitive Dysfunctions in Intellectual Disabilities: The Contributions of the Ras-MAPK and PI3K-AKT-mTOR Pathways." *Annual Review of Genomics and Human Genetics* 18: 115-142.

Participants and other collaborating organizations

Name: **Claudia Bagni**
Project role: Principal investigator
Person Months worked: 2 calendar months
Contribution to the project: Design and supervise experiments and interpret data

Name: **Valentina Mercaldo**
Project role: postdoctoral fellow
Person Months worked: 2 calendar months at 50% effort and 10 calendar months at 80% effort).
Contribution to the project: Valentina and Denise have taken over the project from Esperanza. They are responsible for design and realization of the experiment, as well as analysis of the data.

Name: **Denise Gastaldo**
Project role: PhD student
Person Months worked: 12 calendar months.
Contribution to the project: Valentina and Denise have taken over the project from Esperanza. They are responsible for design and realization of the experiment, as well as analysis of the data.

Name: **Nuria Domínguez**
Project role: PhD student
Person Months worked: 12 calendar months.
Contribution to the project: Help the project with breeding and genotyping mice; preparing brain slices and brain extracts for the experiments.