Breakthroughs in molecular and cellular mechanisms underlying X-linked mental retardation

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Although genetic causes of X-linked mental retardation (XLMR) are heterogenous and complex, recent concerted actions between physicians and biologists have allowed some major difficulties to be overcome and led to the identification of an increasing number of genes involved in these conditions. Indeed, over the past 2 years significant progress has been made in understanding the molecular basis underlying not only XLMR, where there are distinguishing phenotypic or genetic markers (syndromal forms of XLMR), but also non-specific (or idiopathic) mental retardation (MRX). Recent breakthroughs have shown that genes responsible for these latter conditions encode for proteins involved in signalling pathways which regulate cytoskeleton organization, synaptic vesicle transport and, maybe, other cellular functions. Also, they suggest a provocative picture that conceptualizes MRX as disorders resulting from a dysfunctioning of genes required for processes such as the remodelling, establishment and stabilization of connections between neuronal cells. Such processes are crucial for the development of intellectual and cognitive functions. As these functions begin to evolve mainly in post-natal stages through contact with diverse stimuli and environments, a potential therapeutic approach would be the development of drugs that target cellular signalling pathways shown to be implicated in MRX.

INTRODUCTION

Mental retardation, or non-progressive cognitive impairment, is a common and distressing clinical condition that has been a relatively neglected area of research despite its high cumulative frequency. Approximately 2–3% of the population have an intelligence quotient (IQ) <70 (1,2) and 0.3% of individuals are severely handicapped (IQ < 50), yet the underlying cause of mental retardation is established in less than half of all cases. The disorder has a substantial genetic component and there may be a genetic cause in ~50% of severely retarded patients (3). Because of the haploid status of most genes on the X chromosome in males and the ease of gene mapping on this chromosome, mainly X-linked forms of mental retardation (XLMR) have been mapped (4). In some cases, intellectual handicap is part of a complex syndrome associated with developmental brain abnormalities which result in a brain that lacks the normal pattern of organization and connectivity required for normal brain function. Mental retardation is most likely a secondary feature and one would not suspect that these genes were directly involved in the development of human intellectual and learning abilities. In contrast, there is an increasing number of other conditions in which mental retardation is apparently the only event. These conditions are referred to as primary or non-specific mental retardation (MRX) and affected patients do not have any distinctive clinical or biochemical features or brain developmental abnormalities in common other than cognitive impairment. Identification of genes involved in these disorders promises to provide insights into the molecular and cellular mechanisms required for normal cognitive function.

SYNDROMAL FORMS OF XLMR

Progress in identifying genes involved in syndromal forms of mental retardation has been greatly helped because of the presence of specific, distinguishing clinical, radiological or biological features. At least 57 entities have been described with mental retardation and X-linked inheritance (4). In the case of these syndromal forms, mapping information from separate families can be pooled, provided that diagnosis is accurate and that there is no non-allelic heterogeneity. Investigation of candidate genes can be restricted to a relatively small number of families. The first gene, FMR1, involved in the most common form of syndromic mental retardation, the fragile X-A (FRAXA) syndrome, was identified in 1991 (5,6) and it accounts for 15–20% of all XLMR (7). Since this breakthrough, the number of cloned genes involved in syndromal forms of mental retardation has increased rapidly, especially as a result of the development of positional cloning strategies (Table 1). Although the mutational spectrum is variable, many of the reported mutations result in clear loss of function of genes that play a central role in early developmental processes. A potential dysfunction of these latter genes could explain the apparent ‘pleiotropic effects’ and the complex phenotype observed in some syndromal forms of mental retardation. An interesting example

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is ATRX syndrome: XLMR associated with α-thalassaemia. Affected individuals have a typical facial appearance, genital abnormalities, severe psychomotor retardation and an unusual form of α-thalassaemia (8). This last feature is an anaemia that arises due to an imbalance in the production of the α- and β-globin chains of haemoglobin resulting from a reduction in α-globin synthesis. Mutations in an X-linked gene, XH2, showing homology to the SNF2 superfamily proteins, have been shown to be responsible for this syndrome. SNF2 proteins have DNA helicase and DNA-dependent ATPase activity and are involved in a broad range of biological functions: transcriptional regulation, DNA repair and chromosome segregation. A recent study showed that XH2 protein could be considered as a global transcriptional regulator which regulates gene expression by direct interaction with chromatin-associated proteins such as EZH2 (9). Therefore, it seems that XH2 influences gene expression and that α-globin is one of its many normal targets, so that mutations in ATRX not only cause α-thalassaemia but also affect other developmental pathways, including for example the nervous and urogenital systems.

Pleiotropic effects could also result from mutations in genes that play critical roles in signalling pathways involved in the transcriptional activation of genes involved in early developmental processes, cell proliferation and differentiation. A relevant example is the Coffin–Lowry syndrome (CLS), an X-linked disorder characterized by severe mental retardation, facial and digital dysmorphism and progressive skeletal deformations, which results from loss of function mutations in RPS6KA3 (also known as RSK2), encoding a growth factor-induced kinase (10). RSK-2 was shown to mediate growth factor induction of cAMP response element-binding protein (CREB) phosphorylation. Thus, altered RSK-2 function may influence activity of various transcription factors regulated by the Ras–MAPK signalling pathway whereby growth factors activate the transcription factor CREB, a critical regulator of immediate early gene transcription.

Another category of syndromal forms of mental retardation corresponds to disorders where specific distinguishing clinical features are limited to the brain. In these disorders, the intellectual handicap is most likely to be a secondary feature (Table 1). For example, we can reasonably suppose that in X-linked hydrocephalus (11,12) and X-linked subcortical laminar heterotopia and lissencephaly syndromes (X-SCLH/LIS) (13,14) the mental retardation is secondary to the structural abnormality of the brain. In this latter condition, lissencephaly, which affects hemizygous males, there is an absence of gyri (agyria) or a reduced number of broadened gyri (pachygyria) and an abnormally thick cortex overlying a thin periventricular rim of white matter. The brains of affected females with SCLH (or band heterotopia, also referred to as double cortex) show bilateral bands of disorganized grey matter located between the normal cortex and ventricle but well separated from both by a thin band of white matter. This subcortical band of grey matter corresponds to a population of neurons that migrated half way to the cortex, arresting in the subcortical white matter. Positional cloning approaches allowed the identification of a gene highly expressed in fetal brain, called doublecortin, and the demonstration of its involvement in this disorder (15,16). Our recent data show that doublecortin is a developmentally regulated, neuron-specific phosphoprotein, localized in the cell bodies and the leading processes of migrating neurons. Further investigations demonstrate that doublecortin is tightly associated with microtubules, suggesting that it is a microtubule-associated protein (MAP) required for normal corticogenesis (17). Thus, the cortical dysgeneses associated with the loss of function of doublecortin might result from a dysfunction of cytoskeletal dynamics known to be crucial for neuronal migration.

### Table 1. Examples of X-linked syndromic mental retardation

<table>
<thead>
<tr>
<th>Syndrome</th>
<th>Gene</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATR-X, Juberg–Marsidi</td>
<td>XH2 or XNP</td>
<td>Global transcription factor</td>
</tr>
<tr>
<td>Coffin–Lowry</td>
<td>Rsk-2 or RPS6KA3</td>
<td>Growth factor-induced kinase. Activation of Ras/MAPK pathway</td>
</tr>
<tr>
<td>Optiz/G</td>
<td>MID-1</td>
<td>Zinc finger protein. Transcription factor?</td>
</tr>
<tr>
<td>Aarskog–Scott syndrome</td>
<td>FGD1</td>
<td>RhoGEF (regulation of Cdc42). Activation of Ras/MAPK pathway</td>
</tr>
<tr>
<td>Fragile X syndrome (FRAXA)</td>
<td>FMR1</td>
<td>RNA-binding protein mRNA transport?</td>
</tr>
<tr>
<td>HSAS, MASA</td>
<td>L1CAM</td>
<td>Cell adhesion molecule Neurite outgrowth, neuronal migration</td>
</tr>
<tr>
<td>X-LIS/SCLH</td>
<td>Doublecortin</td>
<td>Protein interacting with microtubules required for migration of cortical neurons</td>
</tr>
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HSAS, hydrocephalus with stenosis of the aqueduct of Sivius; X-LIS/SCLH, X-linked lissencephaly and subcortical laminar heterotopia. For a complete list of entries concerning XLMR see Labs et al. (4).

NON-SPECIFIC FORMS OF XLMR

Approximately 65 MRX loci corresponding to individual families are mapped along the X chromosome and can be grouped into 10–12 non-overlapping regions, suggesting the involvement of a minimum number of 10–12 X-linked genes (Fig. 1). Until recently only one gene involved in MRX had been identified: loss of expression of FMR2, a gene of unknown function adjacent to the fragile X-E (FRAXE) site on Xq28, is consistently correlated with FRAXE expansion in some mild mentally retarded patients (18). Over the past 2 years, despite the complexity of the clinical and genetic heterogeneity, positional cloning efforts based either on the investigation of balanced X-autosome translocations, deletion mapping or candidate gene strategy have to date allowed the identification of three additional genes involved in MRX (Fig. 1).

The first one, called oligophrenin 1, encodes a rhoGTPase-activating protein (19). Mutations of this gene are associated with mental retardation and are predicted to cause a loss of...
Recent investigations (unpublished data) using polyclonal antibodies raised against the C-terminal part of oligophrenin 1 allowed its precise cellular localization in primary cultures of neuronal and glial cells. Interestingly, a significant immunolabelling and co-localization with actin cytoskeletal proteins was observed in growth cones of neurites, stress fibres and the cortical cytoskeleton. Additionally, cytochalasin treatment, which disrupts actin cytoskeleton organization, produced a changed oligophrenin 1 distribution mimicking that of actin (P. Billuart et al., unpublished data).

The second gene involved in non-specific XLMR is GDI1 [a rabGDP-dissociation inhibitor (20,21)]. Mutations identified in the GDI1 gene from MRX patients suggest either abolished synthesis of GDI1 or a reduced ability to associate with its target GTPase. As a consequence, the effects of mutations could eventually be to greatly decrease the pool of rab proteins available for synaptic vesicle cycling and subsequent neurotransmitter release. Also, data reported by D’Adamo et al. (20) suggest an alternative or additional role for GDI1. Both the up-regulation of GDI1 and Rab3 expression at the onset of brain differentiation, before any detectable synaptic activity, and the inhibition of neurite outgrowth in hippocampal neurons treated with GDI1 antisense oligonucleotides suggest that GDI1 may be essential for outgrowth of axons and dendrites in vivo during brain development. Whether this role of GDI involves regulation of cytoskeletal proteins remains to be demonstrated. However, this seems likely as cytoskeletal changes play a major role in outgrowth.

The third gene, PAK3, is a member of the large family of p21-activating protein kinases (PAKs). In addition to the nonsense mutation reported by Allen et al. (22), our group has identified an additional mutation co-segregating in a large family with MRX (unpublished data). PAK proteins have been ascribed roles both in actin cytoskeleton regulation and in the Rac/Cdc42-induced activation of the MAP kinase cascades, including the c-Jun N-terminal kinase (JNK) and p38. Concerning the potential role of PAK proteins in neuronal cells, recent data using PC12 cells suggest that PAK(s) may be acting downstream of the GTPases in a signalling system which drives polarized outgrowth of the actin cytoskeleton in developing neurites (23).

In addition to these published data, we have recently identified two new genes involved in XLMR (unpublished data). Their involvement in mental retardation is based either on the detection of mutations or rearrangements that are predicted to cause a loss of function. Comparison of the predicted protein sequences with other sequences in the databases suggests that these proteins are components of signal transduction pathways mediated by cytokines or integrins. Although the organization of integrin-associated actin structures is regulated by complex mechanisms governed by members of the rho family of Ras-related GTPases (24), the potential regulation of cytoskeleton organization by signalling pathways in which these proteins are involved remains to be demonstrated.

**NON-SPECIFIC VERSUS SYNDROMAL FORMS OF MENTAL RETARDATION**

This dichotomy is of course convenient for clinical and diagnostic purposes. However, in the analysis of common genetic disorders one should keep in mind that even the same mutation...
in a single gene can produce a remarkably wide range of associated clinical phenotypes. It is therefore reasonable to expect that a proportion of patients with mild or severe MRX will have mutations in genes already known to cause syndromal forms of mental retardation. We already know that mutations in the FMR1 gene that cause fragile X syndrome may be associated with a very wide spectrum of clinical disorders, ranging from the classical syndrome to mild MRX (25). A broad phenotypic spectrum was also observed for XH2 and RPS6KA3 (also known as RSK2) gene mutations responsible for ATR-X and CLS, respectively (10,26). The recent finding in family MRX19, diagnosed as MRX and mapped to a 42 cM region in Xp22, of a missense mutation in the RPS6KA3 gene (27) is a notable example which confirms the extreme clinical heterogeneity in XLMR phenotypes. Clearly, these data raise important issues when counselling families at risk and whether mutations in genes involved in syndromal mental retardation might be commonly found in patients with non-specific forms of XLMR.

Given the variable mutational spectrum and the considerable scope for variation in the protein regulating processes in which genes responsible for syndromal forms of MR are involved, resulting from differences in genetic background, one might expect to see considerable variation in the effect of mutations in the same gene, and even of identical mutations, from one affected individual to another. However, whether this extreme clinical heterogeneity resulting from mutations in syndromal mental retardation genes is also present for genes involved in MRX remains an interesting question. It stands to reason that one should expect to be able to detect subtle brain abnormalities in some patients and/or that distinguishing clinical features may be associated with mutations in genes known to be involved in MRX.

### Molecular and Cellular Mechanisms Underlying MRX

Proper functioning of the nervous system and development of cognitive functions requires precision in extensive morphological changes necessary to establish functional connections. Dendritogenesis and axonal extension are driven by actin polymerization within the growth cone, a highly dynamic structure at the tip of the axon and dendrite, consisting of filopodial and lamellipodial protrusions that respond to both positive and negative external guidance cues. It is now well established that extracellular cues which function through surface receptors and signalling pathways which involve small GTP-binding proteins of the rho subfamily (rho-like GTPases) play an important role in remodelling of the cytoskeleton and in neuronal morphogenesis (28). The major role of these pathways in neuronal morphogenesis, axonal guidance and neurite growth has been reinforced by the exciting data obtained in vivo. Indeed, genetic analysis of developmental pathways in Drosophila melanogaster, Caenorhabditis elegans and mouse show that models with constitutively activated (or inactivated) rhoGTPases have defects in axonal guidance, neurite outgrowth, neuronal migration and morphogenesis (23,29–31). Contributions of rhoGTPases in development of the nervous system and establishment of functional connections are likely to be mediated through coordinated effects on the organization of the actin cytoskeleton and the regulation of other cellular functions such as gene transcription and adhesion.

At first sight, the complexity of the CNS requires the expression of a great diversity of mRNA and isolated mental retardation or syndromal mental retardation may arise from a large repertoire of mutations affecting any gene expressed in the CNS. However, data related to the genetic causes of non-progressive and non-specific XLMR demonstrate a link between the signalling pathways involved and cognitive functions, and suggest that these genes are not required for major aspects of brain development, such as layering of the cortex, organization of the brain, neuronal migration and the initial steps of neuronal differentiation. These genes are probably required, through their effect on cytoskeleton organization and/or synaptic vesicle cycling (or maybe other functions), for the remodelling, establishment and stabilization of connections: processes which are most likely required for the development of cognitive functions, including intellectual functions, learning abilities, etc. These functions are mainly acquired and modulated in the post-natal stages through contact with all types of stimulus and environment. Although assessment of the appropriate in vivo role in brain function of genes responsible for MRX will require further investigation, their potential involvement in regulating signalling pathways dependent on rho- and rabGTPases is consistent with the above-mentioned physiopathological hypothesis.

Identification of additional genes involved in MRX and syndromal forms of mental retardation without obvious brain developmental abnormalities might provide further evidence that could sustain the proposed cellular mechanisms and processes underlying cognitive impairment.

Although very little is known about qualitative and quantitative changes in synaptic, neurite and axon structures and densities in MRX patients, it is interesting to point out that in animal models deficient for FMR1 (the gene responsible for fragile X syndrome, which is associated with cognitive impairment without cerebral malformation), morphological abnormalities in dendrite spines were reported (32). Another relevant example concerns FGD1 protein (33), the guanine nucleotide exchange factor responsible for faciogenital dysplasia or Aarskog syndrome. It was shown that FGD1 activates the rhoGTPase Cdc42 and induces the polymerization of actin. In addition, FGD1 stimulates the mitogen-activated protein kinase cascade that leads to activation of the c-Jun kinase SAPK/JNK1 (34). Therefore, along with its target protein Cdc42, FGD1 regulates essential signals required during embryonic development. The mental retardation (without morphological brain abnormalities) observed in 50% of Aarskog patients could be explained by a perturbation of the role of FGD1 involved in the regulation of cytoskeleton organization.

Finally, studies designed to assess the prevalence of mutations in each of the genes so far identified showed that mutations in these genes are rare and account for only a very small proportion of MRX. As MRX is a very common disorder which affects ~1 in 600 males and the number of genes on the X chromosome is limited, one can predict the presence of a gene, or a few genes, not yet identified which are frequently mutated and account for a significant proportion of mentally retarded patients. A search for mutations in each MRX gene was performed in several probands from MRX families mapped by the European XLMR Consortium (Fig. 1) in genetic intervals that encompass candidate genes; however, this study did not show any mutations in the cases investigated.
Although these findings cannot exclude the possibility of mutations that might lie in promoters or intronic sequences, an alternative explanation could be the presence in each of the known loci of at least a second gene involved in MRX. This likely prediction suggests that the number of MRX genes is much higher than previously estimated. Therefore, sustained further efforts are required to identify the remaining genes in order to provide reliable molecular diagnosis of this common clinical problem, improve our understanding of the molecular and cellular bases underlying cognitive impairment and identify relevant targets for the development of appropriate therapeutic approaches.

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