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Research Idea

Functional characterization of the several splice variants of *Fmr1*

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Abstract

Background

Fmr1 has been known to be a crucial contributor in neurodevelopment. Events such as alternative splicing in its coding region and the use of different trancription start and end sites in its non-coding regions result in the production of a range of mRNA transcripts. Structural and functional characterization of some of these transcripts have been performed, but several of them remain uncharacterized. Differences in the temporal and spatial expession of these transcripts may play important roles in regulating the development of the fetal and adult brain structures and thus, their physiological functions.

New information

The extensive set of experiments suggested in this study plan exploit mice brain, neuronal cultures and *in vitro* studies. The proposed study for the identification and full characterization of the known and novel transcripts of *Fmr1* is an important initial step towards the assignment of the specific known roles and the determination of the so-far unknown roles of Fmr1 at the various stages of neurodevelopment. This systematic study helps in categorizing the transcripts that can produce stable proteins and further understand their cellular localization and cellular functions individually and in concert with each other. Similarly, identification of the non-coding transcripts helps us in exploring their

roles in the regulatory processes, if any, which might also impact the expression of the coding transcripts in the neuronal cells.

Keywords

FMRP, *Fmr1*, splice variant, alternative splicing, CGG repeats, protein isoform

Overview and background

Fragile X syndrome (FXS) is a neurodevelopmental disorder that results in intellectual disabilities in addition to autistic traits, anxiety and seizures. It is caused by the dysfunctioning of *Fmr1* (fragile X mental retardation gene 1) located on the X-chromosome. *Fmr1* is also identified as a monogenic cause of autism. It encompasses ~38 Kb and consists of 17 exons. Its 5' UTR consists of CGG repeats. In FXS, the number of CGG repeats increases to >200 (full mutation) instead of the healthy number of <55. This increase leads to transcriptional silencing of *Fmr1* and thus, a complete loss of expression of its encoded protein, FMRP. In addition, if the CGG repeat number increases to be within 55-200 (pre-mutation), Fmr1 RNA levels increase in the cells, though FMRP levels are reduced. This condition is linked to two disorders, namely, fragile X-associated tremor/ataxia syndrome and fragile X-related primary ovarian insufficiency. Studies conducted with *Fmr1* KO models have shown that loss of *Fmr1* is associated with an increased density of immature dendritic spines in the neuronal cells. Though, several recent studies have suggested that this phenotype varies with respect to the different brain areas and the different developmental stages of the brain (as reviewed in He and Portera-Cailliau 2012).

FMRP consists of two Tudor domains, a nuclear localization signal (NLS), three K homology domains (KH0, KH1, KH2), a nuclear export signal (NES) and an arginine-glycine-glycine domain (RGG) from N- to C-terminus. The tudor, KH and RGG domains are mainly involved in RNA binding, though they also have protein interaction partners. FMRP is ubiquitous but is highly expressed in the brain and reproductive organs. Being a RNA-binding protein, FMRP has been shown to be involved in the regulation of translation, stability and localization of several mRNA targets (as reviewed in Chen and Joseph 2015, Fernández et al. 2013). In several studies, FMRP has been shown to undergo alternative splicing thus, resulting in the expression of several transcript variants and protein isoforms (Fig. 1). Using brain tissues, it has been shown that these transcripts and isoforms exhibit differences with respect to their spatial and temporal expression (Brackett et al. 2013, Pretto et al. 2014).

As per some recent studies, it has been shown that different transcription start and end sites exist and are used during the transcription of mouse and human *Fmr1*, which along with the alternative splicing events occurring in the coding region, would account for a diverse range of transcripts and thus, would regulate the production of different protein isoforms of Fmr1 (Tassone et al. 2011). This diversity would potentially serve a range of

diverse functions as a result of temporal and spatial expression of *Fmr1* in the brain. Thus, in order to fully understand the functions of *Fmr1* in the brain, it is essential to dissect the roles of its different transcripts and isoforms.

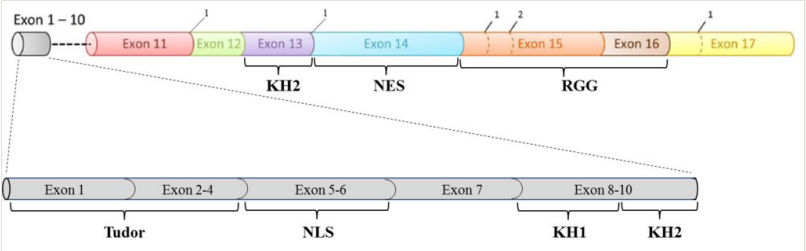

Figure 1.

Diagrammatic representation of the exon structure of Fmr1 and its corresponding functional domains. The splice acceptor sites are marked as 1 and 2.

Objectives

As stated above, in order to fully dissect the role of Fmr1 in the functioning of a healthy brain, it is important to draw a map of the wide variety of transcripts (coding and non-coding) produced by the gene in the different regions of the brain at different stages during development. This detailed information would form the basis of a systematic analysis and finding correlations between the existence/expression of Fmr1 and the different on-going processes in the brain. For such an analysis, it is crucial to identify and functionally characterize all the known and novel transcripts of Fmr1. Henceforth, this task is largely divided into three objectives: (a) identification, cloning and characterization of the various (known and novel) splice variants of Fmr1 expressed in the different regions of the mouse brain, (b) analysis of the known Fmr1 transcripts and isoforms and (c) elucidating the structure of full-length FMRP.

Implementation

I. Identification, cloning and characterization of the various (known and novel) splice variants of *Fmr1* expressed in the different regions of the mouse brain

a. Cloning and sequencing of the splice variants: Pretto et al. in their study have predicted the presence of 24 different transcripts of Fmr1 as a result of alternative splicing in the coding region (Pretto et al. 2014). They along with other studies have validated the presence of different Fmr1 splice variants in the different regions of the mouse brain at different developmental stages ranging from the embryo to adult (Brackett et al. 2013). In addition to the predicted variants, a novel transcript (with spliced exon 3) was also found to be expressed in the cerebellum of the mouse brain (Pretto et al. 2014). Hence, it could be possible that some novel transcripts do exist which are yet to be identified. Thus, as a first

step, using appropriate primers against the various sets of exons, the various splice variants can be isolated and amplified from the mRNA pool of the mouse brain (and individual areas of the brain to isolate low abundance transcripts, if any) and subsequently their cDNAs can be cloned and sequenced. This could be performed at different time intervals during the brain development. This would give us the exact information about the pattern of alternative splicing being used in the coding sequence of Fmr1. A similar extensive study performed by Wang et al. has shown the presence of a range of splice variants of Shank3 in the mouse brain (Wang et al. 2014).

b. Protein expression from the splice variants as a result of variations in coding and non-coding regions: Since, it would be important to know if the splice variants of *Fmr1* are translated/are capable to produce stable proteins in the cells, protein expression from each of these different transcripts could be monitored. (i) If appropriate antibodies would be available at the time, this could be done using Western blotting, or (ii) a fusion tag sequence could be inserted immediately upstream of the mRNA coding sequence and Western blotting against the fusion tag could be used to detect the produced protein. The 5' and 3' UTR regions of the mRNAs can also show variations and thus, can determine the translation efficiency and stability of the mRNAs. Tassone *et al*. have shown the existence and usage of different transcription start and end sites in the Fmr1 mRNAs isolated from mouse and human brains (Tassone et al. 2011). (iii) Hence, the different 5' and 3' UTRs used by Fmr1 mRNAs isolated from mouse brain can be identified using RACE. These UTRs could be cloned and reporter assays (for example, luciferase assay) could be performed to analyze their effect on the translation of the adjoining protein. (iv) *in vitro* translation systems could be used to check the capability of these mRNA/cDNA templates to express the protein. For this, the full-length mRNA possessing the 5'-cap and 3'-tail structures would be used (the cap sequence could be detected using the available methods, for example, as described in Efimov et al. 2001). This information about the UTRs could also help in understanding the roles of spatial and temporal expression of Fmr1 transcripts, if any, in brain function. A recent study has shown the identification of the microRNAs involved in regulating a specific mRNA (Hassan et al. 2013). Similarly, knowledge of the specific 3' UTR of the Fmr1 mRNA splice variants could also be used to identify the regulatory microRNAs associated with them. miRNA has also been shown to bind in the coding region of the target RNA (Fang and Rajewsky 2011, Marín et al. 2013, Xu et al. 2014) and thus, similar studies could be performed for Fmr1 RNA as well.

c. Functional characterization of the protein isoforms expressed from the splice variants: This could be performed by overexpressing each of the splice variants (transiently or stably as required) in neuronal cell cultures (eg. dissociated neuronal cell lines) to check their cellular localization and interactions with other proteins in the cells using pull-down studies, their effects if any, on dendritic spine morphology or on neuronal activity. Differences could also exist with respect to the RNA molecules that associate with these different isoforms of FMRP. Thus, this could also be analyzed using FMRP isoform-specific pull-down or co-immunoprecipitation studies. Similar studies with Fmr1 transcript and isoform expression could also be performed with non-neural and progenitor brain cells (Faulkner et al. 2015, Luo et al. 2010).

II. Analysis of the known Fmr1 transcripts and isoforms

a. Identification of the known isoforms of Fmr1: Bonaccorso *et al*. have shown that the total content of FMRP decreases in the cortex and the cerebellum of the mouse brain during its development and the proportion of decrease varies between the two regions. It was also shown that the total protein content varies between the other regions of the mouse brain as well. The analysis was made using Western blotting with an antibody that recognizes the N-terminus region of FMRP and thus, recognizes its different isoforms (Bonaccorso et al. 2015). The various isoforms detected are of similar molecular weight (~70 KDa) and thus, no proper distinction occurs between the several isoforms. To distinguish between these different isoforms, isoelectric focusing (2-D gel electrophoresis) in combination with western blotting could be performed for the analysis of the different brain regions of the mouse at different time intervals during development. This could be further extended to the sequencing of the identified isoforms.

b. Transcript isoform 7: Pretto *et al*. have shown that the expression of transcript isoform 7 increases during the development of mouse brain ranging from the embryo to the adult stage (Pretto et al. 2014). (i) It could be analyzed if the increase in its mRNA transcript is complemented by an increase in its protein level as well. For this, 2-D gel electrophoresis could be employed as mentioned above in case no specific antibody is available. (ii) Following a detailed analysis of the transcript, an inducible system that could regulate the expression of this transcript/protein could be used (for example, if a siRNA/shRNA specific to transcript 7 could be designed, it could be expressed using an inducible system in the cell culture or in vivo, if possible) to monitor the effect of its absence (for example, on the expression of other interacting proteins, cell morphology) from the cells at different time points during brain development.

c. Group C transcripts (lacking exon 12): It has been shown that transcripts of group C are expressed more during the later stages of brain development and amongst the various transcripts been expressed in the cerebellum, they are expressed maximally (Pretto et al. 2014). Thus, cellular localization studies and protein interaction studies (if the isoforms function individually or as a complex) could be performed for the members of this group taking into account the different regions of the brain. It could also be checked if changes in their cellular expression affect the expression levels of other proteins in the cells.

d. Group A (consisting of all exons), B (lacking exon 14) and D (lacking exon 12 and 14) transcripts: It has been shown that the expression of mRNA transcripts for the members of these groups is very low in the different regions of the mouse brain (Brackett et al. 2013, Pretto et al. 2014). It has also been shown earlier that exclusion of exon 14 results in the expression of shorter isoforms that localize in the cell nucleus (Sittler et al. 1996). Thus, along with protein localization studies, the effect of overexpression of these proteins on the cellular morphology and their interacting partners could also be checked. For the proteins localizing in the nucleus, their interactions with the nucleic acid molecules could be monitored.

III. Elucidating the structure of full-length FMRP

So far, only the structures of the individual domains of FMRP, namely, RGG and N-terminal domains containing KH1-KH2 or Tudor-KH domains are available (Myrick et al. 2014, Vasilyev et al. 2015, Valverde et al. 2007). A structure of full-length FMRP would be beneficial as it would highlight the arrangement of all the domains existing together in the protein and also, the auto-inhibitory blocking of one domain by the other, if it exists. This would be important in understanding the interactions of different interacting partners with the different domains of FMRP. This would also help in understanding the domain arrangements and thus, their interactions in the shorter isoforms of FMRP.

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