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Molecular pathology of Rett syndrome

PhD thesis

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LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine (serotonin)
5-HTT	5-hydroxytryptamine transporter (serotonin transporter)*
5-HTTLPR	5-hydroxytryptamine-linked polymorphic region
APOE	apolipoprotein E gene
ApoE	apolipoprotein E
ARX	aristaless-related homeobox gene
BDNF	brain derived neurotrophic factor*
BF1	brain factor 1
bp	base pair
CDKL5	cyclin-dependent kinase-like 5*
COX2	cyclooxygenase 2
CpG	cytosine-phosphate-guanine dinucleotide
CRH	corticotropin-releasing hormone gene
CT	computer tomography
DLX5	distal-less homeobox 5 gene
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
EEG	electroencephalography
EIEE2	early infantile epileptic encephalopathy 2
FKBP5	FK506-binding protein 5 gene
FOXG1	forkhead box G1*
FXYD1	FXYD domain-containing ion transport regulator 1 gene
GABA	gamma-aminobutyric acid
GABRB3	gamma-aminobutyric acid receptor beta-3 gene
gDNA	genomic DNA
HDAC	histone deacetylase
HMOX1	heme oxygenase 1 gene
HO-1	heme oxygenase 1
HRM	high-resolution melting
ID1	inhibitor of DNA binding 1 gene (also *ID2* and *ID3*)

IGFBP3	insulin-like growth factor-binding protein 3 gene
kb	kilo base pair
MAP2	microtubule-associated protein 2
MBD	methyl-CpG-binding domain
MECP2	methyl-CpG-binding protein 2 gene
MeCP2	methyl-CpG-binding protein 2
MLPA	multiplex ligation-dependent probe amplification
MR	mental retardation
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
NLS	nuclear localization signal
NTNG1	netrin G1 gene
PCR	polymerase chain reaction
PET	positron emission tomography
RFLP	restriction fragment length polymorphism
RTT	Rett syndrome
SGK1	serum/glucocorticoid-regulated kinase 1 gene
STin2 VNTR	serotonin transporter intron 2 variabile number of tandem repeats
STK9	serin/threonine protein kinase 9 gene
Ta	annealing temperature
TRD	transcriptional repression domain
UBE3A	ubiquitin-protein ligase E3A gene
UQCRC1	ubiquinol-cytochrome c reductase core protein 1 gene
UTR	untranslated region
XCI	X chromosome inactivation

* Designation of a gene in the text is the same but written in *italics*.

INTRODUCTION

Mental retardation (MR) is characterized by sub-average cognitive functioning accompanied by deficits in at least two adaptive behaviors, such as self-care, communication, and social skills. The symptoms may appear at birth or later in childhood (Raymond, 2006). Down syndrome and fragile X syndrome are generally the most common genetic causes of MR. In females, the second position is occupied by Rett syndrome (RTT) instead of fragile X syndrome.

The story of RTT begun in 1960´s when two unrelated severely disabled girls, were incidentally sitting next to each other in the waiting room of a Viennese pediatrician Andreas Rett. Their strikingly similar washing hand movements caught the pediatrician´s attention and Rett recollected that he had already seen the same symptoms in a few of his female patients. He also realized that they all suffered the same neurological disorder, distinct from cerebral palsy, the usual designation at the time. He published his findings (Rett, 1966) but the discovery remained overlooked by international medical community. Almost 20 years later, Hagberg and colleagues increased awareness of this condition in the English medical literature and they named it Rett syndrome (Hagberg et al., 1983). After then, RTT became worldwide recognized by pediatricians, neurologists, geneticists, and neuroscientists.

There is a considerable phenotypic overlap between RTT and several neuropsychiatric and neurodevelopmental disorders, especially autism. It has been suggested that a common pathogenic process may induce or at least contribute to these conditions. Since RTT is the only one among pervasive developmental disorders with a known genetic cause, understanding of the pathogenic processes in RTT brains will not only shed more light into neuronal development, plasticity, and functions, but may also provide insight into pathogenesis of other neurodevelopmental disorders. Therefore RTT is considered a prototypical neurodevelopmental disorder.

REVIEW OF THE LITERATURE

Clinical features and stages of Rett syndrome

RTT (OMIM #312750) is a progressive neurodevelopmental disorder, which contributes significantly to a severe intellectual disability in females. The estimated incidence is 1:10,000-15,000 female births worldwide, with no specific ethnical or geographical preference. Male cases, which will be discussed later, are very rare.
The clinical diagnosis of RTT is based on several well-defined criteria, which were set forth in 1988 (Anonymous, 1988) and revised for several times, most recently in 2002 (Hagberg et al., 2002) (Tab. 1).

Tab. 1. Diagnostic criteria for classic Rett syndrome (Hagberg et al., 2002).

Necessary criteria
1. Apparently normal prenatal and perinatal period
2. Apparently normal psychomotor development during the first 6-18 months (but may be delayed from birth in some cases)
3. Normal head circumference at birth
4. Postnatal deceleration of head growth (between the age of 5 months and 4 years) and acquired microcephaly
5. Loss of purposeful hand skills between the age of 6 and 30 months
6. Development of stereotyped hand movements
7. Social withdrawal
8. Lost or severely impaired expressive and receptive language
9. Apparent severe psychomotor retardation
10. Motor dysfunction (gait ataxia, apraxia)
Supportive criteria
1. Breathing disturbances while awake (hyperventilation, breath holding)
2. Bruxism
3. Impaired sleeping pattern (including night screaming)
4. Abnormal muscle tone, sometimes dystonia
5. Peripheral vasomotor disturbances (hypotrophic small and cold, discolored feet and/or hands)
6. Progressive kyphosis or scoliosis
7. Growth retardation
Exclusion criteria
1. Organomegaly or other evidence of storage disorder
2. Cataract, retinopathy or optic atrophy
3. History of perinatal or postnatal brain damage
4. Identifiable inborn error of metabolism or neurodegenerative disorder
5. Acquired neurological disorder due to severe infection or head trauma
6. Evidence of prenatal onset of growth retardation or microcephaly

Prenatal and perinatal periods are usually normal. Affected girls appear to develop normally during the first 6-18 months of life and seem to achieve appropriate developmental milestones. Nevertheless, several studies have shown that even during this period the female infant may display some subtle motor and behavioral abnormalities, as well as hypotonia and feeding problems. General mobility is usually poor but an excess of repetitive hand patting may be seen even during the first year of life. The child is usually quiet and placid, and described as “very good” by parents (Rett, 1966; Kerr, 1995; Burford et al., 2003; Einspieler et al., 2005). The first apparent characteristic features start to manifest in early childhood and they appear gradually over several stages:

I. Stagnation stage (age 6-18 months). Toward the end of the first year of life the psychomotor development begins to slow down. Deceleration of head growth, which eventually leads to microcephaly, occurs in most patients, together with growth retardation and weight loss. The child ceases in the acquisition of skills and this period may last for several months. Girls with RTT may also display some autistic features such as emotional withdrawal, diminished eye contact, and indifference to the surrounding environment (Shahbazian and Zoghbi, 2002; Nomura and Segawa, 2005).

II. Regression stage (age 1-4 years). After the period of developmental stagnation, patients rapidly enter the regression stage. It may occur over a period of days to months and is characterized by reduction or loss of acquired skills, especially purposeful hand use, speech, and interpersonal contact (Nomura, 2005). The voluntary hand use is replaced with repetitive stereotypic hand movements, the hallmark of RTT. Patterns consist of wringing, hand washing, flapping, clapping, patting, and other hand automatisms, occurring during waking hours (Rett, 1966; Nomura and Segawa, 1992; Hagberg, 1995). Additional motor abnormalities include truncal and gait ataxia/apraxia. Irregular breathing patterns, such as hyperventilation, breath holding, and aerophagia, usually develop towards the end of the regression period (Witt Engerstrom, 1992; Kerr, 1995). Seizures occur in about half the patients (Glaze et al., 1987) and are very variable, ranging from relatively easily controlled ones to intractable epilepsy (Jian et al., 2006).

III. Pseudostationary stage (age 4-7 years). After the regression stage, patients stabilize and may even recover some skills. They become more sociable and many of them learn to communicate and express themselves using signs or eye pointing.

Nevertheless, they still have gross cognitive and motor impairments, and often experience seizures (Shahbazian and Zoghbi, 2002).

IV. Late motor deterioration stage (age 5-15 years and older). Communication and social skills continue to improve gradually. Despite persisting serious cognitive impairment, older girls with RTT are usually, in contrast to patients with childhood autistism, sociable and pleased with a company (Mount et al., 2001). The seizures become less frequent and often decrease in severity, presenting minor problems in adulthood. On the other hand, gross motor deterioration continues with age. Hypotonia changes to prominent rigidity and many patients suffer from dystonia in addition to progressive scoliosis. Some patients completely lose their mobility and become confined to wheelchairs during the teenage years (Hagberg and Witt-Engerstrom, 1986; Shahbazian and Zoghbi, 2002). At older age, they often develop Parkinsonian features, too (Roze et al., 2007).

Female RTT patients usually survive into adulthood, some even up to the sixth or seventh decade, but the life expectancy is shorter than in healthy population. Moreover, the incidence of sudden unexpected deaths, accounting for about 25%, is much higher than in controls of similar age. They may occur due to the autonomic nervous system disturbances or cardiac electrical instability (Kerr et al., 1997; Guideri et al., 1999; Kerr and Julu, 1999).

Rett syndrome variants

In addition to the classic form of RTT described above, there are several variants, which do not completely meet all the diagnostic criteria (Tab. 2). They can be milder or more severe than the classic RTT phenotype. The more severe variants include the early-onset seizure variant with the onset of seizures before the age of 6 months (Hanefeld, 1985) and the congenital variant, which lacks the early period of normal psychomotor development (Hagberg and Skjeldal, 1994). Milder variants include the late regression variant manifesting late at preschool or early school age (Hagberg, 2002), the preserved speech variant with preserved communication skills and the normal head size (Zappella, 1992), and finally the “forme fruste”. This variant is the most common atypical form of RTT and is characterized by a very mild phenotype with partially preserved gross motor functions, subtle and easily missed neurological abnormalities (Hagberg, 2002).

Tab. 2. Diagnostic criteria for Rett syndrome variants (Hagberg et al., 2002). Patients have to fulfill at least 3 main and 5 supportive criteria.

Main criteria
1. Absence or reduction of hand skills
2. Loss or reduction of speech
3. Hand stereotypes
4. Loss or reduction of communication skills
5. Deceleration of head growth from early childhood
6. Regression followed by recovery of interaction
Supportive criteria
1. Breathing disturbances while awake (hyperventilation, breath holding)
2. Air swallowing or abdominal bloating
3. Bruxism
4. Abnormal locomotion
5. Impaired sleeping pattern (including night screaming)
6. Inexplicable episodes of laughing or screaming
7. Cold, discolored feet, usually hypotrophic
8. Apparently diminished pain sensitivity
9. Intense eye contact and/or eye pointing
10. Kyphosis or scoliosis

Neuropathology and neurochemistry of Rett syndrome

RTT was initially considered to be a neurodegenerative disorder, but no obvious signs of neuronal loss, inflammation or gliosis have been observed. The macroscopic appearance is generally normal with no apparent alteration in the blood vessels, gyri, and white matter (Jellinger et al., 1988; Reiss et al., 1993). The brains of RTT patients are smaller than in controls of the same age (Jellinger and Seitelberger, 1986), which is consistent with the reduced head circumference. The reduction of brain volume is mostly due to reduced neuronal body size and the cells are also more densely packed, especially in the cortex, thalamus, substantia nigra, basal ganglia, amygdala, and hippocampus (Bauman et al., 1995; Kaufmann and Moser, 2000). Dendrits are less branched with short and sparse spines, but without other abnormalities, indicating delayed neuronal maturation (Belichenko et al., 1994; Belichenko and Dahlstrom, 1995). Hypopigmentation in the substantia nigra also suggests an impaired neuronal development rather than neurodegeneration (Jellinger and Seitelberger, 1986).

Molecular changes observed in RTT brains are consistent with the neuropathological findings, but no common neurochemical pattern has been defined so far. Abnormalities have been reported in most systems. They include decreased levels of

cortical cholin acetyltransferase (Wenk and Hauss-Wegrzyniak, 1999), serotonin, dopamine (Wenk, 1995), substance P (Matsuishi et al., 1997), and nerve growth factor (Lappalainen et al., 1996) in cerebrospinal fluid. On the other hand, glutamate level is considerably elevated (Hamberger et al., 1992). Microtubule-associated protein 2 (MAP2), which is normally expressed during the period of dendritic branching and expansion, has been demonstrated to be reduced in all cortical areas of RTT brains (Kaufmann et al., 1995). Decreased levels of cyclooxygenase 2 (COX2), a protein contributing to cortical development and synaptic activity, were also observed in frontal and temporal regions (Kaufmann et al., 1997).
The neurochemical measurements are influenced by the age of patients as well as the severity of symptoms (Percy, 1992). However, the study of altered levels of neurotransmitters and trophic factors in RTT brains is of great importance and may help in development of innovative supportive therapy.

Genetic basis of Rett syndrome: *MECP2* gene

More than 99.5% of RTT cases are sporadic. Due to the lack of familial cases the mode of inheritance and genetic cause of RTT were unsuccesfully debated for many years. Finally, mutations in the ubiquitously expressed *MECP2* gene, encoding methyl-CpG-binding protein 2, were identified in several RTT patients (Amir et al., 1999).
The *MECP2* gene maps to Xq28 and undergoes X chromosome inactivation (XCI) (D'Esposito et al., 1996; Sirianni et al., 1998). The gene spans 76 kb and is composed of 4 exons (Fig. 1a), which code for the methyl-CpG-binding protein 2 (MeCP2). The first exon was for many years considered a part of 5´untranslated region (5´UTR). Only a few years ago, the new open reading frame containing exon 1 and alternative splicing of exon 2 defining the second MeCP2 isoform were discovered (Mnatzakanian et al., 2004). Exon 4 contains one of the longest known 3´UTR (8.5 kb) in human genome, which is well conserved among mammals. Multiple polyadenylation signals in the 3´UTR generate four differentially expressed transcripts: 1.8 kb, 5.4 kb, 7.5 kb, and 10.2 kb (Coy et al., 1999; Reichwald et al., 2000; Shahbazian et al., 2002; Pelka et al., 2005). The longest transcript is the most abundant one in fetal and adult brains; the shortest transcript occurs at the highest levels in adult muscles, heart, blood, and liver (Shahbazian et al., 2002; Pelka et al.,

2005). Unique expression patterns of each transcript suggest a specific biological significance, such as a role in transcript stability and RNA folding (Coy et al., 1999).

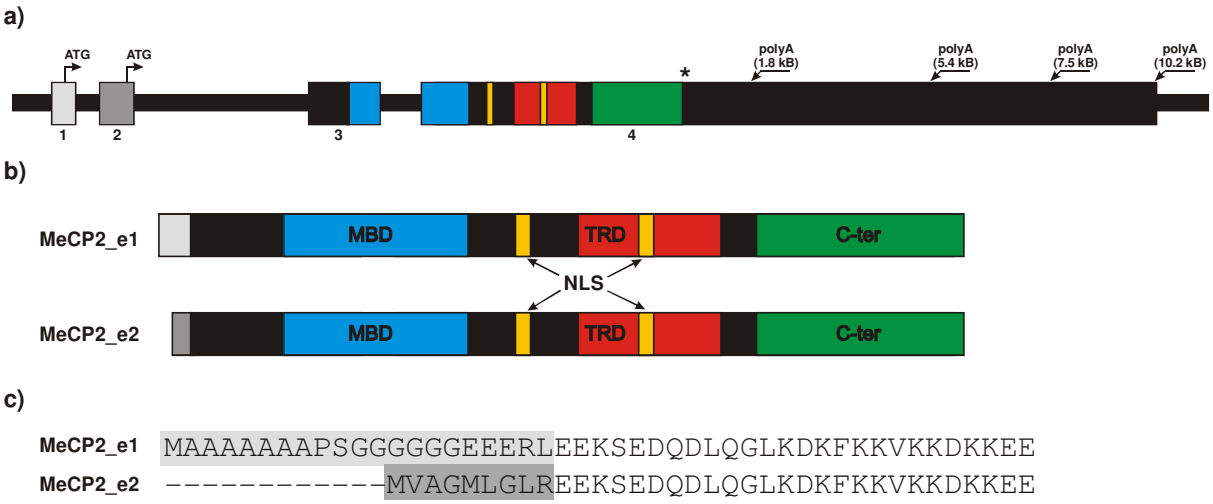

Fig. 1. The *MECP2* gene and the MeCP2 protein.
a) 4 exons of the *MECP2* gene. Arrows indicate the translation initiation codons ATG and alternative polyadenylation sites in 3´UTR with the transcript lengths in the brackets, * indicates the end of translation. Coloured boxes represent regions encoding functional domains.
b) Two isoforms of MeCP2 protein resulting from alternative translation of exons 1 and 2. MBD: methyl-CpG-binding domain, TRD: transcriptional repression domain, NLS: nuclear localization signal, C-ter: C-terminal domain.
c) Alignment of N-terminal parts of MeCP2 isoforms (grey boxes) indicating differences due to alternative translation of exon 1 and 2.

MeCP2 protein

Both isoforms of the MeCP2 protein are constitutively expressed in all tissues, but more widely in the brain. The expression coincides with neuronal maturation and synaptogenesis. It significantly increases during postnatal development suggesting a role of MeCP2 in neuronal surviving and modulation of neuronal activity and plasticity (Cohen et al., 2003; Jung et al., 2003; Samaco et al., 2004; Kishi and Macklis, 2005; Smrt et al., 2007; Swanberg et al., 2009).

The MeCP2-e1 isoform is 498 amino acids long and employs exons 1, 3, and 4. It is about ten times more abundant in the brain than the 486 amino acid long isoform MeCP2-e2, encoded by exons 2, 3, and 4 (Mnatzakanian et al., 2004; Dragich et al., 2007). The possible specific neuronal function of MeCP2-e1 still remains to be elucidated. Nevertheless, the identified causative mutations in exon 1 in contrast to no mutations found in exon 2, strongly support the theory. Apart from different N-

terminal regions, both isoforms share the same amino acid sequence including three functional domains and two nuclear localization signals (NLS) (Fig. 1b,c).

Methyl-CpG-binding domain (MBD), binds to symmetrically methylated CpG dinucleotides with preference for CpGs neighboring A/T-rich motifs. MBD requires only a single methylated CpG for binding to DNA but MeCP2 preferentially associates with whole blocks of methylated CpGs (Klose et al., 2005). MBD also binds to unmethylated four-way DNA junctions (Galvao and Thomas, 2005), implicating a role of MeCP2 in higher-order chromatin interactions (Chahrour and Zoghbi, 2007).

Transcriptional repression domain (TRD) is involved in the repression of downstream genes by recruiting histone deacetylases HDAC1 and HDAC2, corepressor factors (e.g. Sin3A, c-Ski, and N-CoR) (Jones et al., 1998; Nan et al., 1998; Kokura et al., 2001), or components of chromatin remodelling complexes (e.g. Brahma, SWI2/SNF2 DNA helicase/ATPase) (Harikrishnan et al., 2005; Nan et al., 2007).

The C-terminal domain probably facilitates binding to the naked DNA and to the nucleosomal core (Chandler et al., 1999). It also contains evolutionary conserved poly-proline motifs that can bind to group II WW domain splicing factors (Buschdorf and Stratling, 2004).

The original model of MeCP2 function involves deacetylation of core histones by HDAC resulting in the compaction of the chromatin and making it inaccessible to components of the transcriptional machinery (Jones et al., 1998; Nan et al., 1998) (Fig. 2). Interactions between TRD and various cofactors demonstrate that MeCP2 protein is capable to interpret methylated CpGs differently and, depending on the context, mediate multiple downstream responses.

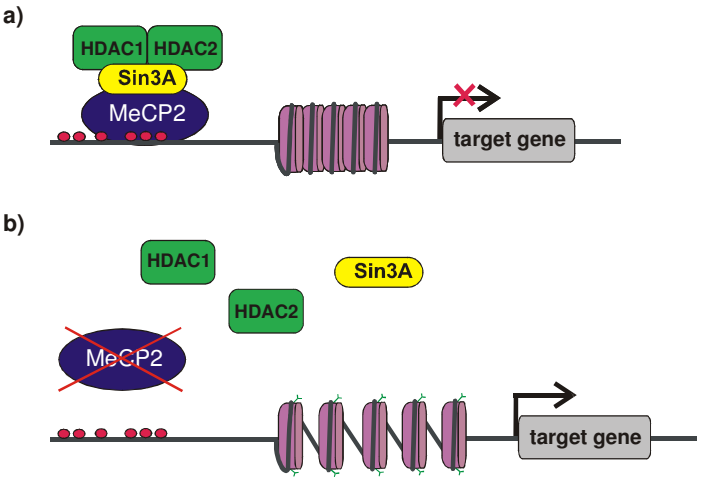

Fig. 2. Model of MeCP2 function.
a) MeCP2 binds to methylated CpGs (red circles) and recruits corepressor proteins such as HDAC1, HDAC 2, and Sin3A. Chromatin remains in condensed and transcriptionally inactive state.
b) Non-functional MeCP2 does not create the repressor complex and histones remain acetylated (blue Ys). Chromatin is in decondesed state, which allows transcription factors to bind to promoter and initiate transcription.

The function of MeCP2 is actually more complex than initially anticipated. MeCP2-directed transcriptional repression is also performed in HDAC-independent manner (Yu et al., 2000), through a direct interaction between the C-terminal domain and chromatin (Nikitina et al., 2007), or through an interaction with a component of the basal transcriptional machinery, transcription factor IIB (Kaludov and Wolffe, 2000). MeCP2 also appears to be an architectural chromatin protein, capable to establish silent domains by promoting chromatin looping without DNA methylation, ATP, and other cofactors (Georgel et al., 2003) (Fig. 3).

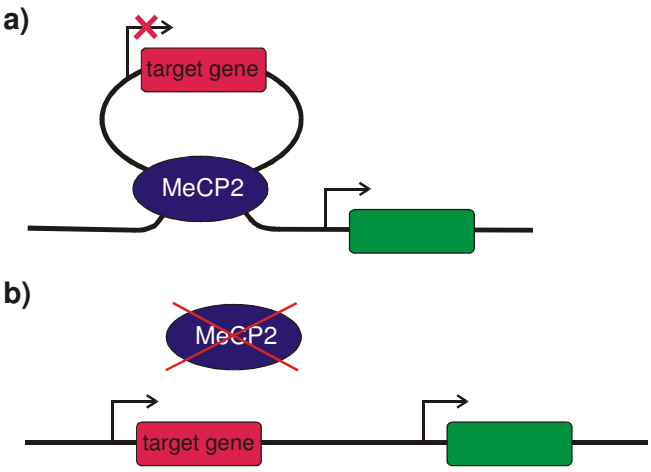

Fig. 3. Gene silencing by establishing of secondary chromatin structures.
a) MeCP2 binds to binding sites surrounding its target gene (red) forming a loop. A repressive environment is established within the loop while genes outside the loop (green) remain transcriptionally active. The loop can encompass one or more target genes.
b) Absent or non-functional MeCP2 does not create the loop and the expression of the genes is not affected.

Recent studies suggest several novel roles for MeCP2, such as regulation of RNA splicing (Young et al., 2005), transcriptional activation of target genes, and others (Chahrour et al., 2008) (Fig. 4).

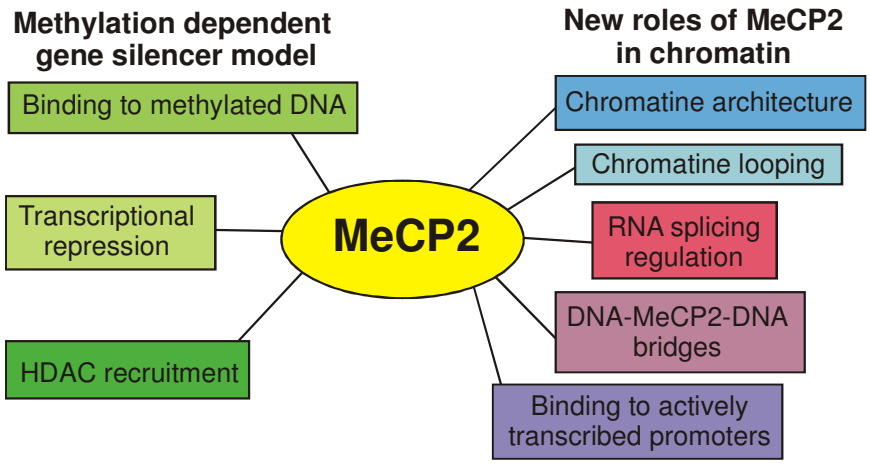

Fig. 4. Representation of multiple MeCP2 roles (Adapted from Hite et al., 2009).

Precise mechanisms, by which is MeCP2 directed and regulated to various functions and interactions, remain to be elucidated. Recent studies report that the majority of MeCP2-binding sites are outside of genes, let alone methylated promoters (Yasui et al., 2007; Hite et al., 2009). Therefore, the fundamental question of what is MeCP2 doing in the nucleus still remains to be answered.

MeCP2 target genes

Due to the ubiquitous expression, MeCP2 was initially believed to act as a global transcriptional repressor (D'Esposito et al., 1996; Nan et al., 1997), but transcriptional profiling studies using brain tissue from *Mecp2*-null mice did not reveal major gene expression changes. It was thought until recently that MeCP2 represses the transctiption of a selected number of genes in certain brain regions or neuronal cells (Colantuoni et al., 2001; Tudor et al., 2002; Ballestar et al., 2005; Jordan et al., 2007). Even this model had to be dramatically re-evaluated. MeCP2 is mostly associated with transcriptionally active genes (Yasui et al., 2007) and apparently regulates transcription in both directions, toward repression and activation (Chahrour et al., 2008). Great effort has been put into the identification of genes, which are controlled by MeCP2, because they provide insight into neuronal functions regulation, RTT pathogenesis, and may lead to a design of rational treatment strategies. It is important to determine whether these genes represent the true MeCP2 targets or are secondarily misregulated due to altered expression of primary MeCP2 targets. The most extensively studied target genes are shown in Tab. 3.

Tab. 3. Some of MeCP2 target genes (Chahrour and Zoghbi, 2007; Matijevic et al., 2009).

Gene	Function
BDNF	neuronal development and survival, neural plasticity, learning and memory
DLX5	neuronal transcription factor (probably involved in control of GABAergic differenciation)
SGK1	hormone signaling (regulation of renal functions and blood pressure)
FKBP5	hormone signaling (regulation of glucocorticoid receptor sensitivity)
UQCRC1	member of mitochondrial respiratory chain
ID1, ID2, ID3	transcription factors (involved in cell growth, senescence, differentiation, angiogenesis)
FXYD1	ion channel regulator
IGFBP3	hormone signaling (regulation of cell proliferation and apoptosis)
CRH	neuropeptide (regulation of neuroendocrine stress response)
UBE3A	member of ubiquitin proteasome pathway, transcriptional coactivator
GABRB3	neurotransmission (GABA-A receptor)

Mutations in the *MECP2* gene

More than 300 different mutations have been identified in the *MECP2* gene so far (www.hgmd.cf.ac.uk, http://mecp2.chw.edu.au/, www.MeCP2.org.uk). The spectrum of *MECP2* mutations is extremely wide including point mutations, small and larger deletions, insertions, duplications, exon or whole *MECP2* gene deletions. 99.5% of all mutations arise *de novo* and mostly originate on the paternal X chromosome (Girard et al., 2001; Trappe et al., 2001). Mutations causing familial cases are very rare and are mostly inherited from a healthy or mildly affected mother with either gonadal mosaicism or favorable X chromosome inactivation.

The mutations are scattered throughout the whole coding sequence with the exception of exon 2, but some clustering may be recognizable. Missense mutations mostly occur upstream of the TRD-coding region, especially in the MBD-coding region. On the other hand, nonsense mutations tend to be located downstream of the MBD-coding region. Frameshift mutations resulting from larger deletions are mostly located in the 3´end of the coding sequence (Fig. 5). The vast majority of point mutations are C>T transitions presumably resulting from spontaneous deamination of methylated cytosines (Lee et al., 2001).

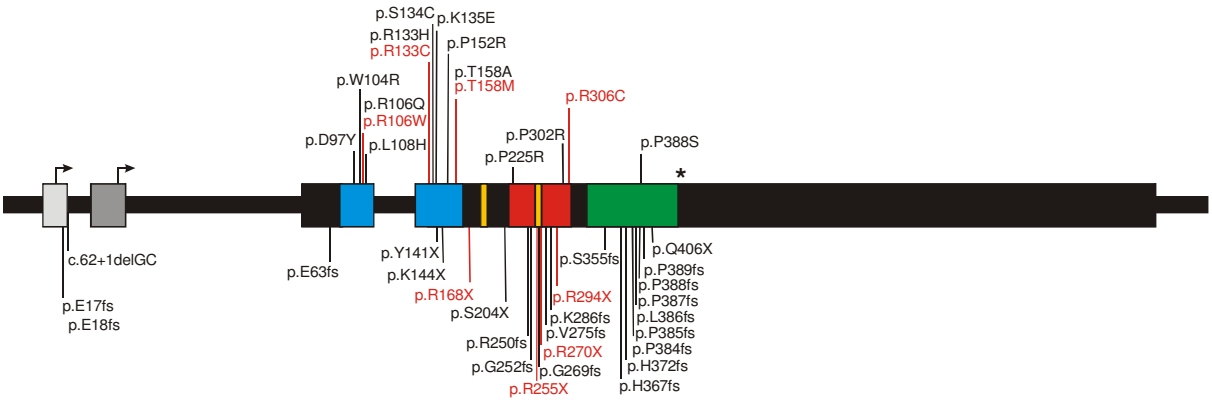

Fig. 5. Some of *MECP2* mutations.
Missense mutations are displayed above, nonsense and frameshift mutations below the *MECP2* gene scheme. 8 the most common mutations, located on 8 CpG hot spots, are highlighted in red. No mutation has been reported in exon 2. Mutations in exon 1 are numbered starting from the ATG start codon in MeCP2_e1 coding sequence. Arrows represent ATG start codons. * represents STOP codon. Colored boxes represent functional domains (legend in Fig. 1). One-letter amino acid codes were used for better lucidity.

MECP2 mutations are found in 90-95% of classic RTT cases and in 20-40% of patients with atypical RTT variants (Amir et al., 2005). The mutations are always heterozygous in female RTT patients and unlikely to act in a dominant-negative mechanism since only one allele is active in each cell due XCI. Mutations located in the functional domains mostly reduce DNA-binding property or transcription regulation capacity of the MeCP2 protein. Other mutations may prevent its transportation to the nucleus, affect the stability, secondary or tertiary structure of the protein. Mutations may also interfere with other functions of the protein, which are not completely investigated, yet. The consequences of *MECP2* mutations are often difficult to predict because the proper activity of this multifunctional protein is currently impossible to test. The pathogenicity of novel mutations is mostly assumed by screening of family members and healthy controls, sequence alignments and evolutionary conservation analyses.

MECP2 mutations in males

Initially, *MECP2* mutations were considered to be lethal in males. However, there have been several reports on boys with clinical features more or less resembling classic RTT, before discovering the causal gene (Philippart, 1990; Christen and Hanefeld, 1995; Jan et al., 1999). The “male Rett variant” was definitely confirmed after the identification of the *MECP2* gene as the causative RTT gene. Boys with *MECP2* mutations fall into three categories: 1) classic RTT, 2) severe neonatal encephalopathy and infantile death, 3) less severe neuropsychiatric phenotypes.

Boys with classic RTT are either a somatic mosaic for *MECP2* mutation (Clayton-Smith et al., 2000; Armstrong et al., 2001; Topcu et al., 2002) or a partial or a complete Klinefelter syndrome (Leonard et al., 2001; Schwartzman et al., 2001). In these patients, the effect of the mutation is mitigated in the same way as in females. Only a fraction of cells express the mutant MeCP2.

The second group consists of the boys with severe neonatal encephalopathy. These patients develop congenital hypotonia, seizures, gastroesophageal reflux, and die from respiratory insufficiency by 2 years of age. They are usually male siblings born into families with recurrent RTT, where asymptomatic mothers carry *MECP2* mutations but do not exhibit the typical RTT phenotype due to favorable XCI (Schanen et al., 1998; Villard et al., 2000; Zeev et al., 2002; Schule et al., 2008).

In the third category are the cases with the least severe impairment. The phenotypes in these boys are very broad, presented with mental retardation, Angelman-like features, various motor abnormalities or neuropsychiatric conditions. Their *MECP2* mutations are different from those found in girls with RTT, presumably because their effect on MeCP2 function is very mild in heterozygous females (Couvert et al., 2001; Cohen et al., 2002; Klauck et al., 2002).

MECP2 duplications

One of the most intriquing findings regarding MeCP2 is the evidence for its dosage-sensitive role in neuronal plasticity and function. In addition to loss of MeCP2 function, a gain in MeCP2 dosage is also detrimental to the nervous system and a tight regulation of the protein level and function in the CNS appears to be crucial (Collins et al., 2004; Moretti and Zoghbi, 2006). Unlike other *MECP2* mutations, duplications of the whole gene have been reported almost exclusively in males. Clinical features include progressive mental retardation, absence of speech, facial and axial hypotonia, spasticity, seizures, recurrent respiratory infections, and often premature death (Van Esch et al., 2005; Friez et al., 2006; Lugtenberg et al., 2006; Kirk et al., 2009; Lugtenberg et al., 2009; Prescott et al., 2009). It has been suggested that similar target genes may be affected due to loss and gain of MeCP2 but in the opposite directions (Chao et al., 2007; Chahrour et al., 2008).

MECP2 mutations in other disorders

It is important to underline that the presence of a *MECP2* mutation does not automatically mean the diagnosis of RTT. *MECP2* mutations have also been identified in other clinical entities, such as autism (Lam et al., 2000; Carney et al., 2003), severe mental retardation with seizures, Angelman-like syndrome in females (Watson et al., 2001; Milani et al., 2005), juvenile-onset schizofrenia (Cohen et al., 2002) or complex forms of mental retardation associated with tremor, epilepsy, and bipolar disease in males (Klauck et al., 2002). The frequency of *MECP2* mutations in these phenotypes is much lower than in RTT, usually less than 2%.

Genotype-phenotype correlation

Clinical manifestation and severity of RTT are widely variable. Genotype-phenotype correlation studies have been conducted to determine whether different *MECP2* mutations can account for the variability of clinical features in RTT patients. Analyzing clinical features in RTT patients provides information on the likely clinical profile for the new cases with specific *MECP2* mutations and may be useful in designing specific therapeutic interventions.

Generally, early truncating mutations lead to the most severe overall phenotype. Milder phenotype is usually associated with missense mutations, except those within the NLS. The mildest are late truncating mutations with intact MBD and TRD (Cheadle et al., 2000; Monros et al., 2001; Huppke et al., 2003; Neul et al., 2008).

The genotype-phenotype correlation has been confirmed even for several individual *MECP2* mutations. The mutations p.Arg270X and p.Arg255X, both located in NLS and truncating the protein in TRD, are considered to be the most severe ones. On the other hand, p.Arg133Cys (located in MBD) and p.Arg294X (located in C-terminal domain) tend to be associated with milder phenotypes and delayed onset of regression. Nevertheless, significant variations in clinical severity between individuals with the same *MECP2* mutation are very common (Scala et al., 2007; Bebbington et al., 2008). Such variations most likely result from differences in XCI patterns. Only one X chromosome is active in each female somatic cell to equalize X-linked gene products between females and males. Therefore, a girl with *MECP2* mutation is typically a mosaic of cells expressing either wild-type or mutant *MECP2* allele. Favourable skewing of XCI (a *MECP2* mutation is in most cells located on inactive X chromosome) can allow an apparently normal or very mild phenotype, whereas a balanced XCI results in the RTT. On the other hand, extremely skewed XCI with the mutated *MECP2* gene located mostly on active X chromosome may cause more severe RTT phenotype (Amir et al., 2000; Zoghbi et al., 2004). Neverthless, the effect of XCI pattern is not ubiquitous and has limitations in explaining all differences of RTT severity. Most studies on XCI use peripheral blood, which is not a perfect model. There is no proof that the same XCI pattern is found in the brain tissue of the same individual. Many patients with the same *MECP2* mutation and random XCI measured in blood lymphocytes exhibit a broad spectrum of clinical severity. It seems that other genetic and non-genetic factors have to be considered. Identification of these factors

will not only provide further insight into pathogenesis of RTT but may also help to design effective treatment approaches. One of the candidate genetic factors is already known. A polymorphism in the brain-derived neurotrophic factor gene (*BDNF*), which is incidentally one of the MeCP2 target genes, has been shown to correlate with the severity and course of RTT. Patients heterozygous for the variant p.Val66Met were slightly more severely affected than those homozygous for the wild-type variant p.Val66Val, especially considering the risk of seizures (Zeev et al., 2009). BDNF plays a major role in neuronal survival, neurogenesis and neuronal plasticity. The substitution probably disrupts folding, dimerization or intracellular trafficking of the protein (Egan et al., 2003; Chen et al., 2004), but the exact role of BDNF in RTT pathogenesis still remains to be elucidated. Looking for other potential modulation factors was one of the aims of this PhD thesis.

Other Rett syndrome genes

The absence of causative *MECP2* mutations in a proportion of clinically well-defined RTT patients suggested the existence of another causal gene. Recently, several mutations have been identified in the cyclin-dependent kinase-like 5 gene (*CDKL5*; OMIM #300203) in patients with atypical RTT associated with intractable early-onset seizures (Tao et al., 2004; Weaving et al., 2004; Mari et al., 2005; Scala et al., 2005; Russo et al., 2009). Mutations in the *CDKL5* gene were initially found in the patients with early infantile epileptic encephalopathy 2 (EIEE2) (Kalscheuer et al., 2003). Other reports include a girl with autistic features and intellectual disability (Weaving et al., 2004) and a boy with severe early infantile-onset neurological disorder (Tao et al., 2004). *CDKL5* (previously known as serine/threonine kinase 9 gene, *STK9*) is located on Xp22.3 and comprises 23 exons. The gene product is a putative serine/threonine kinase of, to date, unknown function (Fig. 6).

Expression profile studies revealed high levels of CDKL5 mRNA in many tissues including brain. Despite its proposed participation in pathogenesis of several disorders, the CDKL5 protein remains poorly characterized. However, several domains and motifs have been predicted (Fig. 6b).

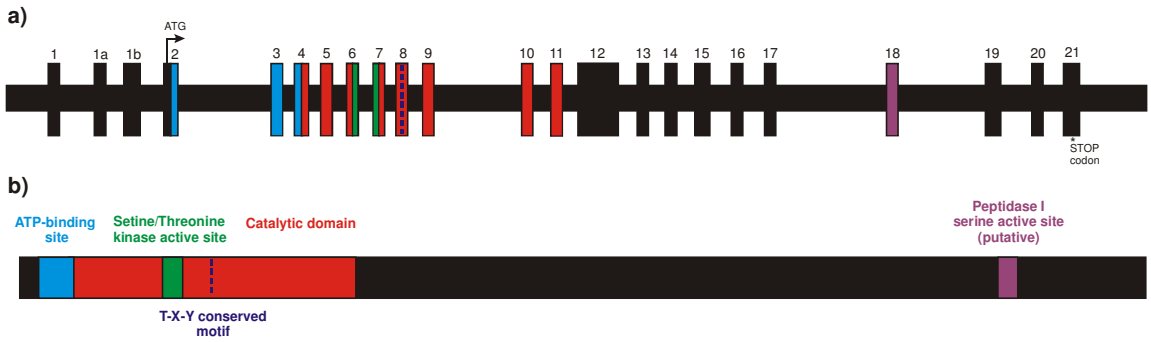

Fig. 6. Schematic structures of the *CDKL5* gene (a) the CDKL5 protein (b).
Arrow indicates the translation initiation codon ATG, * indicates the end of translation, coloured boxes represent functional domains and motifs.

Overlapping phenotypes caused by mutations in the *MECP2* and *CDKL5* genes as well as recent discovery of MeCP2 phosphorylation, lead to a theory that the two proteins participate in a common pathway (Mari et al., 2005). Moreover, the expression profile of the CDKL5 during neuronal maturation and synaptogenesis is very similar to that of MeCP2 (Lin et al., 2005; Mari et al., 2005). *CDKL5* mutations seem to be a rare cause of RTT and the precise pathological mechanism is unknown, nevertheless, the analysis of the *CDKL5* gene should be considered in cases with early-onset seizure variant of RTT.

Isolated reports of mutations in other genes have been also published. Their significance in the pathogenesis of RTT remains equivocal due to low mutation frequency and usually atypical phenotype of affected individuals. Nonetheless, they suggest that RTT and RTT-like phenotypes may be more genetically heterogeneous than previously anticipated.

The *FOXG1* gene is located on chromosome 14 and its product is a transcription factor formerly known as brain factor 1 (BF1). The FoxG1 protein is expressed only in brain and testicles and plays an important role in the initial stages of neuronal development (Yao et al., 2001; Tan et al., 2003). Mutations in *FOXG1* have been reported in several patients with congenital variant of RTT (Ariani et al., 2008; Papa et al., 2008; Mencarelli et al., 2009).

A single case of the disrupted *NTNG1* gene, located on chromosome 1, in a girl with features of RTT has been reported (Borg et al., 2005). The gene product, netrin G1 (also known as laminet-1), is expressed especially in brain and plays an important role in neuronal development (Nakashiba et al., 2000; Nakashiba et al., 2002). Its involvement in the pathogenesis of at least some RTT cases is unconfirmed, yet.

Diagnosis of Rett syndrome

Routine laboratory analyses including blood, urine, and cerebrospinal fluid are usually within physiological range. Neurophysiological tests as well as modern imaging techniques (EEG, MRI, CT, PET etc.) did not reveal a common pathognomic change and their results are strongly affected by the age of patients and the severity of symptoms (Percy, 1992; Glaze, 2005). Before the discovery of the causal *MECP2* gene, the diagnosis of RTT was based only on clinical features. Mutation analysis of the *MECP2* gene provides the confirmation of clinical diagnosis and is available in our laboratory since 2001 (Rosipal et al., 2001).

It is very important to point out that the negative result of routine DNA test does not rule out the diagnosis of RTT. Only a part of the *MECP2* gene is analyzed (coding sequence and exon/intron boundaries) and used molecular methods have their limits in detection of more complex genetic variants. *MECP2* mutation-negative classic RTT cases are most likely caused by mutations in the regulatory elements of the *MECP2* gene. Atypical variants are suggested to be genetically heterogeneous and caused by mutations in other genes.

Treatment of Rett syndrome

To date, there is no treatment known to withdraw the symptoms of the disorder. The complex clinical features of RTT define a unique and demanding clinical situation, which requires complete daily care by parents or caregivers and innovative medical assistance. Pharmacological interventions in patients with RTT have been a challenge for researchers and physicians. The window of relative normality in the first months of life may eventually allow some therapeutic actions before the syndrome is fully clinically developed. At this time, the management of RTT is only symptomatic and supportive, aimed especially to improve motor and cognitive skills and prevent seizures. Alternative forms of communication such as eye and hand pointing, gestures, and body language need to be encouraged. Some of the techniques used to maintain and develop motor functions include physical therapy, horseback riding, music therapy, and water therapy. Some patients require bracing or even surgical intervention to treat scoliosis (Budden, 1995). Seizures are usually successfully compensated by anticonvulsants; L-dopa and dopamine agonists may improve motor

abilities; melatonin has been used to improve sleep rhytms (McArthur and Budden, 1998). Swallowing difficulties, chewing problems and unability to feed themselves contribute to poor weight gain of RTT patients. Frequent small feeds during the day and thickened feeds may be used to maintain growth and weight gain (Weaving et al., 2005).

Several medicaments should be avoided in RTT patients due to higher risk of life-threatening cardiac arrhytmias. These include some prokinetic agents (e.g. cisapride), antipsychotics (e.g. thioridazine), anti-arrhytmics (e.g. quinidine, sotolol, amiodarione), anaesthetic agents (e.g. thiopental, succinylcholine), and antibiotics (e.g. erythromycin, ketoconazole) (Weaving et al., 2005; Williamson and Christodoulou, 2006).

Selected potential modulator genes

Polymorphisms in several genes, which products determine brain development and metabolism, have shown to be implicated in various neurological and psychiatric disorders. We selected several candidates and evaluated their possible association with the severity of RTT phenotype.

5-HTT gene

Serotonin (5-hydroxytryptamine, 5-HT) is often regarded as one of the major neurotransmitters with various roles in neuronal development as well as in functioning and plasticity of the adult brain (Lesch, 2001). It is also worthy of consideration in relation to RTT because of its role in the autonomic regulation. Serotonin transporter (5-HTT) plays a key role in the regulation of serotonergic neurotransmission by determining both magnitude and duration of 5-HT signalling (Lesch and Mossner, 1998). Transcriptional activity of the human *5-HTT* promoter is modulated by two common polymorphisms. A serotonin-transporter-linked polymorphic region (5-HTTLPR) is an insertion/deletion in the promoter region with a short (S) and long (L) allele. It is a major determinant of the *5-HTT* expression. S allele-carriers show a decreased expression level of *5-HTT* compared to L/L individuals (Lesch et al., 1996). The second polymorphism, a variable number of tandem repeats in intron 2 (STin2 VNTR), with alleles STin2.9 (9 repeats), STin2.10 (10 repeats), and STin2.12 (12 repeats) is a transcriptional enhancer. The STin2.12 allele possesses stronger enhancing capacity than STin2.10 and the STin2.9 allele is extremely rare (MacKenzie and Quinn, 1999). Several studies examined the association between these polymorphisms and various neurodevelopmental and neuropsychiatric disorders such as autism, bipolar disorder, depression etc, but the findings are still far from conclusive.

APOE gene

Apolipoprotein E (ApoE) is the major apolipoprotein in brain and an important cofactor in neuronal metabolism. Three major isoforms, ApoE2, ApoE3, and ApoE4, are encoded by alleles ε2, ε3, and ε4 of the *APOE* gene. The ε4 allele is associated

with several pathological processes and disorders of the nervous system, particularly Alzheimer disease (Roses, 1996; Smith, 2000). Further, the analysis of cognitive performances in Down syndrome patients showed that subjects with at least one ε4 allele suffered more severe cognitive decline than those without it. Conversely, the ε2 allele appeared to be significantly neuroprotective (Corder et al., 1994; Del Bo et al., 1997). The *APOE* ε3/ε3 genotype has also shown to be more frequent in Wilson disease patients with delayed onset of neurological and hepatic symptoms compared to the ε3/ε4 genotype (Schiefermeier et al., 2000). The precise mechanisms by which different ApoE isoforms influence brain functions are still not fully understood. It might be caused by loss of trophic or gain of toxic function of the ApoE4 isoform.

HMOX1 gene

Heme oxygenase-1 (HO-1) is an inducibile rate-limiting enzyme in the catabolism of heme into biliverdin, free ferrous iron, and carbon monoxide. Biliverdin is rapidly transformed into bilirubin, an efficient local scavenger of oxidative free radicals (Tenhunen et al., 1968). On the other hand, elevated HO-1 activity may result in pro-oxidant environment due to iron liberation. A delicate balance between the harmful and protective actions of heme, bilirubin, carbon monoxide, and iron determine the role of HO-1 in tissue pathology. In the human brain, the expression of the *HMOX1* gene is increased under critical conditions for cell survival and in responce to growth factors. HO-1 may be related to cytoprotection of neuronal cells and decreased HO-1 activity probably renders cells more susceptible to oxidative stress damage. Humans differ in their ability to mount HO-1 response to exogenous stimuli. The extent of the transcriptional inducibility of the *HMOX1* gene is mostly modulated by a $(GT)_n$ microsatellite repeat in the promoter region of the gene. Transfection and clinical studies show that short alleles (less than 25 GT repeats) cause about 10-fold higher induction of *HMOX1* expression than long alleles (25 GT repeats and more) (Yamada et al., 2000; Exner et al., 2001; Hirai et al., 2003). HO-1 is overexpressed in CNS tissues affected by Alzheimer disease, Parkinson disease, multiple sclerosis, and other neurological CNS disorders due to oxidative stress (Schipper, 2004; Cuadrado and Rojo, 2008; Song et al., 2009). Hypoxia-induced oxidative stress has also been shown to be a part of RTT pathogenesis (Sierra et al., 2001; De Felice et al., 2009), but the possible involvement of HO-1 has not been thouroughly studied, yet.

AIMS OF THE STUDY

The general purpose of the presented work was to study the molecular basis of RTT. Our laboratory represents the RTT diagnostic center for whole Czech Republic and we have also performed DNA analyses in RTT patients from Slovakia, Ukraine, Estonia, and Egypt. The study was focused especially on improving the molecular diagnostics according to the resent research progress.

Specific aims were:

1. to improve the current method of *MECP2* analysis used in the routine molecular genetic diagnostics

2. to establish the molecular analysis of the second RTT gene, *CDKL5*, and provide the possibility of molecular genetic diagnostics of other clinical entities underlined by mutations in this gene

3. to establish a sensitive and cost-effective screening method to precede DNA sequencing

4. to investigate possible association between the phenotypic severity and mutations in the *MECP2* gene

5. to select and study other possible genetic determinants, which might modulate the phenotype of patients with RTT independently of the *MECP2* mutation

SUBJECTS AND METHODS

Ethics

The study was carried out in accordance with the World Medical Association´s Declaration of Helsinki and was approved by the Committee of Medical Ethics at the First Faculty of Medicine, Charles University in Prague. Informed consent from all adult participants and parents or legal guardians of underage patients was obtained.

Patients

346 patients (334 girls and 12 boys) were investigated for mutations in the *MECP2* gene. The patients fell into several categories according to available clinical reports and/or the statement of the attending geneticist or neurologist (Tab. 4.).

Tab. 4. Patients investigated in this study.

Diagnosis	Females	Males
Classic Rett syndrome	86	0
Atypical Rett syndrome	20	0
Male Rett variant	0	6
Child autism	15	2
Angelman syndrome	5	1
MR with Rett-like features, no clinical data*	208	3

* Some cases of classic or atypical RTT might be included into this group. It was impossible to decide into which group these patients belong due to absence of clinical data.

Mutation analysis of the *CDKL5* gene was performed in 10 patients with suspected EIEE2 (1 girl and 9 boys) and in 13 *MECP2* mutation-negative patients (all girls) with early-onset seizure variant of RTT.

Most of patients were diagnosed in departments of clinical genetics, child neurology, and pediatrics. Several patients were ascertained from Institutes of social work for mentally retarded children.

Family members and prenatal diagnosis

83 family members (55 mothers, 22 fathers, 3 sisters, and 3 grandmothers) with affected daughters/sisters/granddaughters participated in the study. The molecular analyses in these participants were focused on:

1) the confirmation of *de novo* origin of newly found *MECP2* mutations, 2) the confirmation of new polymorphisms by their identification in other family members, 3)

the examination of the possibility of somatic mosaicism in healthy mothers of *MECP2* mutation-positive daughters.

9 prenatal diagnoses were performed in 8 families (twice in one family) with the *MECP2* mutation-positive patient.

Molecular genetic methods

DNA extraction

Genomic DNA (gDNA) was extracted from peripheral blood (5-10 ml) anticoagulated with EDTA using the common salting out procedure. Alternatively, when only a small amount of blood (1-2 ml) or dried blood spots were available, QIAamp DNA Blood Minikit (QIAGEN, Hilden, Germany) was used according to manufacturer´s protocol. For prenatal diagnosis, DNA extracted from cultivated amniocytes was used. Concentration and quality of gDNA were evaluated using spectrophotometer NanoDrop ND-1000 (Thermo Scientific, Wilmington, USA).

Mutation analysis of the *MECP2* and *CDKL5* genes

Polymerase chain reaction (PCR)

The pairs of primers were designed using GeneBank reference sequences to amplify coding and flanking intronic regions of the *MECP2* and *CDKL5* genes. Primer sequences are collected in Tab. 5.

Tab. 5. Primer sequences (5´→3´ direction), PCR product, and annealing temperature specifications.

Fragment	Forward primer	Reverse primer	PCR product	Ta
MECP2				
exon 1	tcaatcgcccctcagagca	cacgtcccgcccctgaccc	600 bp	60℃
exon 2	aaaaaggtcgtgcagctcaa	ggccaaaccaggacatatac	227 bp	63℃
exon 3	tggcatgttctctgtgatactt	cctgggcacatacattttcct	481 bp	63℃
exon 4/1	tttgtcagagcgttgtcacc	ctgcacagatcggatagaagac	614 bp	63℃
exon 4/2	ggcaggaagcgaaaagctgagg	ctccctcccctcggtgtttg	616 bp	63℃
exon 4/3	ggagaagatgcccagaggag	gcactgatggcaccgaaaac	628 bp	63℃
CDKL5				
exon 1	gagcccgaaccccaggacaag	gcccgggggactgggtggg	383 bp	63℃
exons 1a+1b	agtggagataggggagagtga	aaaaaccaacagagcaaccag	372 bp	63℃
exon 2	aactgttcattgctcaaaactc	ctctacaaaaaggtaagattgg	353 bp	58℃
exon 3	aagcaat gtcagtatag cagag	acacgcaaagaccactacatta	184 bp	63℃
exon 4	ctggcttcttgctactctgtc	cccacttcttccacactctatg	241 bp	63℃
exon 5	gatgttttcagtgttcttgg	aaaagaatcgggcaaatgtg	263 bp	53℃
exon 6	ctagatgctttgtaaaattg	catgtgaaatactcttaact	267 bp	53℃
exon 7	tcaatcaggagaacatagaa	tcctctaccaatttaagtca	234 bp	53℃
exon 8	tagaatcagcagatgtggaa	ataaatagcccatgcgagaa	239 bp	53℃
exon 9	ccttcttttcacatggaact	aacactgtaatgggaaaaga	447 bp	53℃

Tab. 5. cont.

Fragment	Forward primer	Reverse primer	PCR product	Ta
exon 10	ttttgcttatctgctccctt	cacaaaagaatccacaaacc	234 bp	53℃
exon 11	gaggaaaatgatgatgagat	gctttccttcttttctctaa	299 bp	53℃
exon 12a	gaaggcacatgcttgagaga	gcttggtaggttttggtgtg	314 bp	60℃
exon 12b	aaggtctccctgccaatgaa	cgactctgcttttcattggg	325 bp	60℃
exon 12c	gccactcattcatggaaagc	gctgagcaaagttctcgtgt	299 bp	60℃
exon 12d	accagtaggtacttcccatc	gaagaaaaggggctggtgta	275 bp	60℃
exon 12e	cagtagccattcccattcac	atcacatccctcgtaccacctt	276 bp	58℃
exon 13	gctggttatggtcctagttc	cttatttgtgggagactggg	293 bp	60℃
exon 14	tgtcatcaatgtgtggctta	gcacataaagtgagagaggg	215 bp	60℃
exon 15	aagaaaagtccatcagtgac	gacactaaaaagctcatcca	250 bp	58℃
exon 16	ttatatttgtcacacaatgg	gcaaagtgtaaagtatccat	240 bp	58℃
exon 17	gggtgtggttgcatatctta	tccccgctcctcaggacagtta	255 bp	58℃
exon 18	ttccctccccagccttatggtc	tgtggcactcctggtcacagag	332 bp	60℃
exon 19	tttgtgttcttaaaggcaagtc	cagtagtctagggtcgttatgg	201 bp	60℃
exon 20	tgtctgggagccgatgcctg	cgaggtacagcccaggctga	299 bp	60℃
exon 21	cattagccagagtgcacctg	cacctggccctggtttttga	336 bp	62℃

Primers were synthetized by Generi Biotech (Hradec Králové, Czech Republic).

The PCR amplifications were optimized and carried out according to following protocols (Tab. 6-8).

Tab. 6. PCR mix (all *MECP2* and *CDKL5* fragments except of *MECP2* exon 1).

Chemical	Stock concentration	Final concentration in 1 reaction (25 µl)
PPP Master Mix*	2x	1x
Forward primer	3.2 pmol/µl	0.4 mM
Reverse primer	3.2 pmol/µl	0.4 mM
gDNA	50 ng/µl	50 ng

DMSO (Sigma-Aldrich, St. Louis, USA) was added to PCR mix for amplification of exons 1 and 21 of the *CDKL5* gene to final concentration 6%.
* PPP Master Mix (Top Bio, Prague, Czech Republic)

Tab. 7. PCR mix (*MECP2* exon 1).

Chemical	Stock concentration	Final concentration in 1 reaction (35 µl)
PC2 buffer[a]	10x	1x
ddGTP mix[b]	2 mM	0.2 mM
betaine[c]	5 M	1 M
DMSO[d]	100%	5%
Forward primer	3.2 pmol/µl	0.4 mM
Reverse primer	3.2 pmol/µl	0.4 mM
KlenTaq[e]	5 units/µl	0.5 unit
gDNA	50 ng/µl	50 ng

[a] PC2 buffer (GeneAge Technologies, Prague, Czech Republic)
[b] ddGTP mix: 2.5 ml dATP, 2.5 ml dTTP, 2.5 ml dCTP, 1.875 ml dGTP, 0.625 ml 7-deaza-dGTP (Sigma-Aldrich, St. Louis, MO, USA; stock concentration 10 mM), 40 ml deionized UV-treated water.
[c] betaine (Sigma-Aldrich, St. Louis, MO, USA)
[d] DMSO (Sigma-Aldrich, St. Louis, MO, USA)
[e] KlenTaq polymerase (GeneAge Technologies, Prague, Czech Republic)

Tab. 8. PCR conditions (all fragments).

Step	Temperature	Duration	
1. initial denaturation	94°C	1´30´´	
2. denaturation	94°C	30´´	
3. primer annealing	(see Tab. 5)	30´´	33 cycles
4. primer extension	72°C	45´´	
5. final extension	72°C	5´	

PCR reactions were performed using DNA Engine Dyad Peltier Thermal Cycler (Bio-Rad Laboratories, Waltham, MA, USA) and Tgradient Thermocycler (Biometra, Göttigen, Germany).

Purification of PCR products

The PCR products were purified from agarose gel (Sigma-Aldrich, St. Louis, MO, USA) using QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) or Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) according to manufacturers´ protocols.

DNA sequencing

PCR products were sequenced in both directions on automatic sequencer ABI PRISM 3100/3100-Avant Genetic analyzer (Applied Biosystems, Foster City, CA, USA) using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer´s protocol. 3.2 pmol of primer and 20-30 ng of purified PCR product turned out to yield the best sequencing results. The products of the sequencing PCR were cleaned using the common ethanol precipitation protocol. The obtained chromatograms were analyzed manually using the SeqScape Software v2.5 (Applied Biosystems, Foster City, CA, USA) or Chromas Pro v1.5 (Technelysium, Tewantin, Australia).

Multiplex ligation-dependent probe amplification (MLPA)

Large deletions or duplications, which cannot be detected by common PCR-based methods, were analysed by MLPA, a rapid, high-throughput method of copy number quantification (Fig. 7).

We used the MLPA kit P015-Rett designed for *MECP2* analysis and P189-Rett-like designed for *CDKL5* analysis (both MRC-Holland, Amsterdam, Netherlands). MLPA was performed according to manufacturer's protocol. The probes are designed to hybridise with specific target sequences in the *MECP2* and *CDKL5* genes as well as with several control regions. The P189-Rett-like kit contains several probes specific for two additional genes (*NTNG1* and *ARX*), which are also associated with mental

retardation syndromes. Fragment analysis was performed on ABI PRISM 3100/3100-Avant Genetic analyzer (Applied Biosystems, Foster City, CA, USA) and data were analysed using the original softwares Peak Scanner v1.0. and Gene Mapper v4.0 (both Applied Biosystems, Foster City, CA, USA). Data normalization was performed using the MS Excel application. Aberrant samples were independently rerun in the second MLPA reaction for confirmation.

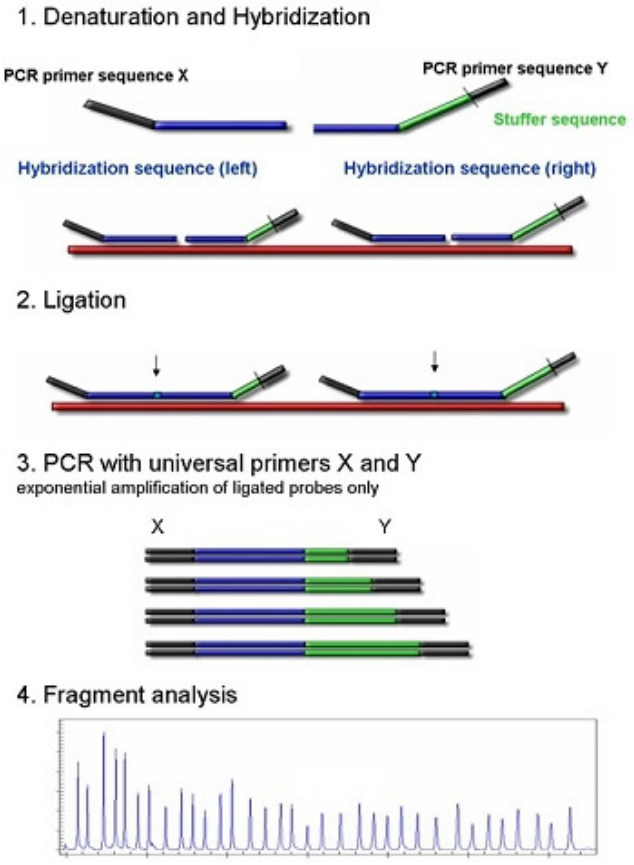

Fig. 7. Schematic principle of MLPA. Each MLPA probe consists of two separate oligonucleotides containing universal forward (X) or reverse (Y) primer sequence. One of each pair of oligonucleotides contains a stuffer sequence with a unique lenght. The two probe oligonucleotides hybridise to immediately adjacent target sequences (1). Only in that case can they be ligated to produce full-lenght probes (2) and subsequently exponentially amplified by PCR (3). The amount of probe ligation products is a measure for the number of target sequences in the sample. The amplification products are separated using capillary electrophoresis (4). Probe oligonucleotides that are not ligated cannot be amplified and will not generate a signal.
(www.mrc-holland.com)

Restriction fragment length polymorphism (RFLP)

Whenever the identified mutation caused creation or loss of restriction site, it was verified by RFLP. PCR products containing the specific mutations were digested with corresponding restriction endonucleases according to manufacturers´ protocols. The mutations and the restriction endonucleases are summarized in Tab. 9.

Tab. 9. *MECP2* mutations verified by RFLP.

MECP2 mutation	Restriction endonuclease	Creation (+)/abolishment (-) of the restriction site
c.189_190delGA[a]	BsmAI	-
c.316C>T	NlaIII	+
c.323T>A	HindIII	-
c.403A>G	Hpy1881	+
c.426C>T	TaqI[b]	-
c.455C>G	NlaIV	-
c.473C>T	HpyCH4III	-
c.502C>T	HphI	+
c.587C>G	BanI	-
c.611C>G	PmlI	+
c.808C>T	NlaIV	-
c.916C>T	HhaI	-

Restriction enzymes were manufactured by New England Biolabs (Ipswich, MA, USA).
[a] Mutation located in exon 1 is numbered starting from the ATG start codon in the MeCP2_e1 coding sequence (AY523575).
[b] TaqI was manufactured by Fermentas (Burlington, ON, Canada).

Long-range PCR

Long-range PCR was used to specify the large deletion c.1102_1461*461del821 in exon 4 of the *MECP2* gene. Additional reverse primer (5´-cagctcacaggttccgagaca-3´) was designed to amplify the end of the coding sequence and the part of 3´UTR region as a single 6.8 kb PCR product (with exon 4/2 forward primer – see Tab. 5.). PCR mix and conditions were the same as described previously in Tab. 6 and 8 (Ta 63ºC), only the primer extension step was elongated to 10 minutes. Purified PCR product was sequenced using available primers to define the exact breakpoint sites.

Cloning

To specify the indel mutation c.1063_1236del174;1189_1231inv43, PCR fragment with the mutation was cloned into the pCR4-TOPO vector of TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA, USA). Plasmid DNA was amplified in Top10 chemically competent *E.coli* cells (Invitrogen, Carlsbad, CA, USA). Cloning, transformation, and plasmid amplification were performed according to manufacturer´s protocol. Amplified plasmids were isolated using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) and sequenced using the universal T7 and T3 primers included in the TOPO TA Cloning Kit for Sequencing.

High-resolution melting analysis (HRM)

Several new primers were designed to generate *MECP2* amplicons of 200-400 bp, which are recommended for HRM (Tab. 10). Primers used for amplification of *CDKL5* exons for sequencing fulfilled the criteria and were not replaced.

Tab. 10. Primer sequences (5´→3´ direction), PCR product, and annealing temperature specifications.

Fragment	Forward primer	Reverse primer	PCR product	Ta	DMSO
exon 2	aaaaaggtcgtgcagctcaa*	ggccaaaccaggacatatac*	227 bp	65ºC	-
exon 3a	tgcctctgctcacttgttct	ggtcatcatacatgggtc	336 bp	57ºC	-
exon 3b	gcaaagcagagacatcagaa	cctgggcacatacattttcct*	285 bp	57ºC	2%
exon 4a	gttgtcaccaccatccgctc	tcgtggtgccgctccctttg	304 bp	66ºC	-
exon 4b	cttcacggtaactgggagag	tcaccatgacctgggtggat	274 bp	57ºC	2%
exon 4c	aaccacctaagaagcccaaa	ctgcacagatcggatagaagac	237 bp	62ºC	4%
exon 4d	aagcgaaaagctgaggccga	gggtgggagcagtggcacgg	396 bp	66ºC	6%
exon 4e	aggaagtggtgaagcccctg	cagccgtcgctctccagtga	337 bp	65ºC	4%
exon 4f	acctccacctgagcccgaga	ccctcccctcggtgtttgt	204 bp	62ºC	2%
exon 4g	cccaaggagccagctaagac	ccaactactcccaccctgaagc	342 bp	62ºC	4%

Primers were synthetized by Generi Biotech (Hradec Kralove, Czech Republic).
* Primers formerly used in the routine sequencing of the *MECP2* gene.

PCR reactions were performed with addition of the DNA saturating fluorescent dye LCGreen Plus (Idaho Technology, Salt Lake City, UT, USA) and optimized to maximal specificity and minimimal primer-dimer formation (Tab. 11, 12).

Tab. 11. PCR mix.

Chemical	Stock concentration	Final concentration in 1 reaction (25 µl)
Plain Combi PP Master Mix[a]	2x	1x
Forward primer	3.2 pmol/µl	0.2 mM
Reverse primer	3.2 pmol/µl	0.2 mM
DMSO[b]	100%	(see Tab. 7)
LCGreen Plus	10x	1x
gDNA	50 ng/µl	50 ng

[a] Plain Combi PP Master Mix (Top-Bio, Prague, Czech Republic)
[b] DMSO (Sigma-Aldrich, St. Louis, MO, USA)

Tab. 12. PCR conditions.

Step	Temperature	Duration	
1. initial denaturation	94℃	1´30´´	
2. denaturation	94℃	30´´	
3. primer annealing	(see Tab. 7)	30´´	40 cycles
4. primer extension	72℃	45´´	
5. final extension	72℃	10´	
6. final denaturation	95℃	30´´	
7. reannealing	25℃	30´´	

PCR reactions were performed using DNA Engine Dyad Peltier Thermal Cycler (Bio-Rad Laboratories, Waltham, MA, USA) and Tgradient Thermocycler (Biometra, Göttigen, Germany).

10 µl of each PCR product was overlaid with 15 µl of mineral oil (Sigma-Aldrich, St. Louis, MO, USA) in a 96-well plate, centrifuged briefly at 2,000 g, and transferred into the LightScanner instrument (Idaho Technology, Salt Lake City, UT, USA). The samples were gradually denatured by increasing temperature (from 65ºC to 98ºC at the rate of 0.1ºC/sec). Melt curves were analyzed using the original LightScanner software v1.5 (Idaho Technology, Salt Lake City, UT, USA).

Analyses of polymorphisms in candidate modulator genes

The pairs of primers were used to amplify relevant polymorphic regions of selected potential modulator genes *5-HTT*, *APOE*[1], and *HMOX1*[2]. Primer sequences and other PCR specifications are collected in Tab. 13.

Tab. 13. Primer sequences (5´→3´ direction), PCR product, and annealing temperature specifications.

Fragment (polymorphism)	Forward primer	Reverse primer	PCR product	Ta
5-HTT				
promoter (5-HTTLPR)	ggcgttgccgctctgaatg	gagggactgagctggacaaccac	529 bp[a]	65℃
intron 2 (STin2 VNTR)	gctgtggacctgggcaatgt	agtgaagactgaaaagacataatc	339 bp[a]	63℃
APOE				
exon 4[b]	acagaattcgccccggcctggtac	taagcttggcacggctgtccaagga	174 bp	56℃
HMOX1				
promoter $(GT)_n$	IR700-agagcctgcagcttctcaga[c]	acaaagtctggccataggac	127 bp[a]	66℃

Primers were synthetized by Generi Biotech (Hradec Kralove, Czech Republic).
[a] The PCR product containing the most common allele of the length polymorphism.
[b] DMSO (Sigma-Aldrich, St. Louis, MO, USA) to final concentration 8% was added to PCR mix.
[c] Primer was labelled with the infrared dye IR700 at 5´end and prepared by East Port Praha (Prague, Czech Republic)

PCRs were carried out according to previously described protocols (Tab. 6, 8).

Genotyping of the polymorphisms was performed using following techniques:

5-HTT polymorphisms: PCR products were resolved in 8% polyacrylamide gel.

APOE polymorphism: PCR products were digested with HhaI restriction endonuclease (New England Biolabs, Ipswich, MA, USA) according to manufacturer´s protocol and resolved in 4% agarose gel.

HMOX-1 polymorphism: PCR products were analysed by fragment analysis using a laser-based automated DNA sequencer LI-COR and original LI-COR software (LI-COR Biosciences, Lincoln, NE, USA).

[1] Analysis of the *APOE* polymorphisms was initially performed in collaboration with Dr. Marie Jachymova, PhD at Institute of Clinical Biochemistry and Laboratory Diagnostics, 1st Faculty of Medicine, Charles University and General University Hospital in Prague. In 2009, the analysis was established in our laboratory by Petra Strachotova, MSc.
[2] Analysis of the *HMOX-1* polymorphism was performed in cooperation with Lubomir Kralik, MSc. (Department of Pediatrics).

PCR products with the known genotype determined by DNA sequencing were used as internal controls in each analysis.

Genotype-phenotype correlation

Severity of the clinical phenotype in the patients with pathogenic *MECP2* mutations was determined using the clinical rating developed from so-called Pineda scale (Monros et al., 2001). It was slightly modified according to available clinical data (Tab. 14).

Tab. 14. Clinical rating based on the Pineda scale used in the genotype-phenotype correlation studies.

Manifestations	Score	Definition
1. Microcephaly	0	Absent
	1	Present
	+1	Microcephaly present at birth
2. Sitting alone	0	Acquired at the age of <8 months
	1	Acquired at the age of 8-16 months
	2	Acquired at the age of >16 months
	3	Never acquired
	+1	Lost acquisition
3. Ambulation	0	Acquired at the age of <18 months
	1	Acquired at the age of 18-30 months
	2	Acquired at the age of >30 months
	3	Lost acquisition
	4	Never acquired
4. Hand use	0	Acquired and conserved
	1	Acquired and partially conserved
	2	Lost acquisition
	3	Never acquired
5. Onset of stereotypies	0	Acquired at the age of >10 years
	1	Acquired at the age of >36 months
	2	Acquired at the age of 18-36 months
	3	Acquired at the age of <18 months
6. Language	0	Preserved and propositive
	1	Phrases
	2	Single words
	3	Lost during regression
	4	Never acquired
7. Non-verbal communication	0	Preserved and intentional finger or eye pointing
	1	Intermittent eye contact or eye pointing
	2	Lost during regression and partially regained
	3	Never acquired
8. Respiratory dysfunctions	0	No dysfunction
	1	Hyperventilation and/or apnea
9. Seizures	0	Absent
	1	Present and controlled with medication
	2	Uncontrolled or infantile spasms (age <6 months)

Each evaluated clinical feature was assigned a score corresponding to the degree of severity (the higher the score, the greater the impairment). The overall severity score

was obtained by summing up the individual scores (9 items, maximum score 26). The patients were divided into several groups depending on the *MECP2* mutation type or modulator gene genotype. The average age of regression onset, the median individual scores for each feature, and the median overall severity score were calculated in each group and compared.

Statistical methods

Non-parametric Mann-Whitney U test for pairwise comparison and Kruskall-Wallis test for comparison of multiple independent groups were used to assess the statistical significance of the observed results in genotype-phenotype association studies. The threshold for the statistical significance was set at 5% ($P<0.05$).

RESULTS AND DISCUSSION

Ad 1. Improving the current method of the *MECP2* gene analysis and characterization of novel *MECP2* mutations

Knowledge of genetic aspects of RTT has been developing considerably since the first report of *MECP2* mutations in 1999. We took several steps to keep the molecular diagnosis up-to-date. Exon 1 of the *MECP2* gene was initially considered to be non-coding. Therefore only exons 2, 3, and 4 used to be routinely analysed in patients with RTT (Rosipal et al., 2001). After the discovery, that exon 1 codes for the N-terminus of a new MeCP2 isoform, which is dominant in the human brain, the sequencing analysis of this exon was established in our laboratory. The analysis of exon 1 was also performed retrospectively in all mutation-negative RTT patients. On the other hand, exon 2 was excluded from the routine sequencing analysis. The reason for this change was the fact that no mutation has been published in this exon. It codes for the MeCP2 isoform that is the minor one in the human brain and its participation in RTT pathogenesis is questionable. Finally, MLPA analysis was introduced to detect large deletions and duplications in the *MECP2* gene. Complete or partial deletions of the *MECP2* gene account for approximately 10% of RTT-causing mutations and also the whole gene duplications are a common cause of neurodevelopmental delay in males. Traditional PCR-based techniques, such as DNA sequencing, fail to detect these mutations. MLPA has further advantage of being a technically uncomplicated and cost-saving method detecting multiple target sequences in one reaction.

Altogether, 334 girls and 12 boys were screened for mutations in the *MECP2* gene by DNA sequencing. MLPA analysis was performed in 117 patients with negative sequencing results. Pathological mutations (all heterozygous) were identified in 80 patients (Tab. 15). The highest mutation frequencies were observed among female RTT patients. One mutation (c.916C>T) was identified in a patient with child autism. Eight mutations were found in a group of patients either with MR and Rett-like features (but not complete RTT phenotype) or without any clinical data available (Tab. 16). No mutation was identified in male patients and investigated family members including 9 prenatal diagnoses.

Tab. 15. *MECP2* mutations identified in this study.

	Nucleotide change	Amino acid change	MeCP2 domain	Number of patients	Reference
	deletion of exon 1[a]	p.Met1?	-	1	novel
	c.48_55del8[b]	p.Glu18ThrfsX21	-	1	novel
exon 3	c.189_190delGA	p.Glu63AspfsX27	-	1	Zahorakova et al. 2007
	c.316C>T	p.Arg106Trp	MBD	5	Amir et al. 1999
	c.323T>A	p.Leu108His	MBD	1	Zahorakova et al. 2007
	deletion of exons 3 and 4[a]	p.Arg9?	-	1	novel
exon 4	c.397C>T	p.Arg133Cys	MBD	6	Amir et al. 1999
	c.401C>G	p.Ser134Cys	MBD	1	Cheadle et al. 2000
	c.403A>G	p.Lys135Glu	MBD	1	Laccone et al. 2001
	c.423C>G	p.Tyr141X	MBD	1	De Bona et al. 2000
	c.430A>T	p.Lys144X	MBD	1	Buyse et al. 2000
	c.455C>G	p.Pro152Arg	MBD	2	Cheadle et al. 2000
	c.473C>T	p.Thr158Met	MBD	12	Amir et al. 1999
	c.502C>T	p.Arg168X	-	4	Wan et al. 1999
	c.611C>G	p.Ser204X	-	1	Buyse et al. 2000
	c.674C>G	p.Pro225Arg	TRD	1	Cheadle et al. 2000
	c.750dupC	p.Gly252ArgfsX7	TRD	1	Zeev et al. 2002
	c.763C>T	p.Arg255X	NLS	5	Amir et al. 1999
	c.806delG	p.Gly269AlafsX20	NLS	2	Wan et al. 1999
	c.808C>T	p.Arg270X	NLS	4	Cheadle et al. 2000
	c.816_832del17	p.Gly273ArgfsX52	TRD	1	Zahorakova et al. 2007
	c.820_823dupAGTG	p.Val275GlufsX57	TRD	1	HGMD 2008
	c.856_859delAAAG	p.Lys286ProfsX2	TRD	1	Hoffbuhr et al. 2001
	c.880C>T	p.Arg294X	TRD	5	Cheadle et al. 2000
	c.904C>T	p.Pro302Ser	TRD	1	Zahorakova et al. 2007
	c.916C>T	p.Arg306Cys	TRD	7	Wan et al. 1999
	c.1063_1236del174; 1189_1231inv43	p.Ser355ThrfsX37	C-ter	1	Zahorakova et al. 2007
	c.1069_1073delAGC	p.ΔSer357	C-ter	1	Zahorakova et al. 2007
	c.1102_1461*461del821	p.His368ValfsX66	C-ter	1	novel
	c.1116_1201del86	p.His372GlnfsX4	C-ter	1	Amir and Zoghbi 2000
	c.1157_1197del41	p.Lys386HisfsX5	C-ter	1	Cheadle et al. 2000
	c.1157_1200del44	p.Lys386GlnfsX4	C-ter	1	Huppke et al. 2000
	c.1162C>T	p.Pro388Ser	C-ter	1	Conforti et al. 2003
	c.1163_1201del39	p.Pro388ArgfsX87	C-ter	1	novel
	c.1164_1207del44	p.Pro389X	C-ter	4	Buyse et al. 2000

Mutations are numbered starting from the ATG start codon in the MeCP2_e2 coding sequence (NT_205965).
[a] Large deletions were identified by MLPA.
[b] Mutation located in exon 1 is numbered starting from the ATG start codon in the MeCP2_e1 coding sequence (AY523575).

Tab. 16. Number and frequency (%) of *MECP2* mutations identified in different groups of patients.

Diagnosis	Number of patients	Number of mutations (%)
Classic Rett syndrome	86	65 (76%)
Atypical Rett syndrome	20	6 (30%)
Male Rett variant	6	0 (0%)
Child autism	17	1 (6%)
Angelman syndrome	6	0 (0%)
MR with Rett-like features, no clinical data	211	8 (4%)

11 novel mutations were identified. Deletions c.48_55del8 in exon 1 and c.189_190delGA in exon 3 are localized close to the 5´end of the coding sequence. mRNAs harboring these mutations are probably degraded before the translation of the aberrant MeCP2 proteins by nonsense-mediated decay.

Deletion c.816_832del17 and duplication c.820_823dupAGTG are frameshift mutations that disrupt the original reading frame and introduce premature stop codons. Both mutations abolish TRD and C-terminal domain of the MeCP2 protein making it inactive and probably unstable.

Transition c.904C>T causes amino acid substitution p.Pro302Ser in TRD. The 3D structure of TRD is not characterized yet, as well as all its binding partners, therefore the exact impact of the mutation on MeCP2 function is impossible to predict. Nevertheless, proline 302 is highly conserved among vertebrates (Fig. 8) and also other substitutions at this position (p.Pro302Ala, p.Pro302Leu, p. Pro302Thr) have been reported in RTT patients.

Fig. 8. Alignment of the prolin 302-containing region of the human MeCP2 protein and its homologues in other vertebrates (http://mecp2.chw.edu.au/mecp2/info/MECP2_homologues.shtml).
Amino acids are numbered according to human MeCP2 sequence.

Complex indel mutation c.1063_1236del174;1189_1231inv43 was exactly defined by cloning and sequencing of each allele separately in the pCR4-TOPO vector. The mutation is located within a “deletion-prone region” of the *MECP2* gene (c.1057-c.1207), where most deletions occur due to recombination between numerous short direct repeat elements. This frameshift mutation causes change in amino acid sequence, which abolishes the C-terminal domain and creates a premature stop codon.

Deletion c.1069delAGC causes in-frame removal of serine at positon 357 (p.ΔSer357) (Fig. 9) in the C-terminal domain. This domain is highly conserved

among mammals, but not in other vertebrates, and is supposedly responsible for additional, to date not fully understood, MeCP2 functions.

Fig. 9. Alignment of the serine 357-containing region of the human MeCP2 protein and its homologues in other mammals (http://mecp2.chw.edu.au/mecp2/info/MECP2_homologues.shtml).
Note: Amino acids are numbered in respective to human MeCP2 sequence.

```
                          330        340        350        360        370
                          |          |          |          |          |
Homo sapiens              GEKSGKGLKTCKSPGRKSKESSPKGRSSSASS-PPKKE---HHHHHHHSES
Bos taurus                GEKSGKGLKTCKSPGRKSKESSPKGRSGSASS-PPKKE---HHHHHHHVEP
Canis familiaris          GEKSGKGLKTCKSPGRKSKESSPKGRSSSASS-PPKKE---HHHHHHHSEP
Mus musculus              GEKSGKGLKTCKSPGRKSKESSPKGRSSSASS-PPKKE---HHHHHHHSES
Rattus norvegicus         GEKSGKGLKTCKSPGRKSKESSPKGRSSSASS-PPKKE---HHHHHHHAES
Monodelphis domestica     GEKSTKGLKPGKSPGRKSKESSPKGRSASTSSSPPKKEQQQQQQQYHHHHYY
```

Large deletion c.1102_1461*461del821 was originally detected by MLPA as a decreased signal of two probes specific to exon 4 at the end of the coding sequence and in 3´UTR (Fig. 10). Exact breakpoint sites were identified by sequencing of long range-PCR product. This deletion as well as another novel mutation c.1163_1201del39 are located in the „deletion-prone region“ and affect the C-terminal domain by frameshift and creation of premature stop codons.

The last two newly identified mutations are large deletions detected by MLPA and comprise exon 1 and exons 3 and 4, respectively. The exact breakpoint sites will be identified by additional molecular analyses in the near future.

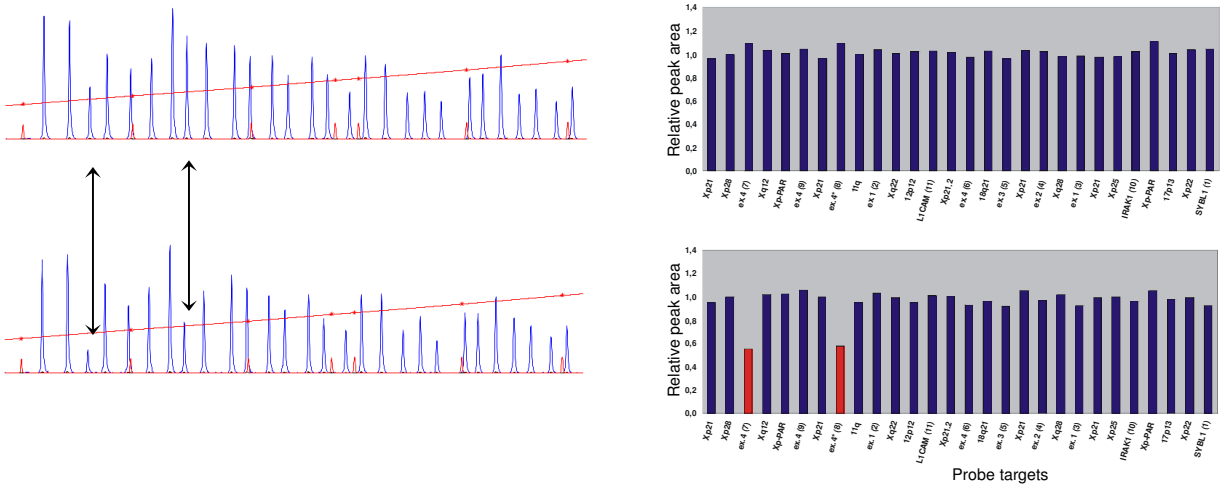

Fig. 10. MLPA analysis in the patient with large deletion in exon 4, which was later specified as c.1102_1461*461del821.
a) Separated MLPA fragments (blue peaks) of a female control (up) and the patient with deletion (bottom). Black arrows indicate peaks with a decreased signal in the patient, which indicate the deletion. Red peaks – size standard.
b) Normalized MLPA data in graphs (female control - up, the patient - bottom). The presence of two copies of a probe target results in a relative peak area equal approximately to 1 (blue bars), while the presence of only 1 copy of a probe target gives approximately 0.5 relative peak area (red bars).

All new mutations of this study were published or submitted into Human Genome Mutation Database (HGMD, www.hgmd.cf.ac.uk) except the last two large deletions, which need to be exactly defined by other molecular genetic methods.

Tab. 17 shows polymorphisms identified in our patients. If samples from family members were available, the paternal origin was determined. Novel polymorphisms were published or submitted into international *MECP2* variation database RettBASE (http://mecp2.chw.edu.au).

Tab. 17. *MECP2* polymorphisms identified in this study.

Nucleotide change	*MECP2* region	Amino acid change	MeCP2 domain	Number of patients	Reference
c.-235G>T[+]	5´UTR	-	-	1	novel
c.-220dupC[+]	5´UTR	-	-	6	Evans et al. 2005
c.18_23dup6	exon 1	p.Ala7_Ala8dup	-	6	Evans et al. 2005
c.210C>T	exon 3	p.Ser70Ser	-	1	Friez et al. 2006
c.377+22C>G	intron 3	-	-	2	Couvert et al. 2001
c.378-17delT	intron 3	-	-	2	Erlandson et al. 2001
c.426C>T	exon 4	p.Phe142Phe	MBD	1	Hoffbuhr et al. 2001
c.582C>T	exon 4	p.Ser194Ser	-	1	Cheadle et al. 2000
c.587C>G	exon 4	p.Thr196Ser	-	2	Shibayama et al. 2003
c.686C>T	exon 4	p.Ser229Leu	TRD	1	Cheadle et al. 2000
c.815C>T	exon 4	p.Pro272Leu	TRD	1	RettBase
c.819G>T	exon 4	p.Gly273Gly	TRD	1	RettBase
c.1161C>T	exon 4	p.Pro387Pro	C-ter	1	RettBase
c.1189G>A	exon 4	p.Glu397Lys	C-ter	1	Hampson et al. 2000
c.1330G>A	exon 4	p.Ala444Thr	C-ter	3	Buyse et al. 2000
c.1335G>A	exon 4	p.Thr445Thr	C-ter	1	RettBase
c.1449G>A	exon 4	p.Glu483Glu	C-ter	1	novel
c.1461*92C>T	exon 4	3´UTR	-	1	Zahorakova et al. 2007
c.1461*227G>A	exon 4	3´UTR	-	1	novel
c.1461*328G>A	exon 4	3´UTR	-	2	Zahorakova et al. 2007
c.1461*359G>C	exon 4	3´UTR	-	1	Zahorakova et al. 2007
c.1461*363G>C	exon 4	3´UTR	-	1	Zahorakova et al. 2007

Polymorphisms are numbered starting from the ATG start codon in the MeCP2_e2 coding sequence (NT_205965).

[+] Polymorphism located in exon 1 is numbered starting from the ATG start codon in the MeCP2_e1 coding sequence (AY523575).

Ad 2. Molecular analysis of the *CDKL5* gene

Analysis of the *CDKL5* gene by DNA sequencing and MLPA was performed in 10 patients (1 girl and 9 boys) with suspected EIEE2 and in 13 *MECP2* mutation-negative patients (all girls) with early-onset seizure variant of RTT. No pathogenic mutation has been detected in our patients so far. The estimated frequency of

CDKL5 mutations in atypical RTT patients with early-onset seizures is 13% and in patients with infantile spasms 17% (Bahi-Buisson et al., 2008). Larger sample of carefully selected patients needs to be analyzed to draw any conclusions about prevalence of *CDKL5* mutations in Czech patients with either atypical RTT or EIEE2.

Ad 3. Establishing the screening method to precede DNA sequencing

HRM is a simple, rapid, and cost-effective screening method with many applications. We optimized the procedure using the LightScanner instrument (Idaho Technologies, Salt Lake City, UT, USA), which allows processing of 96 samples in less than 15 minutes. The analysis was established for *MECP2* exons 2, 3, and 4, and all *CDKL5* exons. Amplicon containing *MECP2* exon 1 is extremely GC-rich with many repeat elements in the coding sequence and flanking non-conding regions and needs further optimization of analysis conditions. The sensitivity and specificity of the method was tested and verified on previously sequenced samples. 16 samples were *MECP2* mutation-negative and 60 samples contained a known heterozygous sequence variant (mutation or polymorphism) in *MECP2* exon 3 or 4. All samples were called correctly with 100% sensitivity. The HRM analysis showed to be a reliable screening method suitable for *MECP2* screening prior to DNA sequencing (Fig. 11).

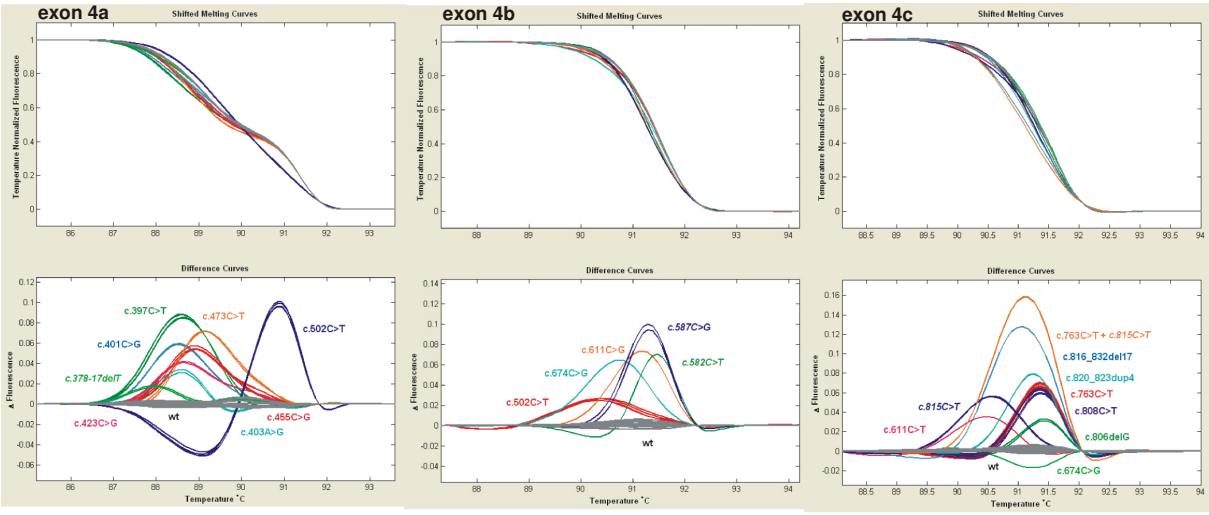

Fig. 11. HRM curves of three amplicons comprising the part of the *MECP2* exon 4.
As the temperature rises and amplicons pass through their melting transitions, fluorescence intensity is reduced due to fluorescent dye release of DNA duplexes. The upper charts represent the normalized melting curves, showing the level of fluorescence vs the temperature; the lower charts show the difference curves and groups of genotypes defined by the original LightScanner software. All mutations and polymorphisms (*in italics*) are heterozygous and clearly distinguished from wild type.

Only the negative control samples were available for *MECP2* exon 2 (30 samples) and *CDKL5* exons (23 samples). Although the samples were interpreted correctly, the overall reliability of HRM in these amplicons could not be definitely evaluated yet due to small amount of analyzed samples and the absence of positive controls. HRM analysis of the *MECP2* exon 2 will continue with random selection of samples for DNA sequencing. The *CDKL5* gene will be analysed by DNA sequencing for diagnostic purposes, at least until several sequence variants will be identified to enable the evaluation of HRM sensitivity.

Based on our observations and published data, HRM is a reliable method for the detection of heterozygous sequence variants in the PCR products up to 400-500 bp. It also offers several advantages over other screening methods. It is a non-destructive method. Samples are easily recovered by a simple purification step and there is no need for re-amplification for further analyses, such as DNA sequencing. It is a very convenient and useful technique of mutation scanning in a large number of samples. 96- or 384-well plate capabilities of the LightScanner instrument permit to process many samples in a short time. It can be also used in cases where expected mutation frequencies are low, such as *MECP2* mutations in autistic and mental retardation patients, to avoid unnecessary sequencing of mutation-negative patients.

Ad 4. Investigation of possible associations between the phenotypic severity and *MECP2* mutations in RTT patients

To determine whether different *MECP2* mutations result in differences in severity of RTT phenotype, several clinical features of patients with confirmed pathogenic mutation were analyzed using a Pineda-based scale. The Pineda scale is one of three the major scoring systems, which were specifically designed for evaluation of the clinical severity in RTT. We chose this scale because, unlike Kerr (Kerr et al., 2001) and Percy (Schanen et al., 2004) scales, it focuses more on the general course and progression of the disorder than on current functioning of the patient. Sufficient data were available in 46 cases out of 79 (the *MECP2* mutation-positive patient with child autism was ruled out of this study). We did not evaluate each mutation separately due to small amount of patients, but three groups according to type and position of the mutation were formed. The average age of regression onset

and median severity score values were calculated and compared between these groups. The statistical evaluation was performed by Kruskal-Wallis test (Tab. 18).

Tab. 18. Comparison of the clinical phenotype in patients with different *MECP2* mutation type.

Manifestations	**Late truncations (7 patients)**	**Missense (20 patients)**	**Nonsense (19 patients)**	**P***
Age at onset of regression	21.4 months	14.1 months	16.2 months	0.249
1. Head growth	0.7	0.7	0.8	0.879
2. Sitting alone	0.3	0.9	0.9	0.369
3. Ambulation	1.3	2.2	2.9	**0.037**
4. Hand use	1.3	1.6	1.3	0.408
5. Onset of hand stereotypies	1.9	2.2	2.3	0.274
6. Language	3.0	3.3	3.2	0.835
7. Non-verbal communication	1.0	1.9	1.7	0.075
8. Respiratory function	0.4	0.7	0.7	0.350
9. Seizures	1.1	0.6	0.6	0.147
Total score	11.0	14.1	14.4	0.131

Average age at regression onset and median score values were calculated for each group. The higher the score, the more severe the impairment. For details of the scoring system, please refer to Tab. 14.
* Kruskall-Wallis test ($P<0.05$)

In agreement with our expectations, truncating mutations located in the C-terminal domain caused generally milder phenotype with later regression onset than missense and nonsense mutations. The most probable reason is that late truncations, when MBD and TRD remain intact, do not abolish the major functions the MeCP2 protein. The average age of regression onset was considerably higher in patients with late truncations than in the other two groups, but the difference was not statistically significant. Statistically significant difference was observed only in ambulation ($P=0.037$), which was the least severely affected in patients with late truncations. According to published studies (Monros et al., 2001; Huppke et al., 2002; Schanen et al., 2004), missense mutations tend to be associated with milder phenotype than nonsense mutations. In our study, patients in both groups had very similar average severity scores in most cathegories, and moreover, missense mutations were associated with slightly earlier average onset of regression and worse hand use. This contradiction is probably only coincidental since a considerable variability among individual patients in both groups was observed. A couple of large studies report an apparent genotype-phenotype correlation in RTT patients with the most common *MECP2* mutations (Bebbington et al., 2008; Neul et al., 2008). In smaller studies, such as ours, the individual differences between patients with the same mutation are more apparent and prove that the prediction of RTT phenotype according to the *MECP2* mutation should be taken with caution. Variability in the clinical severity of

the patients with the same *MECP2* mutation or mutation type is highly probably induced by other factors, such as variabile XCI in brain, *BDNF* polymorphism and others. Identification of these factors will not only provide further insight into pathogenesis of RTT but also help to design effective preventive treatment approaches.

Ad 5. Selection and study of possible genetic determinants, which might modulate the phenotype of RTT patients independently of *MECP2* mutations

We studied the possible association between the phenotypic severity and functional polymorphisms in several candidate genes. The same principle of quantification and comparison of clinical features was applied as in the previous study.

5-HTT gene

Two polymorphisms, which modulate the level of *5-HTT* transcription, were studied: 5-HTTLPR in promoter and STin2 VNTR in intron 2. Since the low-expressing alleles (S and STin2.10) appear to act dominantly (Hranilovic et al., 2004), the patients were divided into S-carriers vs. non-carriers and STin2.10-carriers vs. non-carriers. The results are summarized in Tab. 19 and Tab. 20.

Tab. 19. Comparison of the clinical phenotype of RTT patients in relation to the 5-HTTLPR.

Manifestations	**S-carriers (25 patients)**	**Non-carriers (21 patients)**	**P***
Age at onset of regression	17.1 months	14.8 months	0.299
1. Head growth	0.6	0.9	0.107
2. Sitting alone	0.9	0.7	0.244
3. Ambulation	2.2	2.5	0.639
4. Hand use	1.4	1.5	0.463
5. Onset of hand stereotypies	2.0	2.3	0.106
6. Language	3.1	3.3	0.559
7. Non-verbal communication	1.6	1.8	0.226
8. Respiratory function	0.6	0.7	0.852
9. Seizures	0.8	0.6	0.401
Total score	13.2	14.3	0.413

Average age at regression onset and median score values were calculated for each group. The higher the score, the more severe the impairment. For details of the scoring system, please refer to Tab. 14.
* Mann-Whitney test ($P<0.05$)

Tab. 20. Comparison of the clinical phenotype of RTT patients in relation to the STin2 VNTR.

Manifestations	STin2.10-carriers (29 patients)	Non-carriers (17 patients)	P*
Age at onset of regression	14.9 months	18.1 months	0.306
1. Head growth	0.7	0.8	0.865
2. Sitting alone	1.0	0.5	0.058
3. Ambulation	2.3	2.4	0.981
4. Hand use	1.5	1.4	0.823
5. Onset of hand stereotypies	2.2	2.1	0.694
6. Language	3.4	2.9	0.219
7. Non-verbal communication	1.8	1.5	0.206
8. Respiratory function	0.7	0.7	0.956
9. Seizures	0.7	0.6	0.436
Total score	14.3	12.9	0.361

Average age at regression onset and median score values were calculated for each group. The higher the score, the more severe the impairment. For details of the scoring system, please refer to Tab. 14.
* Mann-Whitney test (P<0.05)

No significant difference was observed between the groups concerning either of two polymorphisms. Nevertheless, patients without the STin2.10 allele showed better communication skills and their ability to sit developed earlier than in STin2.10-carriers. On the other hand, the average age of regression onset was lower than in STin2.10-carriers. It seems contradictory, since the age of regression usually correlates with the overall severity in RTT patients. Despite reported irregularities of the autonomic control in RTT patients, our results do not show that serotonin transporter is a significant modulator of RTT phenotype. Further investigations are needed in larger group of RTT patients, especially with emphasis on the behavioral aspects and autonomous functions. Also other members of serotonergic pathway, such as serotonin receptors, may be involved.

APOE gene

Patients were divided into two groups according to presence or absence of the ε4 allele. The onset of regression occured significantly earlier in ε4-carriers (P=0.047) and they also showed worse non-verbal communication skills (P=0.032) and earlier onset of hand stereotypies (P=0.017) than non-carriers. Patients without the ε4 allele had less seizures and better hand use, although the statistical significance was not confirmed (Tab. 21).

The precise mechanism of ApoE4 involvement in the pathogenesis of RTT and other neurological disorders is unclear. It may interfere with neural transmission and plasticity. An abnormal structure of dendritic spines has been reported in RTT brains.

Independently, an isoform-specific effect of ApoE on the maintenace of dendritic spines has been observed, with ApoE4 being less effective than ApoE3. However, the direct association of ApoE4 on dendrites in RTT brains is without further investigations questionable. Nevertheless, our results show that some differences in the phenotypic severity of RTT, especially the onset of regression, can be explained by different *APOE* genotypes and the *APOE* ε4 allele should be considered as a candidate modulation factor of RTT phenotype.

Tab. 21. Comparison of the clinical phenotype of RTT patients in relation to *APOE* genotype.

Manifestations	**ε4-carriers (18 patients)**	**Non-carriers (28 patients)**	**P***
Age at onset of regression	13.4 months	17.8 months	**0.047**
1. Head growth	0.7	0.8	0.899
2. Sitting alone	0.9	0.7	0.480
3. Ambulation	2.5	2.3	0.753
4. Hand use	1.7	1.3	0.067
5. Onset of hand stereotypies	2.5	2.0	**0.017**
6. Language	3.2	3.2	0.855
7. Non-verbal communication	1.9	1.5	**0.032**
8. Respiratory function	0.7	0.6	0.870
9. Seizures	0.8	0.6	0.205
Total score	14.9	13.0	0.078

Average age at regression onset and median score values were calculated for each group. The higher the score, the more severe the impairment. For details of the scoring system, please refer to Tab. 14.
* Mann-Whitney test (P<0.05)

The results of this part of the study have been accepted for publication in the Journal of Child Neurology.

HMOX1 gene

According to published data, carriers of long *HMOX1* alleles have significantly lower HO-1 induction after the exposure of exogenous stimuli. We divided our patients into two groups. Patients homozygous or heterozygous for L alleles were grouped together and compared with homozygotes S/S (Tab. 22).

No significant difference in the phenotypic severity was observed between the studied groups. However, carriers of L alleles had lower medium score for seizures than non-carriers. The results might be affected by comparing two unproportional groups (8 vs. 38 patients) and significant individual phenotypic variability observed especially in the larger group. When three genotype groups (8 S/S patients, 16 S/L patients, 22 L/L patients) were compared, results regarding the seizures were very

similar, but less statistically significant (Kruskal-Wallis test, P=0.116). Interestingly, 3 patients out of 8 S/S homozygotes suffered from intractable seizures, while only 2 patients among 38 L-carriers had severe seizures. The possible association between *HMOX1* polymorphism and seizures in RTT has to be tested on larger amount of patients.

Tab. 22. Comparison of the clinical phenotype of RTT patients in relation to the *HMOX1* promoter polymorphism.

Manifestations	**L-carriers (38 patients)**	**Non-carriers (8 patients)**	**P***
Age at onset of regression	16.2 months	15.3 months	0.437
1. Head growth	0.7	0.8	0.913
2. Sitting alone	0.9	0.4	0.307
3. Ambulation	2.4	2.1	0.589
4. Hand use	1.4	1.4	0.862
5. Onset of hand stereotypies	2.2	2.1	0.802
6. Language	3.3	2.8	0.137
7. Non-verbal communication	1.7	1.6	0.930
8. Respiratory function	0.6	0.8	0.527
9. Seizures	0.6	1.1	0.064
Total score	13.8	13.1	0.727

Average age at regression onset and median score values were calculated for each group. The higher the score, the more severe the impairment. For details of the scoring system, please refer to Tab. 14.
* Mann-Whitney test (P<0.05)

CONCLUSIONS

Molecular genetic methods provide a definitive confirmation of the RTT diagnosis and offer the opportunity for genetic councelling in the patient´s family. Although the risk of recurrence in RTT family is small, the negative result of prenatal diagnosis may significantly reduce stress during pregnancy and rule out eventual germline mosaicism. Molecular diagnostics also assists in clinically ambiguous cases, such as in very young patients who did not pass through all the stages, yet, or in patients without available data on their early childhood history. Establishing the correct diagnosis helps to predict further evolution of clinical features and enables to plan preventive management and supportive therapy.
The major achievements of the work on the thesis are:

- We improved the methods of the *MECP2* gene analysis, which have been used in the routine molecular genetic diagnostics. We established the multiplex ligation probe-dependent amplification analysis to detect large deletions or duplications in the *MECP2* gene, since these mutations are overlooked by DNA sequencing.

- We confirmed the clinical diagnosis in 79 patients with RTT and identified the *MECP2* mutation in one patient with child autism. The prenatal diagnosis was performed in 8 families and yielded negative results.

- We established the analysis of the *CDKL5* gene, which is the causal gene in some cases of atypical RTT with early-onset seizures and early infantile epileptic encephalopathy 2.

- We established the high-resolution melting as a fast, reliable, and cost-effective screening method performed prior to DNA sequencing. The method was optimized for both genes, *CDKL5* and *MECP2*.

- We analysed severity of clinical phenotype in the patients with identified *MECP2* mutation and confirmed that the mildest clinical outcome is associated

with late truncating mutations, which do not abolish major functional domains of the MeCP2 protein. However, considerable individual differences occur among the patients with the same *MECP2* mutation type. They are most likely caused by other factors and therefore development of clinical phenotype based solely on *MECP2* mutation should be predicted with a caution.

- We selected several candidate genes and studied whether functional polymorphisms in these genes may act as genetic determinants underlining the variabile severity of clinical features in RTT. The most prospective aspirant is the *APOE* gene, which ε4 allele was associated with significantly earlier onset of regression in our RTT patients.

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Original publication unrelated to the thesis:

Martásková D, Šlachtová L, Kemlink D, Záhoráková D, Papežová H (2009). Polymorphisms in serotonin-related genes in anorexia nervosa. The first study in czech population and metaanalyses with previously performed studies. Folia Biol - Prague 55(5):192-197. (IF2008 1.140)

Report on novel mutation:

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