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Methods Article

Title:

A Metabolomic and Systems Biology Perspective on the Brain of the Fragile X Syndrome Mouse Model

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Abstract

Fragile X syndrome (FXS) is the first cause of inherited intellectual disability, due to the silencing of the X-linked Fragile X Mental Retardation 1 gene encoding the RNA-binding protein FMRP. While extensive studies have focused on the cellular and molecular basis of FXS, neither human fragile X patients nor the mouse model of FXS -the *Fmr1*-null mouse- have been profiled systematically at the metabolic and neurochemical level to provide a complementary perspective on the current, yet scattered, knowledge of FXS. Using proton high-resolution magic angle spinning nuclear magnetic resonance (^{1}H HR-MAS NMR)-based metabolic profiling, we have identified a metabolic signature and biomarkers associated with FXS in various brain regions of *Fmr1*-deficient mice. Our study highlights for the first time that *Fmr1* gene inactivation has profound, albeit coordinated consequences in brain metabolism leading to alterations in: *i*) neurotransmitter levels, *ii*) osmoregulation, *iii*) energy metabolism and *iv*) oxidative stress response. To functionally connect *Fmr1*-deficiency to its metabolic biomarkers, we derived a functional interaction network based on the existing knowledge (literature and databases) and show that the FXS metabolic response is initiated by distinct mRNA targets and proteins interacting with FMRP, and then relayed by numerous regulatory proteins. This novel ”integrated metabolome and interactome mapping” (iMIM) approach advantageously unifies novel metabolic findings with previously unrelated knowledge and highlights the contribution of novel cellular pathways to the pathophysiology of FXS. These metabolomic and integrative systems biology strategies will contribute to develop potential drug targets and novel therapeutic interventions, which will eventually benefit FXS patients.

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INTRODUCTION

Fragile X syndrome (FXS) is the most frequent cause of inherited intellectual disability (ID) and the most commonly identified genetic cause of autism (Chelly et al. 2006; Gecz et al. 2009). FXS affects 1/4,000 males and 1/7,000 females world-wide (Hagerman 2008) and is caused by the silencing of the X-linked Fragile X Mental Retardation 1 (*FMR1*) gene positioned in Xq27.3. In FXS patients, a dynamic mutation increasing abnormally the number of CGG repeats in the first exon of the *FMR1* gene leads to their hypermethylation and the subsequent absence of its gene product FMRP (Penagarikano et al. 2007). Although a monogenic disorder, FXS is a disease of complex aetiology accompanied by behavioral (hyperactivity, autism), neurological (susceptibility to epileptic seizures) as well as physical abnormalities (macroorchidism, elongated face, hyperextensible finger joints) (Penagarikano et al. 2007). A mouse model of FXS, knock-out (KO) for the murine homologue of *FMR1,* has been generated, exhibiting learning and behavioral abnormalities that recapitulate the human phenotype (Consortium 1994). The absence of FMRP induces abnormal extra dendritic spines in neurons of FXS patients and *Fmr1*-KO mice that represent the synaptic defect underpinning the ID (Bassell and Warren 2008; Swanger and Bassell 2011). At the molecular level, FMRP is an RNA-binding protein controlling translation as a component of neuronal messenger ribonucleoprotein (mRNPs) particles associated with somatic and synaptic polyribosomes (Davidovic et al. 2006; Khandjian et al. 2004). FMRP is involved in localized synaptic translation, an essential mechanism that controls protein synthesis locally at the synapse to shape dendritic spines (Bassell and Warren 2008). FMRP and its network of mRNA targets and interacting proteins (Bardoni et al. 2006; Khandjian et al. 2005) contribute to several forms of synaptic plasticity involved in learning and memory processes and notably induced by activation of type I metabotropic glutamate receptors (Dölen and Bear 2008). Although perturbations of these neurospecific events involving FMRP represent the molecular basis of the cognitive impairments observed in FXS patients and *Fmr1*-KO mice, the pathophysiology of FXS remains poorly understood. Since FMRP modulates protein synthesis *via* direct regulation of the translation of multiple mRNAs, we hypothesize that *Fmr1*–deficiency could functionally affect protein interaction networks with direct consequences on signaling cascades and cellular metabolism.

To investigate this hypothesis, we performed a comprehensive profiling of the metabolome of the *Fmr1*-deficient brain. In order to connect *Fmr1*-deficiency to its metabolic phenotypes, we developed an integrative systems biology approach to map metabolic markers of *Fmr1*-deficiency directly onto the interactome. Metabonomics is a powerful systems biology approach capturing the variation in low–molecular weight compounds in organs or biofluids in response to pathophysiological interventions or genetic modifications

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(Nicholson et al. 2002). Metabolic profiling methods, such as proton nuclear magnetic resonance spectroscopy (^{1}H NMR) or mass spectrometry, in conjunction with multivariate pattern recognition analyses, are highly effective in understanding disease processes and drug responses in humans and model organisms (Nicholson et al. 2002), particularly in the case of metabolic diseases (Dumas et al. 2006; Dumas et al. 2007). In neuroscience, ^{1}H-NMR metabonomic analysis was used to characterize the neurochemical and metabolic profile of bipolar disorder patients (Lan et al. 2009) and transgenic models of neurological disorders of genetic origin, such as spinal cerebellar ataxia, Huntington's disease or Batten's disease (Holmes et al. 2006; Pears et al. 2005), as well as Rett Syndrome, the major cause of profound ID in girls (Viola et al. 2007). Hypothesis-free metabolic profiling provides genome-scale metabolic modeling strategies particularly suitable to analyze genotype-phenotype correlations following gene inactivation in animal models of human diseases, in metabolic Quantitative Trait Locus (mQTL) mapping (Dumas et al. 2007) studies or in metabolic genome-wide association studies (Illig et al. 2010). Mapping metabolic biomarkers onto biological networks enhances the understanding of complex metabolic signatures at the pathway level, as typically performed by metabolite-set enrichment analysis (MSEA) (Pontoizeau et al. 2011; Xia and Wishart 2010).

Here we developed a novel alternative strategy, which is based on mapping metabolic phenotypes directly onto protein-protein interaction (ppi) networks and analyzing the topology of the resulting network to identify key proteins involved in generating the metabolic phenotype associated in *Fmr1*-deficiency. This novel methodology is particularly suited for studying FXS since *Fmr1*-deficiency directly affects protein networks and also considering that ppi networks are used to unravel the molecular aetiology of complex diseases (Navratil et al. 2011; Navratil et al. 2010).

In this study, we present the first comprehensive metabolomic profiling of the *Fmr1*-deficient brain in the mouse, using ^{1}H high-resolution magic-angle spinning (HR-MAS) NMR-based metabonomics, which is particularly suited for small tissue samples analysis. We then define a robust metabolic phenotype (metabotype) of FXS in cerebellum, cortex, striatum and hippocampus of its mouse model. To understand how *Fmr1*-deficiency mechanistically generates its associated metabolic signature, we present a novel integrative strategy, which we coined integrated Metabolome and Interactome Mapping (iMIM), connecting FMRP to its metabolic endpoints *via* protein-protein interaction (ppi) networks. Concomitantly, we have used network metrics to identify pivotal proteins or FMRP mRNA targets of the network generating the metabotype associated with *Fmr1*-deficiency, and further confirmed their biological relevance *in vivo*. This novel integrative systems biology strategy highlights the role of key regulatory pathways in FXS pathogenesis,

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explaining the complexity of the FXS metabotype. In general, iMIM anchors the regulation of complex metabolic phenotypes in protein interaction networks and signaling cascades.

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RESULTS

Definition of the optimal spatio-temporal coordinates for metabotyping of the Fmr1-deficient brain.

We first assessed the patterns of expression of *Fmr1*, the defective gene in FXS, by Western-blot and immunohistochemistry to define the optimal spatiotemporal coordinates for metabotyping the developing brain of *Fmr1*-deficient mice (*Fig.1*). Western-blot analysis of total brain extracts at different postnatal ages reveals that the *Fmr1* gene-product FMRP is abundantly expressed during the first two post-natal weeks of life (P0 to P14), peaking at 5-12 days of age. Then, FMRP expression strongly decreases as the animal ages to reach lower levels during adulthood (*Fig.1A*). These data are consistent with a study reporting a peak of expression of FMRP in the hippocampus and cerebellum during the second postnatal week of life (Lu et al. 2004). To refine the brain regions that express the highest levels of FMRP, we performed immunolabelling on brain sections from 12-day old wild-type (WT) mice. A strong immunoreactivity was detected in the whole brain (*Fig.1B*), particularly in four brain regions: cerebellum, cortex, hippocampus and striatum (*Fig.1C*), which manifest dysfunction in FXS patients and *Fmr1*-KO mice (Penagarikano et al. 2007). We therefore studied the metabolic consequences of *Fmr1*-deficiency by ^{1}H HR-MAS NMR profiling at this defined post-natal age when FMRP is strongly expressed (*Fig.1).* The choice of this specific postnatal age appears also particularly relevant since morphological and physiological defects in the brain of *Fmr1*-KO mice may appear transiently and are often detected in the second postnatal week of life (Bureau et al. 2008; Cruz-Martin et al. 2010; Larson et al. 2005; Nimchinsky et al. 2001), corresponding to the critical time window of active synaptogenesis in the brain. In addition, we restricted our analysis to males to avoid sex-bias in the assessment of FXS metabolic phenotype.

Identification of metabolic signature of Fmr1-deficiency in mouse brain regions. To derive a metabolic signature associated with FXS, we compared metabolic profiles from intact brain tissues originating from 12-day old wild type and *Fmr1*-null male mice (n=10 for each genotype). Compared to classical ^{1}H NMR spectroscopy, generally used to profile liquid tissue extracts, proton high-resolution magic-angle spinning (^{1}H HR-MAS) NMR spectroscopy was developed to directly profile intact tissues whilst preserving cellular integrity – this is particularly suited for characterization of mass-limited samples such as the mouse hippocampus. Supervised multivariate statistical modeling using orthogonal partial least-squares discriminant analysis (OPLS-DA) enables a significant discrimination between *Fmr1*-deficient and control brain regions (*Fig. 2*). Remarkably, the OPLS-DA three-dimensional (3D) score plot (*Fig. 2A*) shows that WT brain

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samples segregate at the periphery of the model, reflecting the anatomical and functional differences between these brain regions also described in the adult rat brain (Tsang et al. 2005). Conversely, *Fmr1*-null mouse brain regions all tend to project towards a common central area in the model. In fact, WT brain regions have a distinct metabolic phenotype whereas *Fmr1*-null brain regions are more alike: this is suggestive of a relatively undifferentiated metabolic state in *Fmr1*-null brains. Interestingly, cortex and cerebellum display remarkably parallel responses to *Fmr1*-deficiency, suggestive of similar metabolic signatures, whereas striatum and hippocampus display unique metabolic signatures (*Fig. 3*, *Table 1*). We then compared the respective effects of *Fmr1*-deficiency (KO vs. WT) and of region (*ctx* vs. *crb*) on metabolic profiles in an OPLS-based variance components (VC) model (*Fig. 2B*), showing that the metabolic variation originating from anatomic region (explaining up to 89% of metabolic variation for *myo*-inositol, as measured by the signal at δ3.62) is larger than the genetic variation (up to 36% of ethanolamine variation, δ3.83).

The metabolic signature associated with each brain region is derived from model coefficients obtained from a series of pair-wise OPLS-DA models segregating KO from WT for each brain region (*Fig.3*), and shows the existence of brain region-specific metabotypes associated with *Fmr1*-deficiency (*Table 1*). We report here for the first time that, although FMRP is neither an enzyme nor a receptor directly involved in neurotransmitter metabolism or signaling, *Fmr1*-deficiency has dramatic impacts on the levels of six neurotransmitters (gamma-amino butyric acid (GABA), glutamate, acetylcholine (Ach), taurine, alanine, aspartate) and several of their precursors or catabolites (glutamine, acetate, choline, *N*-acetyl aspartate (NAA, *Table 1*). The *Fmr1*-deficient metabotypes also involve the osmolyte and the secondary messengers precursor *myo*-inositol, intermediary metabolites and lipids totally unrelated to neurotransmission (acetoacetate, lactate and carbonyls from fatty acids). At the network level, 25 metabolites undergo significant alterations in the *Fmr1*-null brain (*Table 1*) and may be considered as global FXS biomarkers.

Metabolite-Set Enrichment Analysis (MSEA) of Fmr1-deficiency metabotypes. A Metabolite-Set Enrichment Analysis (MSEA) (Xia and Wishart 2010), an extension of the Gene-Set Enrichment Analysis (GSEA) (Subramanian et al. 2005), was then used to test for metabolic pathways enrichment in each brain region. In the cortex, *Fmr1*-deficiency is significantly associated with: an increase in lipid-oxidized species (mainly carbonyls, such as CH_3-$C\underline{H_2}$-CH_2-CO- and -$C\underline{H_2}$-CO-) and acetoacetate, and a decrease in GABA and glutamate, their precursor glutamine, as well as in *N*-acetyl-aspartate (NAA) and lactate (*Table 1*). The decreased cortical levels of glutamine corroborate a previous study using reversed-phase high performance liquid chromatography (HPLC, (Gruss and Braun 2001)) on *Fmr1*-KO and WT cortex, and we confirmed

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using an enzymatic assay that the cortical levels of glutamate were significantly decreased in *Fmr1*-KO animals (p=0.011, *Fig. 4A*). In addition, the increase in carbonyl groups that we observe in the cortex was previously documented using a biochemical approach (El Bekay et al. 2007). These data strongly support the robustness of ^{1}H HR-MAS NMR to highlight metabolic changes in *Fmr1*-deficient brain. Subsequent MSEA analysis (Xia and Wishart 2010) highlights that significant cortical alterations in glutamine/glutamate metabolism, in synthesis and degradation of ketone bodies, as well as in glutathione metabolism derive from this metabotype (*SI Table 1*). In the cerebellum, decreases in glutamine, GABA, NAA, *myo*-inositol (whereas its stereoisomer *scyllo*-inositol is increased), aspartate and acetate associated with increases in both Ach and its precusor choline (*Table 1*) correlate with significant alteration in glycerophospholipid and β-alanine metabolism (*SI Table 1*). Interestingly, the reduced glutamine levels in *Fmr1*-deficient cerebellum were also detected by others (Gruss and Braun 2001) and we have been able to confirm by ELISA a significant decrease in GABA cerebellar concentrations in *Fmr1*-KO animals (p=0.038, *Fig. 4B*). The hippocampus displays increases in taurine, creatine and *myo*-inositol that evoke alterations in taurine/hypotaurine metabolism and inositol phosphate metabolism (*Table 1* and *SI Table 1*). Of particular notice, increased taurine levels were also reported in hippocampus of *Fmr1*-KO animals by a previous study (Gruss and Braun 2001). Finally, in the striatum, a decrease in kynurenine (*Table 1*) drives significant alterations of tryptophan metabolism (*SI Table 1*) coupled to decreases in alanine, creatine and adenine.

Integrated Metabolome and Interactome mapping of FMR1-deficiency metabotypes. To bring a mechanistic understanding in our genotype-phenotype associations and functionally connect the causal mutation in the *FMR1* gene with the downstream metabolic biomarkers derived from the OPLS-DA models presented above, we developed a novel network biology strategy, which we coined integrated Metabolome and Interactome Mapping (iMIM). Databases and literature datasets were gathered to reconstruct a molecular interaction network (see *SI Table 2* for an exhaustive list of sources and *Methods* for detailed construction of the interactome) encapsulating (*i*) known and putative mRNAs regulated by FMRP resulting from either large-scale screenings or individual characterization of direct RNA-protein interactions, (*ii*) the known FMRP protein-protein interactome, including kinases (*iii*) metabolic (KEGG database (Kanehisa et al. 2008)) and neuronal signaling pathways (NeuronDB, Yale University). Since our iMIM approach uses databases with human annotations, we chose to standardize and use the official human gene and protein symbols in the related paragraphs, eventhough proteins or mRNA interactors might have been identified in a variety of organisms. The topology of the resulting integrated Metabolome Interactome Mapping (iMIM)

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network was then analyzed to visualize functional paths between FMRP and its associated metabotypes, and to model how inactivation of *FMR1* propagates through the cellular network until it reaches its metabolic endpoints (*Fig. 5A*). Considering a given metabolite M and using the Ockham's razor principle, the shortest path between FMRP and M through the interactome network is likely to provide a mechanistic link between *FMR1*-deficiency and its consequence on metabolite M. Since FMRP is neither an enzyme nor a receptor, the shortest path between FMRP and M becomes: FMRP → interacting protein or mRNA-target → enzyme (or receptor) → metabolite M, where the shortest path length (spl) is 3, The spl becomes 2 in the case of a receptor or enzyme being an interacting protein or mRNA target. The average spl computed from the entire knowledge network between FMRP and the 25 metabolites identified as FXS markers by NMR is 3.38, which approaches 3, meaning that FMRP is connected to a perturbed metabolite via one of its mRNA target or protein partner which in turn will interact with a receptor or enzyme. This spl is significantly lower than the average spl obtained from randomly re-sampled lists of 25 biomarkers with 100,000 H_0 iterations (*Fig. 5B*, spl=3.38, p=0.03427), which confirms that *FMR1*-deficiency is selectively connected to the perturbed metabolites identified above.

Centrality-based stratification of proteins in the iMIM network using pivotal betweenness. Based on the iMIM network and the set of all shortest paths, we then assessed the centrality of a given protein or mRNA-target between *FMR1*-deficiency and its metabolic endpoints by estimating their pivotal betweenness (PB). PB is a measure of centrality, meaning that graph edges used by several shortest paths will have a high PB, (see *Methods*, *Table 2* and *SI Table 3*). This continuous variable is directly correlated to the weight of the proteins in the shortest paths of the network and enables a systematic stratification and ranking of these proteins based on their relative importance at the network scale. The iMIM network derived from the metabolic signature of FXS highlights that among the 30 most pivotal proteins, 13 are either FMRP protein interactors -such as the protein FXR2P (PB=8.23×10^{-6} (Zhang et al. 1995)), the homologue of FMRP-, or are encoded by mRNA targets of FMRP (*Fig. 5*), such as the mRNA encoding Super Oxide Dismutase 1 (SOD1, PB=1.35×10^{-5} (Bechara et al. 2009)), Amyloid beta Precursor Protein (APP, PB=1.56×10^{-5} (Westmark and Malter 2007)), Calcium-Calmodulin Kinase 2 Alpha (CAMK2A, PB=7.93×10^{-6} (Iacoangeli et al. 2008)), Elongation Factor 1 Alpha (EEF1A, PB=6.06×10^{-6} (Sung et al. 2003)) and Microtubule Associated Protein 1B (MAP1B, PB=3.93×10^{-6} (Brown et al. 2001; Chen et al. 2003; Iacoangeli et al. 2008; Lu et al. 2004)). Interestingly, poorly characterized putative mRNA FMRP targets such as the mRNA encoding the small GTP-ase and cytoskeleton-regulator RHOA (PB=1.83×10^{-5}; (Chen et al. 2003)) and the calcium regulatory

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protein Calbindin1 (CALB1, PB=1.35x10^{-5}; (Miyashiro et al. 2003)) appear as pivotal in our network (*Table 2*, *Fig. 5*): RHOA connects FMRP to 4 of the perturbed metabolites, while CALB1 relays FMRP to metabolic perturbations of osmolyte and the secondary messenger *myo*-inositol (*Fig. 5*).

Biological confirmation of the in vivo association of FMRP with mRNA encoding pivotal proteins of the iMIM network. The pivotal FMRP mRNA targets highlighted *in silico* by the iMIM network were initially identified in large-scale screenings for FMRP mRNA targets and some still lack extensive biological validation. We therefore sought to further confirm that FMRP physically interacts with iMIM-derived pivotal mRNA targets *in vivo* (Fig. 5A), using UV-crosslinking and immunoprecipitation assays (CLIP, (Ule et al. 2005)). Compared to classical immunoprecipitation, the CLIP approach uses UV-cross-linking irradiation of tissues to generate covalent bonds between nucleic acids and proteins, thereby preserving the labile interaction between mRNA and proteins that are often lost in classical immunoprecipitation. Immunoprecipitation of FMRP mRNA complexes was carried out using the R60 polyclonal antibody raised against the C-terminus of FMRP on *Fmr1*-WT and KO total P12 brain extracts (See *SI* and *SI Fig. 1* for exhaustive characterization of the R60 antibody). As expected, FMRP was not detected in input or immunoprecipitate from *Fmr1*-KO animals, whereas it was recovered in WT input and immunoprecipitate (*Fig. 6A*). In addition, two known interactors of FMRP, its homologues FXR1P and FXR2P (Zhang et al. 1995) present in equal levels in the *Fmr1*-KO and WT input, are only recovered in the WT immunoprecipitate (*Fig. 6A*), confirming the specificity of the immunoprecipitation. RT-PCR analysis of mRNAs extracted from *Fmr1*-WT and KO input and immunoprecipitates was then carried out (*Fig. 6B*). The equal intensity of the bands corresponding to the studied mRNAs reveal that *Mtap1b, Tubb3, Sod1, Rhoa* and *Calb1 mRNAs* are expressed at similar levels in the input fraction from *Fmr1*-KO and WT brain (*Fig. 6B*). *Fmr1* mRNA was detected in WT input and immunoprecipitate (lane 1 and 4, *Fig. 6B*), together with the known mRNA target of FMRP Microtubule Associated Protein 1b *Mtap1b* (lane 4, *Fig. 6A*) corroborating previous studies (Iacoangeli et al. 2008; Lu et al. 2004). Conversely, no amplicon corresponding to the unrelated mRNA encoding beta-Tubulin 3 (*Tubb3*) was detected in either immunoprecipitate. Similar results were obtained with another negative control the mRNA encoding Phosphoglycerate kinase 1 (*Pgk1*, not shown), thereby confirming the specificity of the approach. Interestingly, the mRNA *Sod1* was detected in the WT immunoprecipitate (lane 4, *Fig. 6B*), showing for the first time its *in vivo* association with FMRP in brain. Also, the mRNA *Rhoa* and *Calb1* encoding pivotal proteins of the network were also specifically recovered in the *Fmr1*-WT immunoprecipitates (lane 4, *Fig. 6B*). Altogether, these data confirm the association of FMRP with *Rhoa* and

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Calb1 mRNAs suggested by high-throughput screening and strongly support the fact that these mRNAs are *in vivo* targets of FMRP.

Identification of perturbed pathways in Fmr1-deficient brain. Further Gene Set Enrichment Analyses (GSEA) (Subramanian et al. 2005) were carried out on the iMIM network's pivotal proteins to identify significantly perturbed pathways in *Fmr1*-null mouse brain. GSEA was performed by comparing Gene Ontology (Rivals et al. 2007) (*SI Table 4*) and KEGG (Kanehisa et al. 2008) (*SI Table 5*) annotations of the pivotal proteins (n=257) to those of the entire set of proteins present in the iMIM network. Typical enriched terms from GSEA show that the metabolic signature of the FXS mouse model translates into perturbations of "central nervous system development", "signaling", "function and metabolism" among which alterations of "glutamate signaling pathways", "protein kinase cascade", "actin cytoskeleton organization and biogenesis", "synaptic transmission" coupled to "behavior and learning and memory defects".

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DISCUSSION

Although FXS is a monogenic disorder, *Fmr1*-deficiency genotype/phenotype associations are difficult to assess from a mechanistic point of view, since FMRP is involved in the translation of numerous mRNAs and affects the levels of proteins involved in a wide-range of cellular processes (Darnell et al. 2005). This conceptual difficulty required the development of a novel integrative systems biology method to mechanistically understand the metabolic consequences of *Fmr1*-deficiency. In this study, we profiled the metabolome of the brain of the mouse model of FXS using ^{1}H HR-MAS NMR spectroscopy, to gain knowledge on FXS metabolism. We then developed the integrated metabolome and interactome mapping framework and its associated network metrics, the pivotal betweenness. This strategy allowed us to get new insight into FXS pathophysiology and to identify key FMRP mRNA targets involved in regulating its metabolic phenotype, as discussed in detail below.

I- Comprehensive metabolic profiling of the brain of the mouse model of FXS: identification of Fmr1-deficiency metabolic phenotypes specific to each brain region

Using ^{1}H HR-MAS NMR spectroscopy-based metabolic profiling, we show that the absence of FMRP affects the metabolic phenotype of the developing brain in a region-specific manner, with cortex and cerebellum being the most affected regions. In the wildtype, each brain region has a unique metabolic profile, which translates into a clustering of the samples according to brain region in the OPLS-DA 3D scores plot (*Fig. 2A*). This is suggestive of region-specific functions and metabolisms even at this early postnatal age, and corroborates a previous metabolomic study on adult rat brain (Tsang et al. 2005). This clustering becomes considerably less evident in the *Fmr1*-deficient brain, where samples are projected towards the centre of the model in a more homogenous manner. The incomplete metabolic differentiation observed in the *Fmr1*-deficient brain might be related to the general delayed maturation of the *Fmr1-KO* brain suggested by the immature-looking dendritic spines in Fragile X mice and human patients (Bureau et al. 2008; Cruz-Martin et al. 2010; Larson et al. 2005; Nimchinsky et al. 2001), but it is also directly linked to the functional consequences of the absence of FMRP and the interactions with its mRNA targets and protein partners. However, the events leading to this spine dysgenesis in FXS appear to be a complex mix of abnormal nervous system early development (Callan and Zarnescu 2011), delayed maturation (Bureau et al., 2008; Cruz-Martin et al., 2010) and altered stabilization and pruning (Swanger and Bassell 2011), FMRP being involved in each of these processes via regulation of its mRNA targets and protein partners. This illustrates that the observed delayed maturation of the *Fmr1*-deficient brain and the lack of *Fmr1* function cannot be

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completely disentangled. This prompted us to use novel integrative systems biology approaches to explore the functional consequences of *Fmr1*-deficiency on metabolism.

II- Identification of metabolic pathways perturbed by Fmr1-deficiency

To investigate how *Fmr1*-deficiency results in the metabotypes that we detected in the brain of the mouse model of FXS, we first applied a recent integrative systems biology strategy, testing which metabolic pathways are significantly affected: metabolite-set enrichment analysis (MSEA, (Pontoizeau et al. 2011; Xia and Wishart 2010)) (*SI Table 1*). This analysis contributed to the identification of the principal metabolic pathways perturbed in the brain of the mouse model of FXS.

Fmr1-deficiency drives alterations in neurotransmitters levels. Our results show that FXS brain undergoes global alterations in neurotransmitter metabolism, mainly glutamate, GABA, acetylcholine and taurine. In the *Fmr1*-deficient cortex, the reduction of glutamine and glutamate metabolism we observe might result in a reduction of the amounts of released glutamate with important consequences on the functioning of cortical circuitry and cortical excitability, as previously reported (Gibson et al. 2008). In *Fmr1*-deficient mice, the reduced mRNA levels of *Gad1*, encoding the glutamate-decarboxylase Gad65 that catalyzes the conversion of glutamate into GABA (D'Hulst et al. 2009) (*Fig. 7*) may explain the decreased cortical and cerebellar GABA levels. Given the essential role of GABA for the development of cortex and cerebellum, lower GABA levels might perturb optimal development of these structures, especially at P11-P12, a stage which encompass the time window when GABA shifts from excitatory towards inhibitory effects, as reported in rat (Ben-Ari et al. 2007). On the other hand, our data suggest that the *Fmr1*-deficient hippocampus undergoes increased neuronal inhibition mediated by taurine during early postnatal development (El Idrissi and Trenkner 2004). Finally, a decrease in acetylcholine and its precursor choline suggests a disruption of the cholinergic system in the *Fmr1*-deficient cerebellum (*Fig. 7*), which plays an essential developmental role in the cerebellum (De Filippi et al. 2005). In conclusion, the delicate balance between excitatory and inhibitory inputs appears compromised in the mouse model of FXS at this early postnatal stage corresponding to the time-window of synaptogenesis, with direct consequences on neuronal circuits formation development (Akerman and Cline 2007).

Altered osmolyte and secondary messengers balance. In the *Fmr1*-deficient brain, we observed variations in osmolytes such NAA, *myo*-inositol and taurine. Osmolytes control the ion gradient across membranes and modifications of their levels may facilitate neuronal discharge and epileptiform activity (Liu et al. 2008), as described in the *Fmr1*-deficient mouse (Qiu et al. 2008). In addition, disruption of osmolyte

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balance is widely observed in various neurological conditions, and constitutes a hallmark of brain dysfunctions (Lan et al. 2009; Pears et al. 2005; Viola et al. 2007). Alternatively, decreased *myo*-inositol levels in the hippocampus and increased levels in the cerebellum of *Fmr1*-deficient mice suggest dysregulations of phosphoinositide metabolism since *myo*-inositol is a precursor for these important secondary messengers. Our findings support the idea that *Fmr1*-deficiency might modify neuronal physiology in an unspecific manner, through osmoregulation and secondary messenger signalling machinery.

Disruption of postnatal energy metabolism and neuron/glia metabolic cooperation in the FXS brain. Astrocytes and neurons cooperate metabolically for neuronal energy fueling and biosynthesis of neurotransmitters (*Fig. 7*). In the *Fmr1*-deficient brain, the decreased levels of lactate in cerebellum and cortex and the increase in the ketone-body acetoacetate in cerebellum might reflect a disruption of neuronal metabolic fueling during postnatal brain development, since these metabolites are the essential source of neuronal energy, thus replacing glucose at this specific period to replenish acetyl-coA stores to fuel the tricarboxylic acids (TCA) cycle (Pellerin 2008) (*Fig. 7*). Glutamate and GABA can also enter the TCA cycle *via* conversion upstream or downstream *via* the intermediate metabolite alpha-keto-glutarate (αKG) (*Fig. 7*). Altered glutamine, glutamate and GABA levels also support a disruption of the interplay between glia and neurons controlling the glutamine–glutamate–GABA cycle (McKenna 2007) (*Fig. 7*). Our results highlight the readjustment of TCA cycle and energy metabolism in neurons and glial cells in the absence of FMRP.

Adaptative response to increased oxidative stress. We have identified a marked increase in cortical oxidative stress markers, such as lipid peroxidation products, notably aldehydes and carbonyls species. These species result from free radical attack of unsaturated lipids by superoxide anion, normally detoxified by SOD1. We previously observed this specific metabotype in *sod1*-deficient *C. elegans* (Blaise et al. 2007). Interestingly, we have shown that FMRP binds *in vitro Sod1* mRNA and that its absence decreases the cortical levels of SOD1 (Bechara et al. 2009), a pivotal protein in our network (*Table 2*, *Fig. 5, Fig. 6*). Here, we have shown the association of FMRP with *Sod1* mRNA *in vivo* (*Fig.6B*). These data directly correlate depletion of SOD1 to biochemical and enzymatic markers of enhanced oxidative stress in the brains of *Fmr1*-deficient mice (increase in carbonyl groups in the cortex (El Bekay et al. 2007, and this study). These data indicate that increased oxidative stress is a phenotypic trait of FXS.

III- Development of the iMIM framework and associated network metrics to identify key FMRP targets involved in regulating its metabolic phenotype.

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iMIM and pivotal betweeness. FMRP is a translational regulator and has no direct link with metabolism, apart from SOD1. However it interferes with protein-protein interaction networks *via* regulation of the levels of the proteins encoded by its mRNA targets. To investigate the *Fmr1*-deficiency metabolic phenotype/genotype association, we developed a new approach, which we coined integrated Metabolome and Interactome-Mapping (iMIM). In iMIM, the metabolic markers associated with *Fmr1*-deficiency were mapped onto metabolic pathways and protein-protein interaction networks (*Fig. 5* and *Table 2*). Close analysis by GSEA of the most significantly enriched biological functions in the resulting network show that the metabolic perturbations in the brain of *Fmr1*-deficient mice are associated with various biological processes, typically related to CNS and neuron development, cognitive functions, signal transduction and neurotransmitter metabolism (*SI Table 4-5)*. To identify the key proteins related to these processes that may link *Fmr1*-deficiency consequences to metabolism, we analyzed the network topology by estimating the pivotal betweeness of each protein of the network. We defined this network metric to enable a systematic stratification and ranking of the proteins based on their relative importance at the network scale (*Fig. 5*, *Table 2, SI Table 3, SI Table 4)*. Among the 30 pivotal proteins of the network, 13 are encoded by putative or confirmed mRNA targets of FMRP. To confirm the biological relevance of the identified key regulatory proteins, we validated the *in vivo* the association of FMRP in brain with their corresponding mRNA, notably *Sod1, Rhoa* and *Calb1* (*Fig. 6B*), thereby ascertaining the robustness of our approach.

Identification of key FMRP mRNA targets and proteins and pathways involved in FXS. The two principal branches of the network involve two proteins encoded by the confirmed mRNA target of FMRP: *APP* (Westmark and Malter 2007) and an mRNA target we confirmed *in vivo* : *Rhoa* ((Chen et al. 2003), *Fig. 6B*), which respectively regulate 6 and 4 metabolites. APP plays a central role in synaptic functions and Alzheimer's disease (Kamenetz et al. 2003) and genetic dysregulations of the cytoskeleton-regulators *RHOA* family are linked to ID (Benarroch 2007). These data suggest that alterations of the levels of RHOA and APP proteins in the absence of FMRP may have direct neuronal consequences. Interestingly, APP and RHOA interact with two important enzymes in our iMIM network (*Fig. 5* an *Table 2*) involved in acetylcholine metabolism: respectively acetylcholine esterase (ACHE, *Fig. 7* (Cottingham et al. 2002)), the enzyme degrading Ach into acetate and choline, and phospholipase D1 (PLD1 (Klein 2005)) which provides free choline for the synthesis of Ach in the brain. Our iMIM analysis also reveals that another key enzyme involved in acetylcholine metabolism (choline acetyl transferase, CHAT) is pivotal (*Fig. 3, Fig. 7, Table 2*). These data support the involvement of the cholinergic system in FXS, suggested by the fact that molecules

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targeting the cholinergic pathway rescue some behavioral and molecular phenotypes in the FXS Drosophila model (Chang et al. 2008).

In addition, other pathways involving proteins encoded by poorly characterized FMRP mRNA targets are also highlighted by the network approach. First, the mRNA encoding the neuronal calcium-binding protein Calbindin1 was shown to bind FMRP by filter binding assay (Miyashiro et al. 2003) and we provide evidence that the interaction occurs *in vivo* in brain (*Fig. 6*). Interestingly, Calbindin1 directly targets *myo*-inositol monophosphatase (IMPA1) in spines and dendrites of cerebellar Purkinje neurons (Schmidt et al. 2005). Deregulations of CALB1 levels might therefore directly drive the cerebellar decrease in *myo*-inositol. Intriguingly, the *Calb1-KO* cerebellum displays the similar Purkinje cell spine dysgenesis phenotype as the *Fmr1*-KO mouse (Vecellio et al. 2000), reinforcing the potential involvement of CALB1 in FXS. Also, deficits in the neurotransmitters GABA and glutamate are connected to FMRP by pivotal mRNA targets (*Fig. 5, Table 2, SI Table 3*) encoding respectively the $GABA_B$ receptor subunits GABRD and GABRP (Miyashiro et al. 2003) and the NMDA-glutamate receptor GRIN1 and GRIN2B (Schütt et al. 2009). It is interesting to underline that mutations in both *GRIN1* and *GRIN2B* are involved in forms of syndromic or non syndromic ID (Gecz 2010). Finally, the iMIM network highlights pivotal roles for the mRNA encoding the G-protein regulator GNAI2 (Miyashiro et al. 2003) which is involved in neuronal development (Shinohara et al. 2004), and the mRNA encoding the transcription factor Forkhead box class O 3 (FOXO3) which influences behavioural processes linked to anxiety and depression (Polter et al. 2009). Our iMIM strategy usefully brings to light signaling pathways that are likely to be directly involved into the pathophysiology of FXS.

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Conclusions. Our study defines for the first time a robust metabolic phenotype (metabotype) associated to *Fmr1*-deficiency, involving several neurotransmitters and the readjustment of brain biochemical pathways, suggesting that FXS may not only be a mental disease restricted to alterations in local neuronal functions, but also a metabolic disease. Since FMRP affects mRNA translation and protein-protein interaction networks, we developed a network biology method which we coined integrated Metabolome and Interactome Mapping (iMIM), connecting the causative mutation in *FMR1* to its metabotype, *via* the interactome. Thus we demonstrated that FMRP impacts a variety of transduction cascades, metabolic and signaling pathways, which could be shared with other diseases (e.g. Alzheimer, other IDs). The key regulatory proteins identified by iMIM were functionally validated *in vivo* by UV-CLIP and constitute ideal candidate drug targets for further therapeutic development (Yildirim et al. 2007). Finally, interactome-mapping of metabolic phenotypes is easily amenable to the study of other monogenic disorders or polygenic disorders and should help understanding metabolomic signatures in various mechanistic and signaling contexts.

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METHODS

Animal handling and samples collection. *Fmr1*-knockout (KO) mice of the FBV strain and their wild-type littermates were used in this study. Genotypes were determined by PCR analysis of DNA extracted from tails, according to the original paper describing these animals (Consortium 1994). The day of birth was considered as postnatal day 0 (P0). For western-blot and immunohistochemistry, wild-type FVB mice were sacrified post-natally at various timepoints, the brain was quickly removed from the skull and stored at -80°C for further analysis. For ^{1}H-MAS-NMR analysis, the study was restricted to males aged 11-12 days. Animals were killed by cervical dislocation. The brain was then quickly removed from the skull and dissected, always in the same sequence (first cerebellum followed by cortex, hippocampus and striatum). These operations were typically processed within 5-10 min to limit postmortem changes in the metabolites content of the samples. Right and left samples of each anatomical region were then immediately snapped-frozen in liquid nitrogen and stored at -80°C until further analysis. A total of n=10 knock-out and n=10 wild-type animals were analyzed in this study.

Western-blot and immunohistochemistry. Brain samples were thawed on ice, weighted and extracted in 2 mL / 100 mg of cold extraction buffer (20 mM Tris pH 7.4, 2.5 mM $MgCl_2$, 150 mM NaCl, 0.5% NP40) supplemented with antiprotease cocktail (Roche). Lysates were sonicated on ice twice for 20 s and centrifuged at 10,000 g for 10 min. at 4°C. Supernatant was collected and protein content was determined by spectrometry at 280 nm using Bradford reagent (Biorad). 25 µg of proteins were loaded on a 11% SDS-PAGE and western-blotting was performed as described (Davidovic et al. 2006) using the mAb1C3 anti-FMRP monoclonal antibody (Devys et al. 1993). For immunohistochemistry, a 12-day old wild-type mouse was deeply anesthetized with isoflurane and transcardially perfused with 4% paraformaldehyde in PBS. The brain was removed, postfixed overnight, transferred to cryoprotective solution (HistoPrep, Fisher Scientific), frozen and serially cut into longitudinal sections (15 µm) with a Leica CM1900 cryostat. Immunohistochemistry was performed with the anti-FMRP mAb1C3 antibody revealed by DAB staining using the Vectastain Elite ABC kit, according to the manufacturer's protocol, as described (Devys et al. 1993). Sections were finally counterstained with cresyl-violet and observed with a Leica DMD108 digital microscope.

^{1}H HR-MAS-NMR spectroscopy. All NMR experiments were carried out on a Bruker Avance spectrometer (Bruker GmbH, Rheinstetten, Germany) operating at 700MHz. About 10 mg of brain tissue (1 mm^3) was filled

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in a HRMAS-NMR rotor and spun at 4 kHz for one-dimensional analysis with a high-resolution Magic Angle Spinning (MAS) probe operating at 4°C. To record low-noise high-resolution spectral data, samples were spun for 25 min acquisition to accumulate 1,024 transient scans. 1D and 2D NMR structural assignment was performed using data from literature, HMDB (http://www.hmdb.ca/) and S-Base (Bruker GmbH, Rheinstetten, Germany).

Data Import and Pattern Recognition. ^{1}H HRMAS-NMR spectra were referenced to the centre of the alanine doublet at δ1.48, phased and baseline-corrected using the Topspin 1.3 interface (Bruker GmbH, Rheinstetten, Germany). They were reduced over the chemical shift range of -0.49 to 9.59ppm with exclusion areas around residual water signal (4.61 to 4.99ppm) and its magic angle spinning side band (-0.40 to –0.19ppm), using AMIX (Bruker GmbH, Rheinstetten, Germany) to 10,000 10^{-3} ppm wide regions (buckets), and the signal intensity in each region was integrated. The corresponding bucket tables were then exported to Simca-P 12 (Umetrics, Umeå, Sweden), Matlab and R for statistical analyses, after row profile (constant sum) normalization.

Multivariate Statistics. Principal component analyses (PCA) were run to check the homogeneity of NMR spectra and exclude outliers. Orthogonal partial least square discriminant analyses (OPLS-DA) were run to discriminate the experimental groups of mouse brains by adding a supplementary data matrix **Y**, containing classification information about factors such as genetics and brain region.

Metabolite Set Enrichment Analysis (MSEA). To identify the most significantly affected metabolic pathways, the metabolites affected by *Fmr1*-deficiency were analyzed by Metabolites-Set Enrichment Analysis (MSEA,(Pontoizeau et al. 2011; Xia and Wishart 2010), defined as an extension of Gene-Set Enrichment Analysis (GSEA) (Subramanian et al. 2005) approach to test for metabolic pathways enrichment. For each brain region and each metabolic pathway, a 2x2 contingency table was built by counting the corresponding number of metabolites. An exact Fisher test was then applied to statistically assess the over-representation of discriminant metabolites according to the whole metabolome, *i.e.* the whole set of metabolites assumed to exist in the brain, according to Rivals et al. (2007). To control the false discovery rate associated to multiple testing, the exact Fisher test p-value was finally adjusted using the Benjamini and Hochberg procedure. Only the adjusted p-value is presented in the tables.

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Integrated Metabolome and Interactome Mapping of FMR1-deficiency metabotypes. To functionally connect the *FMR1* genedeficiency in FXS to the candidate biomarker metabolites identified by OPLS-DA analysis, a bottom-up approach combining scientific knowledge extracted from public databases and literature was devised to build the integrated Metabolome and Interactome Mapping (iMIM) network related to *FMR1-KO*. For standardization purpose, human official genes and proteins symbols were used throughout iMIM approach. One part of the network is composed of the *FMR1* gene, the biomarkers ligand receptors (glutamate, GABA, alanine, taurine, acetylcholine, aspartate), but also the target enzymes known to be involved in the metabolism of the biomarkers. A list of 283 receptors and target enzymes linked to the 25 metabolites was extracted respectively from the KEGG database (Kanehisa et al. 2008) and NeuroDB, a database of receptor-ligand interactions developed by Yale University (http://senselab.med.yale.edu/NeuronDB/). To connect *FMR1* dysfunction to the 25 distinct biomarker metabolites, the network integrates metabolite-enzyme interactions (mei) based on the comprehensive metabolic network defined in KEGG but also to metabolite-receptor interactions (mri) available in the comprehensive receptor-ligand database NeuroDB. *FMR1* and this set of receptors and target enzymes were next functionally connected throughout protein-protein interaction (ppi) inferred from a comprehensive mammalian interactome comprising 8,788 proteins and 70,897 interactions (Navratil et al. 2009). According to the functional importance of FMRP in mRNA translation regulation mediated by direct binding of FMRP to its target mRNA, the *FMR1* network was also completed with protein-RNA interactions (pri). A database of 241 interactions including non-redundant pri (n=214) and ppi (n=27) between FMRP and its target mRNA or its binding partners was extensively curated from the litterature (see *SI Table 1* for complete list of references), including FMR1, 42 target enzymes and 3 receptors, 117 target mRNAs of FMRP (including one target enzyme SOD1 and 7 receptors, including GABRP, GABRD, GRIK5) and 25 metabolites.

Gene Set Enrichment Analysis (GSEA). Gene Set Enrichment Analysis was based on Gene Ontology (Biological Process) and KEGG and the Exact Fisher's statistical procedure (Rivals et al. 2007) was used to characterise over-represented functions associated to mRNA targets of FMRP and genes included in the FMR1 knock-out network as compared to the entire GO biological process annotation of the proteome.

Topological analysis of the iMIM knowledge network. The knowledge network was mathematically formalized as an undirected multi-labeled graph Gm,n=(Vm,En) composed of three node types Vm - where m = {"proteins", "mRNA", "metabolites"} and four functional edges En - where n = {"ppi", "pri", "mei", "mri"}.

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Few definitions are listed hereafter.

Shortest Path (sp): To understand how *FMR1*-deficiency propagates throughout the functional knowledge network, shortest paths (sp) from *FMR1* to the target enzymes associated to each candidate biomarker metabolite were measured. The shortest path (sp) problem corresponds to finding a path between two nodes such that the sum of the weights of its constituent edges is minimized. The shortest paths (sp) - also called geodesics - are computed here by using breadth-first search in the *FMR1-KO* iMIM network. In the particular case of the iMIM network, edge weights are not used, *i.e.* all edge weights equal one. The shortest path length (spl) between two nodes is the distance defined by the number of hops between these two nodes within the *FMR1* knockout functional knowledge network. For example, *FMR1* is at a one-hop distance of a target enzyme if they are separated by only one edge (for instance, *FMR1* is directly connected to SOD-1 by a protein-rna interaction).

Pivotal Betweenness (pb): To quantitatively rank the importance of pivotal molecules into the interaction network, a new network metrics based on betweenness centrality measure (Freeman 1977) was defined, called the pivotal betweenness. The node betweenness (b(v)) can be defined by the number of shortest paths going through a node v and was normalized by twice the total number of protein pairs in the network (n*(n-1)).

$$b(v) = \frac{1}{n \times (n-1)} \times \sum_{\substack{i,j,v \in V \\ i- j- v}} \frac{sp_{ij}(v)}{sp_{ij}}$$

In the equation used to compute the betweenness centrality, b(v), for a node v, sp_{ij} is the number of shortest paths going from node *i* to *j*, *i* and *j* □ V and $sp_{ij}(v)$ the number of shortest paths from *i* to *j* that pass through the pivotal node. The pivotal betweenness is a particular case of betweenness, with *i* being *FMR1* and *j* the list of target enzymes. A high pivotal betweenness value for a given molecule indicates that this pivotal molecule is highly central to metabolic biomarker functional path.

Metabolite confirmation assays. Total proteins from *Fmr1*-KO and WT littermates were extracted from cortex and cerebellum using lysis buffer (10mM Tris pH 7.4, 5mM EDTA, 0.1% SDS, 0.5% deoxycholate, 0.5% NP40) and quantified with a Bradford assay according to the manufacturer's recommendations (Biorad). Protein levels in each sample were further verified by Coomassie blue staining. Glutamate levels were then quantified in 40 μg of total protein content using a glutamate enzymatic assay according to the manufacturer's protocol (BioAssay Systems). GABA levels were quantified in 100 μg proteins using a GABA

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ELISA kit (Labor Diagnostika Nord, Nordhom, Germany). Data (n=9 animals per genotype) were analyzed with a one-tailed Mann & Whitney non-parametric statistical test (U-test) and box plots were constructed using the Prism4 software.

UV-crosslinking and immunoprecipitation mRNA interaction confirmation assay. To isolate mRNAs associated with FMRP *in vivo*, UV-crosslinking and immunoprecipitations (CLIP) were performed with total brain extracts from 12-days-old *Fmr1*-KO and WT mice using the protocol described previously (Ule et al. 2005) and the R60 polyclonal antibody directed against the C-terminus of FMRP whose characterization is detailed in supplementary data (*SI* and *SI Fig.1*). For each assay, 10 µg of affinity-purified anti-FMRP antibody was used to immunoprecipitate 1 mg of brain lysate. Approximately 1/100th of the homogenate and 1/20th of the immunoprecipitate were loaded on a 11% SDS–PAGE gel. Proteins transferred onto a 0.45 µm nitro-cellulose membrane were revealed using the mAb1C3 recognizing FMRP (Devys et al. 1993) and the 3Fx antibody recognizing both FXR1P and FXR2P (Khandjian et al. 1998). mRNA were extracted from brain homogenate and immunoprecipitates using Trizol reagent (Invitrogen) according to the manufacturer's protocole and subjected to reverse transcription (RT) using the SuperscriptScriptII RT-PCR system (Invitrogen). RT products were subjected to polymerase chain reaction (PCR), using a PCR Master Kit (Promega) and primers detailed in *Table 3* specific for *Fmr1, Sod1, Mtap1b, Rhoa, Calb1, Tubb3* and *Pgk1* mouse cDNAs. The PCR program consisted in 10 min. of initial denaturation at 95 °C followed by n cycles - 30 s. at 95 °C, 30 s. at 58 °C, 30 s. at 72 °C- and a final elongation step of 10 min at 72 °C. PCR products were visualised on a 1,5% TAE agarose gel and amplicon size was verified using the 1Kb+ DNA ladder (Invitrogen).

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Author contributions. LD, MVC, BB and MED designed the project; LD, MVC, CMB and MED performed the research; VN and MED performed the data analysis; LD, VN, BB and MED analyzed the results; LD and MED wrote the paper.

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TABLES

Table 1. Metabolic signature of *Fmr1*-deficiency. Metabotypes were derived from *Fig. 2* and *Fig. 3* are summarized for each model, by brain regions (OPLS-DA models, *Fig. 3*) or using variance components (factors: genetic or region, *Fig.2b*). ctx, cortex; crb, cerebellum; hip, hippocampus, str, striatum. Bold characters indicate neurotransmitters.

	Brain region				***Component***	
Fmr1-KO metabotypes	**ctx**	**crb**	**hip**	**str**	**genetic**	**region**
Lactate	↓	↓			↓	
Glutamine	↓	↓			↓	
GABA	↓	↓			↓	↑
N-acetyl-aspartate	↓	↓			↓	↓
Glutamate	↓				↓	
Acetotacetate	↑					
$C\underline{H_2}$-CO	↑					
$C\underline{H_2}$-CH_2-CH_2-CO	↑					
Myo-inositol		↓	↑			
Aspartate		↓				
Acetate		↓			↓	
Acetylcholine		↑				↑
Choline		↑				↑
Scyllo-inositol		↑				
N,N,N trimethyl lysine			↓			
Taurine			↑		↑	
Creatine			↑	↓		
Alanine				↓		↓
Beta-amino butyrate				↑		
Kynurenine				↓		
N-alpha acetylornithine				↓		
Adenine				↓		
Inosine					↑	
Purine					↑	↑
Ethanolamine					↓	

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Table 2. Top 30 pivotal proteins derived from interactome mapping of *Fmr1*-deficiency metabotypes. Proteins involved in the interaction network (*Fig. 5*) were ranked by pivotal betweenness (PB). Accession number for the genes encoding these pivotal proteins are provided in the table. Asterisks indicate PB values that appear in the 5% quantile of the PB values distribution. Bold characters indicate proteins directly interacting with FMRP or encoded by FMRP mRNA targets, corresponding reference provided in brackets.

Rank	Human Gene Accession Number	Official Protein Symbol	Full protein name	Reference	Pivotal Betweenness
1	ENSG00000067560	**RHOA**	ras homolog gene family, member A	(Chen et al. 2003)	1.83×10^{-5} *
2	ENSG00000075651	PLD1	phospholipase D1, phosphatidylcholine-specific		1.79×10^{-5} *
3	ENSG00000198805	PNP	purine nucleoside phosphorylase		1.59×10^{-5} *
4	ENSG00000142192	**APP**	amyloid beta (A4) precursor protein	(Westmark and Malter 2007)	1.56×10^{-5} *
5	ENSG00000142168	**SOD1**	superoxide dismutase 1, soluble	(Bechara et al. 2009)	1.35×10^{-5} *
6	ENSG00000104327	**CALB1**	calbindin 1, 28kDa	(Miyashiro et al. 2003)	1.35×10^{-5} *
7	ENSG00000133731	IMPA1	inositol(myo)-1(or 4)-monophosphatase 1		1.35×10^{-5} *
8	ENSG00000087085	ACHE	acetylcholinesterase		1.12×10^{-5} *
9	ENSG00000129245	**FXR2**	fragile X mental retardation, autosomal homolog 2	(Zhang et al. 1995)	8.23×10^{-6} *
10	ENSG00000070808	**CAMK2A**	calcium/calmodulin-dependent protein kinase II alpha	(Iacoangeli et al. 2008)	7.93×10^{-6} *
11	ENSG00000070748	CHAT	choline *O*-acetyltransferase		7.86×10^{-6} *
12	ENSG00000113580	**NR3C1**	glucocorticoid receptor, group C, member 1	(Miyashiro et al. 2003)	7.45×10^{-6} *
13	ENSG00000094755	**GABRP**	gamma-aminobutyric acid (GABA) A receptor, pi	(Miyashiro et al. 2003)	6.73×10^{-6} *
14	ENSG00000187730	**GABRD**	gamma-aminobutyric acid (GABA) A receptor, delta	(Miyashiro et al. 2003)	6.73×10^{-6} *
15	ENSG00000076685	NT5C2	5'-nucleotidase, cytosolic II		6.73×10^{-6} *
16	ENSG00000196839	ADA	adenosine deaminase		6.73×10^{-6} *
17	ENSG00000156508	**EEF1A1**	eukaryotic translation elongation factor 1 alpha 1	(Sung et al. 2003)	6.06×10^{-6}
18	ENSG00000081760	AACS	acetoacetyl-CoA synthetase		5.64×10^{-6}
19	ENSG00000114353	**GNAI2**	guanine nucleotide binding protein, alpha inhibiting	(Miyashiro et al. 2003)	4.88×10^{-6}
20	ENSG00000104951	IL4I1	interleukin 4 induced 1		4.49×10^{-6}
21	ENSG00000104951	AKT1S1	AKT1 substrate 1 (proline-rich)		4.49×10^{-6}
22	ENSG00000104951	NUP62	nucleoporin 62kDa		4.49×10^{-6}
23	ENSG00000128050	PAICS	phosphoribosylaminoimidazole carboxylase		4.49×10^{-6}
24	ENSG00000140443	IGF1R	insulin-like growth factor 1 receptor		4.37×10^{-6}
25	ENSG00000198931	APRT	adenine phosphoribosyltransferase		4.35×10^{-6}
26	ENSG00000171105	INSR	insulin receptor		4.19×10^{-6}
27	ENSG00000118689	**FOXO3**	forkhead box O3	(Brown et al. 2001)	4.11×10^{-6}
28	ENSG00000131711	**MAP1B**	microtubule-associated protein 1B	(Brown et al. 2001; Chen et al. 2003; Darnell et al. 2001)	3.92×10^{-6}
29	ENSG00000131067	GGT7	gamma-glutamyltransferase 7		3.78×10^{-6}
30	ENSG00000141510	TP53	tumor protein p53		3.43×10^{-6}

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Table 3. List and details of primers used to detect mRNAs associated *in vivo* with FMRP immunoprecipitates. Primer sequences originated either from literature or were designed using the Primer3 webtool.

Target mRNA	*Primer name*	*5' 3' Sequence*	*Amplicon size*	*Cycles number*	*Reference*
Fmr1	F	GCTCCAACAGAGGAAGAGGG	306 bp	n=35	(Bechara et al. 2009)
	R	GGGTACTCCATTCACCAGCGG			
Mtap1b	F	TCCGATCGTGGGACACAAACCTG	228 bp	n=45	(Edbauer et al. 2010)
	R	AGCACCAGCAGTTTATGGCGGG			
Sod1	F	ACCATCCACTTCGAGCAGAA	196 bp	n=25	(Bechara et al. 2009)
	R	AGTCACATTGCCCAGGTCTC			
Rhoa	F	TGGTTGGGAACAAGAAGGAC	233 bp	n=45	Primer3
	R	TGAGGCACCCAGACTTTTTC			
Calb1	F	GAAGGAAAGGAGCTGCAGAA	212 bp	n=50	Primer3
	R	TTCCTCGCAGGACTTCAGTT			
Tubb3	F	CGAGACCTACTGCATCGACA	151 bp	n=35	Primer3
	R	CATTGAGCTGACCAGGGAAT			
Pgk1	F	GAAGGGGAAGCGGGTCGTGATG	206 bp	n=35	(Edbauer et al. 2010)
	R	GCAGCAACTGGCTCTAAGGAGTACTTG			

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FIGURE LEGENDS

Figure 1. Expression of FMRP in postnatal brain. (***a***) Western-blot analysis of FMRP expression in total brain extracts (25 µg/lane) at various post-natal stages (0, date of birth to P28, 4 weeks old) and in adult (Ad.). (***b***) Immunohistochemistry on 12-day old mouse brain longitudinal sections reveal a strong expression of FMRP (brown) in the whole brain and in specific regions (***c***) such as cortex (ctx), hippocampus (hip), striatum (str) and cerebellum (crb) (***c***). Nuclei were counterstained with cresyl-violet.

Figure 2. Metabolic signature of *Fmr1*-deficiency in 12-day old brain. The metabolic variation observed in ^{1}H NMR spectra acquired in 12-day old mouse brain samples was modeled using an Orthogonal Partial Least Square-Discrimination Analysis (OPLS-DA). PLS components maximizing the segregation of the groups are computed. Each PLS component corresponds to a combination of the initial ^{1}H NMR spectral variables, known as model coefficients or loadings. Each individual spectrum has new coordinates on the PLS components, known as scores. As a consequence, the three-dimensional OPLS-DA scores plot (***a***) segregates the different sample groups according to brain region (shapes) and *Fmr1*-deficiency status (white and black shapes). (***b***) OPLS variance component model of cortex and cerebellum. Legend: cortex, ctx; cerebellum, crb; hippocampus, hip; striatum, str; ko, *Fmr1*-knockout, wt, wild-type samples.

Figure 4. Quantification of glutamate levels in cortical extracts and GABA levels in cerebellar extracts of *Fmr1*-KO brain vs WT. Glutamate cortical (***a***) and GABA cerebellar concentrations (***b***) are significantly reduced in *Fmr1*-KO extracts compared to WT (p=0,011; p=0,038 respectively. n=9 for each genotype).

Figure 5. Interactome-mapping of *FMR1*-deficiency metabotype. *FMR1* integrated Metabolome and Interactome Mapping (iMIM) Network (***a***). The metabolites significantly affected in the different models (*Table 1*) were mapped onto the interactome, using FMRP mRNA targets, KEGG metabolic pathways and neurotransmitter/receptor databases. The resulting network allows connecting the causal gene *FMR1* to the downstream metabolic consequences of its deficiency. Pivotal shortest paths *via* **enzymes** and **receptors** are respectively represented in **blue** and **green**. (***b***) Statistical validation of the *FMR1* knowledge network under the null hypothesis (H_0). The average spl between *FMR1* and the biomarker metabolites (n=25) was computed and compared to the distribution of average spl obtained after 100,000 H_0 network simulations (*i.e.*

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networks obtained after 100,000 random selection of 25 metabolites from the entire metabolic network). This simulation under the null hypothesis shows that *FMR1* appears significantly connected to the candidate biomarker metabolites, with an average distance of 3.38 hops (n = 100,000 random simulations, p=0.03427).

Figure 6. *Fmr1, Mtap1b, Sod1, Rhoa* and *Calb1* mRNAs interact with FMRP *in vivo.* Western-blot analysis of UV-crosslinking and immunoprecipitation (CLIP) assay performed on *Fmr1* wild-type (WT) and knock-out (KO) total brain lysates using polyclonal antibodies raised against the C-terminus of FMRP (*a*). Input lysate (1/100th) and immunoprecipitates (IP, 1/20th) were probed for FMRP and its interacting protein partners FXR1P and FXR2P respectively by western-blotting, revealing the presence of FMRP in the WT immunoprecipitates (upper panel revealed with 1C3 antibody), together with interacting partners FXR1P and FXR2P (lower panel revealed by 3Fx antibody). RT-PCR analysis of mRNAs associated with FMRP (*b*). Total RNA was extracted from the input brain lysate and immunoprecipitates described in a), and used as template for RT-PCR. RT-PCR products obtained from *Fmr1*-KO (lane 1) and WT (lane 2) inputs and from immunoprecipitates of *Fmr1*-KO (lane 3) and WT (lane 4) brain extracts were separated and visualized by agarose gel electrophoresis. This reveals that the known FMRP mRNA targets *Fmr1* and *Mtap1B* are selectively recovered in the WT immunoprecipitate, while the unrelated mRNA *Tubb3* is not recovered. *Sod1* mRNA is also selectively recovered in the immunoprecipitate together with *Rhoa* and *Calb1* mRNAs. Control PCR (lane 5) was performed in the absence of reverse-transcriptase. Lower DNA molecular weight markers presented on the left of the gels are respectively 100, 200, 300, 400, 500, 600, 800 and 1000 bp.

Figure 7. Schematic metabolic map of *Fmr1*-deficiency in cortex and cerebellum. Astrocytes and neurons cooperate metabolically for neuronal energy fueling and biosynthesis of neurotransmitters, and this cooperation seems compromised in the *Fmr1*-null brain. **Glucose**, **lactate** and **acetoacetate** are neuronal energy substrates (framed in **pink**), which contribute to replenish acetyl-coA stores to fuel the tricarboxylic acid (TCA) cycle. **Glutamate** and **GABA** can enter the TCA cycle *via* conversion upstream or downstream the intermediate metabolite alpha-ketoglutarate (**αKG**). Metabolites affected by *Fmr1-deficiency* are represented in **blue** if decreased and **red** if increased. Metabolites acting as neurotransmitters are represented in yellow frames. Legend: NAA, *N*-acetyl-aspartate; NAAG, *N*-acetyl-aspartyl-glutamate; GAD65, Glutamate Dehydrogenase; CHAT, Choline *O*-Acetyltransferase; ACHE, Acetylcholinesterase.

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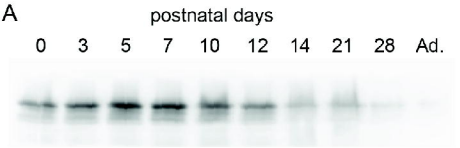
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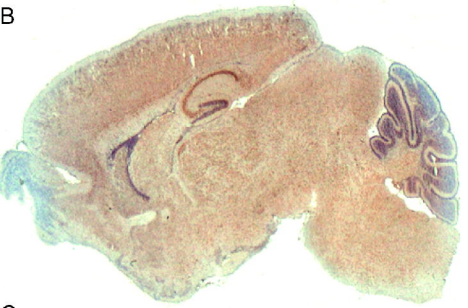
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Figure 1





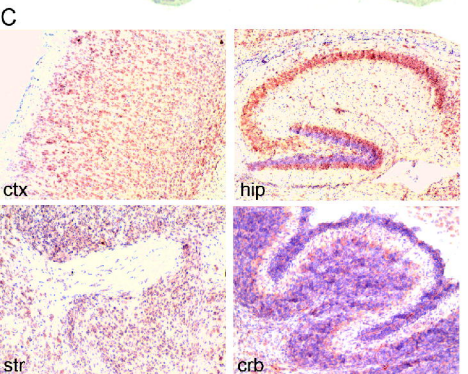


Figure 2

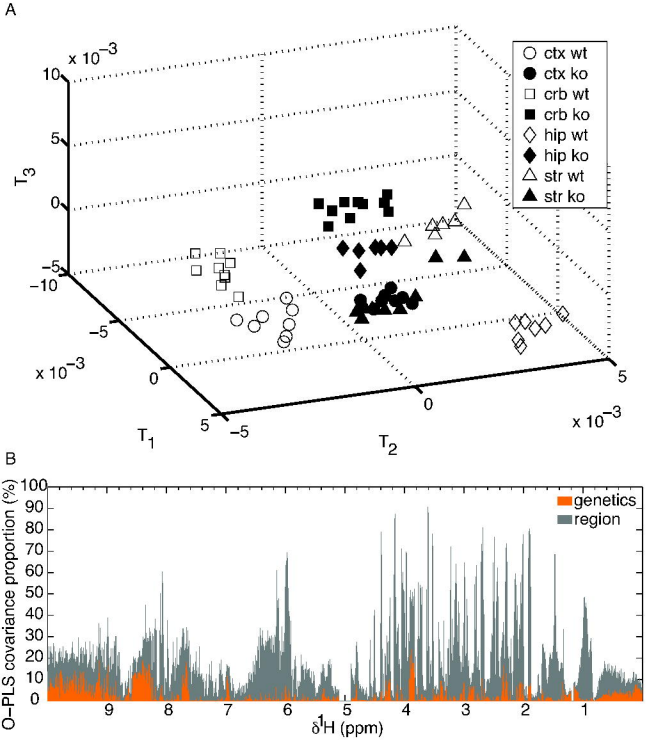

A
x 10−3
10
5
0
−5
−10
T3
ctx wt
ctx ko
crb wt
crb ko
hip wt
hip ko
str wt
str ko
x 10−3
−5
0
5
T1
5
0
−5
T2
x 10−3
B
O−PLS covariance proportion (%)
100
90
80
70
60
50
40
30
20
10
0
genetics
region
9
8
7
6
5
4
3
2
1
δ1H (ppm)

Figure 3

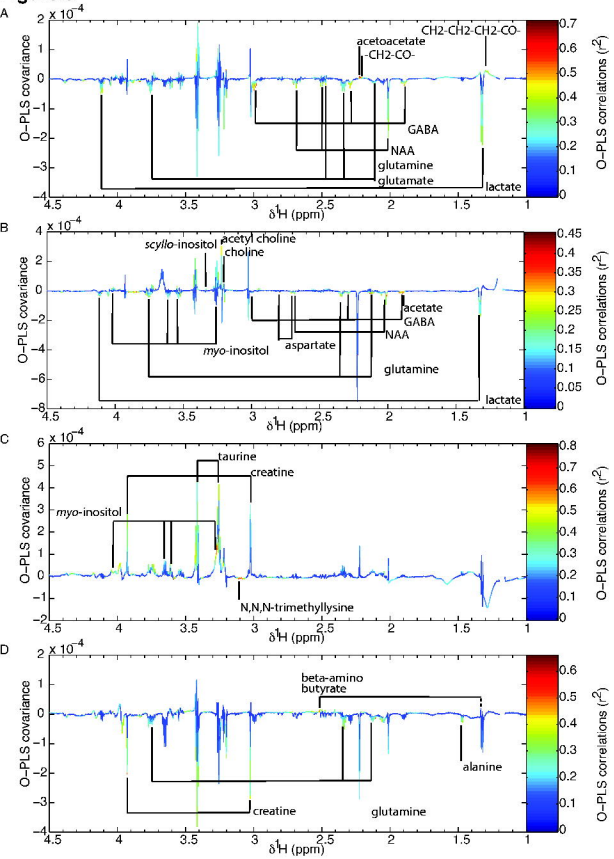

A
x 10^-4
O–PLS covariance
O–PLS correlations (r^2)
δ^1H (ppm)
CH2-CH2-CH2-CO-
acetoacetate
-CH2-CO-
GABA
NAA
glutamine
glutamate
lactate
B
x 10^-4
O–PLS covariance
O–PLS correlations (r^2)
δ^1H (ppm)
scyllo-inositol
acetyl choline
choline
acetate
GABA
NAA
myo-inositol
aspartate
glutamine
lactate
C
x 10^-4
O–PLS covariance
O–PLS correlations (r^2)
δ^1H (ppm)
taurine
creatine
myo-inositol
N,N,N-trimethyllysine
D
x 10^-4
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O–PLS correlations (r^2)
δ^1H (ppm)
beta-amino
butyrate
alanine
creatine
glutamine

Figure 4

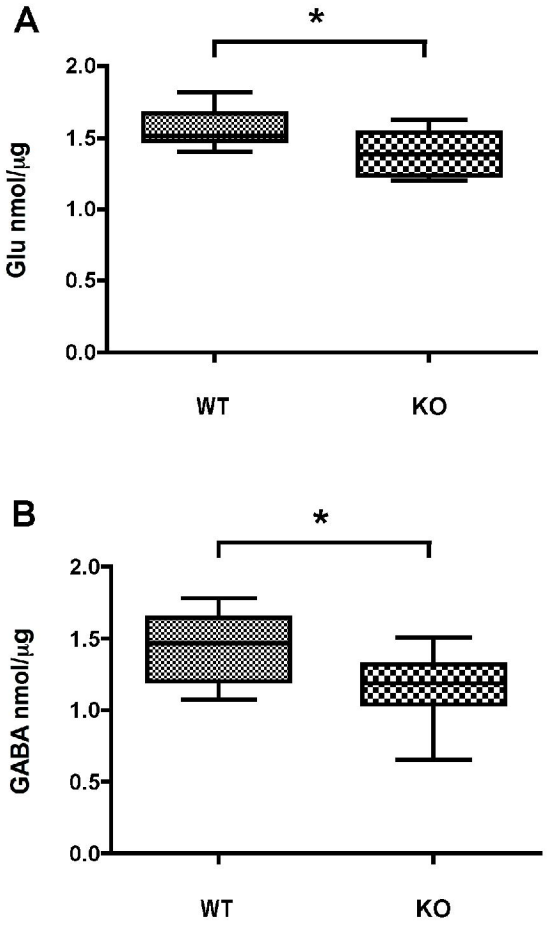
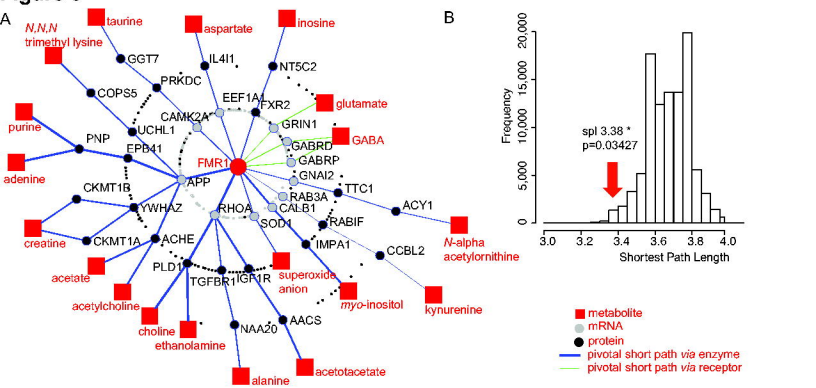


Figure 5


A
B
taurine
aspartate
inosine
N,N,N trimethyl lysine
GGT7
IL4I1
NT5C2
PRKDC
COPS5
EEF1A1
FXR2
glutamate
purine
CAMK2A
GRIN1
UCHL1
PNP
EPB41
GABRD
GABA
FMR1
GABRP
adenine
GNAI2
CKMT1B
APP
RAB3A
TTC1
ACY1
YWHAZ
RHOA
CALB1
SOD1
RABIF
creatine
CKMT1A
ACHE
IMPA1
CCBL2
N-alpha acetylornithine
acetate
PLD1
TGFBR1
IGF1R
superoxide anion
myo-inositol
kynurenine
acetylcholine
choline
ethanolamine
NAA20
AACS
alanine
acetotacetate
Frequency
0
5,000
10,000
15,000
20,000
spl 3.38 *
p=0.03427
3.0
3.2
3.4
3.6
3.8
4.0
Shortest Path Length
metabolite
mRNA
protein
pivotal short path via enzyme
pivotal short path via receptor

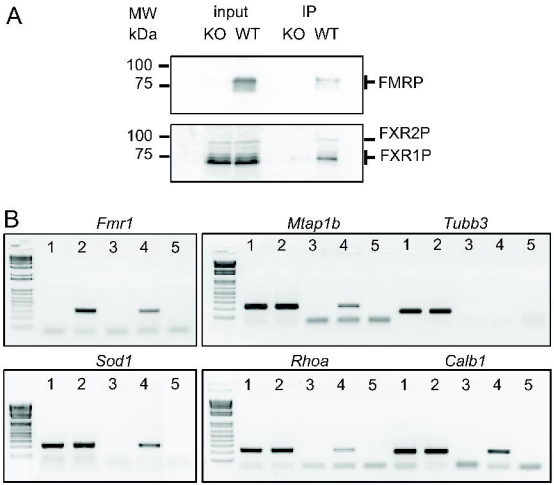
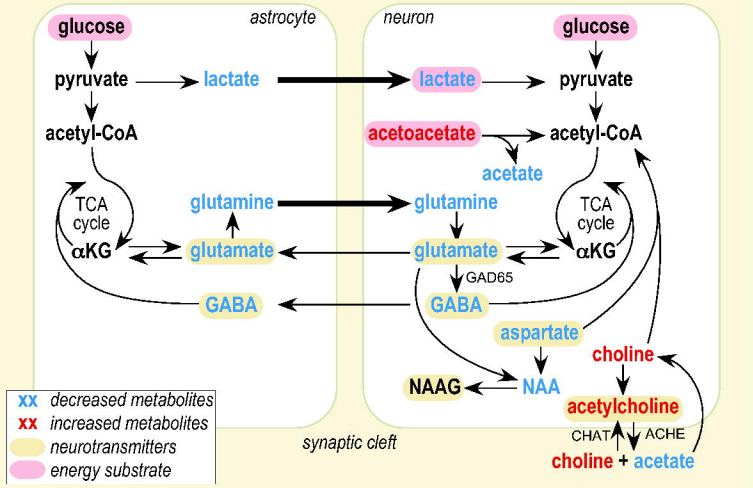

A
MW
kDa
input
KO WT
IP
KO WT
100
75
FMRP
100
75
FXR2P
FXR1P
B
Fmr1
1 2 3 4 5
Mtap1b
1 2 3 4 5
Tubb3
1 2 3 4 5
Sod1
1 2 3 4 5
Rhoa
1 2 3 4 5
Calb1
1 2 3 4 5

Figure 6

Figure 7


astrocyte
neuron
glucose
glucose
pyruvate
lactate
lactate
pyruvate
acetyl-CoA
acetoacetate
acetyl-CoA
acetate
TCA cycle
glutamine
glutamine
TCA cycle
αKG
glutamate
glutamate
αKG
GAD65
GABA
GABA
aspartate
choline
NAAG
NAA
acetylcholine
CHAT
AChE
choline + acetate
synaptic cleft
xx decreased metabolites
xx increased metabolites
neurotransmitters
energy substrate