Contribution of mGluR and Fmr1 functional pathways to neurite morphogenesis, craniofacial development and fragile X syndrome

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Fragile X Syndrome is a leading heritable cause of mental retardation that results from the loss of FMR1 gene function. Studies in mouse and Drosophila model organisms have been critical in understanding many aspects of the loss of function of the FMR1 gene in the human syndrome. Here, we establish that the zebrafish is a useful model organism for the study of the human fragile X syndrome and can be used to examine phenotypes that are difficult or inaccessible to observation in other model organisms. Using morpholino knockdown of the fmr1 gene, we observed abnormal axonal branching of Rohon–Beard and trigeminal ganglion neurons and guidance and defasciculation defects in the lateral longitudinal fasciculus. We demonstrate that this axonal branching defect can be rescued by treatment with MPEP [2-methyl-6-(phenylethynyl) pyridine]. This is consistent with an interaction between mGluR signalling and fmr1 function in neurite morphogenesis. We also describe novel findings of abnormalities in the abundance of trigeminal ganglion neurons and of craniofacial abnormalities apparently due to dysmorphic cartilage formation. These abnormalities may be related to a role for fmr1 in neural crest cell specification and possibly in migration.

INTRODUCTION

Fragile X syndrome is the most common form of inherited mental retardation. The degree of mental retardation is moderate to severe and tends to be worse in males. Behavioural abnormalities of fragile X syndrome include developmental delays, hyperactivity, anxiety and autistic behaviours (1,2). Craniofacial abnormalities in adolescent and adult patients include a long thin face with prominent ears, facial asymmetry, a large head circumference and a prominent forehead and jaw. Common peripheral symptoms include heightened sensitivity to tactile irritation.

Fragile X syndrome is caused by the absence of the RNA binding protein fragile X mental retardation protein (FMRP) (3). Loss of the FMRP is due to a trinucleotide repeat expansion in the 5'-UTR of the FMR1 gene. FMR1 is involved in the control of protein synthesis and FMRP shuttles from the nucleus to the cytoplasm as an mRNP particle (4). It appears that FMRP represses translation of its target mRNAs (5–7), and it may be alleviation of this repression by FMRP loss that leads to the syndrome. FMRP target mRNAs include those of microtubule-associated protein 1B (MAP1B), the FMR1 message itself, FMR1-related genes, FXR1 and FXR2, as well as a number of other mRNAs involved in neuronal development, plasticity/maturaton of synapses and calcium regulation (8–11).

Although fragile X syndrome is not associated with specific brain malformations or neurodegeneration, a subtle neuronal defect has been found in dendritic spines which exhibit a long, spindly ‘immature’ morphology in pyramidal cells of the cortex of fragile X syndrome patients (12,13). This phenotype has been recapitulated in a mouse model (14), along with enhanced long-term depression (LTD) in the hippocampus. LTD is a result of increased activity of the metabotropic glutamate receptor (mGluR) group I type 5 (15). This receptor alters FMRP levels at the synapse and is associated with a protein-synthesis-dependent lengthening of dendritic spines (16,17).

Dendritic spines are actin-rich structures. It has been suggested that an interaction between the FMRP and the Rac1 pathway, which is known to regulate actin dynamics, may be related to FMR1 loss-of-function synaptic defects...
The Rac1 pathway has been linked to FMRP in a study of higher order neurite morphogenesis (20) through knockdown of the Drosophila dfmr1 (dfxr) gene. Lee et al. (20) described an increased level of dendritic branching in Drosophila dendritic arborizing neurons lacking dFmrp. These neurons branch more frequently than normal, whereas overexpression of dfmr1 leads to a reduction in the level of branching. Furthermore, they demonstrated that overexpression of Rac1 causes an increase in branching and this can rescue the effects of dfmr1 overexpression (20).

Mouse models have been generated, which recapitulate several behavioural and physical characteristics of fragile X syndrome, including hyperactivity and macroorchidism (21,22). There has been no report of neurite branching abnormalities in fragile X syndrome patients or in a mouse model, and it has been suggested that such effects may be masked in vertebrates by redundant function from the presence of FMRP-related proteins, FXR1 and FXR2 (19,23). The zebrafish fmr1 gene family has been identified and its expression pattern described (24–26). The zebrafish has orthologues of all three FMR1-related genes, suggesting that any masking or redundancy of FMRP function by related proteins in mouse or human will be replicated in a fish model of the syndrome. Studies in zebrafish may thus be more relevant to understanding the human syndrome than those in Drosophila.

The enhanced mGluR activity in the brains of Fmr1 knock-out mice, as well as the connection between mGluR signalling and human/mouse fragile X syndrome-related behavioural phenotypes, suggests that inappropriate mGluR signalling is the cause of at least part of the disease phenotype (27). Pharmacological treatment with an mGluR5-specific antagonist MPEP [2-methyl-6-(phenylethynyl)pyridine] has recently been shown to rescue some behavioural defects in knockout mice (28). It can rescue behavioural and mushroom body defects in Drosophila lacking dfmr1 activity.

In this study, we establish the zebrafish embryo as a model for fmr1 loss-of-function analysis. Morpholino antisense oligonucleotide repression of fmr1 mRNA translation in zebrafish embryos is observed to produce changes in neurons and in neurite branching in the central and peripheral nervous systems that are not masked by the partially redundant activities of fxr1 and fxr2. Modulation of mGluR signalling can rescue these neurite branching defects. We propose a model for the interaction of Fmr1 and mGluR in control of axonal branching, guidance and fasciculation that has implications for the synaptic morphology component of fragile X syndrome. We also show the effects of fmr1 expression on trigeminal neuron number and craniofacial pattern, which suggest a role for this gene in cranial neural crest specification and possibly migration.

METHODS

Zebrafish

Zebrafish were bred and maintained under standard conditions at 28.5°C (29). Morphological features were used to determine the stage of the embryos in hours (hpf) or days (dpf) post-fertilization according to Kimmel et al. (30).

Morpholinos

For transient knockdown of gene expression, morpholino antisense oligomers (morpholinos, MO; GeneTools, LLC) were prepared to target and inhibit the translation of fmr1 mRNA. Two non-overlapping morpholinos were designed targeting endogenous fmr1 transcript, fmr1MOA (5′-agctgctctagcgccagtt-3′) and fmr1MOB (5′-egcgttcctactagccaggg-3′). A standard control morpholino (ControlMO; GeneTools, LLC) was used to control for morpholino injection-specific effects. All morpholinos were diluted in E3 solution (29).

In initial experiments, fmr1MOA and fmr1MOB were injected individually and were compared with embryos injected with a standard control morpholino (ControlMO). An initial titration of the anti-fmr1MOs was conducted, based on observed gross morphological defects and mortality. These experiments suggested an appropriate fmr1MOA to fmr1MOB ratio, based on their relative optimal concentration. Both morpholinos were then co-injected at this ratio and titrated again to find the optimal injection protocol. The concentration used for complete knockdown was equal part of 0.5 mM fmr1MOA and 0.6 mM fmr1MOB (final concentration 0.25 mM fmr1MOA and 0.3 mM fmr1MOB) and embryos were injected with a total of ~5 nl/embryo. Standard control morpholino was injected as a control at a concentration of 0.55 mM. The rationale is that reduction of individual morpholino concentrations as much as possible while retaining complete knockdown is preferable to injection of higher concentrations of a single morpholino. Efficacy may be improved by having multiple sites on a particular mRNA recognized by morpholinos as opposed to a single site. Secondly, low concentrations of morpholinos with non-overlapping sequences should have less sequence-related non-specific defects than injection of a single sequence morpholino at higher concentrations. As individual injections of fmr1MOA at 0.25 mM and of fmr1MOB at 0.3 mM are not distinguishable from normal or ControlMO injection, it can be inferred that non-specific defects (if any) are not morpholino sequence specific.

As fmr1 mRNA has previously been found to be maternally expressed, all morpholino injections were performed at one-cell stage, and injections into later cell stages resulted in incomplete knockdown (data not shown).

MPEP treatment

After injection, MPEP-treated embryos were developed in 250 ng/ml MPEP dissolved in E3 embryo medium (29).

Western blot

For western blot analyses, 10 dechorionated embryos for each condition (24 hpf) were directly homogenized in SDS lysis buffer (63 mM Tris–HCl, pH 6.8, 10% glycerol, 5% β-mercaptoethanol, 3.5% sodium dodecyl sulphate) and subjected to SDS–PAGE (29). Lysate was probed against a polyclonal mouse anti-Fmrp antibody (Abnova). The membrane was then stripped and probed with β-tubulin as a loading control (Fig. 1A). The final concentrations used were fmr1MO 0.25 mM A/0.3 mM B.
Described by Jowett (31) using single-stranded RNA probes in situ. Whole-mount in situ was performed as described by Jowett (31) using single-stranded RNA probes labelled with digoxigenin-UTP (Roche Ltd, Basel, Switzerland) according to the manufacturers’ protocols. Probes against axial, dlx-1a, dlx-2a, dlx-4a, emx1, emx3, fgfr1, hlx1, lim1, otx2, pax6 and wnt1 transcripts were synthesized from cDNA clones using T7 or T3 RNA polymerase as appropriate. Stained embryos were photographed in 80% glycerol following fixation using a Fujix HC-1000 camera attached to a Zeiss Axiopt microscope.

**Antibody stains**

For whole-mount immunohistochemistry, embryos were fixed in 4% formaldehyde overnight, incubated at 65°C in FST solution overnight (50% formamide, 2× SSC, 0.1% Tween-20), washed in PBT, blocked in ABS (PBS containing 2% DMSO, 0.1% IGEPAL, 2% horse serum, 2% BSA, 0.1% Tween) and incubated overnight in the monoclonal antibody ZN-12 (1:200; Zebrafish International Resource Center, Eugene, OR, USA) or anti-acetylated α-tubulin (1:1500; Sigma, St Louis, MO, USA). Primary antibody was washed off in ABS, and embryos were incubated overnight in donkey anti-mouse secondary antibody conjugated to Alexa-488 (4 µg/ml; Molecular Probes, Eugene, OR, USA). Embryos were imaged with a Leica SP5 spectral scanning confocal microscope and Leica LAS AF software, or with a Zeiss Axioplan 2 microscope with a Zeiss ApoTome attachment. Images were taken with a Zeiss AxioCamMR camera and analysed with Zeiss AxioVision V4.5 software. Image analysis was conducted using the NeuronJ plugin (32) through National Institutes of Health (NIH) ImageJ software.

**Whole-mount Alcian blue skeletal stainings**

Alcian blue-stained cartilage preparations were prepared according to Neuhauss et al. (33) and were imaged in 80% glycerol. Images taken with a Fujix HC-1000 camera attached to a Zeiss Axiopt microscope and were analysed with Zeiss AxioVision V4.5 software.

**RESULTS**

Anti-fmr1 morpholino design, validation and initial characterization

Fragile X syndrome is caused by expansion of a 5’-UTR trinucleotide repeat sequence in the FMR1 gene that silences gene transcription, resulting in the loss of FMRP. To model this in zebrafish embryos, we inhibited translation of the mRNA of the zebrafish FMR1 orthologue, fmr1 (GenBank accession no. AF305882), by injection of morpholino antisense oligonucleotides (MO). Two non-overlapping morpholinos were synthesized and designed to block translation of fmr1 mRNA in zebrafish embryos. In order to demonstrate the rescue with an internal control, bilateral injections were conducted. Embryos injected with fmr1MO at one-cell stage were subsequently coinjected with fmr1 mRNA and high molecular weight tetramethylrhodamine dextran (Molecular Probes) to mark cell lineage into one-cell of a two-cell stage embryo. fmr1MO and fmr1 mRNA injections were separated by approximately half an hour. All embryos were then stained for dlx-2a or fgfr1 marker genes to illustrate bilateral differences, and these embryos were photographed in 80% glycerol using a Fujix HC-1000 camera attached to a Zeiss Axiopt microscope.

**Whole-mount in situ Hybridization**

Whole-mount in situ transcript hybridization was performed as described by Jowett (31) using single-stranded RNA probes. For comparison, also shown are extracts from embryos co-injected with 0.125A/0.15B and 0.05A/0.06B. (B) Ratio of anti-Fmr1 signal to anti-β-tubulin signal, expressed graphically.
expression was completely suppressed (Fig. 1) as assayed by western blotting using a mouse antibody against human FMRP (Abnova). This concentration was subsequently used throughout this study and is henceforth referred to as an ‘fmr1MO’ injection.

Gross morphological phenotypes resulting from fmr1MO injection were analysed (Fig. 2). fmr1MO causes morphological changes in the midbrain/hindbrain boundary (47/60; MHB) and a distinctive lack of organization in the brain (Fig. 2A and B). By 24 hpf, the anterior extension of the head appears incomplete in some embryos (13/60; Fig. 2E and F). At 48 hpf, some embryos display expanded brain ventricles and/or pericardial oedema (6/60). This is generally subtle in nature (Fig. 2C and D).

At the molecular level, fmr1MO injection causes an altered pattern of transcription of the ventral telencephalon/diencephalon marker dlx-2a (17/30; Fig. 3B and D) relative to those injected with controlMO (0/30; Fig. 3A and C). Expression of the MHB marker fgfr1 is also perturbed (11/30; Fig. 3G) relative to controlMO-injected embryos (0/30; Fig. 3F). Whole-mount in situ hybridization with axial reveals an occasional axial defect (3/30; Fig. 2E and F).

To confirm that the neural defects caused by fmr1MO injection result specifically from suppression of fmr1 activity, we attempted the rescue of the defects by co-injection with an fmr1 mRNA modified to lack fmr1MO binding sites. Co-injection of modified fmr1mRNA and fmr1MO at the one-cell stage results in phenotypically normal embryos (dlx-2a 28/30; fgfr1 30/30). This rescue was confirmed by injection of embryos first at the one-cell stage with fmr1MO and subsequently unilaterally at the two-cell stage with a mixture of modified fmr1 mRNA and the lineage tracer tetramethyl-rhodamine dextran (MW 2 000 000). Unilateral injection of mRNA into one-cell of the two-cell stage embryo allows for one side of the embryo to display the mRNA rescue and for the other side of the embryo to act as an internal control for this rescue. Whole-mount in situ transcript hybridization was then used to reveal expression of dlx-2a and fgfr1 (Fig. 3). dlx-2a expression appeared normal in the mRNA-injected half but was reduced in the half lacking modified fmr1 mRNA (13/30; Fig. 3E). In the MHB, fgfr1 is perturbed in fmr1MO-injected embryos (11/30; Fig. 3G) compared with normal/controlMO-injected embryos (0/30; Fig. 3F).

Neurite morphology is altered by the loss of fmr1 and can be returned to normal by MPEP

A number of reports have demonstrated a link between neurite morphogenesis or synaptogenesis and the function of FMRP in mouse, humans (14,34,35) and Drosophila (19,20,35–40). Many of these studies focus on the role of FMRP in postsynaptic sites and dendritic spines.

Recent findings have been made in Drosophila regarding neurite growth/branching defects. Lee et al. (20), Michel et al. (37), Morales et al. (38) and Pan et al. (39), reported the possibility of different functions of Drosophila dfmrp in different neurons. Recently, suppression of mouse FMRP-related behavioural phenotypes (28,41) and Drosophila synaptic/mushroom body fusion defects (41) has been achieved through use of the mGluR antagonist, MPEP. These studies suggest that fragile X syndrome is a consequence of exaggerated response to mGluR activation (27,28,41). Here, we examine whether the loss of fmr1 function affects neurite morphogenesis, whether this can be rescued in vertebrates using MPEP and whether the effects of MPEP alone are equivalent to ectopic expression of fmr1 mRNA.

To determine whether change in fmr1 expression affects neurite morphogenesis, we stained 24 hpf embryos with the monoclonal antibody zn12, which marks a subset of neurons including Rohon–Beard (RB) primary sensory neurons in the developing spinal cord and neurons of the trigeminal ganglion (Fig. 4). In embryos with reduced Fmr1 protein expression due to fmr1MO injection, we found a highly...
Figure 3. Defects associated with injection of fmr1MO can be rescued by injection of fmr1 mRNA. (A–E) Whole-mount in situ hybridization for dlx-2a. (A and B) Lateral view, anterior to left, dorsal to top. (C–E) Dorsal view, anterior to left. (A and B) Expression of dlx-2a in ventral telencephalon and ventral diencephalon is disrupted by injection of fmr1MO compared with controlMO-injected embryos. (C–E) Dorsal view of dlx-2a expression in telencephalon/diencephalon. (E) Unilateral injection of modified fmr1 mRNA and a high molecular weight rhodamine dextran marker (at two-cell stage) into fmr1MO-injected embryos. mRNA-injected side is marked here with an asterix. dlx-2a expression in the telencephalon/diencephalon is unilaterally rescued. (E') Cartoon illustrating unilateral asymmetry in (E). fmr1MO side ventral telencephalon (green) is abnormal compared with fmr1MO and fmr1M RNA-injected side ventral telencephalon (red). Injection of fmr1 mRNA at one-cell stage resulted in the rescue of dlx-2a expression (data not shown). Scale bars (A and B), 200 μm; (C–E), 100 μm.

statistically significant 2-fold increase in neurite branching in RB neurons (Fig. 4C; P < 0.001) and a similar increase in neurite branch terminations, suggesting that branches are generally shorter (42). An 8-fold increase in rare axon-guidance defects was also observed (fmr1MO-injected embryos: 0.16 guidance defects/mm neurite length; controlMO-injected embryos: 0.02 guidance defects/mm neurite length).

Having established a clear and measurable neurite morphological phenotype, we then attempted to rescue this morphology by antagonizing mGluR function with MPEP (Fig. 4B). Treatment of fmr1MO-injected embryos with MPEP resulted in a normal/controlMO phenotype (P < 0.751), suggesting that mGluR function is responsible for branching defects in the absence of Fmr1.

To examine the relationship between mGluR and Fmr1 further, we examined the effect on neurite morphogenesis of MPEP treatment alone and of ectopic/overexpression of Fmr1 following mRNA injection (Fig. 4D and E). Both these treatments resulted in simplification of neurite branching (P < 0.001; P < 0.001) and reduction in branch termination. This suggests that Fmr1 function influences neurite morphogenesis by facilitating mGluR signalling.

Extending our analysis to cells of the PNS, we examined the elaborately arborizing axons of the trigeminal ganglion neurons that make up part of the facial motor and sensory system (Fig. 4H–N). The effects of reduction or overexpression of fmr1 and of MPEP treatment were similar to those seen for RB neurons (Fig. 4A–G), suggesting that fmr1 function is consistent between, at least, these two neuronal populations.

Guidance defects were difficult to examine in neurites of the trigeminal ganglia, although such defects were identified in neurites projecting from the RB neurons. A search for other axon-guidance morphological defects resulted in the identification of a role for fmr1 in neurons in the placode that gives rise to the lateral longitudinal fasciculus (Fig. 5).

To examine functions of fmr1 in the lateral longitudinal fasciculus, we stained injected embryos with antibodies against α-acetylated tubulin at 5 dpf. In fmr1MO-injected embryos, we occasionally observed a dramatic axon defect (3/30; Fig. 5E). A more frequent phenotype of apparent axon defasciculation was also found (13/30; controlMO-injected 0/30; Fig. 5C, D and F). This appears initially as a termination of the lateral longitudinal fasciculus anterior to its normal termination site in the posterior of the tail generally before this reaches the posterior limit of the yolk extension. In contrast, in normal or control embryos, termination of the lateral longitudinal fasciculus occurs in the extreme posterior tail (Fig. 5A and B). Examination of the site of termination of the lateral longitudinal fasciculus leads to the identification of individual axons separating from the fasciculus (Fig. 5F).

A role for fmr1 in cranial neural crest specification and possibly migration

In addition to mental retardation, the characteristic phenotype of fragile X syndrome includes mild craniofacial anomalies such as a long face and prominent jaws (2). Observation of fmr1MO-injected fish has revealed defects in the craniofacial structure possibly related to those described in the syndrome (9/15; controlMO-injected 0/15; Fig. 6A and B). The defect is not prominent until 5 dpf when cartilage has formed. We performed an Alcian blue staining for cartilage (43,44) and found that Meckel’s cartilage was specifically affected. Meckel’s cartilage is both shorter (AP; P < 0.001; Fig. 6E–H) and wider (laterally; P < 0.05; Fig. 6F and G) in fmr1MO embryos than in normal/controlMO-injected...
Figure 4. Neurites of RB and trigeminal ganglion neurons tend to branch and terminate more frequently in embryos injected with fmr1 MO than in controlMO-injected embryos and less frequently in embryos injected with fmr1 mRNA or treated with MPEP. The phenotype caused by injection with fmr1 MO can be rescued with MPEP. (A–E) Lateral view, anterior to left, dorsal to top. (H–L) Dorsal view, anterior to left. Fluorescence imaging of RB and trigeminal ganglion neurons and neurites in embryos stained with the zn12 antibody at 24 hpf. All measurements were taken from the same area of neurites: (A–E) covering the yolk extension, at the posterior end of the yolk extension and (H–L) covering the region immediately anterior to the trigeminal ganglion. Red box indicates 90 × 90 μm² working area represented in (A′–E′) or 100 × 100 μm² working area represented in (H′–L′). (A, A′, H and H′) ControlMO-injected embryos. (B, B′, I and I′) fmr1 MO-injected embryos rescued by the application of MPEP. (C, C′, J and J′) fmr1 MO-injected embryos. (D, D′, K and K′) ControlMO-injected embryos exposed to MPEP. (E, E′, L and L′) fmr1 mRNA-injected embryos. No significant differences in the number of RB neurons were found between treatments, but significant variations were found in the number of trigeminal neurons between treatments (Fig. 7). (A′–E′ and H′–L′) Tracings of neurites in the working area. Tracings were made using the NeuronJ plugin (32) for National Institutes of Health (NIH) ImageJ software. (F and M) Branching frequencies per unit length neurite expressed graphically. (G and N) Termination frequencies per unit length neurite expressed graphically. (F, G, M and N) Significance analysed using Student’s t-test for unpaired samples. * denotes significance at P < 0.05, ** denotes significance at P < 0.01 and *** denotes significance at P < 0.001. Error shown is the SEM. (n = 10 for each treatment; scale bars 50 μm).
embryos. A minority of embryos were more severely affected as far posterior as the ceratobranchial arches (2/60; Fig. 6J).

Two very consistent abnormalities are found in the angles between individual cartilage components in fmr1 MO-injected embryos. First, the angle between ceratobranchial/ceratohyal arch cartilage and basibranchial/hyal cartilage tends to be less acute in fmr1 MO-injected embryos than in normal/controlMO-injected embryos. (Fig. 6F and G). Secondly, the angle that Meckels’ cartilage makes with the rostrocaudal axis (Fig. 6C and D) is also less acute in fmr1 MO-injected embryos than in normal/controlMO-injected embryos (P < 0.001). This phenotype is subtle compared with many other craniofacial defects found in fish (33,45–49).

Figure 5. The lateral longitudinal fasciculus is affected by injection of fmr1 MO. Axons contributing to the fasciculus often defasciculate at a much anterior location compared with ControlMO-injected embryos. A canonical axon-guidance defect is also observed. (A–F) Fluorescent analysis of ControlMO or fmr1 MO-injected embryos stained with antibodies against α-acetylated tubulin at 5 dpf. Lateral view, anterior to left, dorsal to top. (A and C) ControlMO-injected embryos. (B and D–F) fmr1 MO-injected embryos. (A and B) View of the full-length embryo. (C) In ControlMO-injected embryos at 5dpf, the lateral longitudinal fasciculus generally extends to the extreme posterior of the tail. (D) In fmr1 MO-injected embryos at 5dpf, the lateral longitudinal fasciculus often (13/30; ControlMO-injected 0/30) defasciculates before it extends beyond the posterior extent of the yolk extension. (E) More rarely (3/30; ControlMO-injected 0/30), the lateral longitudinal fasciculus in fmr1 MO-injected embryos demonstrates canonical axon-guidance defects. (F) Axon tracing of the lateral longitudinal fasciculus in (D) using NeuronJ (32) in ImageJ, illustrating defasciculation of the lateral longitudinal fasciculus. (A, B, D and F) are composite images. Arrowheads indicate posterior extent of the lateral longitudinal fasciculus. Scale bars (A and B), 200 µm; (C–F), 100 µm.

Figure 6. Craniofacial defects in fmr1 MO-injected embryos and in embryos treated with MPEP appear to be connected with perturbation of underlying cartilage. This is connected to a variety of non-specific effects that contribute to an overall abnormal morphology that is difficult to measure, possibly recapitulating ‘syndromic appearance’ of fragile X patients. (A–E, I and J) Lateral view, anterior to left, dorsal to top. (A and B) Nomarski optics. (C–H) Brightfield; Alcian blue-labelled. (I and J) Fluorescent analysis of injected embryos stained with antibodies against α-acetylated tubulin. (A and B) Embryos treated with fmr1 MO and MPEP exhibit distinct craniofacial abnormalities, appearing in both conditions as shortening of the length of the head and variation in ventral morphology. A number of non-specific abnormalities are common. fmr1 MO-treated embryos tend to be more severely affected. (C–H) This variation in morphology appears to be related consistently to alteration in the morphology of Meckles’ cartilage (anterior limit denoted by arrowhead) and also to the angle Meckels’ cartilage makes with the AP axis. (C–E) Frequency of reduction in the anterior extension of Meckle’s cartilage is quite consistent in fmr1 MO-injected embryos (12/15; ControlMO-injected 0/15; length difference fmr1 MO/ControlMO P < 0.001), but is also found to a lesser extent and greater variation in MPEP-treated embryos (8/15; length difference MPEP/ControlMO P < 0.05). (F–H) Width (lateral extension) of Meckels’ cartilage is consistently greater in fmr1 MO-injected embryos than either MPEP-treated (0/15; width difference fmr1 MO/MPEP P < 0.05) or ControlMO- injected (9/15; width difference fmr1 MO/ControlMO P < 0.001) embryos. Length and width of Meckels’ cartilage are indicated by parallel lines. (H) Rarely, very severely affected embryos may exhibit variations in cartilage as far posterior as the fifth ceratobranchial arch (2/30). (I and J) Craniofacial abnormalities also appear to be linked to a reduction in innervation of the face by cranial nerves (arrow). cb, ceratobranchial; ch, ceratohyal; hs, hyosymplectic; m, Meckel’s; pq, palatoquadrate cartilage (46). Scale bar 200 µm.
Meckel’s cartilage is shorter and wider than normal in fmrMO-injected embryos. However, interpretation of this result is complicated by our ignorance of the long-term (>2 days) efficacy of the injected morpholinos. Unexpectedly, a much less extreme and more variable, short rostrocaudal extension phenotype is also found in embryos treated with MPEP (P < 0.001, Fig. 6E). Meckels’ cartilage width in MPEP-treated embryos is not significantly different from normal or control embryos. Treatment of fmr1MO-injected embryos with MPEP returns the length and width of Meckels’ cartilage to close to normal.

Consistent with the neurite morphology phenotypes reported earlier, we also found a reduced level of innervation of the facial region by the cranial nerves (Fig. 6I and J).

The cranial ganglia and Meckels’ cartilage share a common origin in the neural crest. This suggested that problems in cranial ganglia and cartilage formation might be due to defective neural crest specification, migration or differentiation. To support this, we examined other neural crest-derived structures and found defects in the trigeminal ganglion.

In fmr1MO-injected embryos, we observed that the number of trigeminal neurons was significantly reduced relative to normal/controlMO-injected levels (Fig. 7). Upon examination of MPEP-treated and fmr1mRNA-injected embryos, we found a significant increase in the average number of trigeminal neurons in the ganglion (MPEP-treated P < 0.01; fmr1 mRNA-injected P < 0.05). Furthermore, these treatments increased the number of neurons beyond the maximum observed in normal/controlMO-injected embryos. The trigeminal neurons and Meckels’ cartilage are derivatives of the neural crest cell stream that populates the first branchial arch (50).

To confirm a role of fmr1 in the cranial neural crest, we directly examined the cranial neural crest in 26-somite stage embryos using whole-mount in situ transcript hybridization to reveal the expression of dix-2a (Supplementary Material, Fig. S2). dix-2a was reduced in fmr1MO-injected embryos, indicating that the loss of fmr1 does indeed have an effect on neural crest specification (Supplementary Material, Fig. S2A and B). Unilateral rescue experiments demonstrated differences in the location of neural crest streams between fmr1MO and controlMO-injected embryos (Supplementary Material, Fig. S2C and D). This suggests that fmr1 has a role in specification and possibly migration of a subset of neural crest cells and that the distinctive syndromic craniofacial abnormalities found in patients with fragile X syndrome may be due to defects in the neural crest.

**DISCUSSION**

**Zebrafish is a suitable model system of fragile X syndrome**

In the current study, we used a range of experimental approaches to look at various morphological defects related to the loss of Fmr1 and to the human syndrome connected to the loss of FMRP. Our first aim was to determine whether zebrafish was a suitable model organism in which to model aspects of human fragile X syndrome. We have demonstrated that the zebrafish is an entirely appropriate and easily manipulated fragile X syndrome model in which to examine multiple aspects of the syndrome. Furthermore, we extended our study to demonstrate that the drug MPEP, previously demonstrated to rescue murine Fmr1-knockout behavioural phenotypes, also rescues more basic neurophysiological abnormalities and appears to have a role in alleviating craniofacial defects associated with the loss of zebrafish Fmr1.

The zebrafish will prove an important model organism in the study of fragile X syndrome and associated neural abnormalities, as it appears to recapitulate human craniofacial defects as well as providing a stereotypical pattern of neurons that are simple to image with fluorescent probes in whole embryos. The zebrafish also has a full complement of genes orthologous to the human FMR1 gene family, as well as fmr1 interacting proteins that will be crucial to understanding the context-dependent activities of the transcript and protein. The zebrafish may also be an appropriate model in which to study the RNA-related phenotypes associated with FMRII premutations (51).

**Loss of Fmr1 recapitulates phenotypes found in other model organisms and in the human syndrome**

Given that the primary syndromic phenotype of fragile X syndrome is mental retardation, most work using model organisms to study effects of the loss of FMR1 has focused on some aspect of the neuronal or brain phenotypes. A great deal of work has addressed the role of FMR1 in synaptic defects, LTD, and dendritic spine defects. This is appropriate considering the altered dendritic spine morphology identified abnormality in the brains of fragile X syndrome patients. Neurite morphogenesis defects due to dfmr1 loss has been
found in *Drosophila* (20,39), implicating dfmr1 in branching and neuronal elaboration. However, dfmr1 function has also previously been indirectly connected to both fasciculation and guidance defects through its interaction with CYFIP in both CNS and PNS (19). It appears likely that the behavioural or cognitive defects associated with fragile X syndrome are due, in part, to both synaptic and neurite abnormalities. Furthermore, identifying the mechanisms controlling neurite morphology is important to our understanding of nervous system development in general.

In this study, we have shown that, similar to invertebrates, the loss of FMRP in a vertebrate is connected with neurite branching defects. These defects and other neuritic abnormalities (defasciculation and errors in axon guidance) occur in the context of the full, vertebrate *FMR1* gene family. Thus, it is unlikely that partially redundant function provided by *FXR1* and *FXR2* is masking axonal, fasciculation or guidance defects in fragile X syndrome. Our work also suggests that these various neurite morphological defects may result from a common molecular mechanism causing different morphological outcomes depending on the cellular developmental context. We have also demonstrated that craniofacial abnormalities resulting from the loss of fmr1 activity in zebrafish may be consistent with defects found in fragile X syndrome patients (2). The defect in the width of Meckels’ cartilage at 5 dpf may reflect the human syndromic phenotype of head circumference greater than the 50th percentile, dental overcrowding and jaw defects. Furthermore, it appears that trigeminal innervation of the face in 5 dpf zebrafish larvae is affected by fmr1 knockdown. We speculate that this may be related to the characteristic ‘syndromic appearance’ of patients with fragile X syndrome, as well as to some behaviours identified in the syndrome, such as childhood vomiting or gagging.

**fmr1 appears to affect the fate of the neural crest**

Cranial cartilage develops in the pharyngeal arches from mesenchyme of neural crest origin. The fate of neural crest cells is specified largely by their position on the rostrocaudal axis prior to the onset of migration (52). The first arch (mandibular arch) is populated by neural crest cells from midbrain, rhombomeres 1 and 2, the second arch (hyoid arch) is populated by cells from rhombomere 4 and the third arch by cells from rhombomeres 6 and 7 (53).

Both Meckels’ cartilage and the trigeminal ganglion are derived from the mandibular arch. Meckels’ cartilage, in particular, is derived specifically from the neural crest cells originating in the posterior midbrain (50), whereas the trigeminal ganglion (or cranial nerve V) is derived partially from ectodermal placodal cells and partially from neural crest (54,55) originating in the posterior midbrain and the second rhombomere.

In this study, we have demonstrated that both of Meckels’ cartilage and the trigeminal ganglion are affected by the loss of *fmr1* activity. Furthermore, both Meckels’ cartilage defects and trigeminal ganglion neuron number are rescued to normal by MPEP treatment. Furthermore, we have found that *dlx-2a* expression in migrating neural crest is significantly altered in fmr1MO-injected embryos. These results suggest that *fmr1* has a role in neural crest specification and possibly migration and that *fmr1* and mGluR signalling affect these processes through the same pathway.

**A close association between *fmr1* and mGluR signalling influences various aspects of neuronal architecture and function**

In individuals with fragile X syndrome, dendritic spines that form synapses are abnormally long and appear to be immature (12,34). This has led to the theory that FMRP is involved in synaptic maturation or morphogenesis. Long-term potentiation and LTD are mechanisms of synaptic plasticity in the dendritic spines. These mechanisms are indirectly triggered by local protein synthesis. *FMR1* appears to be one of the transcripts that is translated at the synapse in response to mGluR stimulation (17). In *Fmr1* knockout mice, mGluR-dependent activity is increased, leading to increased LTD (mGluR-LTD) (15,56), and this, along with a number of other features of *FMR1*-related functions, has lead to the development of an ‘mGluR theory of fragile X mental retardation’ (27), which suggests that a significant portion of the disease phenotypes is due to mGluR misregulation.

MPEP is a potent and specific non-competitive antagonist of mGluR receptors. MPEP has been used to rescue behavioural abnormalities in *Fmr1* knockout mice (28) and courtship and mushroom body defects in *Drosophila dfmr1* loss-of-function mutants (41).

Our ability to rescue *fmr1* loss-of-function neurite branching abnormalities in vertebrate embryos with MPEP gives further support to the mGluR theory of fragile X syndrome. In addition, we demonstrated that overexpression of *fmr1* in normal embryos has a similar effect on neurite branching to that of MPEP treatment. This indicates that the effect of mGluR and Fmr1 on neurite architecture is closely linked and extends beyond mutual regulation in synapses. There is also a connection between mGluR signalling and *fmr1* function in specification of trigeminal ganglion neurons. Both overexpression of *fmr1* and treatment with MPEP significantly raise the mean and the maximum number of neurons above that normally seen.

Zebrafish embryos are highly amenable to drug screening. In particular, drugs applied to their growth medium are not metabolized by an intervening placenta. This simplifies interpretation of their effects on developing embryos. Our development of a zebrafish model for the analysis of human *FMR1* loss-of-function phenotypes and our demonstration of the efficacy of MPEP in alleviating these phenotypes support the use of this model in screens for additional drugs that can modulate FMR1 activity and FMRP function.

We have incorporated the findings presented here with those in the literature to construct a model of the role of *FMR1* pathways in neurite morphogenesis. This model integrates *FMR1* function with mGluR signalling.

**A model integrating mechanisms of neurite morphogenesis affected by *fmr1* knockdown, overexpression and pharmacological rescue**

The experiments reported here demonstrate a close similarity in neurite phenotype caused by *fmr1* overexpression and mGluR antagonism. This suggests that both *fmr1* activity and
mGluR signalling directly affect the same pathway(s) controlling neurite morphogenesis. Furthermore, \textit{fmr1} function is involved in not only neurite branching but also axon guidance and defasciculation. It is plausible that there is a common mechanistic link between these phenotypes.

Here, we propose a model that describes how neurite morphologies (branching, guidance and fasciculation) might be dependent on the modulating influences and balance of \textit{fmr1}, Fmr1 and mGluR (Fig. 8). Our model is based on a computational model of neurite outgrowth and branching described by Hely \textit{et al.} (57) and Kiddie \textit{et al.} (58). One of the predictions of that model is that an increase in intracellular calcium leads to an increased likelihood of neurite branching. This is due to the destabilization of structural microtubules and proliferation of path-finding F-actin filaments. Our observations are consistent with this prediction since, if \textit{fmr1} affects neurite morphogenesis through modulation of intracellular calcium, then this provides a mechanism through which \textit{fmr1} may affect other aspects of neurite morphogenesis.

Microtubule assembly and bundling are regulated by microtubule-associated proteins (MAPs). MAP2 functions by binding to microtubules and stabilizing them. This promotes microtubule assembly and bundling into a rigid cytoskeleton. The ability to stabilize microtubules depends on the phosphorylation state of MAP2. Dephosphorylated MAP2 favours elongation by promoting microtubule polymerization and bundling. In contrast, phosphorylated MAP2 tends to separate microtubules, leading to their destabilization. This delays elongation of the neurite and encourages branching (59–61). The ratio of phosphorylated to dephosphorylated MAP2 is determined by the activity of calmodulin-dependent protein kinase II (CaMKII). CaMKII activity is regulated by intracellular calcium levels (57,58).

In this study, we observed a number of defasciculation and axon-guidance phenotypes in zebrafish embryos lacking \textit{fmr1} activity. These phenotypes have been well established, as resulting from changes in the activity of calcium-dependent processes (62–65). Loss of FMRP regulation may lead to perturbed distribution of calcium. This would lead to differential MAP2 phosphorylation and thus differential microtubule assembly.

mGluR signalling and its role in rescuing the \textit{fmr1} morphant phenotypes is consistent with the model described in Figure 8. mGluR is known to control the release of calcium from internal stores and has been associated with the lengthening of dendritic spines (66).

A number of targets of FMRP have previously been shown to be related to the regulation of intracellular calcium levels. Loss of FMRP has been connected to the upregulation of L-type Ca$^{2+}$ channel subunits αB3 and α1D (9,10; Fig. 8, Pathway 1).

FMRP interacts with (but does not appear to regulate) Calretilcin, a low-affinity but high-capacity Ca$^{2+}$ binding protein (10). Calretilcin is a resident endoplasmic reticulum protein that may be transported via FMRP into intracellular stores in neurites to regulate calcium stores (67; Fig. 8, Pathway 2).

It has previously been established that FMRP negatively regulates CaMKIIα (68) and NAP-22 (neuronal axonal membrane protein) (9,11; Fig. 8, Pathway 3). CaMKIIα is a Ca$^{2+}$–calmodulin-dependent protein kinase, which is known to phosphorylate MAP2 (69). NAP-22 is a Ca$^{2+}$–dependent–calmodulin-binding protein present in neurites, which possibly facilitates sensitivity to calcium (70). CaMKII α has also been found to localize to growth cones in the FMRP granule (71) and has previously been established to have roles in phosphorylation of MAP2 and neurite branching (52).

A pathway has also been identified by which FMRP can directly regulate the phosphatase Pp2Ac (protein phosphatase 2A, catalytic subunit c) (18; Fig. 8, Pathway 4) that has an established involvement in the phosphorylation of CaMKIIα (72). Inhibition of Pp2Ac has also been shown to increase the phosphorylation of MAP1B and MAP2 and inhibit their microtubule binding activity (73). Pp2Ac is also a phosphatase of phospho-ADF/cofilin (P-cofilin), a major mediator of Rac1 signalling. A link between FMRP and Rac1 through CYFIP has previously been demonstrated and provides a mechanism through which FMRP may interact with actin cytoskeleton remodelling (18,19). The role of \textit{fmr1} in neurite morphogenesis as described in the present study, and morphogenesis attributed to interactions between Rac1 and actin, may therefore be one and the same. Indeed, Lee \textit{et al.} (20) have previously described that Rac1 overexpression in dendritic arborization neurons also leads to increased dendritic branching in Drosophila. Furthermore, it is reported that decreased dendritic branching caused by overexpression of \textit{dfmr1} can be partially rescued by co-expression of Rac1. This suggests that Rac1 may be affecting neurite morphogenesis through CaMKIIα and MAP2 signalling.

As described earlier, there is considerable evidence connecting \textit{fmr1}, mGluR signalling and calcium levels. As mGluR is known to control the release of calcium from internal stores, antagonism of this activity with MPEP would clearly reduce the levels of calcium released (Fig. 8, Pathway 5). In \textit{fmr1} knockdown cells, this may rescue abnormal neurite branching caused by abnormal calcium regulation. This model is also consistent with the established theory that the absence of FMRP leads to misregulation of protein synthesis at the synapse in response to mGluR activity. Treatment of mGluR activity with antagonists should again reverse these defects attributable to the loss of FMRP function (15,27). The model may also explain the synaptic defects associated with the loss of \textit{fmr1}, as a large rise in intracellular calcium is permissive for LTD in synaptic morphology, and LTD of the synapse is an identified result of the loss of \textit{FMR1} (15).

In conclusion, we have demonstrated that the zebrafish is an appropriate model organism for studies of the genetic basis of fragile X syndrome. It is a vertebrate with single orthologues of all the human \textit{FMR1}-related genes. It is simple to knockdown \textit{fmr1} activity in zebrafish embryos using morpholino antisense oligonucleotides, and this suggests that zebrafish embryos would be an ideal system in which to study combinatorial interactions between the \textit{FMR1}-related genes through simultaneous knockdown. We have described the first identified \textit{fmr1}-related cranio-facial abnormalities in a model of the human syndrome, and we have discovered a new effect of \textit{fmr1} loss on the abundance of neurons in the trigeminal ganglion. These phenotypes may be connected by a role for \textit{fmr1} in the specification and, possibly, the migration of neural crest cells (Supplementary Material, Fig. S2). We have also described effects of the loss of \textit{fmr1} function on neurite morphogenesis in a genetic background.
A proposed mechanism explaining and unifying the neurite morphological abnormalities associated with fmr1 MO knockdown (branching, fasciculation and guidance) and MPEP antagonism of mGluR. fmr1 and MPEP might regulate calcium signalling pathways and influence neurite morphology through modulation of MAP2 phosphorylation. FMRP (yellow hexagon) is translocated into the nucleus (via its NLS) where it forms a messenger ribonucleoprotein (mRNP) complex. The complex is exported into the cytoplasm (via its NES). The Fmrp/mRNP complex can associate with ribosomes and is known to regulate (positively and negatively) a number of transcripts. FMRP can form an mRNA granule that can translocate into F-actin-rich compartments, such as filopodia, spines and growth cones (74–76), likely through an association with MAP1B (MAP1B appears to be negatively regulated by FMRP). It has been speculated that this translocation of the FMRP granule is a form of mRNA and protein transport. In post-synaptic sites, and perhaps in growth cones, the FMRP granule is disassembled or translated in response to stimulation (occurring post-synaptically via mGluR signalling). Alternatively, granule formation might act to repress the translation of a pool of interacting mRNAs (8,16,71,77). These FMRP mechanics have been used to suggest models effectively illustrating the origins of post-synaptic morphological defects (27,78,79). These models have also connected FMRP to the activity of mGluR (inset) (15). Stimulation of mGluRs increases transport of FMRP-containing granules to the neurites and exaggerates granule disassembly (8,74). mGluR stimulation also increases the levels of many post-synaptic mRNAs, including FMR1 mRNA and mRNAs downregulated by FMRP. A reduction in the level of FMRP at synapses has been speculated to reflect the process of granule disassembly (3). This has lead to a theory that fragile X syndrome is a consequence of exaggerated response to mGluR activation (27).
possessing other, partially redundant FMR1-related genes. We have observed whole or partial rescue of these phenotypes using the mGluR antagonist MPEP and have shown that both treatment with MPEP and fmr1 overexpression lead to similar phenotypes. From these observations and the known functions of FMR1, we have proposed a model that connects FMRP, neurite and synaptic morphogenesis and mGluR signalling to a common pathway.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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