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Systematic mapping of Fragile X granules in the developing mouse brain reveals a potential role for presynaptic FMRP in sensorimotor functions

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Abstract

Loss of Fragile X mental retardation protein (FMRP) leads to Fragile X syndrome (FXS), the most common form of inherited intellectual disability and autism. Although the functions of FMRP and its homologues FXR1P and FXR2P are well studied in the somatodendritic domain, recent evidence suggests that this family of RNA binding proteins also plays a role in the axonal and presynaptic compartments. Fragile X granules (FXGs) are morphologically- and genetically-defined structures containing Fragile X proteins that are expressed axonally and presynaptically in a subset of circuits. To further understand the role of presynaptic Fragile X proteins in the brain we have systematically mapped the FXG distribution in the mouse central nervous system. This analysis revealed both the circuits and the neuronal types that express FXGs. FXGs are enriched in circuits that mediate sensory processing and motor planning - functions that are particularly perturbed in FXS patients. Analysis of FXG expression in the hippocampus suggests that CA3 pyramidal neurons utilize presynaptic Fragile X proteins to modulate recurrent but not feedforward processing. Neuron-specific FMRP mutants revealed a requirement for neuronal FMRP in the regulation of FXGs. Finally, conditional FMRP ablation demonstrated that FXGs are expressed in axons of thalamic relay nuclei that innervate cortex, but not in axons of thalamic reticular nuclei, striatal nuclei, or cortical neurons that innervate thalamus. Together, these findings support the proposal that dysregulation of axonal and presynaptic Fragile X proteins contribute to the neurological symptoms of FXS.

Keywords

RNA binding proteins; local translation; axonal protein synthesis

Fragile X syndrome (FXS) is the leading inherited cause of autism and intellectual disability in humans (Pickett and London, 2005; Cohen et al., 2005; Hernandez et al., 2009). FXS

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arises from mutations in the *FMR1* gene, which encodes the RNA-binding protein FMRP (Fragile X mental retardation protein), a critical regulator of protein synthesis in the brain. FXS typically presents as developmental delay with symptoms including cognitive impairments as well as high incidence rates of both autism and epilepsy. Furthermore, the ability of affected individuals to interact with their environment is severely affected by both hypersensitivity to sensory stimuli and impaired motor development.

Understanding the origin of FXS symptoms requires identifying the affected brain circuits and the developmental windows during which FMRP acts. This task is challenging since FMRP is expressed in virtually every neuron, but FXS patients display region-selective abnormalities in brain morphology and function. At the level of brain morphology, boys with FXS exhibit changes in the volume of both cerebellar and thalamic gray matter as well as white matter innervating the frontal lobes (Hoeft et al., 2010). Such heterogeneity is also observed at behavioral and cognitive levels. FXS patients are generally developmentally delayed, but not all domains are equally affected. For example, fine motor development is more severely affected than gross, while expressive language is more delayed than receptive language (Roberts et al., 2009).

FXS symptoms are thought to arise largely from disruptions in functional connectivity resulting at least in part from altered translational regulation of synaptic proteins (Bassell and Warren, 2008; Costa-Mattioli et al., 2009; Zukin et al., 2009; Darnell et al., 2011). FMRP-regulated translation in postsynaptic and dendritic compartments has been extensively characterized. However, several lines of evidence indicate that FMRP also modulates presynaptic function (Akins et al., 2009). Strikingly, FMRP regulates messages encoding approximately one-third of the presynaptic proteome and these transcripts are among the most abundant of FMRP targets (Akins et al., 2009; Darnell et al., 2011). Appropriate synaptic connectivity within hippocampal area CA3 requires FMRP in the presynaptic, but not postsynaptic, neuron (Hanson and Madison, 2007). Mice lacking Fragile X proteins have defective presynaptic short-term plasticity, while *Aplysia* require FMRP both presynaptically and postsynaptically for long-term depression (Zhang et al., 2009; Deng et al., 2011; Till et al., 2011). Finally, *Drosophila* FMRP mutants exhibit altered presynaptic structure and neurotransmission (Zhang et al., 2001; Gatto and Broadie, 2008).

Fragile X granules (FXGs) are endogenous brain structures that contain Fragile X proteins (FMRP and its homologues FXR1P and FXR2P). FXGs localize to axonal and presynaptic compartments in restricted circuits in a developmentally dynamic manner (Christie et al., 2009; and see Results). These granules therefore open a path to link the presynaptic function of Fragile X family proteins to specific circuits and developmental windows in the brain. All FXGs contain FXR2P, which is required for their expression. A large majority of forebrain FXGs contains FMRP, whose loss results in an increased number of FXGs. FXGs in select brain regions contain FXR1P. FXGs are expressed after axonal projections have reached their targets and are instead restricted to developmental epochs corresponding to periods of robust synaptic plasticity. Furthermore, FXGs are upregulated during injury-induced circuit remodeling. A previous survey of selected brain regions showed that FXGs display restricted expression to a subset of circuits including olfactory bulb, neocortex, hippocampus, and cerebellum (Christie et al., 2009).

Here we have systematically mapped FXG expression in the brain. This FXG map reveals additional circuits containing presynaptic Fragile X proteins and provides a comprehensive picture of where in the brain these granules function. FXGs are enriched in circuits underlying sensory and motor processing including in connections between motor cortex and thalamus. In contrast, FXGs are largely absent in prefrontal and visual cortical areas. Within hippocampus, FXGs are expressed in CA3 recurrent associational/commissural fibers but are absent in CA3 feedforward Schaffer collaterals. Neuron-specific FMRP ablation recapitulates alterations in FXG abundance and composition as seen in the full *fmr1* knockout, indicating a requirement for neuronal FMRP in the regulation of FXGs. Conditional FMRP mutants also revealed that FXGs are expressed in thalamocortical and corticocortical, but not corticothalamic connections. Taken together, these data suggest that disruptions in presynaptic Fragile X protein function may play a prominent role in the sensorimotor symptoms and increased incidence of epilepsy seen in FXS patients.

MATERIALS AND METHODS

Animals

All work was performed in accordance with protocols approved by Brown University Institutional Animal Care and Use Committee. Wild type C57BL/6 mice were obtained from Charles River. For analysis of mice in which FMRP was conditionally ablated, mice with floxed alleles of *fmr1* (generous gift of D. Nelson; Mientjes et al., 2006) were crossed to mice in which Cre recombinase is driven by the synapsin 1 promoter (*syn-cre*; Jackson Laboratories). Both of these lines were maintained on a C57BL/6 background. This cross produced wild type (*syn-cre;fmr1^{+/y}*) and conditional knockout (*syn-cre;fmr1^{flox/y}*) mice. The synapsin-Cre transgene expresses Cre recombinase specifically in select populations of neurons including dentate and CA3 pyramidal neurons in hippocampus, neurons in a subset of thalamic nuclei, and a subpopulation of layer IV neurons in neocortex (He et al., 2004). We also observed Cre activity via FMRP ablation in scattered neurons throughout the brain (data not shown).

Tissue

For fresh frozen tissue (Figs. 2,4–7), mice were deeply anesthetized by isoflurane inhalation and decapitated. Intact brains were carefully removed, washed in PBS (100 mM phosphate pH 7.4, 150 mM sodium chloride), embedded in OCT medium (Sakura Finetek) by rapid freezing and stored at -80°C until sectioned. Sections of OCT-embedded brains were prepared using a Leica cryostat at 50 μm , slide mounted, and stored at -20°C until use for immunolabeling. For perfusion-fixed tissue (Fig. 3), C57BL/6 mice were deeply anesthetized by isoflurane inhalation. Animals were then perfused first with 100mM HEPES pH 7.4, 150 mM sodium chloride, 0.5% sodium nitrite, and 1 U/mL heparin followed by PBS containing 4% paraformaldehyde. Intact brains were carefully removed, postfixed overnight in the perfusate, washed for several hours in PBS and cryoprotected in PBS containing 30% sucrose. After the brains had settled they were embedded in OCT, rapidly frozen and stored at -80°C until sectioning. Free-floating sections were collected using a Leica cryostat at 40 μm , rinsed in PBS, and stored at 4°C until use.

Antibodies

Immunostaining and immunoblotting conditions for all antibodies are listed in Table 1. For detection of FXR2P in fresh frozen tissue, we used a rabbit polyclonal antibody, BU38, raised against a KLH-conjugated peptide SAPLERTKPSDSLSGQ, corresponding to amino acids 625–641 of the human FXR2P sequence AAC50292.1. BU38 recognizes a single band in wild type, but not *fxr2* null, mouse brain of the appropriate size by Western and does not cross-react with either of the related proteins FMRP or FXR1P (Fig. 1). The staining pattern in tissue was indistinguishable from that seen with the anti-FXR2P antibody 1G2 in both wild type and *fxr2* null tissue while the preimmune serum gave no specific signal (data not shown).

Western blotting of whole brain lysates

Whole brains from postnatal day 15 (P15) *fxr2* null (Bontekoe et al., 2002) or wild type C57BL/6 (Charles River) mice were Dounce homogenized in RIPA lysis buffer [25mM Tris pH 8, 150mM sodium chloride, 0.1% SDS, 1% NP-40, 0.5% deoxycholic acid, 1X complete protease inhibitor cocktail (Roche), 1mM orthovanadate (NEB), and 1mM AEBSF (Sigma)], incubated for 20 minutes at 4°C, and centrifuged at 10,000g at 4°C for 10min. After boiling in SDS-PAGE sample buffer (60 mM Tris pH 6.8, 2% SDS, 10% glycerol, 735 mM β -mercaptoethanol), 10 μ g of the supernatants were loaded on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes following standard methods. Membranes were blocked for one hour at room temperature in 5% non-fat dry milk in wash buffer (100 mM Tris pH 7.4, 150 mM sodium chloride, 4% normal goat serum, and 0.1% Tween-20). Primary antibodies diluted in 5% milk in wash buffer were incubated overnight at 4°C. Blots were washed four times for five minutes each in wash buffer after primary incubation. HRP-conjugated secondary antibodies were diluted in 5% milk in wash buffer and incubated at room temperature for 90 minutes. Blots were washed four times for five minutes each in wash buffer and signals were detected by chemiluminescence (Amersham ECL Western blotting reagents).

Western blotting of COS-7 lysates

For testing cross-reactivity of BU38 to Fragile X proteins, COS-7 cells were transfected with EGFP-FXR2P (generous gift of K. Murai; Cook et al., 2011), EGFP-FMRP (generous gift of K. Murai; Cook et al., 2011), EGFP-FXR1P (generous gift of D. Radzioch; Garnon et al., 2005), or EGFP using FugeneHD (Promega) according to the manufacturer's instructions. Twenty-four hours after transfection, cells were washed two times with cold PBS and incubated with RIPA buffer for 15 minutes at 4°C. Lysates were then collected and centrifuged at 10,000g for 15 minutes at 4°C. Supernatants (10 μ g) were prepared for Western blotting as above.

Immunohistochemistry of FMRP and neurofilament in free-floating sections

Free-floating sections were treated with 10 mM sodium citrate, pH 6.0 for 30 minutes at 75°C to improve antibody access to the tissue. Tissue was treated with blocking solution [PBST (10 mM phosphate buffer, pH 7.4, and 0.3% Triton X-100) and 1% blocking reagent (Roche)] for 30 minutes and then incubated overnight in blocking solution plus primary

antibodies against FMRP and neurofilament (see Table 1). Tissue was then washed with PBST before incubation in blocking solution plus secondary antibodies. Tissue was washed with PBST followed by PBS and then mounted in mounting medium (4% n-propylgallate, 60% glycerol, 5 mM phosphate pH 7.4, 75 mM sodium chloride). Confocal images were collected using a 63X Plan-Apochromat objective on a Zeiss LSM 510 microscope. Figures were prepared using Photoshop CS5 (Adobe) and CorelDraw X3 (Corel).

Immunostaining of FMRP and FXR2P in sections from conditional knockout animals

Slide-mounted, fresh-frozen sections were allowed to warm to room temperature. They were then immersed in PBS containing 4% paraformaldehyde for ten minutes, rinsed thoroughly with PBS, and then treated with 10 mM sodium citrate, pH 6 at 65°C for 30 minutes. Tissue was then treated with blocking solution for thirty minutes followed by immunostaining as above, using the antibodies 1G2 and 2F5 (Table 1). Images and figures were collected and prepared as above.

Immunostaining of FXR2P for FXG mapping

Slide-mounted, fresh-frozen sections were allowed to warm to room temperature, immersed in methanol (−20°C) for 30 seconds, and air-dried. Tissue was next treated with blocking solution for 30 minutes and immunostained as above using the anti-FXR2P antibody BU38 (Table 1). Images were collected and analyzed as discussed below.

FXG Mapping

Montages of 40X images were collected for each section using Nikon Elements software and a Nikon Eclipse T800 microscope coupled with an Orca ER camera (Hamamatsu). These images have a depth of field of roughly 1 μ m, representing roughly 5% of the postfixation section thickness of ~20 μ m. Individual images were stitched together into a single large image using the FIJI build of ImageJ. Images of entire sections were sharpened in Photoshop CS5 (Adobe) using the Unsharp Mask filter three times with the settings 500%, 2.5 pixels, and 128 levels. This procedure, which highlights bright puncta approximately 3 pixels in diameter, identified a sizable fraction of the FXGs in a spatially accurate manner. In these images, 3 pixels corresponds to structures with an apparent size of approximately 600 nm. However, FXGs are likely to be smaller and may be below the diffraction limit of 250 nm as axons are frequently smaller than 600 nm in diameter. For example olfactory sensory neuron axons expand from 200 to as much as 450 nm in diameter as they traverse the olfactory nerve layer (Cuschieri and Bannister, 1975; Akins and Greer, 2006). In contrast to the expansion of the axons, FXGs do not change in apparent size across the olfactory nerve layer (Fig. 4). FXGs in these images were manually annotated in neuropil and axonal tracts (Fig. 4) using Stereo Investigator (MicroBrightField, Inc.). Examination of unprocessed images indicated that somatic puncta reflected heterogeneity in staining rather than discrete granules and these puncta were therefore excluded. To validate this approach, we compared the processed images of arbitrarily selected brain regions with both the unprocessed images and direct observation of stained sections using a microscope. FXGs identified in the map reliably reflected the presence of FXGs in the tissue, and no populations of FXGs observed in the tissue were absent from the map (see Results and data not shown). As FXR2P staining in cell bodies resembles a Nissl stain, this information was

used to identify brain regions based on an adult mouse brain atlas (Paxinos and Franklin, 2007). Figures were prepared using Photoshop and CorelDraw. For figure composition (but not FXG identification), some images were adjusted using either the burn or dodge tools in Photoshop. The map was generated from sections of one brain. All FXG distributions reported here were confirmed in at least two additional animals.

RESULTS

Neuronal origin of FXGs

In previous work we showed that FXGs are localized in axons and presynaptic specializations. However, FMRP is also expressed in glial cells (Pacey and Doering, 2007), leaving open the possibility that FXGs could also be localized in glial processes. Further, it has been reported that macromolecules can be transferred from glia to neurons (Giuditta et al., 2008). We therefore sought to establish the cellular origin of the FMRP in FXGs. In wild type mouse hippocampus, FXGs in both dentate mossy and CA3 associational fibers contain FMRP and FXR2P (arrows in Fig. 2A–D). We used a conditional mutant approach with a synapsin Cre driver to ablate FMRP in the neurons that give rise to these axons (dentate granule and CA3 pyramidal neurons, respectively; see Methods). This neuron-specific mutation resulted in the loss of FMRP from FXGs in both the mossy and associational fibers (Fig. 2E–H). Further, the abundance of FXGs increased in both populations. This increase in FXG density is consistent with that observed in conventional *fmr1* knockout mice (Christie et al., 2009). Thus, FMRP in neurons alters FXG composition and abundance in a manner equivalent to that seen when FMRP is absent from all cells.

Constructing an FXG map

In order to identify circuits that express presynaptic Fragile X proteins, we developed a method to systematically map FXGs in the brain. Our approach was informed by three key observations: 1) FXG protein components are of neuronal origin (Fig. 2); 2) FXGs are expressed within axon tracts (e.g., within the striatum; Fig. 3) as well as in neuropil (Christie et al., 2009); 3) FXGs are expressed along large extents of individual axons (Fig. 3). We therefore reasoned that identifying both the neuropil regions and axon tracts that contain abundant FXGs would reveal the circuits in which these granules function.

To identify FXGs, we immunostained sections for FXR2P, which is a component of all FXGs (Christie et al., 2009). We then assembled high-resolution montages of these sections. These granules have an apparent size of approximately 500 nm when detected by immunofluorescence microscopy. (However, their actual dimensions are likely to be much smaller; see Methods). To simplify the mapping, we therefore highlighted granules of this size using standardized settings in image processing software (see Methods). The processed images were then manually annotated to score FXGs (white arrows in Fig. 4B–D). To test the validity of this approach, we identified FXGs within the olfactory bulb, where FXG expression has been previously determined (Christie et al., 2009). In agreement with that study, the map generated here showed that FXGs are largely restricted to the olfactory nerve and glomerular layers (Fig. 4). We note that a portion of FXGs that can be identified in raw images are filtered out by the processing method used here (black arrows in Fig. 4B–D).

However, comparison of processed and unprocessed images as well as direct observations of stained sections showed that the FXG map reliably reflected the distribution of FXGs in the tissue (see Methods). This mapping method therefore provides a qualitatively accurate description of FXG expression in the brain.

We next used this mapping approach to identify brain regions enriched in FXGs. FXG expression is restricted to a subset of structures and circuits throughout the brain (Figs. 5,6; Tables 3,4). In a sagittal section of a brain from a P15 wild type mouse, the densest accumulations of FXGs were observed within the olfactory bulb, thalamus, cortex, hippocampus, and brainstem (Fig. 5). To characterize these FXG-expressing brain regions with greater precision, we undertook a systematic analysis of FXG expression. In this approach, a P15 wild type brain was serially sectioned in the coronal plane and FXGs were annotated in every 5th section (Fig. 6). Although only one hemisphere is presented, FXGs were identified in corresponding regions in both hemispheres. In all, 155,344 FXGs were identified across both hemispheres (Table 3). FXG expression in select brain regions is discussed below.

Olfactory Bulb

FXGs were observed in olfactory sensory neuron axons in both the olfactory nerve layer and the glomerular layer (Figs. 4,5,6A–F). In agreement with earlier studies (Christie et al., 2009), FXGs were detected in all glomeruli. However, FXG density varied among glomeruli. Scattered FXGs were also observed in the deep olfactory bulb, particularly in the granule cell layer. In contrast to the main olfactory bulb, FXGs were sparse in the vomeronasal sensory neuron axons innervating the accessory olfactory bulb (Figs. 5,6B–D). FXGs were not commonly observed within either the external plexiform layer or lateral olfactory tract coursing towards piriform cortex.

Anterior Commissure

FXGs were observed in the olfactory limb of the anterior commissure as it coursed between the olfactory bulb and the anterior piriform cortex (Fig. 6E–I). Interestingly, very few FXGs were observed in the caudal anterior commissure as it approached the midline (Fig. 6J–O).

Neocortex

FXGs showed a remarkably restricted distribution in neo-cortex (Figs. 5,6C–CC; Table 4). The greatest FXG density was seen in frontal cortical regions, notably both primary and secondary motor cortex (Figs. 5, 6G–N). Moreover, in these regions FXGs were restricted to layers V and VI. FXGs were largely absent from medial and orbital cortical regions comprising prefrontal cortex. FXGs were rare in medial cortex (including cingulate and retrosplenial cortices) along the entire rostrocaudal axis. In general, FXGs were less prevalent in caudal than in rostral cortex, though a comparatively large number was seen in caudal aspects of the dorsolateral entorhinal cortex (Fig. 6X–CC).

Long-Range Fiber Tracts

We observed numerous FXGs within fiber tracts running deep to neocortex, distributed along the rostrocaudal axis (Fig. 5, 6I–CC). FXGs were detected in the cingulum as well as

in the external capsule. Within the external capsule, there was a relative enrichment in the ventrolateral portion, which was particularly pronounced in the caudal forebrain. At the level of the thalamus (Figs. 5, 6Q–T), FXG-containing fibers were also seen in the striatum and internal capsule. Notably, FXGs were rare in the corpus callosum. Indeed the only midline fiber tract with abundant FXGs was the ventral hippocampal commissure (see below).

Striatum

FXGs were observed in fibers coursing through the striatum, particularly at the level of the ventral nuclear group of the thalamus (Figs. 5, 6J–U). Notably, we did not observe FXGs in the striatal neuropil (Fig. 3); rather, these FXGs were restricted to passing fibers. These fibers are unlikely to be corticospinal axons as FXGs are not observed within the pyramids in the brainstem (Table 4). Our findings in conditional knockout mice indicate that these FXGs are in thalamocortical projections (see below).

Thalamus

FXGs were observed throughout the thalamus (Figs. 5, 6P–Z). These granules were particularly enriched within the reticular nucleus (TRN) and the ventral anterior and lateral relay nuclei (VAL).

Hippocampus

As described previously (Christie et al., 2009), FXGs were observed in area CA3 (Figs. 5, 6Q–Z). Mossy fibers innervating stratum pyramidale and stratum lucidum contained FXGs. FXG expression in mossy fibers was sub-region dependent, as abundance was greater in the ventral as compared to dorsal hippocampus (Fig. 6V–Z and Fig. 6R–U, respectively). FXGs were also observed within the deep stratum oriens. Rostral to the hippocampus, these FXG-containing fibers approached, and were continuous across, the midline at the ventral hippocampal commissure (Fig. 6Q). We interpret these axons to be associational and commissural fibers arising from CA3 pyramidal neurons (see Discussion).

Cerebellum

FXGs were detected within the cerebellar molecular layer (Figs. 5, 6FF–PP), as previously reported (Christie et al., 2009). These FXGs were particularly enriched within the vermis and were less prevalent in lateral cerebellar cortex. Whether this distribution reflects a difference in expression or in developmental stage is currently unclear. We also observed FXGs in the cerebellar white matter located deep to the granule cell layer.

Brainstem

FXGs were present in the reticular formation within the mesencephalon, pons, and medulla (Figs. 5, 6Y–PP). FXGs were also observed within the inferior colliculus (Fig. 6HH–JJ) but were otherwise largely absent from the midbrain. We also observed FXGs in the external cuneate nucleus (Fig. 6MM–PP). Additionally, FXGs were located medial to the external cuneate nucleus in a position consistent with expression in the cuneate nucleus. Brainstem FXGs were not observed in previous studies where FMRP was used to identify these

structures (Christie et al., 2009). We confirmed that FXGs in the brainstem contain FXR2P but not FMRP (data not shown). The gracile nucleus and nucleus thoracicus are located in the spinal cord and were not included in the analyzed tissue.

FXG expression in thalamocortical axons

The FXG map presented above includes fiber tracts and neuropil that comprise multiple axonal populations. In such cases, the map data alone is not sufficient to identify the specific neurons and circuits harboring these granules. For example, FXGs containing both FMRP and FXR2P are observed in motor cortex, external capsule, striatum, TRN and VAL (arrows in Fig. 7B–F). Since motor cortex and VAL of the thalamus are reciprocally connected, axons passing through the striatum and external capsule could be derived from thalamocortical and/or corticothalamic connections (Fig. 7A, G; Stepniewska et al., 1994; Kakei et al., 2001). Furthermore, both thalamocortical and corticothalamic axons give rise to collaterals that innervate the thalamic reticular nucleus (Fig. 7A, G; Jones, 1975).

We turned to a genetic approach to identify which projections in this area express FXGs. Synapsin-Cre mice were useful tools to address this question since they express Cre in VAL, but not in TRN, striatum or the majority of cortex (Fig. 7G–L; see also Methods). In *syn-cre;fmr1^{flox/y}* animals, FXGs in axons of Cre-expressing neurons lack FMRP (Fig. 6). Thus, in this circuit, FMRP would be absent in FXGs arising from VAL, but present in those originating from TRN, striatum or cortex. We observed that FMRP was lacking in essentially all FXGs in the thalamus in both VAL (arrowheads in Fig. 7K) and TRN (arrowheads in Fig. 7L). FMRP was also absent in nearly all FXGs in fiber bundles passing through striatum (arrowheads in Fig. 7J). These findings indicate that FXGs are expressed in thalamocortical, but not corticothalamic axons.

These mutants also allowed determination of the origin of cortical FXGs. We observed two populations of FXGs in motor cortex and the underlying fiber tracts (Figs. 7 H, I): one containing FMRP (arrows) and one lacking FMRP (arrowheads). Taken together with the results discussed above, these findings indicate that the FMRP-containing FXGs in cortex and subcortical fiber tracts are likely to be present in corticocortical axons. Notably, these intracortical connections must arise ipsilaterally as FXGs are not observed within the corpus callosum. The cortical FXGs lacking FMRP are most likely of thalamocortical origin. In sum, these data demonstrate that FXGs are expressed in thalamocortical and ipsilateral corticocortical axons, but are not a feature of corticothalamic fibers.

DISCUSSION

In this study we have mapped the distribution of FXGs in the P15 mouse brain. We have shown that FXGs are expressed in axons within fiber tracts as well as in terminal fields. A general theme of our findings is that FXGs are expressed in many brain regions, but that in each region FXGs are restricted to particular circuits. We further characterized subcircuits within select FXG-expressing regions. In several of these regions, FXG-containing projections show subregion selectivity in their pattern of innervation: neocortical FXGs are highly enriched in motor regions, hippocampal mossy fiber FXGs are more numerous within the ventral hippocampus, and cerebellar FXGs are enriched in the vermis. FMRP is a

component of FXGs in all brain regions examined except the brainstem. Specific removal of FMRP from select neuronal populations recapitulates results seen in conventional *fmr1* null mice. Furthermore, as discussed below, removing FMRP from thalamus demonstrates that cortical FXGs arise from a combination of thalamocortical and intracortical axons. Axonal and presynaptic Fragile X proteins are therefore positioned to contribute to circuit formation and function underlying a variety of behaviors. This contribution could have a significant impact on the brain since FMRP targets encode roughly one-third of the presynaptic proteome (Akins et al., 2009; Darnell et al., 2011). We discuss below how presynaptic Fragile X proteins may contribute to selected circuits.

FXG abundance

FXGs are abundant within select circuits throughout the nervous system. We identified 155,344 FXGs in the analyzed sections. This value is likely to reflect the abundance of FXGs in a typical animal of this age, as a previous analysis across animals indicated reproducible counts of FXG number in olfactory bulb, neocortex, and hippocampus (Christie et al., 2009). Since only every 5th section was surveyed, this measure suggests a conservative estimate of the minimum number of FXGs to be approximately 750,000 in a P15 mouse brain. However, this number is undoubtedly an underestimate of actual FXG density (Fig. 4; see Methods). Moreover, the plane of focus of the objective used to acquire the images was ~5% of the section thickness (see Methods). Therefore the actual number seems likely to be at least an order of magnitude higher and may exceed 10,000,000. Notably, FXGs are plentiful within those axons that contain them (Fig. 3). For example, FXGs are expressed relatively uniformly along the axons of olfactory sensory neurons both within the olfactory nerve layer and within the glomerular neuropil (Figs. 4,5,6; Christie et al., 2009). Within glomeruli, FXGs occur at a density as high as 1.52 per 10 μm^2 in P30 mice (Christie et al., 2009). With a depth of field of approximately 1 μm , this correlates to 1.52 FXGs per 10 μm^3 . By comparison, ultrastructural analysis shows that there are approximately 6 olfactory sensory neuron synapses per 10 μm^3 of glomerular neuropil at this age (Hinds and Hinds, 1976). Thus, in this region there may be as many as one FXG per four synapses, highlighting FXG abundance within these axons. FXGs are therefore positioned to play a significant role in shaping axonal and synaptic architecture, with individual FXGs likely to regulate a highly localized portion of an axonal arbor.

FMRP in FXGs is of neuronal origin

FMRP is expressed by both neurons and glia (Pacey and Doering, 2007; Christie et al., 2009). Since axonal translational machinery including RNA may be transferred from glia to axons (Giuditta et al., 2008), we asked whether the FMRP in FXGs was of neuronal or glial origin. We found that neuron-specific FMRP ablation closely phenocopied removal of FMRP from all cells. FXGs in axons of these neurons did not contain FMRP and the density of FMRP-null FXGs was increased in the neuron-specific mutants (Fig. 2; Christie et al., 2009). Therefore, glial FMRP does not detectably contribute to either the regulation or composition of the axonal FXGs.

FXGs in short- and long-range projections

The expression of FXGs in fiber tracts indicates that presynaptic Fragile X proteins may play a role in long-range communication between brain regions. Tracts containing FXGs show remarkable restriction within the forebrain. For example, FXGs are particularly enriched in fibers innervating motor cortex but essentially absent in those innervating prefrontal cortex. Of note, with the exception of axons in the ventral hippocampal commissure, FXG-containing projections appear to be exclusively ipsilateral. FXGs are highly expressed in thalamocortical circuits (Figs. 5,6), reflecting expression in both thalamocortical and corticocortical axons (Fig. 7). FXGs are particularly enriched in thalamocortical axons innervating motor cortex, but are also present in thalamocortical projections throughout neocortex, consistent with FMRP localization to thalamocortical terminals in barrel cortex at this age (Till et al., 2012).. FXGs may also be expressed in corticofugal projections, as granules in the olfactory limb of the anterior commissure may reflect expression in ipsilateral projections from piriform cortex to the olfactory bulb (Davis and Macrides, 1981). Taken together, these findings suggest that presynaptic Fragile X proteins influence information processing via modulation of both short-range and long-range inputs.

FXG expression in recurrent projections within CA3

Hippocampal CA3 pyramidal neurons project associational connections to both ipsilateral and contralateral CA3 as well as Schaffer collaterals to CA1. FXGs are observed in fibers within CA3 *stratum oriens*, including as these fibers cross the midline in the ventral hippocampal commissure. In contrast, FXGs are not found in CA1 (Figs. 5,6; Christie et al., 2009). Consistent with expression of these FXGs by CA3 pyramidal cells, genetic removal of FMRP from these neurons results in loss of FMRP from FXGs in CA3 *stratum oriens* (Fig. 2). FXGs are thus expressed within CA3 axonal arbors innervating CA3 but not CA1. Presynaptic Fragile X proteins are thus likely to function in recurrent connectivity affecting the output from CA3, while not directly affecting feedforward input into CA1. Loss of this axonal role might be of particular relevance to the increased incidence of epilepsy in FXS patients. It will be of interest to determine whether the FXG-expressing axons in *stratum oriens* terminate on pyramidal neurons or interneurons.

Branch-specific FXG expression in the hippocampus?

The expression of FXGs in CA3 associational fibers, but not Schaffer collaterals, raises two intriguing possibilities. First, FXGs could be expressed in a subset of CA3 neurons that innervate both the ipsilateral and contralateral CA3, but lack true Schaffer collaterals (Lorente de No, 1934; Swanson et al., 1978; Ishizuka et al., 1990). Second, FXGs could be expressed within select branches of individual CA3 neurons. In the latter case, target-derived cues may regulate the subcellular localization of FXGs in those cells that are competent to produce them. Future experiments will address the basis of this subcircuit-specific FXG expression.

FXGs in sensory systems

Loss of FMRP leads to sensory defects in both humans and mice. FXS patients are hypersensitive to sensory stimuli, while *fmr1* null mice undergo severe seizures in response to loud auditory stimuli (Miller et al., 1999; Chen and Toth, 2001; Baranek et al., 2008). FXGs are expressed in both olfactory (olfactory bulb) and somatosensory (external cuneate and cuneate nuclei) systems. The findings in the cuneate and external cuneate nuclei are consistent with previous reports of FMRP expression in axons of dorsal root ganglia neurons (Price et al., 2006). Further, while the solitary nucleus is difficult to delineate, we saw FXGs located lateral to the dorsal motor nucleus of the vagus. This localization is consistent with expression in the rostral solitary nucleus, which receives gustatory information. We did not see FXGs in either the medial nucleus of the trapezoid body or in the optic tract. However, it is notable that within the auditory brainstem FMRP associates with the potassium channel Slack, which is largely presynaptic (Brown et al., 2010). Additionally, FXGs are abundant in the reticular formation, which modulates sensory input to the forebrain, particularly for pain sensation (Mason, 2001). Presynaptic Fragile X proteins are thus positioned to play a role in transmitting sensory information for at least some modalities, including at the initial synapse. Such an action could contribute to the sensory hypersensitivity characteristic of FXS.

FXGs in motor regions

Motor performance is abnormal in the absence of FMRP. FXS patients exhibit stereotyped motor behaviors typical of autistic patients. Affected individuals also exhibit developmental delay of both gross and fine motor skills, with fine motor particularly affected (Largo and Schinzel, 1985; Roberts et al., 2009; Zingerevich et al., 2009). Retrospective analysis of home videos indicates that motor symptoms are present even during infancy (Baranek et al., 2008). Furthermore, at later ages patients have difficulty planning and coordinating movements (Kau et al., 2002). Mice lacking FMRP have temporal defects in oromotor behaviors (Roy et al., 2011). Our findings suggest that dysregulation of presynaptic Fragile X proteins may contribute to these motor symptoms. FXGs are strikingly enriched in several regions involved in motor control including the cerebellum, reticular formation and thalamocortical and corticocortical axons innervating primary and secondary motor cortex. These results raise the possibility that loss of presynaptic FMRP may particularly affect motor function in FXS patients. Future studies will address the contribution of presynaptic Fragile X proteins to cognitive control of motor planning.

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Figure 1.

Specificity of anti-FXR2P. BU38 antiserum was generated as described in Methods. **(A)** BU38 specifically recognized a single polypeptide with an apparent mobility of ~90kD (arrow) in extracts of whole brain from P15 wild type, but not FXR2^{-/-} mice. **(B)** BU38 does not recognize FMRP or FXR1P. Lysates from COS cells transfected with constructs encoding EGFP alone or EGFP-tagged FXR2P, FMRP or FXR1P were probed with BU38 ('FXR2P'). This antibody recognized EGFP-FXR2P (arrow) as well as the endogenous FXR2P (asterisks). The expression of all transfected constructs was verified by probing the same samples with anti-GFP ('GFP').

Figure 2.

Cellular origin of FXGs. FXG expression in hippocampus of wild type (A–D) and synapsin-Cre *fmr1* cKO (E–H) mice. FXGs in wild type mossy fibers in *stratum lucidum* (A; inset in B) contained both FXR2P (green) and FMRP (magenta). FXGs in wild type commissural/associational fibers in *stratum oriens* (C; inset in D) also contained both FXR2P and FMRP. In contrast, FXGs in cKO mice, where FMRP is selectively ablated in neurons, contained FXR2P but not FMRP in both *stratum lucidum* (E; inset in F) and *stratum oriens* (G; inset in H). Note that FXGs in these populations were also more abundant than in the wild type animals. These observations indicate that FXGs are of neuronal origin. Scale bar in A = 10 μ m for A, C, E, G; 2.5 μ m in B, D, F, H.

Figure 3.

FXGs are expressed in fiber tracts and along axons. **(A; inset in B)** FXGs (arrows in B) in the striatum localized to neurofilament-expressing fiber tracts, but were absent in the surrounding neuropil. Single confocal plane for A and B. **(C; inset in D)** FXGs (arrows in D) occurred frequently along neurofilament-expressing axons in neocortex. C is a collapsed z-stack of three optical planes; D is from a single optical plane. NF: neurofilament; FMRP: Fragile X mental retardation protein. Scale bar = 20 μm in A, C; 7 μm in B, 10 μm in D.

Figure 4.

Mapping FXGs in the mouse olfactory bulb. **(A)** Montage of a wild type P15 olfactory bulb section stained with anti-FXR2P. **(B–D)** Inset from **A** showing granules in the olfactory nerve layer. **(B)** Original image. **(C)** Image after processing to highlight granules. **(D)** FXGs were manually annotated (red dots). White arrows in **B–D** indicate granules identified as FXGs by this methodology; black arrows indicate FXGs that were filtered out in processing. **(E)** Original image from **A** inverted and overlaid with FXGs (red) and contours of morphological features (black lines). Note that FXGs are largely restricted to the olfactory nerve layer and glomerular layer. **(F)** FXGs (black) are overlaid upon a drawing of the section in **E**. See Table 2 for abbreviations. Scale bar = 500 μ m in **A**, **E**, **F**; 25 μ m in **B–D**.

Figure 5.

An overview of FXG distribution in the brain. Montage of a sagittal section from a P15 wild type brain. **(A)** FXGs (red) overlaid on an image of the section. **(B)** FXGs (black) overlaid upon contours. FXGs showed a restricted distribution, with highest expression in olfactory bulb, rostral neocortex, hippocampus, thalamus, and brainstem. Inset: Detailed view of FXG distribution in the thalamus. Note the enriched FXG expression in VA, VL, AV and TRN. See Table 2 for abbreviations. Scale bar = 1 mm in main image; 667 μ m in inset.

Figure 6.

Systematic mapping of FXG distribution in the P15 mouse brain. Serial coronal sections were immunostained for FXR2P and imaged, processed, annotated and displayed as described in Fig. 4. A single hemisphere from every fifth section is depicted.

Each section is shown twice: once with red FXGs overlaid upon an inverted fluorescent image and once with black FXGs overlaid upon a drawing of the contours of the section. Portions lost in tissue preparation are indicated with a dashed line. Inset in Q depicts the ventral hippocampal commissure from a section adjacent to the one mapped in Q. Note the restricted distribution of FXGs to distinct brain regions. FXGs expression was selectively enriched in subregions of several structures including olfactory bulb (A-F), motor cortex (F-P), thalamus (P-Z), hippocampus (Q-Z), and brainstem (Y-PP). Note that FXGs were rare in other brain structures including prefrontal cortex (C-J) and corpus callosum (L-W). See Table 2 for abbreviations.

Scale bar for all images = 1 mm.

Figure 7.

Genetic dissection of FXG origin in thalamocortical circuitry. Boxes in A and G indicate where in the circuit images in B–F and H–L, respectively, were acquired: (B, H) motor cortex, (C, I) external capsule, (D, J) striatum, (E, K) VAL, and (F, L) TRN. (A) Basic diagram of circuits connecting the thalamus and motor cortex in wild type mice. Corticocortical connections are also indicated. Cortical neurons (red) form synapses onto each other as well as projecting onto thalamic neurons in the ventral anterior and ventral lateral nuclei (VAL; dark blue) and in the reticular nucleus (TRN; light blue). VAL neurons project to both neocortex and TRN. In wild type brain all FXGs in these regions contained both FXR2P (green) and FMRP (magenta). (G) Circuit diagram from the synapsin-Cre cKO mice. All neuronal components are present as in wild type, but FMRP expression was selectively ablated in VAL neurons (cross-hatching). FXGs in striatum (J), VAL (K), and TRN (L) of the cKO mice contained FXR2P but not FMRP (arrowheads). FXGs in motor cortex (H) and subcortical fiber tracts (I) of the cKO mice consisted of two populations: those containing FMRP (arrows) and those lacking FMRP (arrowheads). Scale bar = 10 μm for B–F, H–L.

Table 1

Antibodies used in this study

Primary Antibody (Dilution/Concentration)	Immunogen	Source	Specificity	Secondary Antibody (Dilution)
Mouse anti-FMRP 2F5-1 (4 µg/mL)	raised by immunizing <i>fmr1</i> null mice with amino acids 1–204 of human FMRP	N/A	recognizes bands of the appropriate size by Western, does not give signal in either blots or sections derived from <i>fmr1</i> null mice (Gabel et al., 2004; Christie et al., 2009).	Alexa-conjugated goat anti-mouse IgG2b (Invitrogen; 1:1000)
Rabbit anti Neurofilament-150 (1:1500)	raised against highly purified bovine neurofilament peptide	Millipore, AB1981	recognizes 150 kDa neurofilament with minimal cross-reactivity to either the 68 or 200 kDa isoforms; stains neurons and axonal tracts (Fig 3)	Alexa-conjugated goat anti-rabbit (Invitrogen; 1:1000)
Mouse anti-FXR2P 1G2 (1:1000)	raised against recombinant FXR2 residues 414–658	Ascites form of the monoclonal antibody 1G2 was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology;	recognizes single band of appropriate size (Zang et al., 2009)	Alexa-conjugated goat anti-mouse IgG1 (Invitrogen; 1:1000)
Rabbit anti-FXR2P BU38 (1:3000)	Raised as described in Methods.	N/A	See Methods and Fig. 1.	Immunofluorescence: Alexa-conjugated goat anti-rabbit (Invitrogen; 1:1000); Western: HRP-conjugated goat anti-rabbit (KPL; 1:2000)
Mouse anti-GFP (1:2)	raised against the full length green fluorescent protein.	NeuroMab N86/8 (supernatant form)	Recognizes a single band in COS-7 cells transfected with GFP (Fig. 1).	HRP-conjugated goat anti-mouse (KPL; 1:2000)

Table 2

Abbreviations used

AC	Anterior commissure
AOB	Accessory olfactory bulb
AV	Anteroventral nucleus of the thalamus
EX CU	External cuneate nucleus
GL	Olfactory bulb glomerular layer
LD	Laterodorsal nucleus of the thalamus
LP	Lateral posterior nucleus of the thalamus
ONL	Olfactory nerve layer
Po	Posterior nuclear group of the thalamus
STR	Corpus striatum (caudate, putamen, globus pallidus, nucleus accumbens)
THA	Thalamus
TRN	Reticular nucleus of the thalamus
VA	Ventral anterior nucleus of the thalamus
VAL	Ventral anterior and ventrolateral nuclei of the thalamus
VL	Ventrolateral nucleus of the thalamus
VPM	Ventral posteromedial nucleus of the thalamus
VM	Ventromedial nucleus of the thalamus

Table 3**FXG number by section**

FXGs manually counted in both hemispheres of the brain pictured in Fig. 6

Section #	Fig. 6 Panel	# of FXGs
1	*	553
2	*	5,860
3	*	8,678
4	*	2,845
5	*	5,381
6	A	3,280
7	B	3,426
8	C	436
9	D	426
10	E	380
11	F	1,395
12	G	1,928
13	H	4,617
14	I	9,254
15	J	7,199
16	K	2,260
17	L	741
18	M	975
19	N	1,075
20	O	1,203
21	P	761
22	Q	6,347
23	R	7,797
24	S	10,233
25	T	2,316
26	U	1,422
27	V	2,301
28	W	5,017
29	X	5,288
30	Y	7,887
31	Z	1,000
32	AA	2,016
33	BB	990
34	CC	891
35	DD	1,609
36	EE	239
37	FF	514
38	GG	1,305

Section #	Fig. 6 Panel	# of FXGs
39	HH	3,102
40	II	2,327
41	JJ	3,211
42	KK	3,586
43	LL	4,118
44	MM	4,728
45	NN	6,491
46	OO	4,170
47	PP	3,766
Total		155,344

* Olfactory bulb sections not depicted in Fig. 6.

Table 4

Summary of FXG distribution in P15 mouse brain

Brain Region	FXG-rich subregions	FXG-poor subregions
Fiber Tracts	Anterior commissure (olfactory limb) External capsule Cingulum	Anterior commissure (anterior and posterior limbs) Corpus callosum Pyramids Corticospinal tract Optic tract Stria medullaris Stria terminalis
Olfactory Bulb	Olfactory nerve layer Glomerular layer	External plexiform layer Internal plexiform layer Granule cell layer Lateral olfactory tract
Neocortex	Primary motor cortex Secondary motor cortex	Prefrontal cortex Cingulate cortex Retrosplenial cortex
Hippocampus	Mossy fibers CA3 associational/commissural fibers (stratum oriens)	Schaffer collaterals
Thalamus	Anteroventral nucleus Ventral anterior nucleus Ventrolateral nucleus Reticular nucleus	
Striatum	Fibers of passage	Striatal nuclei
Ventral Forebrain		Hypothalamus Amygdala Septal Nuclei
Brainstem	Cerebellar molecular layer Cerebellar white matter External cuneate nucleus Reticular formation Inferior colliculus	

See Figs. 5 and 6 for details