The nystagmus-associated FRMD7 gene regulates neuronal outgrowth and development

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Received September 10, 2009; Revised and Accepted October 26, 2009

Mutations in the gene encoding FERM domain-containing 7 protein (FRMD7) are recognized as an important cause of X-linked idiopathic infantile nystagmus (IIN). However, the precise role of FRMD7 and its involvement in the pathogenesis of IIN are not understood. In the present study, we have explored the role of FRMD7 in neuronal development. Using in situ hybridization and immunohistochemistry, we reveal that FRMD7 expression is spatially and temporally regulated in both the human and mouse brain during embryonic and fetal development. Furthermore, we show that FRMD7 expression is up-regulated upon retinoic acid (RA)-induced differentiation of mouse neuroblastoma NEURO2A cells, suggesting FRMD7 may play a role in this process. Indeed, we demonstrate, for the first time, that knockdown of FRMD7 during neuronal differentiation results in altered neurite development. Taken together, our data suggest that FRMD7 is involved in multiple aspects of neuronal development, and have direct importance to further understanding the pathogenesis of IIN.

INTRODUCTION

Idiopathic infantile nystagmus (IIN) is an oculomotor disorder characterized by involuntary oscillations of the eyes that occur in the first 6 months of life. Other associated features may include mildly decreased visual acuity, strabismus, astigmatism, abnormal head posture and occasionally head nodding (1). The prevalence of IIN in the population is estimated to be 24 per 10,000 and currently there is no curative treatment (2). IIN is distinct from other ocular disorders, including albinism and achromatopsia, in which nystagmus accompanies an apparent defect in the visual sensory system. On the contrary, IIN arises independently, leading to speculation that the disorder represents a primary defect in regions of the brain responsible for ocular motor control (3).

The patterns of IIN inheritance are heterogeneous and have been described as autosomal dominant (OMIM 164100), autosomal recessive (OMIM 257400) and X-linked (OMIM 310700). X-linked inheritance, with incomplete penetrance in females, is the most common form and has been mapped to two regions of the X chromosome: Xq26-27 (NYS1) and Xp11.4-p11.3 (NYS2) (4,5). We previously identified 22 mutations in the novel FRMD7 gene (FERM domain-containing 7) (Xq26.2) in 26 families with X-linked IIN (6). Since this initial report, many additional FRMD7 mutations have been associated with IIN, thus confirming the importance of FRMD7 in pathogenesis of IIN (7–9).

The human FRMD7 gene comprises 12 exons and encodes a 714-residue polypeptide (RefSeq accession number NM_194277). However, the functional role of FRMD7 still remains unclear. The FRMD7 protein contains a FERM domain at the N-terminus indicating it may be involved in signal transduction between the plasma membrane and cytoskeleton, similar to other FERM domain contain proteins (10,11). Furthermore, FRMD7 shares close amino acid sequence homology to two other FERM domain containing proteins: FAR1 (FERM, RhoGEF and pleckstrin domain protein 1; chondrocyte-derived ezrin-like protein; NM_005766) and FARP2 (NM_014808).

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Recent work has led to the discovery that both FARP1 and FARP2 play important roles in neuronal development: FARP1 is known to promote the dendritic growth of spinal motor neuron subtypes and FARP2 has been shown to modulate the length and the degree of branching of neurites in developing cortical neurons (12–14). Given the closely shared amino acid sequence homology between FRMD7 and these two proteins, it is postulated that FRMD7 may also play an important role in neuronal development, particularly in regions of the brain associated with ocular motor control. Indeed, we previously observed FRMD7 mRNA expression in the ventricular layer of the forebrain, midbrain, cerebellar primordium and developing neural retina in human embryonic brain at 56 days post-conception (dpc) (6).

In the present study, we have addressed the role of FRMD7 in neuronal development. We demonstrate that FRMD7 expression is spatially and temporally regulated in the developing human and mouse embryonic cortex. In addition, during retinoic acid (RA)-induced differentiation of NEURO2A cells, we detected a strong up-regulation of FRMD7 steady-state mRNA and protein levels within 12 h, suggesting that FRMD7 plays a role in NEURO2A differentiation. Immunofluorescence analysis of RA-differentiated NEURO2A cells revealed that the FRMD7 protein has a prominent concentration in the primary neurite body and the distal tip of the neuronal growth cone. Moreover, by RNAi experiments we demonstrate that FRMD7 plays an important role in the regulation of neurite outgrowth. Overall, our studies suggest that FRMD7 may be involved in multiple aspects of neurite outgrowth and specification, and have direct importance to further understanding the pathogenesis of IIN.

RESULTS
FRMD7 expression is spatially and temporally regulated in the embryonic brain

We previously demonstrated that FRMD7 mRNA levels are expressed in the human embryonic brain. In particular, we noted strong expression of FRMD7 mRNA in the ventricular zone (VZ) of the forebrain at 56 dpc (6). The human embryonic VZ contains the neural stem cells for most excitatory neurons contributing to the adult neocortex (15,16). During corticogenesis, neuroepithelial progenitors at the apical surface of the VZ divide to give rise to progenitors, post-mitotic neurons and macroglia. Since neurogenesis proceeds in a temporally and spatially controlled fashion this prompted us to examine in detail the expression profile of FRMD7 in the developing human cerebral cortex.

We examined the expression of FRMD7 in cerebral cortex from five different stages of human embryo development [Carnegie Stage (CS) 15, CS16, CS19, CS22, CS23] and two stages of human fetal development [9 weeks post-conception (wpc) and 14 wpc] by in situ hybridization. Strong hybridization signals were observed in the VZ of the forebrain at CS16 and CS19, in both the telencephalon and diencephalon (Fig. 1A and B). Furthermore, at CS22 and CS23, FRMD7 mRNA remained expressed in VZ, but was also observed in the intermediate zone and cortical plate (Fig. 1C). During these early stages of development, neuroblasts, generated by asymmetrical cell division from neuronal precursor cells, begin to migrate from the VZ to the cortical plate using the processes of radial glial cell as a guide. The expression of FRMD7 mRNA in the VZ and intermediate zone at these stages is consistent with a role in the asymmetrical neuronal precursor cell division and radial migration of newborn neurons. By 9 wpc, limited cells in the ventricular layer show expression of FRMD7 mRNA whilst the majority of FRMD7 expression was observed in the cortical plate (Fig. 1D). Similarly, by 14 wpc, expression of FRMD7 mRNA was limited in the VZ, however, strong expression was observed in the cortical plate and subplate (Fig. 1E). In these stages of human corticogenesis, many neurons begin to send processes to their targets for the formation of functional networks. Therefore, these results suggest that FRMD7 may also play an important role in axonogenesis or dendritogenesis.

To confirm this expression profile of FRMD7 holds true at the protein level, we performed an immunohistochemical analysis in the developing mouse cerebral cortex. At early stages of development, E13 and E15, strong immunoreactivity was observed in the ventricular and intermediate zones (Fig. 2A and B). In contrast, at later development stages, E17 and P0, FRMD7 expression appears to be restricted to the cortical plate (Fig. 2C and D). This temporal and spatial expression profile of the FRMD7 protein in the developing mouse cortex is consistent with the mRNA expression profile we observed in the human embryonic and fetal cortex. Furthermore, the expression profile we observed here is similar to the expression pattern previously observed for FARP2 in the developing mouse cortex (17). Taken together, these data provide strong evidence that FRMD7 plays a role in neuronal development.

Intact brain contains a complex mixture of cell types, including neurons and many different types of glial cells. To determine whether FRMD7 is expressed in neurons, we made use of primary cultures enriched in brain cortical neurons. Western blot analysis revealed that FRMD7 is present in cultured cortical neurons, and its expression increases at later times in culture (Fig. 2E), strengthening the suggestion that FRMD7 plays a role in the maturation of neurons and their morphological differentiation.

Finally, we further examined the importance of FRMD7 in brain development by examining the expression of FRMD7 in brain and other tissues from neonatal mice. Here, Western blot analysis revealed that FRMD7 is highly expressed in the early post-natal cortex, hippocampus, cerebellum, olfactory bulb and eye (Fig. 2F). In contrast, little or no expression was observed in the liver, kidney, skeletal muscle and heart muscle. These data confirm the importance of FRMD7 expression in neuronal tissue, and demonstrate that within the brain, FRMD7 continues to be expressed during early post-natal development. This suggests that FRMD7 may also play a role in the adapting post-natal brain.

FRMD7 mRNA and protein levels are elevated during differentiation of NEURO2A neuroblastoma cells

To address whether FRMD7 is involved in differentiation we examined FRMD7 mRNA and protein expression in neuro2A (NEURO2A) neuroblastoma cells during RA-induced differ-
Figure 1. FRMD7 mRNA expression in the developing human cortex. Sections from embryonic and fetal human brain were hybridized with the FRMD7 antisense probe. (A) CS16 brain (B) CS19 brain. Intense signals (purple deposit) were detected in the ventricular zone of the forebrain. (C) CS22 brain. Intense signals were detected in the ventricular zone and cortical plate. Expression was also detected in scattered cells in the intermediate zone. (D) 9 wpc brain. Limited cells in the ventricular zone showed expression, however, an intense signal was observed in the cortical plate and expression was also detected in the intermediate zone. (E) 14 wpc brain. Expression in the ventricular zone was almost absent however an intense signal was observed in the cortical subplate. Expression was also detected in the cortical plate. No signal was detected when sense probes were hybridized to adjacent sections (Supplementary Material, Fig. S1). CP, cortical plate; IM, intermediate zone; SP, subplate; VZ, ventricular zone. Scale bars (A) 400 μm and 100 μm (B) 1.6 mm and 200 μm (C) 800 μm and 100 μm (D) 800 μm and 100 μm (E) 800 μm and 200 μm.

Figure 2. FRMD7 protein expression in the developing mouse brain. Sections from embryonic and post-natal mouse tissue were examined by immunohistochemistry with anti-FRMD7 antibody. (A) E13 brain and (B) E15 brain. Strong immunoreactivity was observed in the ventricular and intermediate zones during early stages of development. (C) E17 brain and (D) P0 brain. Strong immunoreactivity was restricted to the cortical plate during later stages of cortical development. CP, cortical plate; IM, intermediate zone; PP, preplate; VZ, ventricular zone; WM, white matter. All scale bars = 50 μm. (E) Western blot showing FRMD7 is present in cultured cortical neurons, and its expression increases at later times in culture (DIV, days in vitro). (F) Western blot analysis of FRMD7 expression in neonatal mouse tissue. High expression is noted in the cortex, hippocampus, cerebellum, olfactory bulb and eye.
entiation. NEURO2A cells are a well-recognized model system for studying signaling pathways mediating neurite outgrowth (18,19). Under normal growth conditions, most NEURO2A cells spontaneously develop a basal level of neurites. However, under treatment with RA, NEURO2A undergo differentiation to a neuron-like phenotype (20). Specifically, RA-treated NEURO2A cells develop non-uniformly oriented microtubules typical of dendrites (19). Therefore, it is believed that differentiation of NEURO2A cells with RA treatment closely approximates the events that occur during neuronal development in post-mitotic neurons.

A time-course experiment was performed to determine FRMD7 expression during RA-induced differentiation of NEURO2A cells. Interestingly, within 12 h of treatment with RA, FRMD7 mRNA and protein expression levels were significantly increased in differentiating NEURO2A cells compared with the undifferentiated cells (Fig. 3). Moreover, an increase in FRMD7 protein expression was observed within 12 h of differentiation and was sustained during 72 h post-differentiation (Fig. 3). This rapid up-regulation of expression suggests that FRMD7 is important for neuronal differentiation and neurite development.

Expression of FRMD7 in RA-induced primary extensions and growth cone regions within differentiating neurons

We next sought to establish the subcellular localization of endogenous FRMD7 in neurons using immunofluorescence staining and confocal microscopy in undifferentiated and differentiated NEURO2A cells. In undifferentiated cells, we observed that the FRMD7 protein is highly colocalized with actin around the cell periphery (Fig. 4A). Similarly, in differentiated cells, FRMD7 colocalizes with actin in the cell body but is also observed in the neurite processes. Closer examination of the neurite processes reveals that FRMD7 is highly localized to the actin-rich regions of the primary extension and is almost absent from the actin-rich secondary extensions arising from the primary neurite (Fig. 4B). Interestingly, FRMD7 is also present at the actin-rich distal end of the growth cones in differentiated cells, suggesting a possible role of FRMD7 in the regulation of growth cone guidance (Fig. 4C).

Downregulation of FRMD7 in NEURO2A cells results in altered neurite development

Given that our present studies reveal that FRMD7 protein levels are up-regulated during RA-induced neurite outgrowth and that FRMD7 is highly co-localized with actin-rich regions in the NEURO2A primary neurite extension and the neuronal growth cone, it seems likely that this protein is an important modulator of neurite outgrowth in differentiated neurons. Therefore, we used an RNAi approach to determine whether FRMD7 is essential for normal neuronal morphogenesis. For these experiments, NEURO2A cells were transfected with commercially available pGIPZ-shRNAAmir vectors, containing either a sequence targeted to a unique site of the mouse FRMD7 gene (shRNA-FRMD7) or a non-silencing sequence (scramble). Selecting for the vector encoded puromycin resistance gene enabled for the generation of stable cell lines, corresponding to the shRNA-FRMD7 construct and the non-silencing control. Quantitative PCR and western blot analysis were used to confirm a decrease of FRMD7 mRNA and protein levels in undifferentiated shRNA-FRMD7 transfected cells compared with non-silencing control cells (Supplementary Material, Fig. S2A and Fig. 5A). Importantly, this knockdown of FRMD7 was maintained during RA-induced differentiation (Supplementary Material, Fig. S2B and Fig. 5A).

Having established that the shRNA-FRMD7 transfected cells expressed reduced levels of FRMD7 protein, we next sought to examine how this change would affect neurite development in undifferentiated and differentiated NEURO2A cells. ShRNA-FRMD7 transfected cells were compared with scramble control cells, under undifferentiated and RA-induced differentiated conditions, for a variety of morphological changes. Statistics were gathered on the percentage of cells with neurites (an outgrowth with a length more than half the diameter of the cell body), percentage of cells with neurite branching (neurite branching is defined as a neurite with secondary neurites arising from the primary neurite), average number of neurites per cell and average neurite length (neurite length from cell body to distal tip).

In non-differentiating conditions, we observed a significant increase in the number of cells with neurites (scramble = 20.9 ± 1.8%, shRNA-FRMD7 = 28.9 ± 1.8%), the average number of neurites/cell (scramble = 0.5 ± 0.1, shRNA-FRMD7 = 1.1 ± 0.1) and the percentage of cells with neurite branching (scramble = 1.0 ± 0.3%, shRNA-FRMD7 = 2.3 ± 0.5%) (Fig. 5B and Table 1). However, no significant change in average neurite length was observed (Fig. 5C and Table 1).
We next compared RA-treated shRNA-FRMD7- to non-silencing control-transfected NEURO2A cells. Once again, there was a significant increase in the number of cells with neurites (scramble = 84.6 ± 1.3%, shRNA-FRMD7 = 90.3 ± 1.2%), the average number of neurites/cell (scramble = 2.5 ± 0.1, shRNA-FRMD7 = 2.8 ± 0.1) and the percentage of cells with neurite branching (scramble = 27.1 ± 1.9%, shRNA-FRMD7 = 35.52 ± 1.9%) (Fig. 5B and Table 2). However, the shRNA-FRMD7 cells also exhibited a large reduction in the average neurite length (scramble = 89.4 ± 3.7 μm, shRNA-FRMD7 = 51.7 ± 3.0 μm) (Fig. 5B and C). Overall, our data demonstrates that knockdown of the FRMD7 protein results in significant alterations in neurite development, including a strong reduction in neurite length in the differentiated neuron.

**Downregulation of FRMD7 leads to alteration of F-actin/G-actin dynamics in differentiated NEURO2A cells**

To further investigate the alterations in neurite development we used Rhodamine-phalloidin staining to examine the F-actin content in differentiated shRNA-FRMD7 and non-silencing control NEURO2A cells. In differentiated shRNA-FRMD7 NEURO2A cells, a noticeable increase in F-actin was observed compared to the differentiated scramble cells (Fig. 6A). This increase in polymerized actin was reflected by the increased number of neurites per cell, as well as increased filopodia and lamellipodia in the neurite processes. To quantify the observed increase in F-actin, we
determined the ratio of F-actin/G-actin in shRNA-FRMD7 and scramble cells using an *In Vivo* Actin Dynamic Assay kit. A significant increase in the F-actin/G-actin ratio was observed in the differentiated shRNA-FRMD7 cells compared with the differentiated scramble cells (Fig. 6B and C). Taken together these results indicate that down regulation of FRMD7 leads to a disturbance in the F-actin/G-actin dynamics within differentiated NEURO2A cells.
**Table 1.** Neurite outgrowth of NEURO2A cells in 10% FCS/DMEMa

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<th>Scramble</th>
<th>shRNA FRMD7</th>
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<tr>
<td>Cells with neurites (%)</td>
<td>20.9 ± 1.8</td>
<td>28.9 ± 1.8</td>
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<tr>
<td>Cells with neurite branching (%)</td>
<td>1.0 ± 0.3</td>
<td>2.3 ± 0.5</td>
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<tr>
<td>Average number of neurites/cell</td>
<td>0.5 ± 0.1</td>
<td>1.1 ± 0.1</td>
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<tr>
<td>Average neurite length (µm)</td>
<td>20.7 ± 0.6</td>
<td>21.3 ± 0.6 (ns)</td>
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*aCells were treated with 10% FCS/DMEM for 72 h, and neurite parameters were quantified. Data are mean ± SEM of three independent experiments. N = 250 cells counted for each experiment. P < 0.05 unless indicated.

**Table 2.** Neurite outgrowth of NEURO2A cells treated with 10 µM retinoic acid

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<thead>
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<th>Scramble</th>
<th>shRNA FRMD7</th>
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<tr>
<td>Cells with neurites (%)</td>
<td>84.6 ± 1.3</td>
<td>90.3 ± 1.2</td>
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<tr>
<td>Cells with neurite branching (%)</td>
<td>27.1 ± 1.9</td>
<td>35.2 ± 2.7</td>
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<tr>
<td>Average number of neurites/cell</td>
<td>2.5 ± 0.1</td>
<td>2.8 ± 0.1</td>
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<tr>
<td>Average neurite length (µm)</td>
<td>89.4 ± 3.7</td>
<td>51.7 ± 3.0</td>
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*aCells were treated with 10 µM retinoic acid, 0.2% FCS/DMEM for 72 h, and neurite parameters were quantified. Data are mean ± SEM of three independent experiments. N = 250 cells counted for each experiment. P < 0.05.

**DISCUSSION**

The underlying cause and pathophysiology of IIN remain unclear. We previously identified FRMD7 as the first gene to be associated with IIN (6). However, despite its identification, very little is known about the function of FRMD7, or indeed the role it plays in the pathogenesis of IIN.

It has been postulated that FRMD7 plays an important role in neuronal development based on sequence homology with other FERM protein family members. Consistent with this suggestion, we previously demonstrated that FRMD7 mRNA is expressed in the human embryonic brain (6). However, it was not known whether FRMD7 expression is spatially and temporally regulated in the developing human cortex. Here, we have revealed that the expression profile of FRMD7 changes considerably during development of the cortex. We have shown that FRMD7 mRNA is expressed in the ventricular and intermediate zones of the forebrain during early development stages (CS16–CS22), suggesting a role for FRMD7 in asymmetrical cell division and the radial migration of newborn neurons. In later development stages (9 wpc and 14 wpc), we have shown that FRMD7 is highly expressed in postmitotic cells within the developing subplate and cortical plate, whereas little expression remains in the VZ. During these later stages of corticogenesis, the majority of neurons extend to sites of final positions and send processes to their targets for the formation of functional networks. Indeed, subplate neurons are believed to pattern the connectivity in cortical circuits during early periods of cortical development (21,22). Therefore, our observations suggest that FRMD7 may also play a role in axonogenesis or dendritogenesis. Interestingly, a similar pattern of mRNA expression has been observed for FARP2, a FERM-domain containing protein shown to be involved in the regulation of neurite development (17). Taken together, our current findings that FRMD7 expression is spatially and temporally regulated in the developing cortex indicate that FRMD7 may play an important role during multiple stages of neuronal development.

On the basis of our observations of FRMD7 in the developing brain, we have investigated the role of FRMD7 in the regulation of neurite development in NEURO2A cells. NEURO2A neuroblastoma cells are capable of differentiating to a neuron-like phenotype with the addition of chemicals such as RA, a derivative of vitamin A (20). Specifically, NEURO2A cells differentiated with RA exhibit primarily dendrite-like processes containing non-uniformly orientate microtubules, making them an excellent model system for studying signaling pathways mediating neurite outgrowth (18,19). Our data shows that FRMD7 steady-state mRNA and protein levels are dramatically increased in NEURO2A cells within 12 h of RA-treatment, indicating the importance of this protein during neuronal differentiation. Moreover, we have demonstrated that down-regulation of FRMD7 in differentiating NEURO2A cells leads to a significant reduction in overall neurite length. Thus we have shown that FRMD7 functions in the regulation of neurite outgrowth in NEURO2A cells and more specifically, appears to be required for the elongation of neurite processes during differentiation. These observations correlate with the suggestion that FRMD7 plays an important role during neuronal development, and indicate the potential role that FRMD7 may play a role in neuronal guidance and pathfinding.

The precise mechanism by which FRMD7 contributes to neurite outgrowth requires further investigation. The FRMD7 protein contains a FERM domain, shared with ezrin, radixin and moesin, and known to regulate cell adhesion and morphogenesis through interactions between membrane-associated proteins, such as hyaluronan receptor CD44, and the actin cytoskeleton (23). In the present study, we have revealed that the subcellular localization of FRMD7 in undifferentiated and differentiated NEURO2A cells is primarily within the actin-rich regions of the cell body and neurite processes. In addition, FRMD7 was noted to co-localize with actin in the primary outgrowth but not in small secondary neurite branches arising from the primary neurite, in RA-induced dendrite-like processes. These results indicate that FRMD7 may be involved in signal transduction from the plasma membrane receptors to the actin cytoskeleton; however, this requires further investigation. In neuronal development, two other FERM domain containing proteins, which share close amino acid sequence homology with FRMD7, have been found to play important roles in neuronal development: FARP1 is known to regulate the dendritic growth of spinal motor neuron subtypes and FARP2 has been shown to modulate the length and the degree of branching of neurites in developing cortical neurons (12–14). Both FARP1 and FARP2 function as guanine nucleotide exchange factors (GEFs), regulating Rho GTPases, which are established modulators of cytoskeletal dynamics within neurons. Indeed, one of the most essential steps in the formation and movement of the neuronal growth cone is the recruitment and activation of the Rho GTPases and their regulators, the GEFs and GAPs (24). Interestingly, in the present study, we have demonstrated
that, in differentiated NEURO2A cells, FRMD7 is localized strongly in actin-rich regions within the growth cone of dendrite-like processes, with decreased levels found within other regions of the process. Furthermore, we observed a disturbance in the F-actin/G-actin ratio in the differentiated shRNA-FRMD7 NEURO2A cells indicating the process of actin polymerization might be perturbed. It remains to be determined whether FRMD7 also functions as a GEF. However, the FERM domain itself is known to be an upstream regulator of Rho family members, interacting with both inhibitory regulators (e.g. Rho GDI) and stimulatory regulators (e.g. Dbl) (25,26). Therefore, it is possible that FRMD7 is involved in the regulation of neuronal cytoskeletal dynamics through Rho GTPase signaling at the growth cone.

In summary, these data show that FRMD7 protein expression are regulated during neuronal development. Additionally, we demonstrate that the subcellular localization of FRMD7 is restricted primarily to the cell body of the neuronal cell and to the area of the primary dendrite extensions and distal tip of the growth cone in dendrites. Moreover, we find that down-regulation of FRMD7 disturbs neurite outgrowth as a consequence of RA-induced differentiation of NEURO2A cells. These findings have important implications for understanding the role of this protein in neuronal development and the pathogenesis of IIN.

**MATERIAL AND METHODS**

**Human tissue and in situ hybridization**

Human embryonic and fetal tissues were collected by the MRC-Wellcome Trust Human Developmental Biology Resource (HDBR), Newcastle, with appropriate maternal consents and ethical approval by the Newcastle and North Tyneside Research Ethics Committee. Tissue sections from stages CS15 (~33 dpc), CS16 (~37 dpc), CS19 (~47 dpc), CS22 (~54 dpc), CS23 (~56 dpc), 9 wpc and 14 wpc were examined (CS15, n = 2; CS16, n = 1; CS19, n = 2; CS22, n = 1; CS23, n = 1; 9 wpc, n = 1, 14 wpc, n = 1). Staging was performed as described (27,28), whereas fixation and embedding for non-radioactive in-situ hybridization were as described (29,30). *In situ* hybridization studies were performed in collaboration with the HDBR. Digoxigenin-labeled riboprobes were generated as previously described (6).

**Cell culture and differentiation**

Cortical neurons were prepared from E17 stage mouse embryos as previously described. The dissociated neurons were cultured in Neurobasal medium supplemented with 2 mM l-glutamine, B27 supplement (50X serum-free supplement) and 1% penicillin-streptomycin (Invitrogen-Gibco).
in a 37°C incubator containing 5% CO2/95% humidified air. NEURO2A cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS) and 1% penicillin-streptomycin (Invitrogen-Gibco). For differentiation experiments, NEURO2A cells were subjected to RA treatment (10 μM RA in 0.2% FCS/DMEM). For microscopic observations, cells were plated on 12 mm glass coverslips at a density of 10 000 cells/cm².

shRNA and control stable cell lines

To knockdown FRMD7 expression in the NEURO2A cell line, we used a commercially available pGIPZ-lentiviral shRNAmir vector containing a hairpin sequence targeting FRMD7 (Open Biosystems, Huntsville, AL, USA). The FRMD7 hairpin sequence was as follows with underlined sequencing corresponding to region of the mouse FRMD7 gene targeted: TGCTGTGGA CAGTGAGCCGCTTTGGTATGTGCTTCCAATAATAGTGAGCCACAGATGTATTTGGAAACATAACACAAGGCTTTG CCTACTGCGCTCGGA. The shRNA-containing lentiviral vector was co-transfected with lentiviral packaging into HEK-293T cells (Open Biosystems) to produce shRNA-carrying lentivirus particles. Culture supernatants were collected at 48 and 72 h after transfection and lentivirus particles were concentrated using PEG (System Biosciences). NEURO2A cells were transduced by the resulting concentrated viral particles. For the generation of stable cell lines, 10 μg/ml puromycin treatment was begun 96 h after transduction. A pGIPZ lentivirus containing a non-silencing shRNA control (scramble) with no homology to known mammalian genes was used as the negative control for the knockdown experiments.

Immunohistochemistry and immunofluorescence

Immunohistochemistry (IHC) of formalin-fixed, paraffin-embedded tissues from BALBc mice were performed with the ABC-Staining System according to the manufacturer’s protocol (VectorElite Universal Kit). Sections were incubated overnight at 4°C in a humidified chamber with a 1:150 dilution of polyclonal antibody to FRMD7 (Atlas antibodies). Control IHC experiments (data not shown) were performed without primary antibody and with anti-FRMD7 blocked with saturating levels of its specific peptide antigen. All sections were counterstained with Gill’s haematoxin. For immunofluorescence, isolated cells were first fixed in 4% paraformaldehyde prior to blocking in 5% goat serum in 0.1% PBS-Tween (vol/vol). Cells were then incubated with primary antibody (1:100 anti-FRMD7 (Atlas antibodies); 1:1000 anti-actin (Sigma); 1:1000 anti-βIII tubulin (Promega)) overnight at 4°C. Cells were washed three times for 10 min each with 1× PBS, incubated for 1 h with secondary antibody (in blocking solution), washed three times with 1× PBS, and counterstained with DAPI and mounted for confocal microscopy. Rhodamine-phalloidin was included for F-actin visualization.

Immunoblotting

Whole-cell lysates were prepared by lysing cells and tissue in RIPA lysis buffer. The insoluble material was excluded by centrifugation. The resulting supernatant was mixed with SDS-PAGE loading buffer and was subjected to SDS-PAGE on a 10% gel. After electrophoresis, the proteins were transferred to nitrocellulose membrane, and the membrane was incubated with the appropriate primary antibody. Following incubation with the primary antibody, the membrane was exposed to a horseradish peroxidase-conjugated secondary anti-rabbit antibody, subjected to SuperSignal Chemiluminescent reagent (Pierce Biotechnology, Inc.), and exposed to film. The following antibodies were used: 1:700 anti-FRMD7 (Atlas antibodies), 1:10 000 anti-GAPDH (Santa Cruz).

RNA extraction and qPCR

Total RNA was derived from cells using the Qiagen RNAeasy kit (Qiagen) and tested for purity (A260/280 ratios) and integrity (denaturing gel electrophoresis). Real Time PCR amplification was performed using specific primers and probes for mouse FRMD7 and GAPDH genes (TaqMan® Gene Expression Assays, Applied Biosystems). Thermal cycling was performed using the ABI PRISM® 7000 Sequence Detector System (AB, Applied Biosystems).

Neurite outgrowth quantification

Neurite outgrowth was observed 24–72 h post-RA-induced differentiation of NEURO2A cells. Phase-contrast images of NEURO2A cells were viewed at ×40 and images were captured on Zeiss AXIOVERT 200 M microscope. Statistics were gathered on the percentage of cells with neurites (a neurite is defined as an outgrowth with a length more than half the diameter of the cell body), percentage of cells with neurite branching (neurite branching is defined as a neurite with secondary neurites arising from the primary neurite), average number of neurites per cell and average neurite length (neurite length from cell body to distal tip). The data presented are the mean of three independent experiments. At least 250 cells per transfection were scored for neurite outgrowth. The NEURO2A data shown represent the mean (SEM), with P < 0.05 via Students’ unpaired t-test.

F-actin quantification

The concentration of F-actin in differentiated scramble and shRNA-FRMD7 NEURO2A cells was measured with an Actin Polymerization Assay Kit, according to the manufacturer’s protocol (Cytoskeleton Inc.). Cells were homogenized in F-actin stabilization buffer, pH 6.9 plus protease and phosphatase inhibitors. Phalloidin at 1 μM and cytochalasin D at 10 μM were used as positive and negative controls, respectively. Equal amounts of protein were centrifuged, first at 600g to separate the nuclear fraction and then at 100 000g for 60 min at 37°C. The supernatants, corresponding to F-actin fractions, were placed on ice. Pellets, corresponding to F-actin fractions, were re-suspended in an equal volume of ice-cold H2O plus 1 μM cytochalasin D for 1 h to dissociate F-actin. SDS sample buffer was added to each of the samples and the fractions were separated by 12% SDS-PAGE, transferred onto nitrocellulose membrane and visualized with anti-actin antibody (1:500). The ratio of F-actin/G-actin within each sample was subsequently determined.
Statistical analysis

All experiments were performed in triplicate for each condition, and from at least three different cell culture preparations. Results are expressed as mean ± SEM. Statistical analysis was performed by one-way ANOVA with Bonferroni post-test to compare all conditions or by paired/unpaired Students’ t-test using Prism 5.0 software (Graph Pad Software, San Diego, CA, USA). P < 0.05 were regarded as significant.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS

The human embryonic and fetal material was provided by the Joint MRC Wellcome Trust Human Developmental Biology Resource (http://www.hdbr.org) at the IHG, Newcastle-upon-Tyne, UK. We thank the consenting women who made this study possible and A. Farnworth who gained consent on our behalf. The authors gratefully acknowledge J. Edwards (Medical Research Council Toxicology Unit) for assistance with immunocytochemistry, R. Snowden (Medical Research Council Toxicology Unit) for assistance with cell sorting, and S. Galavotti and M. Guerra-Martin (Medical Research Council Toxicology Unit) for technical support. We also thank D. Read and members of the P.N. laboratory for scientific discussion.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by the Medical Research Council (MRC) and The Ulverscroft Foundation (Registered UK Charity no. 264873).

REFERENCES