4 DISCUSSION

4.1 Selection of X-chromosomal candidate genes for further studies

It is estimated that there is a causal link between chromosome anomaly and phenotype in only 50% of disease-associated balanced translocation carriers; therefore, both clinical and molecular data were considered in order to select only the most promising cases for further studies. Given the predominance of males with mental retardation and the established link between the X chromosome and numerous forms of MR, molecular characterisation of X chromosome anomalies was the obvious starting point in this study. Localisation of the X chromosome breakpoints and subsequent in silico analyses aimed to determine whether any X-linked genes or obvious regulatory regions were disrupted in our translocation patients. Causative genetic defects are most frequently reported among mentally retarded individuals who have a severe phenotype (Van Buggenhout et al. 2001); therefore, MR severity was also considered. Analysis of available clinical and preliminary molecular data from three patients with X;autosome balanced translocations enabled us to select a single candidate MRX gene. Our evaluation of each case is described below.

4.1.1 Patient 1 with translocation t(X;7)(q22;p22)

Our clinical data for Patient 1 and subsequent molecular analysis resulted in the exclusion of this case from further studies. This patient was diagnosed with ovarian dysgenesis and mild mental retardation, and carried a balanced translocation t(X;7)(q22;p22). There is an established link between Xq abnormalities and ovarian problems (Schlessinger et al. 2002); however, given the difference in nature of the two disorders, they were considered independently, and the ovarian dysgenesis is not relevant to this study. With regards to clinical data, Patient 1 was a rather poor candidate: with an IQ of 70, she is considered only mildly, or borderline, mentally disabled, and no other available clinical information suggested that the cognitive phenotype might be more severe. However, molecular analysis of the X chromosome breakpoint region was considered. Localisation of the breakpoint by FISH mapping led to the identification of two genes in the vicinity of the breakpoint, the retinal guanylyl cyclase GUCY2F, and the NTF2-related export factor NXT2. GUCY2F is expressed in the photoreceptor layer of the retina. According to previously published work, it is absent in all other tissues examined, including the cerebral cortex, midbrain, and cerebellum (Lowe
et al. 1995), and for this reason, it is unlikely that it plays a role specifically in cognitive function. We showed, however, that \textit{NXT2} is expressed in multiple tissues, including the adult and developing brain, and previous studies suggest that it binds to the TAP/NXF family of nuclear export proteins that are critical for nuclear mRNA export (Herold et al. 2000). Based on its expression in brain and its function in a fundamentally important cellular process, \textit{NXT2} was considered the more promising candidate gene for further studies. However, fine-mapping of the breakpoint revealed that the gene is not disrupted, and analysis of \textit{NXT2} transcripts indicated no difference between patient and controls. These data argue against a central role for the X chromosome breakpoint in the mental retardation present in this patient. It is possible that the chromosome 7 breakpoint plays causal role in the observed mental disability; however, given the mild phenotype, this breakpoint was not investigated in detail, and further studies involved other translocation cases.

\subsection*{4.1.2 Patient 2 with translocation t(X;15)(q13;p11)}

Patient 2, a second female with a balanced X chromosome translocation, was also excluded from further studies for specific reasons. Unlike Patient 1, Patient 2 was diagnosed with moderate to severe mental retardation, and more detailed clinical information was available. Moreover, we found that the breakpoint-spanning BAC clone carried the ATP-binding cassette transporter gene \textit{ABCB7}, which has been associated with MR (Bekri et al. 2000). However, FISH mapping of cosmid clones in the region showed that this gene was not disrupted by the breakpoint, and analysis of \textit{ABCB7} transcripts indicated no difference between patient and controls. This is in line with the absence of anaemia in the patient, and suggests that the clinical abnormalities are not specifically related to the \textit{ABCB7} gene. Likewise, the absence of known genes in the chromosome 15p11 region (Genome Browser July 2003 update) argues against a causative role for this breakpoint in the disorder. We cannot exclude the possibility that the breakpoint on Xq13 alters the function of a set of X-chromosomal genes quite far from the breakpoint and therefore not considered in our study. Indeed, distant position effects causing specific disorders have been documented (for review, see Kleinjan and van Heyningen 1998). On the other hand, it is important that the parental karyotypes are unknown in this case; we therefore cannot be sure that the chromosome rearrangement in the patient is \textit{de novo}. Thus, it is possible that the patient inherited the translocation from one of her phenotypically normal parents, and that the mental disability
results from a mutation in a different gene, or some unidentified environmental damage. These possibilities cannot be conclusively ruled out; therefore, without direct evidence suggesting a causal relationship between translocation and disorder, cases were not considered favourably. For Patient 2, further investigations are required to determine if such a causal relationship exists.

4.1.3 Patient 3 with translocation t(X;7)(p11.3;q11.21)

Unlike those involving Patients 1 and 2, preliminary studies on Patient 3, also a female carrying an X;autosome balanced translocation, did suggest that the X chromosome breakpoint is the cause of the mental disability. Breakpoint localisation by fluorescence in situ hybridisation indicated that the X chromosome breakpoint lies in the region of sequence overlap between two fully-annotated genomic clones. The ZNF41 gene, a member of a large family of transcriptional repressors, spans this region, and expression studies in the patient cell line indicated that ZNF41 transcripts are essentially absent, whereas they are readily detectable in control cell lines. Moreover, this patient was diagnosed with severe mental retardation, and previous studies had confirmed that neither of her phenotypically normal parents carry the translocation. The possibility that her disability was caused by the most common known genetic form of MR, Fragile X syndrome, had also been excluded. A severe mental disorder associated with loss of function of an X-linked gene that is expressed in multiple tissues, combined with the additional information that the chromosome abnormality is not inherited, and the exclusion of more common genetic causes for the disorder, made this case an obvious starting point for further studies, the implications of which are discussed in detail in the next section.
4.2 Understanding the role of ZNF41 in mental retardation

As discussed earlier, studies on Patient 3 provided the impetus for further examination of the ZNF41 gene. Subsequently, we found additional ZNF41 alterations in unrelated individuals with MR, which further supports our hypothesis that ZNF41 plays a critical role in brain development and function. In this section, we discuss the implications of our results and explore the functional relationships between ZNF41 and other MRX genes.

4.2.1 ZNF41 alterations are associated with cognitive dysfunction

Screening a panel of patients with apparent X-linked inheritance of MR led to the identification of two variations that were not present in controls. One of these is a proline to leucine amino acid exchange (P111L), which lies in the linker region of ZNF41, between the repressor domain and the zinc finger motifs. In light of the unique structure of proline, it is plausible that the tertiary structure of the protein is modified or destabilised by this exchange, which could have functional consequences. The second is an intronic splice-site mutation, which may have significant implications, as specific ZNF41 variants cannot be detected in the patient lymphoblastoid cell line. Both affected families are relatively small, and DNA from only immediate family members is available; as a result, our molecular data cannot definitively ascertain whether or not these genetic alterations play causative roles in MR. However, neither of these alterations was found in over 400 control chromosomes screened. We consider, therefore, that a causal relationship is highly probable in both cases.

No potentially disease-causing mutations were found in any other MRX patients tested, but it should be noted that our mutation detection technique relied primarily on amplification of exonic sequence from genomic DNA. Therefore, other potential splicing defects caused by alterations in the ZNF41 gene well within intronic sequence would not have been detected. It is also important to recognise that the frequency of mutations in any single MRX gene identified to date is relatively low. For example, a mutation in ARHGEF6, one of the 14 known genes implicated in monogenic forms of non-syndromic MR, was found in only one MRX family out of 119 screened (Kutsche et al. 2000). Based on the combined results of our study, we consider ZNF41 an important candidate gene for cognitive function. The exact mechanism by which ZNF41 mutations result in mental retardation is not yet clear; exploration of this question is the focus of this section.
4.2.2 Zinc finger genes, transcriptional repression and MR

Krueppel-type zinc finger genes are evolutionarily conserved transcriptional regulators characterised by their Cys\textsubscript{2}His\textsubscript{2} zinc-binding motif, which is typically responsible for sequence-specific DNA binding. ZNF41, absent in our patient, is a member of the subfamily of Krueppel-type zinc finger proteins harbouring a highly conserved N-terminal domain known as the Krueppel-associated box (KRAB). KRAB domains are present in approximately one third of all human zinc finger proteins (ZFPs) (Bellefroid et al. 1991) and are responsible for potent transcriptional repression (Margolin et al. 1994; Friedman et al. 1996; Kim et al. 1996; Moosmann et al. 1996; Moosmann et al. 1997). While the specific functions of ZNF41 are not fully understood, various related genes play an established and important role in human development and disease (Ladomery and Dellaire 2002b). Moreover, it is interesting to note that in two other female patients with severe non-syndromic MR and balanced translocations, the disorders likely result from the disruption of two related X-chromosomal zinc finger genes. Lossi et al. (2002) localised the X chromosome breakpoint just upstream of the Krueppel-like factor 8 (\textit{KFL8}), also known as \textit{ZNF741} gene, and confirmed that \textit{KLF8} transcripts are absent in the patient cell line. Similarly, \textit{ZNF81} transcripts are absent in another patient with MR and an associated balanced translocation (personal communication, Kleefstra et al. submitted). This case is especially pertinent given that \textit{ZNF81} is one of the closest known \textit{ZNF41} homologues. Additionally, the Wilms’ tumour suppressor gene \textit{WT1}, which harbours four Krueppel-type zinc fingers, has been implicated in several urogenital developmental disorders, including WAGR Syndrome, which is associated with mental retardation (Call et al. 1990; Rose et al. 1990; Gessler et al. 1992). Of particular relevance for this study, however, are the biochemical studies that illustrate the links between the highly conserved KRAB/ZFP subfamily of zinc finger proteins and chromatin remodelling. As outlined in the introduction, other MR-associated disorders have been linked to defects in processes that govern chromatin structural modification (for review, see Hendrich and Bickmore 2001).

Repression of transcription by a KRAB/ZFP requires binding of the corepressor KAP-1 (also known as TIF1\textbeta and KRP-1) (Friedman et al. 1996; Kim et al. 1996; Moosmann et al. 1996; Peng et al. 2000a; Peng et al. 2000b). KAP-1 is a molecular scaffold that coordinates gene-specific silencing. It recruits HP1-like proteins, homologues of non-histone heterochromatin-associated proteins with a well-established epigenetic gene silencing
function in *Drosophila* (Ryan et al. 1999) and interacts with the novel histone H3 Lys9-specific methyltransferase SETDB1 (Schultz et al. 2002). This interaction enhances HP binding to *cis*-regulatory sequences *in vivo*. Interestingly, within the primary sequence of SETDB1, Schultz et al. identified a methyl CpG binding domain that is related to that found in the MR-associated methyl CpG binding protein MeCP2. Like *ZNF41*, *MECP2* is ubiquitously expressed, and yet loss of functional protein results in a neurological phenotype. Although the mechanism by which *MECP2* mutations cause mental retardation is not clear, it is well established that MeCP2 binds to methylated CpGs and represses transcription (for review, see (Ballestar and Wolffe 2001), and it has recently been shown that MeCP2 associates with an unidentified methyltransferase that specifically methylates Lys9 of histone H3 (Fuks et al. 2003), as does the KAP-1/KRAB/ZFP binding partner SETDB1.

Further characterisation of the KRAB/KAP-1 repressor module has indicated that, in addition to the KRAB domain, a bipartite domain of the plant homeodomain (PHD) finger and a bromodomain, located within the C-terminal portion of KAP-1, are also required for effective gene silencing (Schultz et al. 2002). Interestingly, the point mutations in this study were modelled after naturally occurring mutations in the PHD finger of the human *ATRX* gene, which has been implicated in both X-linked alpha-thalassemia/mental retardation syndrome (Gibbons et al. 1995) and Juberg-Marsidi syndrome, which is also associated with MR (Villard et al. 1996a). In light of the fact that the PHD finger of KAP-1 is required for KRAB/ZFP-associated transcriptional repression, the association between the plant homeodomain-containing ATRX and MR is especially interesting. *ATRX* mutations, like *KAP-1* mutations, probably inhibit the PHD-dependent formation of multiprotein complexes required for chromatin remodelling. It is conceivable, therefore, that *ATRX* mutations and loss of KRAB/ZFP/KAP-1 function lead to MR via similar defects in regulation of chromatin structure, which likely overlap with the functional defects caused by *MECP2* mutations.

### 4.2.3 KRAB/ZFPs and evolution: *ZNF41* is a candidate gene for higher cognitive functions

Perhaps also of significance is the observation that many human members of the KRAB ZFP family have no obvious mouse orthologues. In a recent study on a conserved cluster of these genes on chromosome 19, it was ascertained that for 21 functional human KRAB ZFPs, there are only 10 corresponding mouse genes. The authors suggest that
duplication events occurring after human-rodent evolutionary divergence are responsible for this phenomenon, and they put forward the hypothesis that sequence and functional variation in these duplicated proteins plays a role in establishing species-specific traits (Shannon et al. 2003). In light of this, it is interesting that in silico analysis of both publicly available EST sequences, and alignment of fully annotated human and mouse genomic sequence (Genome Browser April 2003 and February 2003, respectively) suggests that ZNF41 is one of the KRAB zinc finger genes for which there is no mouse counterpart. It is enticing to propose that this subset of KRAB ZFPs may be specifically important for some of the psychological and cognitive traits that distinguish humans from lower mammals.
4.3 Selection of autosomal candidate genes for further studies

Unlike individuals with disrupted genes on the X chromosome, translocation carriers with affected autosomal genes generally maintain one functional copy on the normal chromosome. A phenotype results, therefore, if such breakpoints have dominant and detrimental effects. This applies in the case of haploinsufficiency, when a single gene dose is functionally inadequate, or it reflects a dominant negative effect, when a modified protein interferes with the function of the normal copy. Autosomal gene disruptions that function as recessive defects may also lead to disorders, but only in the subset of translocation carriers in which the second copy of the relevant gene is inactivated, by imprinting for example, or as a result of an independent mutation in the intact copy. It follows from this, that, while breakpoints affecting the X chromosome provide a logical starting point in the search for both recessive and dominant gene defects, characterisation of disease-associated autosome abnormalities favours the identification of genetic disorders with a dominant basis.

Also relevant is the fact that for autosomal candidate genes, verification of a genotype-phenotype correlation is more challenging. Given the heterogeneity of MR, which is already clear from studies that are confined to the X chromosome, screening for mutations in autosomal genes is simply not feasible in light of the number of patients that would be required. Instead, we rely on functional characterisation of selected genes to validate their association with a disorder.

Taking both of these points into consideration, autosomal genes are particularly good candidates when a chromosome abnormality results in translation of a modified protein. Especially in cases for which the relevant gene is implicated in neuron function, such modified proteins are excellent contenders for dominant negative effects that result in mental retardation; moreover, they provide an obvious starting point for the required functional studies.

4.3.1 Patient 4 with translocation t(2;14)(p22;q13)

Patient 4, diagnosed with severe mental retardation, agenesis of the corpus callosum, and microcephaly, carries a balanced translocation t(2;14)(p22;q13), that likely interrupts two uncharacterised transcripts. On chromosome 2, FISH mapping of genomic clones indicated that the breakpoint lies within a region consisting of several overlapping intron-spanning EST sequences. In silico analysis of these sequences suggested that a novel gene, of unknown
function and expressed in multiple tissues, is disrupted by the breakpoint. Cytogenetic studies on chromosome 14, in contrast, highlighted a particularly promising candidate gene, the forkhead transcription factor *FOXG1B*, specifically expressed in the brain and known in the mouse for its essential role in the developing telencephalon (Xuan et al. 1995). Further breakpoint characterisation, however, indicated that the known sequence of this gene is not directly affected by the breakpoint, which lies approximately 5-8kb distal to end of the *FOXG1B* 3'UTR. We cannot exclude the possibility that the altered chromosome environment influences *FOXG1B* function, but the absence of direct evidence for a causal link between this gene and the disorder precluded it from further investigation within this study. Moreover, an independent transcript, represented by Unigene cluster Hs.92556 is disrupted by the chromosome 14 breakpoint, further adding to the genetic complexity of this case. In contrast to studies on Patient 5, discussed in the next section, our preliminary analysis of Patient 4 did not suggest a specific genotype-phenotype correlation, from which we could initiate functional studies. For this reason, Patient 4 will be assessed in more detail in future investigations.

### 4.3.2 Patient 5 with translocation t(Y;4)(q11.2;q21)

Patient 5, like Patient 4, presented with a severe phenotype. His disorder was first recognised at the age of thirteen months, when he began to lose acquired motor and cognitive skills, and developed seizures that did not respond to medications. Molecular studies on his balanced translocation t(Y;4)(q11.2;q21) focussed on chromosome 4, and led to the identification of a breakpoint-spanning clone lying within the coding sequence of the 160 kb-spanning c-Jun N-terminal kinase 3 (*JNK3*) gene. JNK proteins, also referred to as stress-activated protein kinases (SAPKs), are a subset of MAP kinase signalling molecules best known for their role in the apoptotic process (Kyriakis and Avruch 2001), but also implicated in neuronal differentiation (Coffey et al. 2000; Waetzig and Herdegen 2003). There are three known JNK proteins (JNKs 1, 2 and 3), all of which are expressed in the central nervous system, but JNK3 is the only member of this subfamily with predominant expression in the brain; it is logical, therefore, to expect that this gene has specific importance for neuronal development and function. Moreover, both the *Jnk3* +/− and −/− mice exhibit abnormal responses to seizure induction (Yang et al. 1997), which provides an additional link to the patient phenotype. These two points, together with the fact that JNKs are known downstream...
effectors of specific Rho-GTPases that have been implicated in MR (Minden et al. 1995; Olson et al. 1995; Ramakers 2002), provided the impetus for further characterisation of the disrupted *JNK3* in the patient. The implications of our results are discussed in more detail in section 4.4.
4.4 Understanding the role of a truncated JNK3 in MR

Fine-mapping of the chromosome 4 breakpoint in Patient 5 indicated that JNK3 is disrupted by the breakpoint following coding nucleotide 802. This results in a truncated JNK3 protein with 267 out of a possible 422 or 464 amino acids (corresponding, respectively, to JNK3α1 and JNK3α2). The conserved serine-threonine kinase domain is disrupted, which most likely abolishes its catalytic activity. Expression studies in the patient cell line, however, confirmed that the truncated protein is present, indicating that the corresponding transcript is not subject to nonsense-mediated mRNA decay. Taken together with the established role of JNK3 in neurons, these data suggest that the chromosome aberration causes the phenotype in Patient 5. Whether this occurs by simple loss of JNK3 activity, or by effects of the mutant JNK3 on normal JNK signalling, or a combination of both, is the focus of this discussion.

4.4.1 A truncated JNK3 likely interferes with normal JNK3 function in neurons

In light of the fact that the patient retains one functional copy of JNK3, it is important to note that certain critical amino acid residues remain intact in the truncated protein. For example, the mutant protein retains residues 221-223 (TPY), which, in the normal JNK3, is the site of phosphorylation by upstream MAP kinase kinases MKK4 and 7 (Ip and Davis 1998). Moreover, residues for approximately 70% of the conserved kinase domain are present. While little is known about the specific regions of JNK3 required for binding to its various interaction partners, it is likely that a truncated JNK3 will not behave like the wild type in protein-protein interactions. Moreover, over-expression of both wild type and mutant proteins in several cell types indicated striking differences in protein expression within the cell. Such aberrant cellular localisation not only disturbs JNK3 itself, but probably also has effects on any other proteins that bind the mutant JNK3. It is likely, therefore, that signalling through the normal JNK3 is modified by the truncated protein in the patient, and that the phenotype is not a result of simple haploinsufficiency. We discuss this idea in more detail in the next two sections.

4.4.2 Links between Rho-GTPase signalling, neurite outgrowth, and JNKs

Pathophysiological studies have provided insights into the biological processes that Rho-GTPases regulate. Analysis of brain tissue from patients with cognitive impairment suggest that the disorder might result at least in part from abnormalities in dendritic
branching. Both Huttenlocher (1974) and Purpura (1974) observed a reduction of dendritic branching in tissues from patients with MR, and this phenomenon (reviewed by Ramakers 2002) is now well accepted. While it has long since been established that Rho-GTPases are critical for regulation of the actin cytoskeleton by influencing formation of lamellipodia and filopodia, their role specifically in regulation of neurite outgrowth has more recently come to the forefront. In one of the earliest of such studies, transgenic mice expressing constitutively active human Rac1 developed ataxia, and on examination of Purkinje cells in the deep cerebellar nuclei, reduced axon terminals were observed. In addition, dendritic spines in the developing and mature cerebella were reduced in size but increased in number (Luo et al. 1996a). In another early study, the importance of GTPases in regulation of neurite outgrowth was demonstrated using a combination of constitutively active and dominant negative mutants of Rho, Rac, and Cdc42 (Threadgill et al. 1997).

Since then, numerous studies support an increasingly accepted model in which activation of Rac1 and Cdc42 induce respective formation of lamellipodia and filopodia, resulting in neurite outgrowth and spine formation, whereas activation of RhoA tends to have an inhibitory effect on these processes (Luo 2000). Moreover, several studies have shown that inhibition of RhoA promotes neurite outgrowth (Luo et al. 1996b; Kozma et al. 1997; Li et al. 2000; Dergham et al. 2002). Although there is no known direct interaction between Rho proteins and JNKs, it is well accepted that signalling through GTPases activates the mitogen activated protein (MAP) kinase cascade, which is characterised by sequential activation of a series of kinases (Kyriakis and Avruch 2001). In general, MAP kinase kinase kinases (MAP3Ks) activate the MAP kinase kinases (MAP2Ks or MKKs), which subsequently activate the MAP kinases. JNKs belong to the MAP kinase subgroup of these signalling molecules, and thereby make up one of several distal branches of the GTPase-mediated signal transduction network. Indeed numerous studies illustrate this connection.

The links between the Rho-GTPases and JNK were first reported in studies in which activation of Rac1 and Cdc42 induce respective formation of lamellipodia and filopodia, resulting in neurite outgrowth and spine formation, whereas activation of RhoA tends to have an inhibitory effect on these processes (Luo 2000). Moreover, several studies have shown that inhibition of RhoA promotes neurite outgrowth (Luo et al. 1996b; Kozma et al. 1997; Li et al. 2000; Dergham et al. 2002). Although there is no known direct interaction between Rho proteins and JNKs, it is well accepted that signalling through GTPases activates the mitogen activated protein (MAP) kinase cascade, which is characterised by sequential activation of a series of kinases (Kyriakis and Avruch 2001). In general, MAP kinase kinase kinases (MAP3Ks) activate the MAP kinase kinases (MAP2Ks or MKKs), which subsequently activate the MAP kinases. JNKs belong to the MAP kinase subgroup of these signalling molecules, and thereby make up one of several distal branches of the GTPase-mediated signal transduction network. Indeed numerous studies illustrate this connection.

The links between the Rho-GTPases and JNK were first reported in studies in which activation of Rac1 and Cdc42, but not RhoA, led to up-regulation of the JNK signalling pathway (Minden et al. 1995). While JNK/SAPKs are best known for their role in the stress response and apoptosis (for review, see Ip and Davis 1998), more recent studies have directly implicated these kinases in neuronal differentiation and neurite outgrowth. For example, Waetzig et al. (2003) demonstrated that over-expression of JNK3 enhances neurite outgrowth in PC12 cells. Yu et al. (2003) observed that JNK1 is activated upon retinoic acid-induced
neurite outgrowth in a human neuronal cell line, and that both differentiation and JNK activation are blocked by a dominant negative form of MAP kinase kinase 4 (MKK4), an upstream regulator of JNK. These data support the hypothesis that JNKs are not only critical in the stress-response, but also play an active physiological role in the differentiating neuron, and together with studies that link JNKs to Rac1 and Cdc42 but not RhoA, support a model in which Rac1/Cdc42-mediated positive regulation of neurite outgrowth is JNK-dependent. This model is especially attractive in light of our results. A non-functional JNK protein such as the JNK3 mutant present in Patient 5 could, for example, sequester JNK binding partners involved in this cascade, thereby preventing normal JNK activation and inhibiting neurite outgrowth.

The molecular links between specific Rho-GTPases and the JNK subset of downstream cascades have been investigated in numerous contexts, thereby resulting in an array of somewhat contradictory conclusions. Considering our data, however, specific intermediate components of the GTPase-JNK cascade acquire particular importance as candidate regulators of neurite outgrowth and neurological function. This concept is the focus of the next section.

4.4.3 JNKs within the context of Rho-GTPase-mediated signalling and MR

Of particular relevance to our understanding of how this cascade functions in neurons is that, in addition to the upstream kinases within the MAP kinase cascades, both the p21-activated kinase (PAK) and the cyclin dependent kinase (Cdk) families serve as intermediary links between Rho-GTPases and JNKs. The human PAK1 gene, like its homologue in yeast (Ste20), is a serine-threonine kinase that has been shown to influence JNK signalling following activation by Rac1 and Cdc42 (Brown et al. 1996). These studies were not done in neuronal cells; however, this link between human GTPases and JNKs via the PAK family has specific implications in neurons. Three out of the six known members of the PAK family, PAK1, PAK3 and PAK5, show predominant expression in the brain (Manser et al. 1995; Burbelo et al. 1999; Dan et al. 2002); moreover, mutations in PAK3 are responsible for MRX (Allen et al. 1998), and activation of PAK5 has been shown to promote neurite outgrowth in neuroblastoma cells (Dan et al. 2002). Also relevant is the fact that the LIM domain kinase LIMK1, also implicated in cognition (Frangiskakis et al. 1996), is a direct target of the PAK kinases. It is plausible, for example, that several of the brain-expressed PAKs have critical
and specific effects on the predominantly CNS-expressed JNK3. In addition, Cdk5 plays an established role in neurons, in association with its neuron-specific regulator p35. When over-expressed together in neurons, these two proteins enhance neurite outgrowth (Nikolic et al. 1996). Moreover, they form a complex with active GTP-bound Rac in neuronal growth cones (Nikolic et al. 1998). Interestingly, in an independent study by another group, JNK3 was identified as a direct phosphorylation target of Cdk5 (Li et al. 2002). Together these three studies provide a second neuron-associated link between the JNKs and the Rho-GTPases.

However, the resulting model is not a simple one in which activated Rac/Cdc42 results in activated PAK and Cdk5, which in turn stimulate JNKs to promote neurite outgrowth. While active PAK1 was initially implicated in specific activation of JNK signalling (Brown et al. 1996), the interaction between Cdk5 and JNK3 described by Li et al. (2002) is one of negative regulation. They demonstrated that Cdk5 inhibited JNK3 activation of downstream transcription factors, and that JNK3 was correspondingly upregulated in Cdk5-/- mice, suggesting that JNK3 and Cdk5 have antagonistic functions. Interestingly, Nikolic et al. (1998) found that the p35/Cdk5 complex also has a negative regulatory effect on PAK1 activation, suggesting that it plays a similarly antagonistic role with respect to signalling through PAKs. In light of this result, it is also interesting that the PAK effector LIMK1 is a target of the Rho-associated kinase ROCK (Ohashi et al. 2000), which is activated by RhoA, but not Rac1 and Cdc42 (Nikolic 2002). Although RhoA is typically associated with inhibition rather than activation of neurite outgrowth, it is clear that there is complex cross talk between the RhoA and the Rac/Cdc-mediated cascades, and that a correct balance is required for normal function.

Also perhaps relevant are the studies that implicate the upstream MAP kinase kinases MKK4 and 7 (sometimes referred to as JNKKs) in positive regulation of the JNK proteins (for reviews, see Ip and Davis 1998, and Gutkind 2000). These early data were obtained primarily from experiments in non-neuronal cells, however, and their relative importance in neurons has not yet been assessed. It is noteworthy that studies on signalling through the PAK family of kinases are often contradictory, especially with respect to downstream effects on JNKs, perhaps due to cell-type specific regulatory factors. While Brown et al. (1996) demonstrated a stimulatory effect of PAK activation on JNK signalling, other groups have shown the opposite. Teramoto et al., for example, demonstrated that over-expression of PAK1 in fact diminished rather than enhanced JNK activation in some cell types (Teramoto
et al. 1996b). The same group proposed an alternate mechanism by which Rac1 and Cdc42 might activate JNKs. They identified the mixed lineage kinase MLK3, a member of the MAP3K family, and showed that it binds to active Rac1 and Cdc42 and, through this interaction, mediates specific activation of JNKs via the MAP kinase cascade (Teramoto et al. 1996a). Taken together, these results suggest that activation of JNKs by the Rho-GTPases is mediated through a complex signalling network that relies on both stimulatory and inhibitory functions of multiple upstream kinases. Those aspects of this network discussed above are depicted schematically in Figure 31.

Other proteins that serve to link the JNK proteins with GTPases include several JNK scaffolding proteins. One such example is provided by studies on the scaffolding protein POSH (Plenty Of SH3s). POSH binds directly to activated Rac/Cdc42 and also to the JNK-activating mixed lineage kinases (MLKs) (Xu et al. 2003). As mentioned earlier, these proteins have been previously implicated in Rho-GTPase-mediated JNK activation. More importantly with respect to our study, this group showed that POSH forms a complex with JNKs, together with the M KKs 4 and 7, and they suggested that it plays a critical role in recruiting necessary components of the JNK signalling pathway, enabling them to interact with each other. Although the proteins discussed in this study are not neuron-specific proteins, the experiments were carried out in both neuron-like cells and primary neurons, affirming the relevance of these results within the context of neuronal development and function. Another protein with a proposed JNK-scaffolding function is the WASP-family verprolin homologous protein-1 (WAVE-1). WAVE-1 has been implicated in cognition in mice (Soderling et al. 2003), and it complexes with both Rac1 and PAK1 (Wu et al. 2003). The WAVE-1 protein is specifically expressed in brain (Soderling et al. 2003), and it has also been shown to interact with MEGAP/srGAP3 (also known as WAVE-associated RacGAP protein, or WRP), a GTPase-activating protein that has independently been linked to cognition through its association with two independent forms of MR (Soderling et al. 2002; Endris et al. 2002). In addition, the JNK interacting protein (JIP) family serves a JNK scaffolding function. JIPs 1 and 2 are related in sequence and are highly expressed in the brain. They have been shown to interact with MAP3K12, MLK2, MLK3, M KK7, and all 10 known JNK isoforms (for review, see Yasuda et al. 1999).
Figure 31: Signalling cascades that link the Rho-GTPases with the JNK proteins
While active Rac1 and Cdc42 generally stimulate neurite outgrowth (green arrow), active RhoA has a predominantly inhibitory affect on this process (red arrow). Active Rac1 and Cdc42 are implicated in activation of the MAP kinase cascade through both MAP3 kinases (MAP3K) and mixed lineage kinases (MLK), which promotes JNK activation and apoptosis. Rac1/Cdc42 activation is also associated with activation of p21-activated kinase (PAK) and cyclin-dependent kinase (Cdk). The combined activity of these two kinases results in a balance of stimulatory and inhibitory signals, which may promote JNK activation in only specific circumstances. RhoA activation, which for the most part inhibits neurite outgrowth, results in activation of the Rho-associated kinase (ROCK), which activates the LIM-domain kinase (LIMK). LIMK is also activated by PAK; therefore, like JNK, it is regulated through a balance of multiple signals.

Perhaps of greatest significance, however, are studies that directly implicate proteins in JNK3 binding. JIP3, also known as JNK/stress activated protein kinase-associated protein 1 (JSAP1) is by sequence unrelated to JIPs 1 and 2, but is likewise known for its role as a scaffold for JNKs. JIP3/JSAP1 exhibits predominant expression in the brain, and it has particular affinity for binding to JNK3, in comparison with JNKs 1 and 2 (Ito et al. 1999; Kelkar et al. 2000). It is also phosphorylated by JNK3 (Ito et al. 1999). Another JNK scaffold, β-arrestin 2, is of relevance for similar reasons. It interacts with both JNK3 and its upstream regulators MAP3K5 and MKK4, and facilitates JNK3 phosphorylation (McDonald et al. 2000; Miller et al. 2001). Moreover, β-arrestin 2-mediated JNK activation is specific for JNK3 (McDonald et al. 2000), perhaps in part because the unique N-terminus of JNK3 (not present in JNKs 1 and 2) plays a role in this binding interaction (Whitmarsh et al. unpublished results, personal communication). While the well-established JNK activation of apoptosis occurs through nuclear localisation of JNK and subsequent phosphorylation of the transcription factor c-Jun (for review, see Mielke and Herdegen 2000), McDonald et al.
(2000) demonstrated that β-arrestin 2 also stimulates activation of JNK3 in the cytosol, and that active phosphorylated JNK3 colocalised with β-arrestin 2 in subcellular compartments. Together with the JNK3-specific nature of the JNK-β-arrestin 2 interaction, this result strongly supports the expanding theory that regulation of apoptosis is only one of a diverse set of functions executed by the JNK proteins in neurons. It is reasonable to suspect that JNK3 may have a specific role in the CNS together with any or several of these brain-expressed scaffolding proteins, and that this role may be independent of the apoptosis-related roles of JNKs in multiple cell types. Further evidence in support of this theory is provided by a study in which JNK3 over-expression clearly promoted neurite outgrowth (Waetzig and Herdegen 2003).

Another interesting direct link between JNKs and neural development is the fact that JNK3 binds and phosphorylates the neuronal growth-associated protein SCG10 (Neidhart et al. 2001). This protein is enriched in growth cones where it destabilises microtubules, and like JNK3 and JSAP1, it exhibits essentially neuron-specific expression. Although several families of MAPK proteins are able to phosphorylate SCG10 (Antonsson et al. 1998; Neidhart et al. 2001), only the JNK family has been shown to bind SCG10 (Neidhart et al. 2001). Moreover, this JNK-SCG10 interaction was demonstrated using JNK3; therefore, like the results of experiments demonstrating strong JNK3 interactions with both JSAP1 and β-arrestin 2 described earlier, it has immediate implications for our understanding of the disorder in our patient. These protein-protein interactions with demonstrated specificity either for JNK3 itself or for exclusively neuron-related processes are depicted below in Figure 32.

**Figure 32: JNK3 binding partners and scaffolding proteins**
Depicted here in orange and blue are the proteins POSH and JIPs 1 and 2, which serve as scaffolds for the MAP kinase cascade proteins (shown in yellow and green). JNK3 has been shown to bind with specific affinity for both β-Arrestin 2 and JSAP1, two additional scaffolding proteins (depicted in beige). SCG10 (also in beige) is phosphorylated by JNK3 and plays an established role in neuronal growth cone dynamics.
4.4.4. Proposed model for a mutant JNK3 in the aetiology of MR

Given the role of JNK activation in GTPase-mediated cascades, and the established function of JNK3 in regulation of neurite outgrowth and neuronal apoptosis, partial loss of function of JNK3 could lead to neuronal malformations that result in MR and neurodegeneration. However, studies on both heterozygous and homozygous *Jnk3* knockout mice, which show no obvious developmental phenotype, do not support a model based exclusively on haploinsufficiency. Mice with reduced or absent JNK3 protein are resistant to induced seizures, but pathological studies on the brains of these mice showed no morphological changes indicative of defective neuritogenesis (Yang et al. 1997). This result is in line with our hypothesis that the truncated JNK3 in our patient, which maintains approximately 60% of the residues present in the wild type isoforms, plays a dominant role by disrupting normal signalling cascades critical for neurite outgrowth and differentiation. As discussed earlier, the role of GTPase-mediated signalling in neurite outgrowth, together with the evidence that JNKs serve as critical effectors in this cascade, perhaps through their interactions with several other neuronal proteins, provides the molecular basis for this theory.

In section 4.4.2 we outlined the complexity of GTPase-mediated effects on neurite outgrowth, highlighting the fact that this process depends on both stimulatory and inhibitory effects of numerous kinases and likely requires a fine balance. In section 4.4.3 we described several important JNK interacting proteins, several of which have been shown to bind tightly and specifically to JNK3. Two of these binding partners, SCG10 and JSAP1, have been shown to accumulate in the growth cones of developing neurites (Antonsson et al. 1998; Kelkar et al. 2000) and therefore provide an obvious link between JNK activation and neuritogenesis. It is plausible that a truncated JNK3 sequesters one or more JNK3 binding partners, thereby preventing them from functioning optimally within the normal GTPase-JNK cascade, and perhaps simultaneously disrupting the balance of Rho-, Rac-, and Cdc42-mediated effects that is necessary for proper neural development. One could also speculate that Cdk5, which phosphorylates JNK3 at its N-terminus, is also to some extent occupied by the mutant JNK3, thereby preventing the p35/Cdk5 complex from inhibiting the apoptotic function of the wild type JNK3 and serving its classical cell survival function.

Very likely, the phenotype in Patient 5 results in part from a dominant effect of the truncated protein on the normal JNK3 interactions we have outlined, perhaps in combination
with additional imbalances in unidentified JNK3-mediated cascades. Together with a simultaneous partial loss of any JNK-dependent stimulatory effects on neurite outgrowth, it is easy to see that such alterations could have detrimental effects on neural development.